Pacemakers and/or dynamic plasticity of rhythm generation in the in vivo respiratory network - The Physiological Society Paton Lecture

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Eupneic breathing of mammals is not simply an alternation between inspiratory lung inflation and expiratory lung deflation as a passive relapse of the lungs. Normal quiet breathing in vivo involves a precisely co-ordinated interplay between inspiratory and post-inspiratory muscle contractions, variable but rare expiratory muscle contractions, and a concomitant adjustment of sympathetic cardiovascular and vagal cardiac activities, all together being organized by a common cardio-respiratory control system. As we do not only breathe quietly but possibly also want to dine, sing and dance, for example, such in vivo control cannot just rely upon autonomous pacemaker cells rigidly driving a ventilator pump, but requires in a highly dynamic plasticity of neural network control.

To increase the options for such varying behavioural phenotypes, mammals have developed quite a complex network involving diverse assemblies of distinct classes of respiratory neurones localized in the ventral region of the brainstem that extends in a rostro-caudal direction from the pons to the cervical spinal cord. A commanding "kernel", however, is localized in the pre-Bötzinger Complex (pre-BötC) just caudal to the compact division of the nucleus ambiguus. Its vital role was demonstrated by the finding that in vivo rhythmic respiratory output from the brainstem disappears when the pre-BötC is lesioned. Such findings allowed the isolation of a spontaneously active respiratory network in "reduced" preparations, such as the "en bloc" brainstem-spinal cord preparation or the "rhythmic" brainstem slice of neonatal rat or mouse containing the pre-BötC. These preparations endogenously generate a "respiratory-like" rhythmic activity. The activity pattern is, however, quite different to the normal (eupneic) pattern as seen in more intact preparations like the in situ network in the arterially perfused working heart-brainstem preparation of rat and mouse or the fully intact in vivo preparations of anaesthetized mini-pig, cat, rat or mouse. A clear distinction between the "quality" of the various preparations can be drawn by the capacity of the network in the preparation to alter inspiratory and post-inspiratory activity patterns and oscillatory frequency to changes in behaviour or metabolic demands as well as their ability to demonstrate dynamic plasticity in response to modulating influences.

The lecture will start with the cellular biophysics and continue with various aspects of synaptic interaction and integration to explain how under in vivo conditions potential pacemakers are kept under harsh control. It will then address the repertoire of metabotropic receptors acting through separate and convergent signal pathways onto ionotropic receptors and describe the molecular and cellular processes underlying a vital network plasticity that includes unexpected changes in network configuration to maintain rhythmicity. The final aspect will deal with translational approaches to pharmacotherapies treating life-threatening disturbances of network functions.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

The Beginnings of Optogenetics – The Physiological Society’s Bayliss-Starling Prize Lecture

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Light-sensitive proteins encoded in DNA can serve as selective optical interfaces for observing and controlling genetically targeted neurons in functioning circuits, in vitro and in vivo. Light-emitting sensors of neuronal activity (reporting calcium increase, neurotransmitter release, or membrane depolarization) have begun to reveal how information is represented by neuronal assemblies, and how these representations are transformed during the computations that inform behaviour. Light-driven actuators control the electrical activity of central neurons in freely moving animals and establish causal connections between the activities of specific neurons and the expression of particular behaviours. The combination of finely resolved optical field sensing and finely resolved optical field actuation is opening new dimensions for the analysis of the connectivity, dynamics, and plasticity of neuronal circuits, and perhaps even for replacing lost - or designing novel - functionalities.

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My genes made me eat that! - The Physiological Society Public Lecture

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The recent and rapid increase in the prevalence of obesity in most developed and developing countries has correctly focused attention on environmental determinants of that secular trend. However, a fuller understanding of the factors determining any individual person’s adiposity requires appropriate consideration of inheritance. Studies of twins, adoptees and adopted twins provide incontrovertible evidence that heritable factors play a major, perhaps even the major factor, in determining a person’s fatness. Until recently, the precise mechanisms whereby such genes might influence fatness were obscure. However, in the past decade we have witnessed an explosion of information regarding the molecular mechanisms underlying the control of mammalian energy balance. That information, much of it originating from animal models, is beginning to demonstrate its clear relevance to human energy balance.
Stephen G. Waxman, M.D., Ph.D.

These examples underscore the continuing relevance of the human nociception and suggest new therapeutic strategies. Humans, and which both establish the role of this channel in pain, and the “man on fire” syndrome, erythromelalgia, in.

We will describe loss-of-function and gain-of-function mutations which produce insensitivity to hyperexcitability that underlies neuropathic pain. Finally, we will discuss changes in sodium channel expression and irreversible worsening, and the role of other sodium reverse sodium-calcium exchange which triggers axonal degeneration and irreversible worsening, and the role of other sodium channels in restoring conduction along demyelinated axons, which permits remission (recovery of function) in multiple sclerosis and neuropathic pain as model diseases. We will discuss the role of specific sodium channels in driving reverse sodium-calcium exchange which triggers axonal degeneration and irreversible worsening, and the role of other sodium channels in restoring conduction along demyelinated axons, which permits remission (recovery of function) in multiple sclerosis.

We will also discuss changes in sodium channel expression following axonal injury which contribute to DRG neuron hyperexcitability that underlies neuropathic pain. Finally, we will describe loss-of-function and gain-of-function mutations of the Nav1.7 sodium channel which produce insensitivity to pain, and the “man on fire” syndrome, erythromelalgia, in humans, and which both establish the role of this channel in human nociception and suggest new therapeutic strategies. These examples underscore the continuing relevance of the original studies on sodium channels, carried out in a model system, the squid.

Stephen G. Waxman, M.D., Ph.D.

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suggesting an additional involvement of ATP and P2X receptors in the generation of the NO signal and negative feedback inhibition of CB receptors. The reasons for this complex pattern of regulation of CB function by multiple neuroactive agents are presently unclear. Conceivably, they may contribute to the mechanisms underlying CB plasticity during patterned stimulation, e.g., chronic sustained hypoxia and chronic intermittent hypoxia, as experienced during cardiorespiratory disorders such as chronic obstructive pulmonary disease and sleep apnea.

Studies in the author's laboratory were supported by grants from the Canadian Institutes of Health Research.

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Analysis of the RyR-Channel Stochastic Dynamics in the Electron-Conformational Model

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Methods: We have proposed a simple, biophysically reasonable electron-conformational theory for the ryanodine receptor channel (RyR) to be a main element governing the calcium dynamics in a cardiac cell [1,2], starting with the well-known model theory of the photo-induced structural phase transitions [3]. The main feature of our model is that, in addition to a fast electronic (dichotomic) degree of freedom, the RyR channel is characterized by a slow classical conformational coordinate, $Q$, that obeys the Langevin dynamics. Two minima of a conformational potential (CP) are related with a closed and open RyR state, respectively. We took into account the calcium induced direct electronic Franck-Condon transitions between the CP branches and different relaxation mechanisms.

Results: We have performed a series of computer simulations of a single RyR stochastic gating under steady-state conditions and made a Hurst analysis of the channel’s conformational coordinate $Q(t)$. Dynamics of our system appears to be strongly correlated ($H \approx 1.0$) for relatively short times compared with the characteristic times of conformational relaxation to the metastable minimum of CP, and weakly correlated ($H \approx 0.5$) for larger time intervals.


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Chronic hypoxia in utero (CHU) increases superoxide production in adult rat skeletal muscle vasculature

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We have shown that chronic hypoxia in-utero (pregnant dams breathing 12% O₂ during the second half of pregnancy) may lead to a reduction in nitric oxide (NO) bioavailability in the vasculature of the adult offspring. Superoxide anions (O₂⁻) are implicated in reducing NO bioavailability by directly reacting with NO to produce peroxynitrite. Superoxide dismutase (SOD) catalyses the dismuting of O₂⁻ into H₂O₂ and O₂, thus protecting NO bioavailability. In light of this, we have now investigated the role of SOD in skeletal muscle vasculature in CHU offspring.

Under anaesthesia (Alfaxan; 3-6ml hr⁻¹kg⁻¹ i.v.), we recorded arterial blood pressure (ABP) and femoral blood flow (FBF) in 9-10 week old normal (N, n=12) rats and in age-matched CHU (CHU, n=8) offspring. Femoral vascular conductance (FVC) was calculated (FBF/ABP) and integrated FVC (IntFVC) used to give an indication of vascular tone. Variables were recorded during air breathing and during 10min periods of acute systemic hypoxia (breathing 8% O₂) before and during infusion of the cell permeant SOD inhibitor Diethyldithiocarbamate trihydrate (DETC; 5mg⁻¹kg⁻¹,min⁻¹ i.v.)

Baseline ABP was similar in both groups (N:136±3, CHU:143±5mmHg) as was baseline IntFVC (N:7.9±1.1, CHU:6.3±1.1 CU) resulting in similar FBF (N:5.4±0.6, CHU:4.6±0.9ml⁻¹min⁻¹kg⁻¹). As previously shown, responses to acute hypoxia were similar in both N and CHU with both showing a fall in ABP, an increase in IntFVC which maintained FBF at control levels. DETC caused a small increase in ABP, although not statistically significant (N:137±3, CHU:149±3 mmHg). DETC also caused a significant reduction in IntFVC (N:0.6±0.7, CHU:1.8±0.6) in CHU, but not N rats, which was mirrored by changes in FBF (N:0.2±0.4, CHU:1.1±0.3).

Inducing acute hypoxia during DETC infusion caused ABP to fall. However, it remained at a level higher than under control conditions in both N and CHU (N:79±5, CHU:77±3). There was no difference in the magnitude of muscle vasodilatation induced by hypoxia as indicated by a similar increase in IntFVC.

SOD normally dismutates O₂⁻ into the vasodilator H₂O₂, therefore, SOD antagonism results in the build up of O₂⁻ and induces vasoconstriction. The reduction in FVC in air breathing CHU rats during DETC administration suggests increased basal production of O₂⁻ anions that is not apparent in N rats. It is possible that this O₂⁻ production is through uncoupling of nitric oxide synthase. However, in keeping with our previous findings with shorter periods of hypoxia, the acute response to hypoxia and is similar in both N and CHU rats. Further, there is no apparent difference during DETC infusion suggesting the increased O₂⁻ production in CHU rats is only evident during air breathing. Preliminary findings in the carotid artery suggest there may also be differences in other vascular beds, warranting further work.

The respiratory profiles of Dexmedetomidine in paediatric patients following cardiac surgery

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Dexmedetomidine is a potent sedative, analgesic and sympatholytic agent. The sympatholytic effects of Dexmedetomidine are associated with decrease in endogenous concentrations of noradrenaline resulting in dose-dependant decreases in arterial blood pressure and heart rate that lead to perioperative haemodynamic stability with a decrease risk of adverse perioperative cardiac events. Series of case reports by Tobias et al enhanced the focus on the usage of Dexmedetomidine among pediatric patients. The objective of this study was to assess the respiratory profiles including respiratory rate, SPO2 and PaO2/ FiO2 ratio of pediatric cardiac patients exposed to Dexmedetomidine. It is hoped that we may evaluate the potential benefits of Dexmedetomidine in pediatric cardiac patients. The study was randomized controlled double blinded comparing Dexmedetomidine sedation to institution’s standard for postsurgical sedation. The study was approved by the institution’s ethical committee. Patients were randomized in a 1:1 fashion into two study arms, Group A received infusion of dexmedetomidine between 0.1 to 0.7 mcg/kg/hr (n= 25) and Group B, as control group received midazolam between 0.5 to 2 mcg/kg/min (n: 25). The drug infusion was performed under strict protocol by the anesthesiologist involved. The study drugs were started after patients arrived in ICU with stable haemodynamic. Results showed no significant differences in respiratory rate; O2 saturations; arterial pH and PaCO2 between both groups compared (p>0.05). Although the arterial partial O2 tension (PaO2): fractional inspire O2 (FiO2) ratios were slightly lower in dexmedetomidine group, they was not significantly different from the control group (p>0.05). Our observation tends to show that dexmedetomidine does not have clinically important adverse impacts on respiration in the postsurgical paediatric patients who requires intensive care. Hence it appears that dexmedetomidine is safe for post operation sedation in selected paediatric heart surgery.
affected by dietary restriction. This work evaluated the alterations in MAP and heart rate (HR) of Fisher rats fed for 35 days after weaning with regular (15%) or low (6%) protein diets, before and after systemic administration of the angiotensin converting enzyme inhibitor (ACE) or AT1 receptor antagonist. Under ketamine (80 mg/kg) plus xylazine (7 mg/kg) anesthesia, polyethylene cannulas were inserted into the femoral artery to record arterial pressure and into the vein for drug injections one day before the experiments. All procedures and experimental protocols were conducted in accordance with the Brazilian Society for Neuroscience and Behavior instructions for the use of animals in research. MAP levels in malnourished rats were greatly dependent on RAS because the inhibition of angiotensin converting enzyme with enalapril or the blockade of AT1 receptors with losartan produced greater fall in the blood pressure of malnourished rats compared to control rats (-37±2 mmHg vs. -3±2 mmHg, n=8 and -41±5 mmHg vs. -3±1 mmHg, n=8, respectively). To further investigate the relative contributions of angiotensin II acting directly on smooth muscle cells versus through the sympathetic nervous system to maintain blood pressure in malnourished rats, experiments were carried out with peripheral α1 adrenergic and AT1 receptors blockade. The blockade of α1 receptors with prazosin after losartan further decreased the MAP in both groups (-29±2 mmHg in control, n=8 and -17±2 mmHg in malnourished, n=8). Prazosin was less effective than losartan to reduce MAP in malnourished rats when compared to control rats that underwent the same treatment judged by the changes due to AT1 blockade versus AT1 plus α1 blockade (-8±3 mmHg and -29±2 mmHg respectively in control, n=8 vs. -44±5 mmHg and -17±2 mmHg respectively in malnourished, n=8). When prazosin was given first, malnourished rats also presented greater fall in MAP compared to control (-38±3 mmHg vs -8±2 mmHg respectively). The subsequent administration of losartan produced further and similar fall in MAP for both groups (-25±3 mmHg in control and -22±4 mmHg in malnourished). The present results suggest that ongoing production of angiotensin II and its action on AT1 receptors are critical factors supporting the blood pressure in malnourished rats and that α1 receptors activation could be under strong influence of angiotensin II.

FAPEMIG and CNPq.
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The aim of this project was to determine if there are gender differences in the RSNA response immediately following a myocardial infarct (MI).

The University of Auckland Animal Ethics Committee approved all experiments. Male and female Wistar rats were anaesthetised with urethane (1000–1500 mg/kg I.P) and α-chloralose (80–120 mg/kg I.P). Rats were intubated and ventilated. The left renal artery was exposed and the arterial nerves dissected for recording of RSNA along with arterial pressure (femoral artery) and ECG. A left intercostal thoracotomy was performed to expose the heart, the pericardium was removed. After a one hour period of baseline recording the left coronary artery was ligated using a 6-0 suture. In the sham group the chest was opened, pericardium removed, a suture inserted around the coronary artery but not tied.

In male rats (n=7) coronary ligation resulted in abrupt increase (32 ± 12%, mean±sem) in RSNA before subsiding to lower steady state within 10 minutes of the infarct for the next 2 hours (13 ± 4% above the baseline level, P<0.05, ANOVA). In contrast in female rats (n=7) no significant changes in RSNA were observed in response to myocardial infarction. No change in RSNA was observed in sham operated rats (n=10). Arterial pressure responses were also different between the male and female rats; two hours post-infarct arterial pressure was 10 ± 5mmHg below that seen in the sham group (P<0.05), whereas arterial pressure in the male rats was not significantly different to the shams. Baseline baroreflex control of RSNA differed between the sexes with the females displaying no significant changes in RSNA were observed in response to myocardial infarction. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Heart rate variability is largely dependent on cardiac vagal activity and its loss is an independent risk factor for arrhythmias and cardiac mortality. Thus, it is important to elucidate the factors

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In a paper by Stojicic et al (2008) we found that central V2 vasopressin antagonist modulates cardiovascular homeostasis. To further assess the role of central vasopressin V2 receptors we performed experiments in adult male Wistar rats overexpressing V2 receptors in hypothalamic paraventricular nucleus, the major integrative site of stress response. Under xylazine/ketamine anesthesia (0.4ml 10% ketamine IP, 0.1ml 2% xylazine IP per animal) TA-11-PA C40 DSI implants were introduced in aorta followed by metamizol treatment (200mg, IP). After ten days rats were exposed to stereotaxic surgery (anaesthetics as above); glass micropipettes containing 1:1 mixture of the replication-deficient adenoviral vectors Ad.CMV.eGFP (titer, 1.5x1010 pfu/ml) plus Ad.CMV.V2 (2.5 x1010 pfu/ml) or Ad.CMV.eGFP (control), were injected in PVN (Qiu et al. (2007)). Seven days elapsed before full expression of transfected genes and beginning of the 10 minute air-jet stress protocol. Only animals in which eGFP expression was observed within the PVN were subjects of cardiovascular signal analysis and one-way ANOVA statistical significance evaluation. All experiments were performed in accordance with Directive 86/609/ECC. Systolic (SBP), diastolic blood pressure (DBP) and heart rate (HR) were derived from the arterial blood pressure digitalized at 1000Hz, as maximum, minimum and inverse of interbeat interval, respectively. Evaluation of the spontaneous BRR was performed by using the method of sequences and calculation of baroreceptor reflex sensitivity (BRS) and effectiveness index (BEI). Time spectra were created using fast Fourier transform algorithm on 15 overlapping 2048 point time series involving 410-s registration period. Spectra were analyzed in very-low-frequency (VLF: 0.0195-0.195Hz), low-frequency (LF: 0.195-0.8Hz) and high-frequency (HF: 0.8-3Hz) range. Basal values of cardiovascular parameters did not differ between groups.

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These results indicate that gender exerts a major influence on the resulting sympathoactivation occurring after MI. We suggest that different treatment strategies may justified based on gender after MI.

This work was supported by the University of Auckland and the Health Research Council of New Zealand

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Upregulation of vasopressin V2 receptors in paraventricular nucleus of rats modulates cardiovascular response to acute panic

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In a paper by Stojicic et al (2008) we found that central V2 vasopressin antagonist modulates cardiovascular homeostasis. To further assess the role of central vasopressin V2 receptors we performed experiments in adult male Wistar rats overexpressing V2 receptors in hypothalamic paraventricular nucleus, the major integrative site of stress response. Under xylazine/ketamine anesthesia (0.4ml 10% ketamine IP, 0.1ml 2% xylazine IP per animal) TA-11-PA C40 DSI implants were introduced in aorta followed by metamizol treatment (200mg, IP). After ten days rats were exposed to stereotaxic surgery (anaesthetics as above); glass micropipettes containing 1:1 mixture of the replication-deficient adenoviral vectors Ad.CMV.eGFP (titer, 1.5x1010 pfu/ml) plus Ad.CMV.V2 (2.5 x1010 pfu/ml) or Ad.CMV.eGFP (control), were injected in PVN (Qiu et al. (2007)). Seven days elapsed before full expression of transfected genes and beginning of the 10 minute air-jet stress protocol. Only animals in which eGFP expression was observed within the PVN were subjects of cardiovascular signal analysis and one-way ANOVA statistical significance evaluation. All experiments were performed in accordance with Directive 86/609/ECC. Systolic (SBP), diastolic blood pressure (DBP) and heart rate (HR) were derived from the arterial blood pressure digitalized at 1000Hz, as maximum, minimum and inverse of interbeat interval, respectively. Evaluation of the spontaneous BRR was performed by using the method of sequences and calculation of baroreceptor reflex sensitivity (BRS) and effectiveness index (BEI). Time spectra were created using fast Fourier transform algorithm on 15 overlapping 2048 point time series involving 410-s registration period. Spectra were analyzed in very-low-frequency (VLF: 0.0195-0.195Hz), low-frequency (LF: 0.195-0.8Hz) and high-frequency (HF: 0.8-3Hz) range. Basal values of cardiovascular parameters did not differ between groups. Exposure of rats to air-jet stress increased SBP(137.81±5.36mmHg, p<0.05, and 144.23±5.14mmHg, p<0.05) DBP(107.94±3.36 mmHg, p<0.01 and 107.92±5.85mmHg, p<0.01), HR(447.92±16.59Hz p<0.01 and 465.57 Hz p<0.01), LF SBP(3.97±0.56mmHg2/Hz, p<0.05 and 4.58±0.7256mmHg2/Hz, p<0.05) and HF SBP(1.79±0.36 mmHg2/Hz, p<0.05 and 3.20±1.75 mmHg2/Hz, p<0.001) in both groups of rats. In HR spectra, only transfected rats exhibited significant increase of LH HR(81.59±47.12 mmHg2/Hz, p<0.05) and HF HR(19.5±63.2 mmHg2/Hz, p<0.05) and reduced LF/HF HR ratio. In control rats exposure to air-jet stress reduced BRS, whereas in transfected rats BRS remained close to baseline values. The results indicate that overexpression of V2 receptors in PVN of rats increases vagal control of the heart and preserves the baroreceptor reflex functioning during stress. Qiu J et al.(2007). J Neurosci 27(9):2196-2203.


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Central drives to cardiac ganglion neurons of rat: an intracellular analysis in situ

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Heart rate variability is largely dependent on cardiac vagal activity and its loss is an independent risk factor for arrhythmias and cardiac mortality. Thus, it is important to elucidate the factors...
that modulate cardiac vagal ganglionic transmission in health and in cardiovascular disease states. Although cardiac vagal ganglionic transmission has been studied in a number of in vitro preparations (Edwards et al 1995) there have been no intracellular recordings from cardiac ganglion neurones in a functionally intact setting to permit the detailed analysis of synaptic integration and modulation at this site.

Using the working heart-brainstem preparation (Paton, 1996) from Wistar rats (aged 4-5 weeks, initially anaesthetised with Halothane), we have obtained the first intracellular recordings from functionally connected cardiac vagal ganglion cells in situ. The atria were dissected from the ventricles, pinned flat and stabilized with a nylon mesh foot. Intracellular recordings of 33 cardiac ganglion cells were made with sharp microelectrodes (0.5M KCl; 80-120 MOhm). Stable recordings were obtained for periods of over 30 minutes allowing examination of intrinsic properties, spontaneous firing pattern and responses to vagus nerve (right) evoked and cardiorespiratory reflex activation: peripheral chemoreflex (NaCN, 0.03% i.a.; baroreflex, by increasing pump flow; diving response, 10% C saline applied to snout. Several different classes of neurone were identifiable on the basis of their intrinsic electrophysiology, reminiscent of Edwards et al. (1995), and distinct patterns of spontaneous and reflex evoked activities. Active neurones showed EPSPs and/or action potentials (AP) that occurred most commonly in the post-inhibitory phase. In these neurones activation of bradycardic reflexes (baro-, chemo-, nasotrigeminal) increased EPSP frequency and/or AP with atrial rhythm slowing. Spontaneous, vagus- and reflexly-evoked AP appeared mostly triggered by suprathreshold unitary EPSPs rather than by summation of subthreshold EPSPs. Vagus stimulation evoked EPSPs at a latency of 30-40 ms.

In conclusion, it is possible to obtain high resolution, stable intracellular recordings from physiologically intact cardiac vagal ganglion cells, which exhibit appropriate patterns of ongoing and reflex excitatory synaptic drives. Future studies will elucidate how cardiac vagal transmission may be modified in health and disease. EDWARDS, F. R., HIRST, G. D., KLEMM, M. F. & STEELE, P. A. (1995) Different types of ganglion cell in the cardiac plexus of guinea-pigs. J Physiol, 486 (Pt 2), 453-71.


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Equine activity rhythms exhibit circadian and ultradian characteristics under different environmental conditions

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Activity rhythms are regulated by the hypothalamic circadian pacemaker in response to daily changes in photoperiod. In some mammalian species, both circadian (~24 h) and ultradian (<24 h) activity patterns are observed and are strongly influenced by environmental stimuli (1). Evidence to date suggests that horses housed in stalled conditions display diurnal (daytime) activity rhythms (2). However, this contrasts with observations of feral horse populations where ultradian activity bouts throughout the 24-hr cycle have been suggested (3). The true endogenous nature of a circadian rhythm can only be confirmed under constant conditions in the absence of time cues. This study determined the activity patterns of horses in their natural environment (Pasture) and under both a light/dark (LD) and constant dark (DD) stabled environment. Six mares of lightweight breed were fitted with Actiwatch-L (Respiricons, Bend, OR) monitors (for measurement of activity and light intensity) and were maintained for successive 48-h periods at pasture, in individual stalls within a lightproof barn under LD, and finally in DD. Actiwatch data were used to create ClockLab (Actimetrics, Evanston, IL) compatible files. ClockLab’s batch analysis function was used to compute average activity counts/min for each mare in each treatment interval. A bout analysis function was used to quantify the ultradian structure of activity data identifying distinct bouts of high activity. Initial examination of the actigraphs revealed distinct ultradian activity patterns with a mean of 9 bouts/day (S.D.±3.1). One-way repeated measures ANOVA was used to analyse; average counts/min, activity bouts/day, average bout length and percentage of activity counts/light phase (subjective day in DD) across the three treatments (Pasture, LD, DD). Results reveal significantly higher activity counts/min at pasture compared to LD and DD (p<0.001). Mares at pasture demonstrated reduced bouts/day compared to LD and DD (p<0.001) and increased bout length compared to DD (p<0.01). However, mares demonstrated a greater percentage of activity within the light phase in DD compared to pasture and LD (p<0.001). In addition, cosin analysis (4) of the time series data identified a significant 24-h component of the activity rhythms with significantly increased robustness (goodness of fit values) associated with DD (p<0.05). In summary, mares display activity patterns that are weakly circadian and predominantly ultradian in nature. It is proposed that the DD condition, investigated in horses for the first time, permits greater unmasking of endogenous circadian periodicities in the absence of environmental stimuli such as social cues. Elucidating the nature of activity rhythms in the horse will have implications for future studies investigating diurnal variations in performance parameters in the equine athlete.


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The maximal rate of lipids oxidation is strain dependant and correlated with performance in running mice
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In mice model, the most currently exercise performance criteria used in running is the maximal running speed (Vpeak) and is considered as an aerobic index (Hoydal et al., 2007). Furthermore links between performance and the lipid metabolism was not explored in mice, despite the fact that the maximal rate of lipid oxidation (Lipox max) has been reported to be a major performance factor and to be training sensitive in man (Brooks and Mercier, 1994, Jeukendrup and Wallis, 2005). Therefore, the purpose of this study is to test the hypothesis that the lipids metabolism and also the anaerobic metabolism, between VO2max and Vpeak are, as VO2max, consistent performance factors and could be highly heritable, explaining the high performance difference according the mice strains (Lightfoot et al., 2001).

Seven FVB (high performer strain) and 7 C57BL6 (low performer) ran an incremental exhaustive run on treadmill in a metabolic chamber until maximal speed (Vpeak) which was taken as the performance criteria. The respiratory gas exchange (RER) allowed determination of Lipox max and the associated speed (Vlipox).

We found that VO2 increased rapidly and significantly at the beginning of the exercise with increasing speed, but VO2 plateaued early until the end of the exercise (51.7 ± 3.4 vs 48.1 ± 3.2 ml.kg-0.75.min-1, in FVB and C57 p=0.07). Vpeak was significantly higher than vVO2max in FVB vs. C57 (167±25 vs. 135±44% of vVO2max, p = 0.011). The Vpeak (rI = 0.72, g2 = 0.56), Lipox max (rI = 0.53, g2 = 0.36) and the cross over speeds (rI = 0.83, g2 = 0.71) were highly heritable in contrast with VO2max (rI = 0.30, g2 = 0.18) and the Accumulated Oxygen Deficit (AOD) (rI=0.11 g2 = 0.05). However, the anaerobic metabolism, estimated with the AOD, was a consistent factor of performance. Furthermore Lipox max (r = 0.80, p = 0.0006) was highly related with the performance (Vpeak) while VO2max (r = 0.57, p = 0.04) and AOD (r = 0.56, p = 0.04) were moderately.

Thus the use of Vpeak for aerobic capacity assessment can not be performed independently of the strain and the anaerobic capacity is then a factor of performance eventhought AOD was not heritable. Indeed, at end exercise, RER was higher for the FVB strain, indicating a greater part of glucose oxidation, and a relative higher metabolism sollicitation for this strain. In contrast with human model, in both strain, the mice had a high Vlipox set between vVO2max and Vpeak. This means that the mice do not rely to the aerobic glycogen metabolism but rather on the lipids and the anaerobic metabolism (glycolysis and phosphagene) (Craig et al., 1995, Pederson et al., 2005). In conclusion, this study demonstrated, for the first time, that the lipid metabolism was not only a consistent performance factor but were also highly heritable, and must also be determined when evaluating aerobic performance in mice.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
We demonstrate that the endocannabinoid, AEA, prevents the Aβ1-40 –induced lysosomal membrane destabilisation, and the subsequent release of the lysosomal enzyme, cathepsin-L. Similarly, AEA prevents the Aβ1-40-evoked changes in LAMP1 and LAMP2 expression. Modification of the lysosomal branch of the apoptotic cascade by endocannabinoids could be a therapeutic strategy of relevance for AD.

An age-related decrease in T2 relaxation time in the cortex was observed with a significant difference between the values in young and aged rats (p<0.05, ANOVA, n=6). An age-related increase in the expression of MHC II was observed and analysis of staining intensity in cortex identified a significant increase in sections prepared from middle aged and aged rats, compared with young rats (p<0.05, ANOVA, n=4). A significant increase in CD68 immunoreactivity was observed in sections prepared from aged, compared with young, animals (p<0.05, ANOVA, n=4). These data reveal a positive correlation between T2 relaxation time and microglial activation in the cortex of aged rats and identify that some changes in cell surface markers of microglial activation are evident in middle age.

This work was supported by the Health Research Board Ireland.

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The corticostriatal system in dissociated cell culture

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The cortex provides a massive input to striatal neurons but some of its properties make it extremely difficult to study single synapses between cortical fibres and striatal cells. For instance, each striatal cell may receive in the order of 5,000 cortical inputs, from an almost identical number of cortical neurons. However, the neighbouring striatal cells are extremely unlikely to respond to the same set of neurons since the likelihood of sharing more than about 1% of the inputs is vanishingly small (Kincaid et al., 1998). We therefore have developed methods to culture cortical cells from green fluorescent protein expressing transgenic mice of the same developmental stage (E14.5 from QBM Cell Science, Ottawa, Canada). It is then possible to record from pairs of neurons in culture and to be sure that one is cortical and the other striatal. We have used conventional whole cell patch clamp recording methods in cultures (10-28 days in vitro) recorded in artificial media of composition (NaCl 136; KCl 5; MgCl 1; CaCl 2.5; Hepes buffer 10; Glucose 10, all mM) at 36°C. The glass micropipettes had a resistance of 6-12MΩ inter-


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-100 to +40 mV for 10 s before stepping to a +40 mV test pulse for 500ms. In 13 cells transfected with Kv2.1 only, the mean V1/2 of inactivation was –55 ± 3 mV compared to –30 ± 3 mV in the cells transfected with Kv2.2 (n=11). When native SMC were studied with the same protocol, the V1/2 of inactivation of the Kv current was –56 ± 3 mV (n=9).

These electrophysiological and pharmacological data suggest that Kv2.1 channels underlie the delayed rectifier Kv current in rabbit urethral SMC.


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ICa(TTX) is present in rat left ventricular myocytes but not in the Xenopus oocytes expressing hNaV1.5

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A slow inward current is often reported in the range of -40 mV in ventricular myocytes in the presence of low (or no) extracellular Na+ and normal extracellular Ca2+ and is variably attributed to ‘slip-mode conductance (SMC)’ of the phosphorylated Na+ channels and to a subtype of TTX-sensitive Ca2+ channel [Na(null) or ICa(TTX)] (1,2). It is also reported that ICa(TTX) is expressed specifically in the ventricular myocytes away from the region of chronic infarct and not in those from normal heart (3) or that it may be due to L-type Ca2+ current overlapping Na+ current (4). The present study was carried out on isolated rat left ventricular myocytes (rLVM) obtained from the myocardium away from the infarct and on Xenopus oocytes expressing hNaV1.5 to assess, (i) the status of ICa(TTX) in rLVM, and (ii) whether SMC contributes to ICa(TTX). The ventricular myocytes were isolated from the normal (n=10), sham (n=14), and infarcted (n=19) rat heart by collagenase dispersion technique (5). Whole-cell patch-clamp experiments on rLVM from all the three groups showed an inward current at depolarizing steps of –40 mV from h.p. of -80 mV or –130 mV in the presence of 0 [Na]o or 1 mM [Ca]o, or 5 mM [Na]o and 1 mM [Ca]o. TTX (≥ 10 μM) added to the bathing solution blocked this inward current. Two electrode voltage clamp experiments on Xenopus oocytes expressing hNaV1.5 (n=9), with or without beta1 subunit, did not show any inward current in the range of ~60 mV to +40 mV when bathed with 0 [Na]o and 0.1-65 mM [Ca]o containing solution. Injection of Sp-cAMP, an activator of PKA, into the oocytes expressing hNaV1.5 also did not facilitate Ca2+ inward current through these channels. These observations indicate that ICa(TTX) is present in rLVM from both the normal heart and the heart with chronic infarction but is not observable in Xenopus oocytes expressing hNaV1.5.


I am thankful to Prof N.S. Dhalla (Winnipeg, Canada)and Prof. W. Schreibmayer (Graz, Austria) for providing me facilities for experiments on rats and Xenopus oocytes respectively

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Electrophysiology of Rabbit Cultured Synoviocytes

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The secretion of hyaluronan into the synovial cavity is crucial for normal joint function, both for tissue lubrication and synovial fluid conservation, but the underlying cellular mechanisms are poorly understood. Essentially nothing is known about synoviocyte electrophysiology and the purpose of this study was to use synoviocytes cultured from the rabbit knee joint to investigate the passive electrical properties of these cells and to establish which ionic currents are expressed. The synovium was microdissected from healthy New Zealand white rabbits immediately after they had been killed by lethal injection of pentobarbitone. The synovial lining from lateral and medial sides of the suprapatellar zone was microdissected from the underlying areolar subsynovium and chopped into 0.5-1mm3 pieces in order to commence a primary explant culture. This was maintained until confluency was reached, whereupon tissue fragments were removed. The synovial lining cells were cultured and reseeded and cells were used for experimental purposes upon reaching passage six.

Resting membrane potential, measured in zero current clamp mode using K+-filled electrodes immediately after establishing a gigaohm seal, ranged from ~30mV to ~66mV with a mean of –45 ± 8.6mV (SD, n = 40). Input resistance was measured in 33 cells in voltage clamp mode by measuring the passive current responses to a series of hyperpolarizing and depolarizing voltage steps from a holding potential of ~60mV. This ranged from 0.54 to 2.6 GΩ with a mean of 1.28 ± 0.57 (SD). Cell capacitance averaged 97.97 ± 5.39pF (SD, n = 30) as calculated by integrating the capacitative current evoked by small hyperpolarizing and depolarizing steps and dividing by the amplitude of the voltage change. When cells were voltage clamped at ~60 mV and stepped from ~80 to ~50mV in 10mV steps, a family of outward currents was evoked which showed clear outward rectification. These currents were reduced from a peak of 1625 ± 238pA at +50mV to 776 ± 127pA (SEM n=9) in the presence of 1mM TEA. The more selective Kv1 blocker margatoxin (100nM) reduced the peak outward current from 1482 ± 329
to $355 \pm 93\text{pA}$, n=8). Alpha-dendrotoxin (100nM) reduced the peak outward current from $1189 \pm 182\text{pA}$ to $312 \pm 25\text{pA}$ (n=5) while 50nM kappa-dendrotoxin reduced peak outward current from $2450 \pm 623\text{pA}$ to $162 \pm 27\text{pA}$ (n=4). In conclusion we have demonstrated that isolated cultured synoviocytes from the rabbit express a current that has all the properties of a delayed rectifier potassium current and the pharmacology indicates that this is of the Kv1.1 subtype. This current undoubtedly has a role in regulating cell membrane potential and its modulation is likely to control calcium influx and thus hyaluronan secretion (Ingram et al, 2008).


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**C16**

**Contribution of Kinetically Distinct HCN Isoforms to Murine Pacemaking: A Computational Study**

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**Aim:** Pacemaking in the sino-atrial node (SAN) is modulated by hyperpolarization-activated cyclic nucleotide (HCN) gated channel, If. Several isoforms of the HCN family have been identified in murine SAN which show vast differences in time kinetics. This computer modelling study evaluates the functional role of individual HCN isoforms on murine pacemaker activity.

**Methods:** Murine SAN If consists of HCN1, HCN2 and HCN4 [1]. All isoforms have similar steady state activation [2] with a half-activation of approximately -63.7 mV. The molecular expression pattern of HCN1, HCN2 and HCN4 in SAN cells has been shown to be 5:25:70 respectively [3]. The primary biophysical differences in the individual isoform function have been identified to be a quantitative difference in time kinetics and cAMP sensitivities [4]. Based on these biophysical properties, Hodgkin-Huxley models for each isoformal component of If were developed and incorporated into our recently developed murine SAN mathematical cell model. The contributions of individual isoforms to model action potential (AP) were evaluated by blocking the isoformal channels individually. Model responses were defined as AP features including minimum diastolic potential (MDP), AP duration at 50% (APD50), CL and diastolic depolarisation rate (DDR) [5]. Blocking of total If and Control cases were also simulated.

**Results:** Blocking of If or its isoforms had marked effects on CL and DDR with other AP features largely unaffected. The results are summarised in the table. Blocking total If increased the overshoot potential (OS), AP duration at 50% (APD50) and at 90% repolarisation (APD90), cycle length (CL) and diastolic depolarisation rate (DDR) [5]. Blocking of total If and Control cases were also simulated.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>MDP (mV)</th>
<th>OS (mV)</th>
<th>APD50 (ms)</th>
<th>APD90 (ms)</th>
<th>CL (ms)</th>
<th>DDR (ms/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35.77</td>
<td>13.68</td>
<td>50.07</td>
<td>70.12</td>
<td>191.71</td>
<td>6.28</td>
</tr>
<tr>
<td>If Control</td>
<td>-64.36</td>
<td>56.95</td>
<td>57.54</td>
<td>70.87</td>
<td>218.24</td>
<td>6.23</td>
</tr>
<tr>
<td>HCN1 Control</td>
<td>-57.85</td>
<td>54.71</td>
<td>56.64</td>
<td>70.47</td>
<td>195.20</td>
<td>6.24</td>
</tr>
<tr>
<td>HCN2 Control</td>
<td>-57.45</td>
<td>52.78</td>
<td>59.02</td>
<td>75.84</td>
<td>231.98</td>
<td>6.33</td>
</tr>
<tr>
<td>HCN3 Control</td>
<td>-56.47</td>
<td>54.73</td>
<td>55.05</td>
<td>70.33</td>
<td>166.65</td>
<td>6.24</td>
</tr>
</tbody>
</table>

**Conclusions:** If regulates murine SAN pacemaking during the slow diastolic depolarisation. Although the slowly activating HCN4 is the most extensively expressed, HCN2 is the isoform majorly contributing to murine pacemaking electrical activity. On the other hand, the contribution of HCN1 is small due to its small conductance. The HCN isoforms have different functional impacts on murine pacemaking.

Summary of model responses upon blocking of If and its individual HCN isoforms.

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**C17**

**Role of calcium-dependent and independent reactive oxygen species generation in pancreatic acinar cell death**

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The relationship between cytosolic calcium [Ca\(^{2+}\)] and mitochondrial function plays an important role in cellular physiology and pathology, yet is currently poorly defined. We have previously shown that oxidative stress promotes apoptotic pancreatic acinar cell death (Criddle et al., 2006) and have now investigated the influence of [Ca\(^{2+}\)] on ROS generation, mitochondrial function and cell fate in isolated murine pancreatic acinar cells.

Isolated murine pancreatic acinar cells were exposed to taurinehydrochloric acid sulphate (TLC-S; 500\muM) or the quinone oxidant menadione (MEN; 30\muM) as a Ca\(^{2+}\) chelator.

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TLC-S caused a large, sustained rise of [Ca\textsuperscript{2+}]c above basal levels and a NAC-sensitive generation of ROS (n=16). Mitochondrial function was inhibited by application of TLC-S; a large, sustained increase of [Ca\textsuperscript{2+}]mt was observed with a concomitant decrease of NAD(P)H (n=18). Both the sustained rises of [Ca\textsuperscript{2+}]c and ROS were abolished by BAPTA-AM pre-treatment, indicating the Ca\textsuperscript{2+}-dependency of TLC-S-induced ROS generation (n=14). In contrast, MEN-induced elevation of ROS was unaffected by BAPTA pre-treatment, but blocked by NAC (n=16), in accord with a Ca\textsuperscript{2+}-independent redox cycle mechanism. When cells were treated with TLC-S or MEN for 30 minutes, apoptotic cell death was increased 3.2-fold and 5.5 fold from control levels (n=1132, 832), respectively, in a manner that was abolished by treatment with NAC, indicating a ROS-dependency of the actions of both compounds.

Reactive oxygen species, generated by distinct Ca\textsuperscript{2+}-dependent and independent mechanisms, are important mediators of apoptosis in the pancreatic acinar cell. Criddle DN et al. (2006) J. Biol. Chem. 281, S2.

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C19

Localised mitochondrial depolarisation evoked by subcellular release of a membrane-permeant, chemically-caged uncoupler in freshly isolated smooth muscle cells

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Numerous cellular functions are influenced by mitochondrial activity, including the organelle’s ability to produce ATP, accumulate Ca\textsuperscript{2+} and produce reactive oxygen species. Mitochondria are distributed throughout the cytosol and, as such, these small organelles (~1-5 μm) may display particular influence over processes in their immediate neighbourhood, e.g. on nearby Ca\textsuperscript{2+}-sensitive channels. Indeed, mitochondrial activity within sub-cellular microdomains has been implicated in the regulation of channels both on the plasma membrane and the internal calcium stores, the endoplasmic- or sarcoplasmic-reticulum (ER or SR). We have shown previously that mitochondrial uptake of Ca\textsuperscript{2+} promotes the activity of inositol-1,4,5-trisphosphate-sensitive Ca\textsuperscript{2+} release channels (IP\textsubscript{3}R) on the SR in smooth muscle cells. This suggests a localised regulation of IP\textsubscript{3}R by mitochondria located close to the channel, yet it has been difficult to study how mitochondria, acting in restricted regions, control cell-wide activity.

We have developed a mitochondrially targeted UV-activated caged dinitrophenol. UV irradiation in vitro caused it to undergo photolysis, a product of which displayed the absorbance properties of free dinitrophenol (DNP, an uncoupler of the mitochondrial membrane potential, ΔΨ\textsubscript{m}, from ATP synthesis). Smooth muscle cells freshly isolated from guinea-pig colon were loaded with mitochondrially targeted UV-activated caged dinitrophenol (200 nM) plus the ΔΨ\textsubscript{m}-sensitive dye tetramethylrhodamine ethyl ester (TMRE, 10 nM) for 30 min. High-speed epifluorescent imaging detected localised decreases in TMRE fluorescence specifically in regions (as small as 5 μm diameter) exposed to UV laser light for periods <1s. The regions of decreased TMRE fluorescence only spread slightly over the course of an experiment (~20 min), i.e. the uncoupling remained localised to the site of photolysis. No changes in TMRE fluorescence were observed to UV light in the absence of the caged uncoupler. These results indicate that, in freshly isolated colonic smooth muscle cells, mitochondria are individual, electrically-independent units that do not move throughout the cell. The caged dinitrophenol has the benefits of being membrane permeable (hence alleviating the requirement for micro-injection or patch-clamping) and targeted to mitochondria, thus refining the capability to rapidly depolarise ΔΨ\textsubscript{m} in very small sub-cellular regions and determine the role of those mitochondria in cellular signalling processes.

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C20

Calcium elevation in mitochondria is the main Ca\textsuperscript{2+} requirement for mPTP opening

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We have investigated in detail the role of intra-organelle Ca\textsuperscript{2+} content during induction of apoptosis by the oxidant menadione while changing and monitoring Ca\textsuperscript{2+} load of ER, mitochondria and acidic organelles. Menadione causes production of reactive oxygen species, induction of oxidative stress and subsequently apoptosis. In both pancreatic acinar and pancreatic tumour AR42J cells, menadione was found to induce repetitive cytotoxic Ca\textsuperscript{2+} responses due to release of Ca\textsuperscript{2+} from both ER and acidic stores. Ca\textsuperscript{2+} responses to menadione were accompanied by elevation of Ca\textsuperscript{2+} in mitochondria, mitochondrial depolarisation and mPTP opening. Emptying of both the ER and acidic Ca\textsuperscript{2+} stores did not necessarily prevent menadione-induced apoptosis. High mitochondrial Ca\textsuperscript{2+} at the time of menadione application was the major factor determining cell fate. However, if mitochondria were prevented from loading with Ca\textsuperscript{2+}, then apoptosis did not occur irrespectively of other Ca\textsuperscript{2+} stores' content. These results were confirmed by ratiometric measurements of intra-mitochondrial Ca\textsuperscript{2+} with pericam. We conclude that elevated Ca\textsuperscript{2+} in mitochondria is the crucial factor in determining whether cells undergo oxidative stress-induced apoptosis.


Possible Link Between Estrogen Levels, Estrogen Receptors and the Tumor Suppressor Gene p53 During Gestation in Rat Placenta

A. Elfarra, M. Al-Bader, S. Mohan and L. Jacob

Introduction: Estrogen is essential for initiation and maintenance of pregnancy in the rat. However, high levels of estrogens during pregnancy may have a specific growth-retarding effect on the placenta (1). Consequently, there has to be a control mechanism that enables the placenta to proliferate regardless of the otherwise high inhibitory levels of circulating estradiol. This may partially be mediated through a decrease in estrogen receptor (ER), which has been reported before (2), and a parallel decrease in p53 expression as a link between the expression of these two genes has been reported (3). Therefore, we hypothesize that a decrease in placental ER protein expression correlates with a decrease in placential p53 expression during pregnancy. This study was designed to investigate whether the placential expression of p53 changes during pregnancy in rat.

Methodology: Pregnant Sprague-Dawley dams were stunned and killed by cervical dislocation at 16, 19 and 21 days gestation (dg). Placental tissues from each litter were collected (four pregnancies at each gestational age [n = 4] and three to four placential tissues per pregnancy were pooled). Gene and protein expression of p53 were studied using real-time PCR (RetT-PCR) and Western blotting and immunodetection methodologies. Taqman probes specific for p53 and for two housekeeping genes, 18S and beta-glucuronidase (BGLUC) were used. For p53 protein expression, tissues were homogenized to obtain nuclear and cytosolic fractions (verification of nuclear and cytoplasmic fractions was done using Histone H4 antibody and glucose-6-phosphate dehydrogenase (G6PD) assay, respectively. P53 and actin were analyzed in these fractions in addition to the total homogenate fraction.

Results: Placenta weight increased significantly between 16 dg and 19 dg (p=0.001), and 16 dg and 21 dg (p=0.01), while there was no significant increase between 19 and 21 dg. Both 18S and BGLU were found to be suitable housekeeping genes as their expression was not changed with gestation. The expression of p53 decreased significantly by 19 dg (p<0.05) and increased by 21 dg (p<0.05). As for protein expression, p53 was detected in both homogenate and nuclear fractions with very faint bands in the cytosolic fraction at 16 dg only. There was a trend for p53 protein to decrease by 19 dg in both homogenate and nuclear fractions, however, this was not statistically significant.

Conclusion: According to our results, we found that the expression of p53, at least at the mRNA level, decreased at 19 dg allowing the placenta to increase in weight while at 21 dg the p53 expression increased suppressing placental growth. This was reflected in the weights that we obtained.

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3% vs. 36 ± 7%; p<0.05). Similarly, incubation of omental vessels from normal pregnant women in plasma from women with PE resulted in impaired relaxation to BK (45 ± 7% vs. 86 ± 3%; p<0.01). This impaired relaxation was reversed by co-incubation of the PET plasma with PJ34 (85 ± 2% vs. 45 ± 7%; p<0.05). In contrast, treatment with DR2313 did not significantly affect the impaired vasorelaxation induced following overnight incubation with PET plasma. The present study demonstrates that plasma-derived factors appear to mediate the vascular dysfunction documented in both the RUPP rat model of PE and the clinical condition. Furthermore, this work demonstrates a role for the overactivity of PARP in mediating this vascular dysfunction. Sankaralingam S. et al. (2006) Acta Obstet. Gynecol. 81-86P. Soriano FG. et al. (2001) J. Mol. Med. 437-442P. Crews JK et al. (2000) Hypertension 367-372P.

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C23

4-Aminopyridine sensitivity in human placental chorionic plate arteries
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Voltage gated potassium channels (Kv) alter vascular tone by effecting changes in membrane potential and regulating Ca2+ influx. Several Kv channel subtypes are expressed in the fetoplacental vasculature, but the functionally important Kv channels have not been identified [1, 2]. Kv channel subtypes have different sensitivities to block by 4-aminopyridine (4-AP) and we showed previously that the basal tone of chorionic plate arteries was significantly increased to block by 4-aminopyridine (4-AP) and we showed previously that the basal tone of chorionic plate arteries was significantly increased by 1μM 4-AP [2]. Here, we test the hypothesis that different Kv channel subtypes contribute to regulation of fetoplacental vascular tone by determining the sensitivity of chorionic plate artery (CPA) constriction to 4-AP, using pressure myography. Term placentas (N=8) were obtained post-delivery (vaginal or Caesarean section) from uncomplicated pregnancies. Biopsies were placed into ice-cold HCO3--buffered physiologic salt solution (PSS). CPAs were mounted on a pressure myograph, equilibrated for 30 minutes at an intraluminal pressure of 20mmHg (to reproduce pressure in vivo) with intraluminal flow (20μl/min; 37°C, 5%O2/5%CO2). Contraction was assessed with 120mM potassium solution (KPSS) and U46619 (10^-10-2x10^-6M) added to the bath. Post wash, a concentration response curve to 4-AP (1-5000μM) was then assessed in the continued presence of 5000μM 4-AP. Baseline arterial diameters were 272±24μM. KPSS reduced baseline CPA diameters by 39±8%. 4-AP reduced baseline diameter of CPAs at concentrations above 5μM (Figure 1; *P<0.05 Wilcoxon Signed Rank Test). 4-AP did not affect maximum U46619 constriction at 10^-6M (data not shown).

In conclusion, 4-AP-induced constriction of chorionic plate arteries at concentrations above 5μM, confirming and extending our previous observations that Kv channels contribute to maintenance of chorionic plate artery tone [2, 3]. Future studies with more specific blockers could address the contribution of 4-AP-sensitive Kv channel subtypes to regulating fetoplacental vascular resistance and blood flow in normal and compromised pregnancies.

Figure 1: Scatter plot represents % change in baseline diameter of chorionic plate arteries in response to cumulative concentrations of 4-AP (N=8; line at median).

Wareing M et al. (2006). Biol Reprod, 75, 518-23

This work is supported by Action Medical Research and Tommy’s

C24

Prenatal vs. postnatal influences on fat and lean mass in sheep
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It has been suggested that poor prenatal growth may predispose to increased adiposity later in life. In this retrospective analysis, a large cohort of sheep was utilised to interrogate the a priori hypothesis that intrauterine growth restriction leads to fatter adolescent offspring and this effect is modified by their subsequent postnatal growth rate. Data for five flocks of pedigree Suffolk sheep (n=13,630) were available in which multiple variables were recorded as standard (for inclusion in the Signet pedigree database, MLC) including birth weight, weaning weight at 8 weeks of age and weight and body composition at 20 weeks of age. Body composition was assessed in two ways: 1) by ultrasound at the 11th rib by a skilled operator (n=7,671) and 2) by computed tomography (CT, n=220). Postnatal growth was assessed in absolute (g/day-1) or relative (g/day-1, kg-1 birth weight) terms. Data are presented as estimated marginal means ± S.E.M. and were analysed by General Linear Mixed Models using Genstat v11. P<0.05 was accepted as indicating a statistically significant effect. Birth weight or postnatal growth were modelled as continuous or categorical variables; categories conforming to 1kg increments in birth weight from <3kg to ≥8kg. In this cohort of sheep there was a 5-fold natural variation in birth weight (Mean 5.13, range 2-10). Lambs born relatively
small (IUGR, -25D. or <3kgs) tended to exhibit postnatal growth acceleration during the first 8 weeks of life only. Ultrasound determined fat mass correlated well with CT determined fat mass ($r=0.72, P<0.0001$). There was a strong positive relationship between birth weight and/or relative postnatal growth and fat mass at 20 weeks of age (Figure 1). This effect was apparent and similar in both sexes. Adjustment for random effects in the model such as flock and shared genes from either parent (ewe and ram) did not materially affect the conclusions. In a large cohort of sheep, natural variations in birth weight as a result of unknown environmental aetiologies had a pronounced effect on body composition in the young, adolescent offspring. Being born of relatively high birth weight is strongly linked to increased fat mass later in life. Similarly, a tendency to exhibit relative postnatal growth acceleration during early life is also, independently, associated with increased fat mass later in life. The data recapitulate observations in human epidemiological studies suggesting that the early developmental environment is key to determining later body composition; observing similar effects in species with widely different metabolic function and life history gives biological plausibility to programming of later body composition by early environment.

The authors wish to acknowledge the Meat and Livestock Commission and the School of Veterinary Medicine and Science, University of Nottingham.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

ECG alterations and microtubule proliferation following monocrotaline induced right ventricular heart failure in rats
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Microtubules are load bearing and load-modulated components of the cardiac cytoskeleton whose proliferation may impede contractile function in animal and human right heart failure (Cooper, 2006). We wished to test whether there was a proliferation of microtubules in a non-invasive model of pulmonary hypertension induced by monocrotaline (MCT). Male Wistar rats were injected with 60 mg/kg of MCT or an equivalent volume of saline (CON). Telemetry was used to measure the electrocardiogram (ECG) in vivo, in conscious, unrestrained animals on a weekly basis until day 21 then daily. Surgical implantation of telemetry devices was performed using isoflurane anaesthesia by inhalation (up to 5%) and maintained with current UK legislation.

MCT treated rats had increased heart weight:body weight (CON 4.1 ± 0.2 vs. MCT 5.5 ± 0.2 mg/g (mean ± SEM)) and RV weight:LV weight (CON 0.38 ± 0.03 vs. MCT 0.68 ± 0.06 g/g), consistent with the development of right ventricular hypertrophy/failure (n=6, CON and MCT t-test P=<0.001). Measurement of ECG parameters using radiotelemetry indicated modification of T-parameters in MCT treated animals e.g. a prolonged QT interval (CON 49.7 ± 2.0 vs. MCT 76.2 ± 2.5 ms, P<0.001, t-test) and time from the peak to the end of the T-wave (Tpe, CON 25 ± 1.8 vs. MCT 33.1 ± 1.7 ms, P<0.007, t-test) (CON n=6, MCT n=7). In MCT treated rats there was an increase in the polymerised fraction of β-tubulin in the RV compared to control rats (CON 0.55 ± 0.02 vs. MCT 0.6 ± 0.01 a.u, P=0.003 2-WAY ANOVA, n=6 each group, 3 replicates). There was a concomitant increase the expression of mRNA encoding α-tubulin in the RV of MCT rats (CON 0.6 ± 0.1 n=7 vs. MCT 17.3 ± 4.2 a.u n=9, P<0.001, 2-WAY ANOVA).

We conclude that MCT treatment results in; ECG changes consistent with prolongation (QT) and increased global dispersion (Tpe) of the action potential; increased microtubule proliferation as evidenced by the increase in α- and β-tubulin. These changes may be relevant to alterations seen in the mechanical and electrical activity of right heart failure.


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Remodelling of the extracellular matrix in the rat sinoatrial node in congestive heart failure
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The extracellular matrix (ECM) includes structural proteins (e.g. collagen types 1 and 3 and elastin), adhesive proteins (e.g. fibronectin), and matrix metalloproteinases (MMPs; responsible for collagen degradation). MMP activity is regulated by endogenous inhibitors (TIMPs). Four TIMP isoforms (TIMP1-4) are
known to be present in the heart. In the heart, the ECM is responsible for connecting myocytes, aligning contractile elements, transmitting force and preventing myocardial rupture. Although it is well known that there is a remodelling of the ECM in the working myocardium in heart failure (Graham et al., 2008), it is not known whether there is a similar remodelling in the sinoatrial node (SAN; pacemaker of the heart). The aim of the present study was to investigate the effect of heart failure on the ECM and the factors that control the ECM (e.g. transforming growth factor β1, TGFβ1, and tumour necrosis factor α, TNFα). Sprague-Dawley rats, aged five weeks, received a single subcutaneous injection of monocrotaline (60 mg/kg), which induced inflammation of the pulmonary artery and pulmonary arterial hypertension. After three weeks, the experiments were ended after the animals developed congestive heart failure as confirmed by echocardiography. All experiments were conducted in accordance with the Japanese Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973). Quantitative PCR was used to measure the expression of transcripts in the SAN (and also the right atrium, RA, and right ventricle, RV).

In the heart failure rats (as compared to control rats), mRNAs for collagen type 1 (constitutes 80% of total collagen) and elastin were significantly increased in the SAN, RA and RV; collagen type 3α1 mRNA was significantly increased, but only in the RA and RV. Fibronectin mRNA was significantly increased in all three tissues. MMP2 mRNA was significantly increased in the RA only. TIMP-1 was significantly increased in all three tissues; TIMP2 did not show any changes; TIMP4 was significantly decreased in the SAN and RV. Fibroblasts synthesise collagen, and vimentin mRNA (a marker for fibroblasts) was significantly increased in the SAN and RV. While some of this change occurs naturally with age, it is significantly accelerated in the SHR. Both of these models are significant for strain and strain-age interaction. The layer width in terms of myocyte diameters shows a significant increase at 18 and 24 months in the SHR (significant for strain, age, and strain-age interaction).

It is clear that significant morphologic changes occur in the tissue structure of the SHR around 12 months with increasing collagen and remodelling of the laminar organisation. High-resolution analysis of the change in collagen at this time-point indicates that the increase is due to both scarring and increasing endomysial collagen. While some of this change occurs naturally with age, it is significantly accelerated in the SHR.

**Age-Related Changes in Left-Ventricular Structure in the Spontaneously Hypertensive Rat**

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High-resolution three-dimensional images of myocytes and collagen have been acquired across the left ventricular wall in ten normal Wistar-Kyoto (WKY) and thirteen spontaneously hypertensive (SHR) rat hearts using extended-volume confocal microscopy,1 with at least 2 hearts of each strain imaged at each age endpoint of 3, 12, 18 and 24 months. Tissue blocks were prepared as described previously2 and imaged at 1 μm resolution. The resulting 3-D image volumes were approximately 4 mm × 1 mm × 0.3 mm with isometric voxels of size (1 μm)³. A higher resolution image volume (560 μm × 560 μm × 330 μm, (0.4 μm)³ voxel size) was also acquired from the mid-wall of one heart of each strain at 12 months of age. Collagen volume fractions were computed on each block using a modified Top-hat filter that identifies regions that are bright relative to the local background. Specific myocardial structural components (myocytes, blood vessels, interlaminar spaces and different collagen structures (endomysial, perimysial, perivascular, scar)) were segmented in the midwall of each image volume by digitally tracing structural boundaries. Laminar width was measured along lines perpendicular to the myolaminae in the midwall region at 200 μm intervals. To identify laminar remodelling independent of changes in cell size, the laminar widths were normalised by the average myocyte diameter in each block.

Results are presented in Figure 1, where the collagen fractions (A) and layer widths (B) are expressed as group mean ± SEM. The myocyte areas (C) are expressed as box plots (first, median and third quartiles, with whiskers at 10% and 90%) to avoid making assumptions about the underlying statistical distribution. Subsequent analysis was performed using a 2-factor ANOVA with statistical significance based on a value of P ≤ 0.05. Collagen fraction increases with age from a similar baseline in both strains, but is more pronounced in the SHR. Myocyte cross-sectional area increases with age in the SHR, but not in the WKY. Both of these measures are significant for strain and strain-age interaction. The layer width in terms of myocyte diameters shows a significant increase at 18 and 24 months in the SHR (significant for strain, age, and strain-age interaction). It is clear that significant morphologic changes occur in the tissue structure of the SHR around 12 months with increasing collagen and remodelling of the laminar organisation. High-resolution analysis of the change in collagen at this time-point indicates that the increase is due to both scarring and increasing endomysial collagen. While some of this change occurs naturally with age, it is significantly accelerated in the SHR.

**Figure 1. Comparison of left-ventricular tissue structure in SHR (dark, filled) and WKY (white, open) hearts. (A) Collagen fraction. (B) Layer width, measured as number of myocyte diameters. (C) Myocyte cross-sectional area.**
Ischaemic Preconditioning In Hearts From Apolipoprotein E Knockout Mice Fed High Fat Diet

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Exposing the heart to brief periods of ischaemia followed by reperfusion (ischaemic preconditioning) is a powerful tool for protecting the heart from further ischaemic insults. Understanding the mechanisms involved in this phenomenon is vital for the translation of preconditioning techniques into clinical applications. Many preconditioning studies utilize healthy heart models to evaluate the effectiveness and mechanisms of preconditioning, however it is essential to assess the effects of preconditioning protocols on the compromised or diseased heart in order to fully appreciate the potential of preconditioning to improve outcome of patients with coronary disease. When fed a high fat diet for 24 weeks, male apolipoprotein E knockout mice (apoE −/−) develop atherosclerotic lesions in the coronary arteries and show coronary artery disease similar to that seen in humans. Previous work [1] has shown that the hearts from these mice are resistant to ischaemic insult compared to healthy apoE −/− hearts from mice fed a standard chow diet. The aim of this study is to evaluate the ability of the diseased apoE −/− heart to be protected by ischaemic preconditioning techniques. Hearts from 8 month old male apoE −/− mice fed a high fat diet for 24 weeks were excised and perfused in Langendorff mode with Krebs-Henseleit buffer. Following a 20 minute stabilization period, hearts were either subjected to a preconditioning protocol of 2 cycles of 5 min ischaemia and 5 min reperfusion (ischaemic preconditioned, IP), or were continually perfused for 20 min (control). Hearts were subjected to 60 minutes global ischaemia followed by 60 minutes reperfusion. Functional recovery (left ventricular developed pressure, LVDP) was assessed and creatine kinase release upon reperfusion was measured as an indicator of myocardial injury. There was no significant difference in functional recovery between control and IP hearts after 60 minutes reperfusion (% LVDP 58.9 ± 8.6 % for control vs. 64.5 ± 8.3 % for IP hearts, data are mean ± S.E. for n=6 hearts in each group, p=0.65 unpaired t-test). There was also no difference in myocardial injury as determined by creatine kinase release into the coronary effluent upon reperfusion. The results presented here indicate that apoE −/− hearts with coronary artery disease are resistant to further cardioprotective effects of ischaemic preconditioning. These data suggest that these diseased hearts may require optimal preconditioning protocols or that they could be chronically preconditioned to resist ischaemic insult.

Chronic ENaC Blockade Rescues Na+ Induced High Blood Pressure in 11β-Hydroxysteroid Dehydrogenase Type 2 Heterozygote Mice

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11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) confers mineralocorticoid specificity to the mineralocorticoid receptor (MR) by inactivating glucocorticoids. Mutations in HSD11B2 cause the rare hypertensive disorder of Apparent Mineralocorticoid Excess (AME) and polymorphisms in the general population have been linked to Na+ sensitive hypertension [1]. We have previously shown that a high Na+ diet increases blood pressure (BP) in hsd11b2 heterozygote (hsd11b2+/-) mice [2]. Here we have investigated the role of the epithelial sodium channel (ENaC) in this response. Male hsd11b2+/- (n=6) & wild-type (hsd11b2+/+; n=6) mice were housed in metabolic cages and fed control diet (0.25% Na+) for 3 days before mini-pumps delivering the potent ENaC inhibitor benzamil (0.7 μg/day/g) were implanted under isoflurane anaesthesia (2% with O2 by inhalation). After recovery, measurements were made for 2 days before high Na+ diet (2.5% Na+) was given: Na+ excretion was measured for a further 7 days, after which mice were moved to normal cages for a further 2 weeks. Mice were then anaesthetized (Inactin, 100mg/kg IP) for measurement of arterial BP. Daily sodium excretion was averaged over the baseline and cumulative sodium balance calculated for the first 7 days on 2.5% Na diet. Data are mean ± SE; statistical comparisons were made using either t-test or ANOVA, as appropriate.

Baseline daily Na+ excretion was similar in hsd11b2+/- and hsd11b2+/+ mice on 0.25% Na diet (11.3±1.7 vs 12.6±2 μmol/24h/g). Benzamil administration caused a significant natriuresis in both groups (hsd11b2+/- = 16±0.7 and hsd11b2+/+ = 14.3±1.3 μmol/24h/g; P<0.01 vs baseline), confirming ENaC blockade. New analysis of our previous data shows the hsd11b2+/- mice in positive cumulative Na+ balance during the first week on a 2.5% Na+ diet (134.2±48 μmol; P<0.05) vs hsd11b2+/+ mice are in neutral balance. Benzamil treatment caused a negative sodium balance in both hsd11b2+/- and hsd11b2+/+ mice after 7 days on 2.5% Na+ diet (-122±10 μmol vs -111±41 μmol). Benzamil also prevented the Na+-induced rise in BP in hsd11b2+/- mice, which now had a similar BP to hsd11b2+/+ animals at the end of the high sodium regimen (83.5±5 vs 83.7±4 mmHg).

In summary, dietary Na+ loading induces a BP rise in mice with reduced 11β-HSD2 activity due to impaired renal sodium excretion. Chronic ENaC blockade prevents sodium accumulation in
repeated experiments. These data suggest ENaC may be upregulated in hsd11b2+/- mice due to inappropriate MR activation by glucocorticoids. The resulting unregulated increase in Na+ reabsorption could be contributing towards the increased BP observed in hsd11b2+/- mice on a high Na+ diet.


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Role of thiazide-sensitive sodium transporter in the development of angiotensin II dependent hypertension

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Inappropriate modulation of the renin angiotensin system (RAS) can lead to deranged blood pressure homeostasis. To identify important mechanisms underlying the development of angiotensin II dependent hypertension, we inserted the mouse Ren2 gene into the Fischer rat genome under the control of an inducible Cyp1a1 promoter (1). Induction of the transgene causes an increase in blood pressure, associated with a reduction in urinary sodium excretion (2). The aim of this investigation was to establish the contribution of renal sodium handling to the development of hypertension in the Cyp1a1 Ren2 transgenic rat. Expression of the Ren2 transgene was induced by daily gavage of indole 3 carbimol (I3C) at the dose of 100mg/kg Bwt for either 1 or 3 days (with a non-induced group as control), after which rats were anesthetized (Inactin, 120mg/kg, IP) and prepared for renal clearance experiments as described (2). A urine collection was made to establish baseline sodium excretion, renal blood flow and glomerular filtration rate. In the same rats, hydrochlorothiazide (2mg/Kg Bwt/h) was administered to inhibit NCC and a second urine collection made. At the end of the experiment, a 1ml blood sample was taken and rats killed by an overdose of Inactin. In a separate cohort of rats, HCTZ was chronically administered via osmotic minipump (4mg/d; 50% DMSO in saline), implanted under halothane anaesthesia. Baseline Systolic blood pressure (SBP) was obtained using tail cuff before any induction of the Ren2 transgene and then SBP was measured during the 3 days of induction by I3C daily gavage. Statistical comparisons were made by using one-way ANOVA (Tukey posthoc test).

BP in the non-induced Ren2 transgenic rats was (125±4.8 mmHg; n=6). Induction of Ren2 expression for either 1 day (146.9±2.2 mmHg; n=9) or 3 days (171.5±5 mmHg; n=6) days caused a significant increase in BP. Neither glomerular filtration rate nor renal blood flow was affected by transgene induction. Excretion of sodium was significantly lower in the 1 day induced group compared to the non-induced group (0.5±0.1 vs. 0.95±0.16 μmol/min, p<0.05). A further incremental drop in sodium excretion was found after three days of induction (0.36±0.09 vs. 0.95±0.16 μmol/min, p<0.05). Acute injection of HCTZ caused a significant natriuresis in all three groups of rats: the net effect of thiazide on sodium excretion was greater in the 1 day (10.8±1.5 μmol/min, NS) and 3 day (12.9±1.4 μmol/min, p<0.05) group than in the non-induced controls (7.2±0.84 μmol/min). Chronic administration of HCTZ significantly blunted the hypertensive response to transgene induction (145.1±4.1 mmHg; n=6).

These data suggest that thiazide-sensitive sodium transport contributes to the development of angiotensin II dependent hypertension.


I would like to thank Gillian Brooker for technical assistance.

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The impact of a low sodium diet on the renal functional responses to angiotension 1-7 (Ang1-7) in anaesthetised rats

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Angiotension converting enzyme isoform 2 (ACE2) converts angiotensin II (AngII) into Ang1-7 which has vasodilator and natriuretic and diuretic actions at the kidney. Burgeleva et al (2005) reported that the influence of Ang1-7 on renal haemodynamic and excretory function was enhanced in rat models of hypertension in which plasma renin activity was elevated. This study examined whether activation of the endogenous renin-angiotension system, by placing animals on a low sodium diet, would enhance the haemodynamic and excretory actions of Ang1-7 at the kidney.

Groups (n=5 or 6) of male Wister rats (220-330g) were maintained on a normal (0.3% Na+) or a low sodium (0.03% Na+) diet for 2 weeks. Following anaesthesia, 1ml chloralose/urethane (16.5/250 mg/ml) i.p., cannulae were placed in a femoral artery, to monitor mean arterial pressure (MAP) and vein, to allow infusion of saline (0.9% NaCl) containing inulin (2g/l) at 3ml/h. The left kidney was exposed via the flank, its ureter cannulated for urine collection, the renal artery cleared to fit an ultrasonic flow probe to measure renal blood flow (RBF), and a small cannula was inserted 4.0 to 4.5 mm into the cortex to lie at the cortico-medullary border and saline infused at 1 ml/h. After a 2h stabilisation period, four 20 min clearances were performed, two before and two 30 min after starting an infusion of Ang1-7 at a concentration of either 9x10-7 M or 3x10-6 M. Data, means±S.E.M., were deemed significant when P<0.05 (Wilcoxon Signed Rank Test).
Control values for MAP, glomerular filtration rate (GFR), RBF and urine flow (UV) were similar in all groups at 92±3 mmHg, 2.50±0.15 ml/min/kg, 4.5±0.5 ml/min and 21.9±2.1 μl/min/kg while fractional sodium excretion (FENa) was reduced (P<0.05) in the low sodium diet group. 0.75±0.14 versus 1.45±0.32%. Infusion of Ang1-7 at 9x10-7 and 3x10-6 M increased GFR by 20% and 28%, UV by 23% and 71% and FENa by 25% and 95%, respectively (all P<0.05) in rats on a normal sodium diet. In the rats on a low sodium diet, both doses of Ang1-7 increased (all P<0.05) GFR by 45% and 31%, UV by 200% and 225% and FENa of 350 and 320%, respectively, which were larger (P<0.05) than the responses in the rats on a normal sodium intake. Ang1-7 has vasodilator properties, as it raised GFR but this was not related to the dose of peptide infused or the dietary sodium status. By contrast, in rats on a normal sodium intake, the diuretic and natriuretic responses to Ang1-7 were dose related suggesting a tubular action. Interestingly, the excretory responses to Ang1-7 were markedly enhanced in rats on the low sodium diet and appeared maximal as a dose-response relationship was not evident. These findings demonstrate that manipulation of dietary sodium intake impacts on the ability of Ang1-7 to determine sodium excretion. Burgelova M et al. (2005). Kidney Int 67,1453-1461.

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High sodium diet modifies renal vascular responses in Wistar rats

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High salt intake is a known risk factor in the development of hypertension and related kidney damage. Changes in renal blood vessel function and morphology may also be directly related to consequences of high salt intake other than hypertension, such as hypernatremia, altered reactivity to vasoactive agents, oxidative stress or inflammatory process. The aim of our study was to examine the impact of high salt diet on the reactivity of intrarenal vasculature estimated on basis of changes in intrarenal regional perfusion. The experiments were approved by the First Warsaw Ethical Committee. In male rats maintained on standard (STD, 0.25% Na w/w) or high sodium diet (HS 4% Na w/w) for 3 weeks, systolic blood pressure (SBP, tail cuff method), plasma sodium (PNa) and osmolality (Posm) were measured repeatedly (HS, n=15; STD, n=6). After 3 weeks rats were anaesthetized (sodium thiopental, 100 mg/kg, i.p.) and 10-min infusions of acetylcholine (Ach, 5 and 10 μg/kg/h) and noradrenaline (NA, 10 and 30 μg/kg/h) were given via renal artery, in random order (HS, n=6; STD, n=4). Total renal blood flow (RBF, Transonic probe) and cortical (CBF), outer- and inner medullary (OMBF, IMBF) blood flows (laser-Doppler technique) were measured. The mean values of all the parameters were compared using ANOVA and Student t test for dependent or independent samples, as appropriate.

From 16th day of HS diet, SBP became higher than in STD rats (163±3 vs. 140±13 mmHg, P<0.05). PNa did not change significantly but Posm increased during high sodium intake. RBF, CBF and IMBF (but not OMBF) tended to increase in HS rats, which resulted in a reduction of the OMBF/IMBF ratio. In HS rats the increase in CBF in response to Ach (10 μg/kg/h) was significantly smaller than in STD rats (33±10 vs. 57±15 Perfusion Units, P<0.05). For outer and inner medulla, the vasodilator response to Ach (5 μg/kg/h) was visibly impaired compared to that in STD rats (15±10 vs. 40±18 PU for OMBF and 6±7 vs. 32±20 PU for IMBF, respectively).

In HS rats the dose dependence of the response to NA was visibly diminished for RBF and completely abolished for CBF. The decrease in CBF in response to the higher dose of NA was significantly less in HS than in STD rats (6±1 vs. 25±3 %, respectively, P<0.001). Our measurements of intrarenal regional perfusion confirm that changes in Na intake alter renal vascular reactivity to vasoactive agents. On high Na diet the ability of the renal cortex and medulla to vasodilate was distinctly impaired. The relative resistance to vasodilatation in the renal medulla, the region crucial for the control of arterial blood pressure, could contribute to the rise in the pressure observed in HS rats. On the other hand, high Na intake appeared also to limit the cortical vasoconstrictor response to NA, which could help maintain glomerular filtration and renal excretion.

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Contrast hydrotherapy following two modes of high-intensity cycle exercise: Blood lactate clearance and subsequent exercise performance

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Contrast hydrotherapy comprising immersion in alternating cold and warm water baths during recovery from exercise is postulated to facilitate lactate clearance by inducing fluctuations in muscle blood flow,. This study compared a 30 min passive non-immersed recovery (PASS) with two separate cold (8°C) to warm (40°C) water contrast ratios (CON 1:1 and CON 1:4) to examine blood lactate (BLa) clearance and subsequent performance following two different modes of high-intensity cycle exercise. Ethical approval was granted by the Health Sciences Research Ethics Committee, Trinity College Dublin.

Wingate test (WAnT) Protocol: Eight active, male volunteers (25±3 yr; 82±6 kg; 180±9 cm) completed three trials separated by 7 days. For each trial, subjects completed three 30-s Wingate tests. Post recovery, the Wingate tests (WAnT1, WAnT2, WAnT3) were repeated.

Repeated Intermittent Sprint (RIS) Protocol: Eight active, male volunteers (23±1 yr; 81±5 kg; 184±4 cm) completed three RIS
trials separated by 7 days. They completed an incremental test to establish Pmax. Intermittent 30-s workloads were calculated as 40% Pmax (recovery) and 120% Pmax (sprint). Post recovery, RIS protocol was repeated to failure.

Both groups completed the same randomised recovery interventions: CON 1:1 (2.5 min cold: 2.5 min warm); CON 1:4 (1 min cold: 4 min warm) and PASS (seated, non-immersed passive recovery). Data were analysed using a two-way repeated measures ANOVA with Holm-Sidak post hoc analysis (data presented as mean±SD).

BLa concentration for the WAnT group was significantly lower at 1 and 2.5 min of recovery in CON 1:1 and CON 1:4 compared with PASS (651±76W). For WAnT2, MP was significantly higher (P<0.05) in CON 1:1 (451±35W) and CON 1:4 (657±52W) compared with CON 1:1 and PASS (674±43, 628±57W). A similar finding was observed for WAnT3. For the RIS group, there were no significant differences in BLa between recovery interventions, however total work performed post-recovery was significantly higher (P<0.01) for CON 1:1 and CON 1:4 compared with PASS (92719±25765, 90503±25599, 74529±22486kJ respectively).

Despite no overall difference in BLa clearance, contrast hydrotherapy had a positive effect on exercise performance in both WAnT and RIS groups. Although BLa was lower during the initial phase of contrast water immersion for the WAnT group, this was not the case for RIS groups. Although BLa was lower during the initial phase of contrast water immersion for the WAnT group, this was not the case for RIS groups.

Aerobic fitness in humans is under system control by the angiotensin 2 pathway

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Blood supply is critical to fuel muscle contraction and limits exercise performance. The presence of a silenceer sequence in intron 16 (insertion, I) in the ACE (angiotensin converting enzyme) gene, which is the enzyme that converts the vasoconstrictor angiotensin (Ang) into its active form Ang2, has been associated with enhanced endurance capacity (Montgomery et al. 1998 and Scanavini et al. 2002). However, conflicting evidence exists on the association of ACE genotypes with regard to the presence (or absence) of the I sequence and human performance (Rankinen et al. 2000, Amir et al. 2007 and Zhao et al. 2003).

Adaptations of capillary supply lines in muscle contribute and explain the altered endurance phenotype in ACE genotypes, with an ACE insertion sequence, due to reduced ACE gene expression in muscle.

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Adaptations of capillary supply lines in muscle contribute and explain the altered endurance phenotype in ACE genotypes, with an ACE insertion sequence, due to reduced ACE gene expression in muscle.

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Maximal strength training of the plantar flexors: can changes in the brain be detected using functional magnetic resonance imaging?

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The maximal strength training protocol used in the present study has previously been shown to enhance muscular strength and neural drive. Results suggested that these neuronal changes were located at the supraspinal level. This hypothesis was tested
Maximal isometric plantar flexion training enhances strength at the whole brain level, a significant training effect was not detected. holding (p<0.05, corrected for multiple comparisons) at the 0.61; -0.53, respectively). These results suggest that 1) NIRS can reliably track changes in pre-frontal cortex HbO2 and muscle HHb during isokinetic muscular endurance fatigue testing; and 2) the peak pre-frontal cortex HbO2 that occurred before termination of the test, simultaneous with a plateau in muscle HHb, would suggest that central factors were implicated in the maximal knee extension muscular exercise fatigue in this study.

We would like to thank the volunteer subjects for their participation in this study. Financial Support: NSERC (JPN); University of Applied Science (MK-B).

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How many myosin heads act on a single actin filament at any instant in working muscle?

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If N is the number of myosin heads acting on a single actin filament in vertebrate striated muscle, working at any instant to produce tension, stoichiometry gives 150 ≥ N ≥ 1; the actual value of N is clearly an important parameter. Stiffness measurements have long been used to estimate N, though a warning was sounded [1] after X-ray measurements showed that a sizeable amount of the muscle compliance arose from the actin and myosin filaments. The usual approach compares the instantaneous stiffness measured during quick stretch
or quick release in rigor and contraction. Immediately after this response, though, the time scales of the tension response are very different: in rigor the tension persists for several tens of ms; in contraction it changes with a half time of a few ms. This stiffness approach makes three inherent assumptions: (i) in rigor N = 150 (here resisting extension rather than producing tension); (ii) the rigor stiffness is the same as when the interactions are producing tension; (iii) the filament compliance is the same in contraction as in rigor. However Kawai and Brandt showed that the stiffness of rigor (crayfish) single fibres could change by a factor of two or more depending on the approach to rigor [2]. Therefore the assumptions are probably over-optimistic, especially given the differences in ionic composition of the solutions used to induce the various states. For all these reasons we think it wise to accept the warning of Goldman and Huxley [1], that ‘stiffness cannot be used safely as a measure of cross-bridge attachment’, and we choose to discount numbers derived from stiffness measurements. It is a pity that there is no independent experimental method to estimate N.

We present a model (derived from other physiological and biochemical data) where the average time between impulses on an actin filament is one or two ms and that gives an inherent natural explanation of AV Hill’s force-velocity equation from first principles [3]. The model gives physical meaning to Hill’s constants ‘a’ and ‘b’ and it also implies that the size of the individual contractile impulse in situ between an actin filament and one myosin head must be approximately 140 pN.ms (an impulse has the dimensions of force x time). The impulses normally act sequentially in time along the filament [4]. In response to a fast perturbation, however, extra impulses may occur within the same time frame [5]. Our impulse model provides an alternative estimate of N; accumulating biochemical evidence supports our view that N is of the order of 1 in normal contraction and 8-9 in transient recovery after quick release. Goldman YE & Huxley AF (1994) Biophys J 67, 2131-2133


Elliott GF & Worthington CR (1997) Int J biological macromolecules 21, 231-275


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Effect of doubling the volume of resistance exercise on myofibrillar protein synthesis (MPS) and anabolic signaling in muscle of postabsorptive young and old men

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We have previously reported that older men show a blunted dose response relationship between increases in MPS and intensity of resistance exercise in the post absorptive state (Kumar et al 2009). We have now tested the hypothesis that doubling the volume of exercise would increase the MPS response in overnight fasted men, both young (n=5, 24±6 y, body mass index (BMI) 23±2 kg.m-2) and elderly (n=6, 70±5 y, BMI 24±2 kg.m-2). The subjects performed, at a 3 months interval, first 3 and then 6 sets of 8 repetitions of isotonic unilateral leg extension at 75% of their one repetition maximum (1 RM). Muscle biopsies were taken from the vastus lateralis of the exercised leg under local anaesthesia (1% lignocaine sc) before, immediately after and 1, 2 and 4 h after exercise. After separation of myofibrillar protein, incorporation of [1,2-13C]leucine was measured (by gas chromatography-combustion-mass spectrometry) and MPS calculated using plasma labeling of α-ketoisocaproate as surrogate precursor.

Figure 1.

In young men doubling the volume of exercise at 75% 1RM had no additional effect on the response of MPS in the post absorptive state; however, in older men, it resulted in a more rapid and greater response. The results suggest that there is increased latency of response to exercise in muscle of older men.

Figure 1. Time course of responses of MPS (%/h) to exercise at 75% 1RM of 3 or 6 sets of leg extension exercise in post absorptive young and older subjects. Mean ± SEM. * = P<0.05 vs. basal (ANova with Bonferroni post hoc adjustment). NB protein synthesis is measured over 2.5 h in the basal pre-exercise state and then over the periods shown post-exercise.


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Identification of aerobic-anaerobic transition in male rowers using surface electromyography during graded incremental exercise

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It has recently been shown that non-linear changes in myoelectric data relative to exercise intensity can be used to identify the aerobic-anaerobic threshold (Condotti et al. 2008, Farina et al. 2007. However these studies restricted their analysis to cycling tasks. The aim of this study was to assess the use of surface electromyography (EMG) as a non-invasive determinant of the metabolic response to incremental rowing exercise. The
relationships between EMG threshold (T_{EMG}) and more commonly used variables for detection of the aerobic-anaerobic threshold namely; blood lactate threshold (T_{Lac}) and onset of blood lactate accumulation (OBLA) were assessed.

Eleven male club-level rowers (age 21±4yr, height 1.88±0.04m, mass 84±7kg, VO_{2max} 60.9±5.8mL.kg^{-1}.min^{-1}) performed graded tests to volitional exhaustion on a Concept II ergometer. This ethically approved study involved intermittent exercise bouts at fixed workloads (start load 120W, duration 3 min, rest 1 min, increment 40W) during which EMG data were recorded from Rectus Femoris (RF), Vastus Lateralis (VL), Biceps Femoris (BF) and upper portion of Trapezius (UT). The rest period between increments facilitated earlobe blood sampling for lactate determination. Root mean square EMG (rms-EMG) for each muscle were calculated using a 50ms averaging window over 10 consecutive stroke cycles during the final minute of each exercise increment. Individual loads at EMG threshold were identified using the V-slope method and compared against load at T_{Lac} and OBLA using a repeated measures ANOVA. Pearson’s correlation analysis showed strong association between blood lactate and rms-EMG activity in RF (r=0.81), VL (r=0.63) and BF (r=0.70), and lower association in UT between blood lactate and rms-EMG activity in RF (r=0.81), Isolated EDL muscle from 4-week old Wistar rats were mounted at fixed length on a muscle holder connected to a transducer. The muscles were incubated in buffer with 0 Ca^{2+} either at rest or stimulated at 1 Hz for 120 min (0.2 ms pulses, 24 V/cm). Following incubation the muscles were allowed to recover for 40 min in either Ca^{2+}-free buffer or normal Krebs Ringer (NKR) containing 1.27 mM Ca^{2+}. Maximum tetanic force was measured prior to incubation, immediately after incubation and at 20 and 40 min recovery (90 Hz, 0.5 sec). Mean values ± SD are given. Statistical difference between groups was ascertained using a t-test for non-paired observations. Stimulation in Ca^{2+} free buffer led to a 60% loss of tetanic force. If muscles were allowed to recover in Ca^{2+} free buffer no force recovery was observed, however if muscles recovered in NKR a complete force recovery was observed. The amount of Ca^{2+} taken up during recovery was measured using ^{45}Ca and corresponded to 132±5 nmol/Ca^{2+} g wet wt. (n=3). Addition of the cation channel blocker SKF 96365 reduced the uptake of Ca^{2+} by 60% (to 48±36 nmol/Ca^{2+} g wet wt., n=3, P<0.05) and prevented force recovery. Stimulation at 1 Hz for 120 min led to a marked uptake of ^{45}Ca in accordance with previous studies (3). SKF reduced this stimulation induced uptake. From this study it is concluded that SOCE can be observed in whole isolated muscles and that this uptake is important for the maintenance of contractile function. The effect of SKF 96365 on the stimulation induced uptake of Ca^{2+} suggest involvement of SOCE or ECCE.


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Uptake of Ca^{2+} is necessary for maintenance of contractile function and is partly inhibited by the cation channel blocker SKF 96365 in rat skeletal muscle

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It has previously been shown that excitation is associated with an uptake of Ca^{2+} in rat skeletal muscle (3). The mechanism behind this uptake has remained elusive. Store-operated Ca^{2+} entry (SOCE) or excitation coupled Ca^{2+} entry (ECCE) may serve as possible entry mechanisms. The molecular basis for SOCE has now been elucidated and involves the Ca^{2+} sensing protein STIM1 in the SR membrane and the pore forming Orai in the cellular membrane (4). ECCE seems to involve the ryanodine receptors (RyR) in the SR membrane and the dihydropyrimidine receptors (DHPR) in the cellular membrane. ECCE differs from SOCE that it does not require emptying of intracellular Ca^{2+} stores but depends on depolarization of the membrane (2). STIM1 haploinsufficiency has been shown to affect tetanic force in mice (5). Both SOCE and ECCE is blocked by SKF 96365 (1). The aim of this study was to investigate whether SOCE is important for the maintenance of contractile function in rat skeletal muscle and to investigate whether SOCE or ECCE is involved in the observed excitation-induced uptake of Ca^{2+}. Isolated EDL muscle from 4-week old Wistar rats were mounted at fixed length on a muscle holder connected to a transducer. The muscles were incubated in buffer with 0 Ca^{2+} either at rest or stimulated at 1 Hz for 120 min (0.2 ms pulses, 24 V/cm). Following incubation the muscles were allowed to recover for 40 min in either Ca^{2+}-free buffer or normal Krebs Ringer (NKR) containing 1.27 mM Ca^{2+}. Maximum tetanic force was measured prior to incubation, immediately after incubation and at 20 and 40 min recovery (90 Hz, 0.5 sec). Mean values ± SD are given. Statistical difference between groups was ascertained using a t-test for non-paired observations. Stimulation in Ca^{2+} free buffer led to a 60% loss of tetanic force. If muscles were allowed to recover in Ca^{2+} free buffer no force recovery was observed, however if muscles recovered in NKR a complete force recovery was observed. The amount of Ca^{2+} taken up during recovery was measured using ^{45}Ca and corresponded to 132±5 nmol/Ca^{2+} g wet wt. (n=3). Addition of the cation channel blocker SKF 96365 reduced the uptake of Ca^{2+} by 60% (to 48±36 nmol/Ca^{2+} g wet wt., n=3, P<0.05) and prevented force recovery. Stimulation at 1 Hz for 120 min led to a marked uptake of ^{45}Ca in accordance with previous studies (3). SKF reduced this stimulation induced uptake. From this study it is concluded that SOCE can be observed in whole isolated muscles and that this uptake is important for the maintenance of contractile function. The effect of SKF 96365 on the stimulation induced uptake of Ca^{2+} suggest involvement of SOCE or ECCE.


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Are the projections from the periaqueductual grey to pontine noradrenergic neurones excitatory or inhibitory?

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The periaqueductal grey (PAG) is a midbrain site involved in the modulation of nociception and is believed to control nocicep-
tion, in part, by activating pontospinal noradrenergic (NA) neurones. Connections between the PAG and noradrenergic cell groups have previously been demonstrated (Bajic et al., 2001), however, the neurotransmitter phenotype is as yet unknown. To address this issue we have injected viral vectors to the ventrolateral PAG (VL-PAG) to anterogradely label projections to pontine noradrenergic (NA) neurones in combination with immunocytochemistry for glutamate (vGlut1&2) and GABA (VGat) transporters.

Male Wistar rats (n=3) received injections of AAV-CMV-EGFP (250nl) under anaesthesia (ketamine 60mg.kg-1/medetomidine 25μg.kg-1 i.p.) into the VL column of the PAG. After 8 days all animals were terminally anaesthetised with sodium pentobarbital (70mg kg-1 i.p.) and perfusion fixed with 4% formalin, brains were removed and 40μm sections were cut. Sections were processed immunocytochemically to identify dopamine β-hydroxylase and visualise terminals containing EGFP and VGlut1&2 or VGat. Injection sites and terminal labelling were determined using confocal imaging and 3D reconstruction software (Volocity 4, Improvision).

AAV-CMV-EGFP produced strong terminal labelling in the pons with a predominantly ipsilateral distribution. Terminals showing co-localisation with Vglut or Vgat were identified in A5, A6 and A7 cell groups indicating excitatory or inhibitory amino acid (EAA or IAA) projections from the VL-PAG. Quantification of terminals closely apposing NA cell bodies in these regions showed that the LC received the densest innervation of EAA and IAA profiles. However after analysis of the density of innervation per NA neuron, the A7 cell group received the largest number of EAA and IAA profiles per soma (3.5±1.8 and 3.8±1.5 profiles/soma respectively). By contrast the A6 cell group received moderate EAA and IAA innervation (1.3±0.4 and 1.1±0.3 profiles/soma). The A5 territory received more IAA (3.5±2.2 profiles/soma) than EAA innervation from the VL PAG (1.5±1.0 profiles/soma).

In conclusion, we show that VL PAG projections to A5, A6 and A7 have terminals closely apposed to NA neurones. These terminals express vesicular transporters for EAA and IAA suggesting that there are both excitatory and inhibitory phenotypes. The A7 region is densely innervated by glutamatergic and GABAergic profiles, which may identify the neurotransmitter phenotype of previously documented symmetrical and asymmetrical synapses made by PAG projections to A7 (Bajic et al 2001). We have also shown that the A5 region receives a strong inhibitory input from the PAG, which may be responsible for the depressor response that occurs on activation of VL PAG. These data provide evidence for a specific and differential control of pontine NA neurones by the VL PAG.


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Activation of the capsaicin receptor TRPV1 desensitises the nociceptive ion channel TRPA1 in rat dorsal root ganglion neurones

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Transient Receptor Potential A1 (TRPA1) is an ion-channel receptor implicated in a number of nociceptive pathways including cold-induced pain and the detection of noxious stimuli such as isothiocyanates. It is specifically activated by cinnamaldehyde and mustard oil. TRPA1 is often co-expressed with the heat/capsaicin receptor TRPV1 in primary sensory neurones. To clarify how TRPV1 activation affects the activity of TRPA1, we have examined the effects of capsaicin on the cinnamaldehyde sensitivity of dorsal root ganglion (DRG) neurones.

Adult Sprague-Dawley rats were killed by 100% CO2 inhalation followed by decapitation, and dissociated DRG neurones were cultured with nerve growth factor for 12-24 hours. Capsaicin (1 uM) and cinnamaldehyde (200 uM) responsiveness of individual neurones was evaluated using Calcium Green-1 microfluorimetry (ΔF/F0). TRPA1 activation in DRG neurones was significantly reduced as evidenced by a diminished sensitivity to cinnamaldehyde (ΔF/F0: 0.03 ± 0.003, mean ± SEM, n=299, vs. control 0.3 ± 0.02, n=367; P<0.0001, Student’s unpaired t test).

Next, we determined whether the observed capsaicin-mediated inhibition was dependent upon TRPV1 activation. DRG neurones were pre-incubated with 10mM capsazepine, a TRPV1 antagonist, before evaluating capsaicin-induced inhibition of TRPA1. Capsazepine reduced the response of TRPV1 to capsaicin (ΔF/F0 decreased from 0.45 ± 0.03, n=299, to 0.14 ± 0.02, n=156; P<0.001), and reduced the capsaicin-induced inhibition of TRPA1 (response to cinnamaldehyde increased from ΔF/F0 = 0.03 ± 0.003, n=299, to 0.16 ± 0.03, n=156; P<0.0001). In order to determine whether the capsaicin-induced desensitisation of TRPA1 occurs via a calcium-dependent mechanism, we depolarised DRG neurones using 50 mM KCl. This concentration elicits an increase in [Ca2+]i similar to that induced by 1 µM capsaicin in TRPV1-expressing neurones, and desensitised TRPA1 to a similar degree: the response to cinnamaldehyde (ΔF/F0 = 0.02 ± 0.002, n=35) was comparable to that following capsaicin-mediated inhibition.

We conclude that the desensitising effect of capsaicin on TRPA1 is dependent on a TRPV1-mediated increase in [Ca2+]i. This mechanism could be a potential pharmacological target for modulating TRPA1-mediated nociceptive pathways.

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**C45**

K\(_{Ca}\) channels regulate stretch-evoked afferent firing from muscle spindles

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Muscle spindles constantly report skeletal muscle length and movement. Mechanosensory transduction occurs at annulospiral terminals around intrafusal muscle fibres. We reported that Ca\(^{2+}\)-dependent glutamate release from synaptic-like vesicles in these terminals modulates spindle firing (Bewick et al., 2005). To test if voltage-gated Ca\(^{2+}\) channels (VGCCs) or Ca\(^{2+}\)-dependent K\(^+\) channels (K\(_{Ca}\)) are also functionally important, we tested whether channel-selective neurotoxins affected spindle afferent discharge.

Adult Sprague-Dawley rats (male, 300-370 g) were killed (Schedule 1, Animal (Scientific Procedures) Act, 1986) and 4th lumbrical nerve-muscle preparations excised and placed in gassed (95%O\(_2\)-5%CO\(_2\)) Liley`s saline. Spindle afferent discharges were recorded on passant with Ag wire electrodes and firing frequency (mean ± SE, n) determined for the first 0.5 s of the "hold" phase of stretch-and-hold cycles (~10% muscle length) for 'n' preparations. The significance of differences between pre-drug and with-drug means was evaluated by paired t-test, with a threshold of P < 0.05.

Unlike inorganic Ca\(^{2+}\) channel blockers Co\(^{2+}\) and Ni\(^{2+}/Cd\(^{2+}\), which abolish responses (Bewick et al., 2005), P/Q type VGCC inhibitors enhanced firing. \(\omega\)-Agatoxin-I-IVA (200 nM, P/Q type) increased firing to 301% of control (92.7 ± 15.8 imp/s vs 278.6 ± 23.9 imp/s, 6; P < 0.0001). \(\omega\)-Conotoxin-MVIIIC (1 μM, Q type) enhanced firing to 202% (98.40 ± 21.83 imp/s vs 199.60 ± 24.62 imp/s, 5; P < 0.002). Conversely, \(\omega\)-conotoxin-GVIA (1 μM; N type) had no significant effect (176.92 ± 14.77 imp/s vs 196.92 ± 10.47 imp/s, 6; P = 0.1). L-type blockers Taicatoxin (50 nM, 4) and Nifedipine (10 μM, 5) increased afferent firing (131.88 ± 10.05 imp/s vs 302.13 ± 25.17 imp/s and 192.00 ± 13.09 imp/s vs 271.70 ± 9.56 imp/s, respectively) but also caused spontaneous muscle twitching, suggesting actions on skeletal muscle dihydropyridine receptors. Thus, only P/Q type channel blockers increased afferent discharge in the absence of other effects.

P/Q type channels often regulate K\(_{Ca}\) (BK or SK) channel opening (Edgerton & Reinhart, 2003). Therefore, selective K\(_{Ca}\) channel blockers were applied. Charybdotoxin (200 nM; SK & BK), iberiotoxin (200 nM; BK selective) and apamin (200 nM; SK selective) all increased afferent discharge (to 195%, 4; 224%, 4 & 160%, 6 of control, respectively; all P < 0.02). Conversely, NS1619 (BK channel activator) inhibited firing (1 μM, 179.75 ± 14.35 imp/s vs 33.25 ± 18.44 imp/s, 6). This was not due to voltage-gated potassium channel or action potential conduction block, since NS1619 did not affect Nervus saphenus compound action potential amplitude (100 μM, 4). The data suggest Ca\(^{2+}\) entry through P/Q type channels activates K\(_{Ca}\) channels to regulate firing frequency in spindle 1a afferents.


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**C46**

Motor unit rotation in a variety of human muscles

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To study the phenomena of substitution and rotation among motor units of a muscle, motoneurone pools of seven different muscles were investigated. Intramuscular motor unit activity and surface EMG (electromyogram) were recorded from one of the following muscles: abductor digitii minimi, first dorsal interosseus, extensor digitorum communis, flexor and extensor carpi radialis, tibialis anterior and soleus. The subject was asked to discharge a discernible motor unit at a comfortable constant or rhythmically modulated rate with audio and visual feedback. Results are reported from a total of 42 sets of motor units from all seven muscles. We observed that when a subject fired a motor unit for a long period, frequently an additional motor unit started to discharge after a few minutes. When the subject was asked to keep activity down to one unit, very often it was Unit 1 that dropped and Unit 2 continued to fire. While unit 2 had fired for a few minutes, Unit 1 resumed firing without any conscious effort by the subject. When the subject was then asked to retain just one unit, it was Unit 2 that dropped. Rhythmic modulation of firing rate of a tonically firing unit showed that while the threshold of this unit increased, the threshold of a phasically discharging unit decreased substantially, and rotation occurred. Quantification of firing times of lower and higher threshold units showed that the total discharge time was significantly longer for lower threshold than for higher threshold units. This finding indicates that even when lower threshold units do rest, they recover faster to contribute for a longer time to the total contraction. The increase in threshold of a tonically discharging unit is suggested to arise from inactivation of Na\(^+\) and Ca\(^{2+}\) channels and the decrease in threshold of higher threshold units is suggested to arise from an increase in persistent inwards currents that may occur during prolonged contractions. Whether a unit stops or starts to fire is suggested to depend on a balance between the strength of the net excitatory motor command, persistent inward currents, and inactivation of voltage gated channels. Rotations among low threshold motoneurones would ensure sustained contractions in small as well as larger muscles.

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Muscle movement while maintaining posture at the human wrist

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The ability to control the static posture of a joint depends on the elastic stiffness of the load being held (Chew et al., 2008). Here we investigate at the wrist the muscle and tendon contributions to this postural load-dependence. The Human Research Ethics Committee of the University of New South Wales approved the studies. Subjects (N = 6) used the wrist flexor muscles to hold as still as possible three loads of different elastic stiffness (Chew et al., 2008). One had positive-stiffness (i.e. spring like; 0.1 Nm.deg^-1) so that more force was required as the wrist flexed, one had negative stiffness (inverted-pendulum like; -0.1 Nm.deg^-1) and the third had zero stiffness (isotonic). Each load was held with the wrist in the same posture and at this position each load required the same wrist torque (0.70 Nm) for static balance. Flexor carpi radialis were observed by ultrasound (Acuson 128XP, L7384 linear array), which provided a 38 mm parasagittal view of the full depth of the muscle with a temporal resolution of 25 Hz. Spatial cross correlation of image luminance was used to measure longitudinal tissue velocity of the proximal and distal tendon plates. Using measured changes in wrist angle and the moment arm of the wrist, changes in length of the tendon and contractile portions of the muscle were determined.

For the positive- and zero-stiffness loads, the ranges of wrist movement over 30 s were approximately one degree whereas for the negative-stiffness load it was about five degrees. The relationship between contractile length and wrist angle at frequencies below 2 Hz varied with load stiffness. For the positive- and zero-stiffness loads, the muscle was shorter at more flexed wrist angles, with a higher gain for the positive-stiffness relationship. With the negative-stiffness load, the contractile length was longer at more flexed wrist positions. At ~ 2 Hz, a resonant peak in torque and wrist movement is reflected in the tendon length but not in the contractile portion. These load-stiffness effects are not apparent at higher frequencies (> 4 Hz) where the inertial properties of the load dominate and torque and angle are antiphase. A 10 Hz "tremor" oscillation in the EMG, contractile length and tendon length was minimal or absent in wrist angle because the tendon lengthened and shortened antiphase with the contractile portion.

We conclude that there is no simple relationship between muscle length and joint angle. Rather, it is a dynamic function of frequency strongly dependent on the elastic stiffness of the load. At the point of resonance, the load and joint oscillate on the end of a compliant tendon without significant transfer of the movement to the contractile portion, thus constituting an effective proprioceptive and control blindspot. This muscle-tendon behaviour is a major determinant of the limits of human postural control.


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Comparison of corrosion casts of brainstem vasculature of normotensive and hypertensive rats
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In the spontaneously hypertensive rat (SHR) hypertension is associated with raised sympathetic outflow. Why sympathetic activity is raised is unclear but if blood flow to the brain is impeded, blood pressure increases (Cushing effect) and there is a parallel increase in sympathetic outflow (Knapp, 1965). Our hypothesis is that the brainstem is highly sensitive to a correlate of perfusion such that flow reduction drives a proportionate increase in sympathetic outflow and arterial pressure (Paton et al., 2009). We postulate that cerebrovascular conductance is reduced in the SHR compared to the normotensive Wistar Kyoto rat (WKY).

Having measured BP by tail cuff we prepared vascular corrosion casts of age-matched WKY and SHR rats according to the method of Krucker et al. (2006). Animals were killed according to the UK Animals (Scientific Procedures) Act 1986. On cessation of the circulation, a cannula was placed in the left ventricle and vascular constriction inhibited by flushing with a zero calcium, high magnesium phosphate buffer containing 100 μM hydralazine. After perfusion fixation (formaldehyde, 4%), the vasculature was filled with a urethane-based resin (Pu4ii, VasQtec). Perfusion pressure during flushing, fixation and resin infiltration was the systolic pressure previously recorded from each animal. Resultant casts were labelled by maceration using sodium hydroxide (10%) and hydrochloric acid (10 mM) at 55 oC. Casts were freeze-dried, sputter coated with gold and examined in a scanning electron microscope (SEM; FEI, Quanta 400).

Our preliminary results relate to n=1 for each strain. Measurements of arterial diameter were made using software tools intrinsic to the SEM. Mann-Whitney U tests were used to make statistical comparisons of median values. Median values for basilar and vertebral artery diameter were smaller in the SHR compared to the WKY. Similarly, the diameters of branches arising from these arteries tended to be smaller in the SHR compared to the WKY. It should be noted that in the SHR, the diameter of the right vertebral (rv) artery (174 μm) was very much smaller than that of the left vertebral (lv) artery (272 μm), whereas, in the WKY, the diameters of the two arteries were similar (271 vs 291 μm). The fourth power of the vessel radius relative to the principle feeding artery (basilar, rv or lv) was calculated as an index of conductance and comparisons made within and between animals. This revealed that vascular conductance is substantially lower in the SHR than the WKY (P<0.05).

Our preliminary findings are consistent with the hypothesis that the brainstem of the SHR has a lower vascular conductance than the WKY. Our findings resonate with a study of human tissue at post-mortem in which a reduction in calibre of the rv artery was strongly and inversely correlated with ante-mortem blood pressure (Dickinson, 1960).


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i.p.) followed after surgery with 10% atipamezole hydrochloride (1.5ml/kg, i.m.) increased from 124±5 to 159±2 mmHg (mean±SEM, P<0.05, ANOVA) and was associated with enhanced JAM-1 immunofluorescence in NTS (381±49 vs. 138±17 units/section in control, P<0.05). When arterial pressure reached a plateau at 6 weeks (211±2 mmHg), JAM-1 immunofluorescence in NTS was not increased further (P<0.05).

We next assessed whether up regulation of JAM-1 was specific to the brain. WKY, SHR, SP-SHR, pre-hypertensive SHR and Goldblatt rats (male, n=4 each group) were anaesthetised with halothane (5%) and decapitated. Brainstem blood vessels were isolated by ultracentrifugation (Mrsulja et al. 1976) along with tissues from lung, liver, kidney, spleen and heart. In all animal groups, JAM-1 protein levels were higher in all organs studied as determined by western blotting (P<0.05, ANOVA).

These data indicate that up regulation of JAM-1 is a generalised phenomenon associated with early and pre-hypertensive stages of both genetic and renovascular hypertension and further supports its possible causative role in this pathological condition and, hence, as a novel prognostic indicator.


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Similar cardiovascular autonomic changes during the development of renovascular and angiotensin II (ANGII) induced hypertension in rats

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Hypertension remains a serious clinical problem. Numerous rodent models of hypertension have been developed to allow studies into possible causative mechanisms. Two such models are the chronic ANGII infusion and renovascular hypoperfusion (two kidney one clip; 2K1C). Although both models depend on activation of ANGII type 1 receptors, it is not known when and if the autonomic nervous system is engaged in the development and/or maintenance of the resultant hypertension.

The development of hypertension in both models was documented using 24 hour radio-telemetry recording of arterial pressure (AP) and heart rate (HR) in conscious freely moving rats. Hey Presto software (Waki et al. 2006) was used to calculate spontaneous baroreflex gain (sBRG) and indices of autonomic function from the AP and HR variabilities by spectral analysis.

ANGII model: Rats (male, 250–350g, n=12) were anaesthetised with a mixture of ketamine (60mg kg-1) and medetomidine (250μg kg-1, both i.m.) and radio-transmitters installed. Continuous recordings of AP and HR were made for 3 days prior to, and 10 days during, osmotic minipump driven infusion of ANGII (800ng/kg/min, s.c.). 2K1C model: Rats (male, 150–180g) were anaesthetised as above and radio-transmitters installed plus the left renal artery was partially obstructed with a silver clip of 0.2 mm width (n=6) or sham surgery (n=6). Recordings of AP and HR were made for 6 weeks. Values are means ± S.E.M., compared by ANOVA.

Both models exhibited similar alterations in autonomic indices especially when the hypertension plateaued. For both the 2K1C and the ANGII group AP rose to similar levels (e.g. 185±15 mmHg vs. 103±10 for sham rats and 150±3 vs. 91±3 mmHg pre-infusion, p<0.05 respectively). Additionally, HR was elevated (ANGII: 409±10 vs. 368±5 bpm pre-infusion, p<0.05; 2K1C: 468±11 vs. 382±11 sham treated, p<0.05), very low frequency of systolic blood pressure increased (ANGII: 6.8±0.5 vs. 5.4±0.2 mmHg2-pre-infusion, p<0.05; 2K1C: 7.0±0.3 vs. 3.9±0.3 mmHg2 sham treated, p<0.05), high frequency of the pulse interval was reduced (ANGII: 10.6±1.5 vs. 15.0±1.1 ms2-pre-infusion, p<0.05; 2K1C: 9.6±0.5 vs. 16.7±0.9 ms2 sham, p<0.05) and sBRG was reduced (ANGII: -1.1±0.3 vs. -2.0±0.1 bpm/mmHg pre-infusion, p<0.05; 2K1C: -0.83±0.05 vs. -2.5±0.17 bpm/mmHg, sham, p<0.05).

Thus, these established models of experimental hypertension are associated with failure of the parasympathetic component of the baroreflex and increased sympathetic vasomotor activity. These data suggest that chronically increased peripheral levels of ANGII, resulting from exogenous infusion or renal hypoperfusion, may cause hypertension via mechanisms involving central modulation of both cardiovascular autonomic activity and baroreflex function.


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Increased brainstem vascular resistance precedes onset of hypertension in the spontaneously hypertensive rat

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Using the spontaneously hypertensive rat (SHR) as an animal model of essential hypertension we are working on a hypothesis that the condition is due, in part, to increased sympathetic activity that occurs in response to brainstem hypoperfusion. There is good evidence of increased sympathetic tone in adult humans with essential hypertension and in the SHR even at a prehypertensive (<5 week) age (see 1). Brainstem hypoperfusion causes an acute rise in blood pressure in humans and other species (1,2,3) and in humans, chronic hypertension correlates more closely with narrowing of the vertebral arteries than any other vessel (4). There is also evidence of anaerobic metabolism in the SHR brainstem and increased vertebral and basilar arterial wall thickness at the prehypertensive age (5,1). However, the functional significance of the latter histological changes in large vessels to resistance and flow remain unclear and were functionally investigated in the present study.
Using a novel in vitro perfusion preparation, vascular resistance within the basilar arterial tree was measured in male 4-5 week SHR (n=7) and Wistar (n=9) rats. Under deep halothane (5%) anaesthesia, rats were decapitated at the 1st cervical level and decorticated. The brainstem with cerebellum was removed in cold (4°C) artificial cerebrospinal fluid (ACSF) and pinned down in a perspex chamber, ventral side up. The basilar artery was cannulated at its caudal end with a double-lumen theta glass pipette and tied in place with a fine silk suture. The preparation was bathed in rat intrapulmonary arteries (IPA) of the rat, mounted on a myograph; experiments were performed on intact or permeablised arteries, H2S caused a contraction additive to α-toxin at 25°C, and in some cases were permeablised with (IPA) were mounted on a myograph containing physiological saline solution (PSS) and gassed with air/5% CO2 (pH 7.4, 37°C), and in some cases were permeablised with α-toxin at 25°C, and clamped to pCa 7.1. Intracellular calcium was measured using fura-PE3. All drugs were added before H2S. IPA slightly pre-constricted with U46619 exhibited a triphasic constriction-relaxation-constriction response to H2S, which persisted in the presence of the β-adrenoceptor blocker phenotolamine (10 μM, n=4) or the purinergic receptor blocker suramin (300 μM, n=3). The transient initial constriction (phase 1) and the subsequent sustained constriction (phase 2) were retained in endothelium-denuded IPA (n=4); however the intervening relaxation was suppressed by removal of the endothelium or treatment with L-NAME (300 μM; n=4&7). Phase 1 but not phase 2 was decreased by thapsigargin (1 μM; n=5; P<0.05) or ryanodine (100 μM, n=4; P<0.01). Phase 1 was unaffected by the Ca2⁺ channel antagonists nifedipine (3 μM, n=2) or diltiazem (10 μM, n=3). In IPA bathed in Ca²⁺-free PSS (100 μM EGTA), H2S elicited a rise in [Ca2⁺]i that was suppressed by cyclopiazonic acid (30 μM), n=2). In tension experiments, the Rho kinase inhibitor Y-27632 (1 μM, n=5; P<0.05) but not the broad spectrum protein kinase C inhibitor Gö6983 (3 μM, n=3) inhibited phase 2, while in permeablised arteries, H2S caused a contraction additive to that induced by pCa 7.1 (100 μM NaHS, n=4; P<0.05; 300 μM NaHS, n=6; P<0.05). Notably, the increase in tension induced by H2S in IPA under slight preconstriction was not associated with a concomitant increase in [Ca2⁺]i. We conclude that the constriction-relaxation-constriction response elicited by H2S in rat IPA is due to 1) an initial endothelium-independent contraction associated with Ca²⁺ release from ryanodine sensitive stores, 2) an endothelium and NO-dependent relaxation, and 3) finally a Rho kinase-dependent but endothelium-independent contraction that is not associated with an increase in [Ca2⁺]i. In view of some similarities to the response to hypoxia, we speculate that H2S elicits its response in IPA in part via inhibition of cytochrome oxidase, this mimicking hypoxia.


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In intact IPA constricted with 10 μM PGF2α, 8-Br-cGMP (cGMP analogue) elicited relaxation with an EC50 of 20±13μM and maximum relaxation of 102±% (n=6). In permeabilised arteries at pCa 6.7 the potency of 8-Br-cGMP was much greater, with an EC50 of 18±4μM and maximum relaxation of 64±% (n=5). 8-Br-cGMP (100nM) also caused a rightward shift of the Ca2+ dose-response curve, with an increase in EC50 from 165±5 nM to 316±14 nM (n=44; p<0.05), indicating calcium desensitisation. Complete inhibition of Rho kinase with Y-27632 (10μM) did not prevent this shift (EC50; Y-27632: 205±2 nM, n=8; Y-27632+8-bromo-cGMP: 306±26 nM (n=6). This suggests that cGMP does not act via Rho kinase. Interestingly, 8-Br-cGMP-induced relaxation of permeabilised IPA was suppressed not only by the cGMP antagonist Rp-8Br-cGMP (25μM) (control: 77±3%; Rp-8Br-cGMP: 44±3%, n=4-8, p<0.01), but also by the cAMP antagonist Rp-8Br-cAMP (25μM) (43±5%, n=4; p<0.01); there was no increase when these were applied together (43±9%, n=3), suggesting that cGMP and cAMP may be acting sequentially.

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Relationship Between Glutathione Redox State And The Vasoconstrictor Response To Hypoxia In Rat Pulmonary Arteries
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Hypoxia elicits constriction of pulmonary arteries (hypoxic pulmonary vasoconstriction, HPV) but dilation of systemic arteries. It is proposed that hypoxia-induced changes in the pulmonary artery smooth muscle cell redox state and/or reactive oxygen species trigger HPV, although there is controversy as to whether this involves a net oxidation or reduction. Glutathione is the major thiol-disulfide redox buffer of the cell, so measurement of GSH and GSSG levels should provide an indication of overall cytoplasmic redox state. We measured the effect of hypoxia on GSH and GSSG in rat small intrapulmonary arteries (IPA) and aorta, using the approach of Griffith (1980), and investigated the effect of agents which should cause cell oxidation on HPV and GSH/GSSG ratio. The redox potential (expressed in mV) of the GSH-GSSG couple was calculated using the Nernst equation.

Under normoxic conditions, the GSH-GSSG redox potential was -176±4mV (n=18) in IPA and -150mV (n=16) in aorta. After 45 min of hypoxia imposed by gassing the solution with 0, 1 and 2% O2, the redox potential in IPA was decreased to -159±6mV (n=14, p<0.05), -162±4mV (n=20, p<0.05) and -174±4mV (n=10, ns), respectively. Conversely, the redox potential of aorta after 45 min of hypoxia was -142±11 mV (n=12), -155±3mV (n=12) and -171±4mV (n=10, p<0.01), in 0, 1 and 2% O2, respectively. Thus, hypoxia tended to oxidize IPA.

Glutathione reductase (GR) converts GSSG to GSH, whilst oxidizing NADPH to NADP+. NADPH is reformed from NADP+ by glucose-6-phosphate dehydrogenase. The conversion of GSH back to GSSG is mediated by glutathione peroxidase (GPx); peroxide is reduced to water in a coupled reaction. Carmustine (100μM), a GPx blocker, completely abolished the sustained phase of HPV (n=8), and shifted the cell redox potential during normoxia from -172±3mV (n=8) to -154±6mV (n=6). Conversely, mercaptosuccinate (MS; 100μM), a blocker of GPx, increased the sustained phase of HPV by 47±13% (n=8, p<0.05). Neither carmustine nor MS affected the initial transient phase of HPV. Ebselen (30μM), a GPx mimic, attenuated both transient (44±9%, n=3) and sustained phases (39±18%, n=3) of HPV. Dehydroepiandrosterone (100μM), which blocks glucose-6-phosphate dehydrogenase, shifted the normoxic cell redox potential to -154±6mV (n=6, P<0.05 vs control) and blocked sustained HPV by 43±13% (n=3). Diethylmaleate (1 mM), which depletes glutathione, shifted the cell redox potential to -105±6mV (n=3), and blocked sustained HPV by 36±6% (n=6). In summary, hypoxia caused an oxidizing shift in the redox state in intact IPA. Moreover, several substances which reduce total glutathione levels in tissue and caused cell oxidation consistently inhibited sustained HPV.


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Potential selective role for CXCR7/CXCL12 signalling in the lung in response to hypoxic stress
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Pulmonary hypoxia is a common complication of chronic lung diseases leading to the development of pulmonary hyperten-
Acoustic over-exposure changes the firing pattern of dorsal cochlear nucleus neurons

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Acoustic over-exposure (AOE) triggers deafness in animals and in humans and provokes auditory nerve degeneration. One month after AOE, there is an increase in the cellular excitability within the dorsal cochlear nucleus (DCN) and this is considered as a possible neural correlate of tinnitus. The origin of this phenomenon is still unknown but it is suggested that it is triggered by plastic adjustments within the DCN arising at the early stages of deafness (Kaltenbach JA. 2007). The purpose of the present study was to determine how DCN excitability was affected 3-4 days after AOE. Wistar rats (14-16 days old) were anesthetized (0.15mg/kg fentanyl; 5.1 mg/kg fluanisone; 2.5 mg/kg midazolam) and exposed to a 110 dB SPL 15-kHz noise stimulus for 4 hours (AOE). In vivo auditory brainstem response recordings (anaesthesia as above) and in vitro whole-cell current-clamp recordings from brainstem slices containing the DCN were made 3-4 days post AOE. Auditory brainstem response recordings showed that the hearing thresholds were significantly elevated (between 20-30 dB SPL) for frequencies above 15 kHz. Control fusiform cells (the main output of the DCN) fired with a regular firing pattern as assessed by the coefficient of variation of the interspike interval distribution of 0.11 (n=5). Three to four days after AOE, 30% of fusiform cells exhibited bursting irregular discharge patterns (coefficient of variation of the interspike interval distribution of 1.8 ± 0.6, n=5; unpaired T test p<0.05). Control granule cells (interneurons projecting onto fusiform cells) fired with a high gain (slope of 2.5 ± 0.4 Hz/pA, n=10) that was decreased 3-4 days after AOE (to 1.4 ± 0.2 Hz/pA, n=9; unpaired T test p<0.05). This was accompanied by a decrease of their membrane resistance (from 1.9 ± 0.3 GΩ, n=10 to 1.1 ± 0.2 GΩ, n=10; unpaired T test p<0.05) and more hyperpolarized resting potentials (from -43 ± 4 mV, n=9 to -57 ± 4 mV, n=10; unpaired T test p<0.05). Data obtained in fusiform cells and in granule cells recorded in control condition and after AOE were fitted with a leaky integrate-and-fire model. In conclusion we suggest that the DCN excitability changes occurring 3-4 days post AOE trigger the increased DCN excitability observed at a later stage and this represents the initial stages of tinnitus.


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Synaptic release evoked by depolarization in mouse cochlear inner hair cells imaged in situ in the isolated temporal bone

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The ribbon synapse of cochlear inner hair cells (IHCs) is the first relay point of the auditory system. Each contact made by a single IHC onto the afferent nerve can operate with a high degree of temporal precision. To understand the underlying mechanisms, we have developed techniques to image vesicle
ward K+ currents. Under such conditions inward calcium cur-

Corti. All ages of preparation can be used. FM1-43 (2 μM) was

added to the bath to be taken up rapidly by IHCs from their

intact apical surface and trafficked to the cell base at an

effective rate of 0.08 μm s⁻¹. After 300 s regions corresponding
to ribbon sites were identified using a 2 photon (2P, 840nm) laser

scanning microscope. Images of FM1-43 ‘hotspots’ were

acquired as a series of frames (61 ms/frame) or as lines scans

(1.8 ms/line).

On stimulation by transepithelial extracellular current designed
to depolarise the basolateral terminal, hotspots destained by

the presumed ribbon sites. Cell depolarization by 60 mV (from

Vh = –70 mV) decreased the fluorescence by 1-2%. The fluo-

crescence intensity recovered with a time constant of 220 ms.

In a few cases there was also a small fluorescence rebound, sug-

gesting redistribution of dye. The imaged destaining rate is

consistent with the inference (Goutman & Glowatzki, 2007)

that only a small number of vesicles (10-20) may be released at

each site on short 200 ms depolarizing commands.


212-215.

Goutman, JD & Glowatzki, E (2007) Proc natl Acad Sci USA

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C60

Fast NMDAR-mediated EPSCs in the auditory brainstem of

mouse contribute to nitric oxide signalling in matured synapses

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Synaptic NMDARs provide voltage-dependent depolarization and a Ca²⁺ influx signal allowing downstream activation of Ca²⁺-dependent processes. In tandem with AMPARs which mediate most fast glutamatergic EPSCs, NMDARs mediate a slow EPSC in this dual component glutamatergic synapse. Synaptic NMDAR currents are large during early development but decline in magnitude as synaptic pathways mature. This has lead to the suggestion that NMDAR have no role at mature auditory brainstem synapses. We have shown that NMDAR-mediated nitric oxide production occurs at the mature (P35) calyx of Held
synapse (1). Therefore, the present study characterises the changing properties of NMDAR from P9 to P35 and shows that they do contribute to synaptic signalling at the mature synapse. CBA/Caj mice (P9–P35) were killed by decapitation in accordance with the UK Animals (Scientific Procedures) Act 1986. Brainstem slices containing the superior olivary complex were prepared as previously described (2) and patch clamp experiments were performed at 36°C on principal neurons of the medial nucleus of the trapezoid body (MNTB). Synaptic activity was achieved by midline stimulation using a bipolar electrode. We confirm the decline in NMDAR-mediated EPSC magnitude by P18, and show that this maturation process can be divided into two phases. First, there is an early decline in NMDAR current amplitude (P9: 3.7±0.8 nA to P35: 0.3±0.1 nA, n=4-5) and acceleration in kinetics, so that NMDAR-mediated EPSCs decay with a dominant fast time-constant (P9: 36.3 ms at P35: 16.7±4.1 ms, n=4-5) at mature synapses. Quantitative PCR showed increased NR2A mRNA expression after P10, consistent with faster kinetics and a declining contribution from NR2B subunits, since EPSCs from P18/P21 showed no block by the NR2B-prefering antagonist ifenprodil (10 μM, Ctrl: 0.48±0.06 nA vs ifen: 0.44±0.1 nA, n=4). Second, the NMDAR-mediated EPSC exhibits decreased voltage-dependent block by [Mg2+]o, consistent with incorporation of NR2C subunits into synaptic channels. In addition, sensitivity to extracellular Zn2+ was not detected, and the Zn2+ chelating agent, TPN, had no effect. We conclude that NMDARs are not eliminated from the calyx of Held synapse, but that channel expression evolves from an immature state where large conductance channels with slow kinetics are replaced by small conductance channels with fast kinetics and reduced Mg2+ block. These results confirm the relevance of NMDAR channels throughout development and suggest that the declining NMDAR-EPSC is a shift from ‘gross’ electrical signalling to a Ca2+-dependent intracellular signalling (e.g. activation of tightly coupled neuronal nitric oxide synthase). Results are reported as mean±SEM. Significance was tested using two-tailed Student’s t-test. Differences were considered statistically significant (*) at p<0.05.

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**An extracellular-facing sensing histidine closes the CIC-2 chloride channel through a long-range effect on the pore**

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The activity of the CIC-2 chloride channel has a biphasic response to extracellular pH, with activation by moderate acidification followed by abrupt channel closure at pH values lower than ~7. We recently identified H532 as the sensor coupling extracellular acidification to complete closure of the channel (Niemeyer et al., 2009). This extracellularly-facating histidine is located at the N-terminus of transmembrane helix 7 that, according to a homology molecular model of the channel, connects via a short loop with Y555, a conserved residue that has been suggested to take part in the gating of certain CIC proteins at an intracellular gate located at the cytoplasmic end of the pore (Bell et al., 2006; Jayaram et al., 2008). We have now investigated whether amino acids in the environment of H532 are important in the acidification-induced channel closure and whether the H532 sensing function depends upon inner pore residue Y555. The homology model for CIC-2 suggests that extracellular loop I-J is in the vicinity of H532. Several charged residues in I-J were neutralised without any effect upon the inhibition of CIC-2 by acidification (mutants examined E302V, E301Q, R311Q and D315N). Three aromatic residues of loop I-J, however, had a marked effect upon acidification-induced inhibition of CIC-2. These were F308, F312 and F316. Double mutants F308A-F312A and F308A-F316A were completely indifferent to acidification. As shown in Figure 1, when Y555 was mutated to F there was a complete disappearance of CIC-2 inhibition by acidification. Our present data highlight the importance of extracellular residues of the I-J loop F308, F312 y F316. Given the closeness of H532 to these residues and their chemical nature, it is tempting to speculate that some aromatic-aromatic, perhaps of the π-π type, interaction occurs between H532 and the mentioned F residues. Protonation of H532 might lead to the disruption of this putative network of interactions and to channel closure. Finally our result showing that CIC-2-Y555F fails to be blocked by extracellular acidification suggests that the effect of H532 charge change might be exerted through a long-range action on a putative intracellular gating mechanism located near the intracellular pore entrance.

**Fig. 1. Effect of extracellular pH on Y555F mutant of CIC-2.** The data (triangles) are averages with SEMs, n=3. The intra- and extracellular solutions contained 35 and 140 mM chloride respectively. The potential used was -130 mV. Data for CIC-2-H532F are from Niemeyer et al. (2009) and are shown for comparison. Bell SP et al. (2006) Biochemistry 45, 6773-6782

Jayaram H et al. (2008) Pro Natl Acad Sci USA 105, 11194-11199

Niemeyer MI et al. (2009) J Physiol 587, 1387-1400

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Acid-sensitive $K_{2p}$ channel heterodimerisation

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Acid-sensitive background two-pore potassium channels ($K_{2p}$) are leak channels that are open at all membrane potentials and are important contributors to the control of cellular excitability. The members of acid-sensitive $K_{2p}$ channels; $K_{2p}3.1$ and $K_{2p}9.1$ share 54% homology with each other, and 51% homology with $K_{2p}15.1$ (Kim et al., 2000). $K_{2p}3.1$, $K_{2p}9.1$ and $K_{2p}15.1$ are widely expressed, and demonstrate co-expression in an array of tissues including the pancreas, placenta, kidney and lung. $K_{2p}3.1$ and $K_{2p}9.1$ show functional expression in heterologous expression systems, they are potassium selective and are strongly inhibited by acidic pH. A single nucleotide polymorphism was found at position 95 of the selectivity filter of $K_{2p}15.1$, when cloned in 2001 (GYG to EYG), and to date both isoforms have been shown to be non-functional when expressed in COS-7 cells (Kim et al., 2001).

Here we investigated the interaction between both $K_{2p}15.1$ EYG and $K_{2p}15.1$ GYG and other acid-sensitive $K_{2p}$ channels. We used two-electrode voltage-clamp to measure the functional activity of the acid-sensitive $K_{2p}$ heterodimers. Xenopus laevis oocytes expressing $K_{2p}3.1$ or $K_{2p}9.1$ exhibited a pH-dependent current, typical of acid-sensitive $K_{2p}$ channels (4.6 $\mu$A (±2.0 $\mu$A, n=25) and 5.2 $\mu$A (±3.8 $\mu$A, n=27) respectively at a membrane voltage potential of 60mV at pH 7.6). A small current (1.2 $\mu$A ± 1.1 $\mu$A, n=9) was seen for $K_{2p}15.1$ EYG when expressed in oocytes, but $K_{2p}15.1$ GYG (0.45 $\mu$A ± 0.28 $\mu$A, n=13) was no different from water injected controls at 60mV at pH 7.6 (0.35 $\mu$A ± 0.24 $\mu$A, n=20). Co-expression of $K_{2p}15.1$ EYG or $K_{2p}15.1$ GYG alongside $K_{2p}3.1$ or $K_{2p}9.1$ did not alter the current recorded. AK2P3.1ΔV411 mutant channel tagged with green fluorescent protein (eGFP) was used as a tool to look at the heterodimerisation using immunofluorescence microscopy. The $K_{2p}3.1$ΔV411 mutant is unable to bind cytosolic protein 14-3-3, and this loss of interaction has been shown to prevent surface expression of the channel (O’Kelly et al., 2002). Here the mutant $K_{2p}3.1$ΔV411 channel was expressed in COS-7 cells, and was able to overcome intracellular retention by co-expressing with wild type $K_{2p}3.1$, $K_{2p}9.1$ or $K_{2p}15.1$. COS-7 cells transfected with the eGFP-tagged $K_{2p}3.1$ΔV411 mutant alongside wild type channels were analysed using flow cytometry to quantify the level of surface expression. In addition the interaction between the three acid-sensitive $K_{2p}$ channels was characterised at a biochemical level using co-immunoprecipitation. Together this data provides the first evidence that $K_{2p}15.1$ EYG is functional, and that $K_{2p}15.1$ interacts with $K_{2p}3.1$ and $K_{2p}9.1$. Given that these channels are co-expressed in a number of tissues the physiological significance of these results is far reaching.


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P2X₂ receptor operates as a symmetrical trimer

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P2X receptors are ligand-gated ion channels gated by extracellular ATP. Several approaches indicate that P2X receptors have a trimeric structure; this is different from other ion channel families, such as glutamate and nicotinic receptors. Whole cell and single channel patch clamp recordings were used to study the functional contribution of each channel subunit in rat P2X₂ receptors expressed in HEK293 cells. We used a previously described reporter mutation [T339K] in the second transmembrane domain of the receptor (Cao et al., 2007). This mutation markedly reduced unitary conductance (7.3 ± 0.4 pS, n = 8, at -120mV), compared to wild type (49.3 ± 4 pS, n = 7, t-test, p < 0.01) channels. Wild type P2X₂ receptors typically show strong inward rectification when activated by ATP (at sub-EC₅₀ concentrations, 3 μM); however, P2X₂[T339K] channels exhibit both inward and outward rectification in whole cell recording.

First, we co-expressed wildtype P2X₂ and P2X₂[T339K] subunits in HEK293 cells, and we measured single channel conductance and rectification. After co-expression of wild type and [T339K] subunits, we observed four levels of unitary conductance (I: 8.7 ± 0.1; II: 17.3 ± 1; III: 29.4 ± 1.2; IV: 52.8 ± 1.9 pS, n = 18). The appearance of new levels (II and III) intermediate between those of wild type and [T339K] homomers suggests the assembly of two species of heteromeric channels. Second, we expressed the series of P2X₂ trimeric concatamers that contained the [T339K] substitution in one or more of the first, second or third subunit of the concatamer (i.e. position 1, 2 or 3). Concatamers with three wild type or three [T339K] subunits had unitary conductance similar to those observed with the corresponding monomers (i.e. Thr in position 1, 2 and 3: 45.1 ± 2 pS (n = 9) and with Lys in position 1, 2 and 3: 7.9 ± 0.3 pS, n = 7). Concatamers with one Lys-containing subunit had a unitary conductance similar to level III (in position 1: 26.4 ± 0.8 pS; position 2: 29 ± 1 pS; position 3: 22.9 ± 1.4 pS, n = 4 - 8, ANOVA p > 0.05). Concatamers with two Lys-containing subunits had unitary conductance similar to level II (positions 1+2: 15.4 ± 1.1 pS; positions 1+3: 13.2 ± 0.7 pS; positions 2+3: 13.5 ± 2.3 pS, n = 4 – 8, ANOVA p > 0.05). Thus, the opening levels of the concatamers were independent of the position (i.e. subunit) that carried the mutation [T339K]. Measurement of the rectification shown by concatamers also indicated a progressive contribution by each subunit that was also independent on the position of mutation. In summary, these experiments show that the rat P2X₂ receptors operates as a symmetrical trimer in which each of three subunits contribute equally to the permeation properties of the open channel.


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Lysophosphatidic acid promotes keratinocyte migration through Orai1- and lipid raft-mediated calcium mobilization and NFAT activation

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Lysophosphatidic acid (LPA) enhances cell motility in many cell types and is thought to promote metastasis as well as wound repair. LPA is well known to elicit mobilization of intracellular calcium ([Ca²⁺]ᵢ) but the physiological consequences downstream of this phenomenon remain incompletely explored. Here we tested the wound healing-related hypothesis that LPA-activated calcium fluxes affect the motility of normal human epidermal keratinocytes through the calcium-responsive NFAT signalling pathway. Moreover, we characterized LPA-evoked calcium mobilization, investigating if LPA-induced calcium signalling was modulated by the extracellular calcium concentration [Ca²⁺]ₒ and analyzing the involvement of Orai1 channels and lipid rafts. The effect of LPA on keratinocyte migration was assessed over 14h using a 2-dimensional wound motility assay and a 3-dimensional chemotactic migration assay. As expected, 10 μM LPA significantly promoted both 2- and 3-dimensional cell migration. Pre-treatment for 1h with the NFAT pathway inhibitor cyclosporin A (CsA, 1 μM) significantly impaired LPA-induced migration, indicating that LPA induces keratinocyte migration through the NFAT pathway. As monitored by Fluo-4 imaging, LPA stimulation of keratinocytes in 60 μM [Ca²⁺]ₒ evoked short-lived (<3 min) transient [Ca²⁺]ᵢ elevations due to store release, while in 1.2 mM [Ca²⁺]ₒ, LPA triggered a peak elevation of [Ca²⁺]ᵢ followed by a plateau elevation extending over 10 min, indicating store release coupled to extracellular calcium influx. As expected, manganese quenching blocked calcium influx. Interestingly, calcium influx was blocked by diethylstilbestrol (DES) but not by the SK&F96365 or MRS-1845. Transient expression of dominant/negative Orai1 R91W and lipid raft disruption using methyl-β-cyclodextrin treatment also impaired LPA-evoked calcium entry. NFAT activity was assessed using a luciferase reporter assay and by monitoring the nuclear translocation of GFP-tagged NFAT2. Both assays revealed modest activation of NFAT by LPA-evoked store release. LPA-evoked store release coupled to influx in 1.2 mM [Ca²⁺]ₒ resulted in a much more robust activation of NFAT, which was blocked by addition of DES, expression of mutant Orai1 R91W and lipid raft disruption. Our data thus indicate that LPA promotes keratinocyte migration by triggering [Ca²⁺]ᵢ influx, which activates a NFAT signalling cascade via Orai1 and lipid rafts, firstly suggesting an involvement of NFAT in epidermal wound healing.
The Ca\(^{2+}\)-stimulated adenylyl cyclase type 8 (AC8) generates dynamic patterns of cAMP signal in response to local Ca\(^{2+}\) events to coordinate the actions of Ca\(^{2+}\) and cAMP (1). Previous studies have revealed that AC8 is uniquely sensitive to sub-\(\mu\)M Ca\(^{2+}\) rises mediated by capacitative Ca\(^{2+}\) entry (CCE). In contrast, AC8 displays little sensitivity to Ca\(^{2+}\) release from ER stores and to other forms of Ca\(^{2+}\) entry, including ionophore-mediated entry (2). Part of the selectivity of AC8 for CCE is thought to rely on its targeting to lipid rafts alongside CCE channels (3).

Here, we have tethered a genetically-encoded Ca\(^{2+}\) sensor, GCaMP2 (4), to the N-terminus of AC8 to directly monitor Ca\(^{2+}\) changes within the immediate vicinity of the AC when exposed to a range of Ca\(^{2+}\) stimuli in HEK293 cells. Comparisons were made between the GCaMP2-AC8 sensor and GCaMP2-AC2 (AC2 is a Ca\(^{2+}\)-insensitive, non-raft targeted AC). All experiments were performed at RT using a CCD-camera imaging system. In situ calibrations provided K\(_d\) values of 316nM for GCaMP2-AC8 and 240nM for GCaMP2-AC2 with Hill coefficients \(\sim\)3.0 (consistent with previous data for untagged GCaMP2 (4)). To compare the response of the targeted sensors to different modes of Ca\(^{2+}\) increase, the muscarinic agonist, carbachol, was used to trigger ER store depletion and CCE was then evoked upon addition of 2mM external Ca\(^{2+}\). GCaMP2-AC2 detected a 7.26 \pm 0.91 fold larger Ca\(^{2+}\) signal during IP\(_3\)-mediated Ca\(^{2+}\) release than during CCE (n=34 cells). In GCaMP2-AC8 expressing cells this ratio was decreased to 1.73 \pm 0.20 (n=87; p<0.01) with \(\sim\)50% of cells detecting no Ca\(^{2+}\) rise during IP\(_3\)-mediated release. Furthermore, the rate of signal increase during CCE was faster for GCaMP2-AC8 (26.8 \pm 2.0s to peak) than for GCaMP2-AC2 (77.4 \pm 5.2s to peak) suggesting that AC8 resides much closer to sites of CCE when exposed to a range of Ca\(^{2+}\) stimuli than AC2.

Differential distribution of AC8 and AC2 into sub-domains of the plasma membrane that differ, not only in terms of their lipid composition (raft vs. non-raft), but also by their exposure to distinct local Ca\(^{2+}\) signals, serves to optimise interplay between dynamic Ca\(^{2+}\) events and AC8-mediated cAMP production.


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### Selectivity of adenylyl cyclase type 8 for specific modes of Ca\(^{2+}\) increase is attributable to its localization in a protected microdomain

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### Clopidogrel-induced calcium transients in isolated rat dorsal root ganglia neurons

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Extracellular signalling by purine nucleotides exerts a wide range of biological effects including platelet aggregation, exocrine and endocrine secretion and nociceptive transduction. Yet, some subtypes of ionotropic purinergic receptors have been shown to be predominantly localised in the subpopulation of small nociceptive sensory neurons in dorsal root ganglia (DRG). The aim of this study was to investigate the effects of clopidogrel, the potent antithrombotic agent with high P2Y12 receptor antagonist action on intracellular calcium signalling ([Ca\(^{2+}\)]\(_i\)) in isolated rat sensory neurons.

DRG neurons were loaded with 5 \(\mu\)mol Fura-2 AM and Ca\(^{2+}\) responses were assessed by using the fluorescent ratiometry (excitation at 340 and 380 nm, and emission at 510 nm). All data were analyzed by using an unpaired t test, with a 2-tailed P level of <0.05 defining statistical significance. Clopidogrel caused a dose-dependent increase in the [Ca\(^{2+}\)]\(_i\). The mean 340/380 nm ratio was 0.75 \pm 0.02 (baseline, n=6), 0.89 \pm 0.01 (10 nM clopidogrel, P<0.01, n=6); 0.85 \pm 0.03 (baseline, n=11), 1.10 \pm 0.01 (100 nM clopidogrel, P<0.01, n=11); and 0.76 \pm 0.02 (baseline, n=32), 1.08 \pm 0.02 (1 \(\mu\)M clopidogrel, P<0.001, n=32), respectively.

These results indicate that antagonism of the ADP receptors P2Y12 by clopidogrel cause a dose dependent increase in [Ca\(^{2+}\)]\(_i\) indicating involvement of P2Y12 receptor subtype specific purinergic signalling in these sensory neurons which might have importance in nociceptive and neuropathic pain.

This study was financially supported by a grant from Firat University Research Fund.
Modulation of InsP₃ receptor-dependent Ca²⁺ signalling by the antiapoptotic proteins Bcl-2 and Mcl-1

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Members of the Bcl-2 protein family play a central role in the regulation of apoptosis. Some of their physiological effects are mediated by modulation of the ER localized inositol trisphosphate receptor Ca²⁺ release channel (InsP₃R). In previous studies we determined that Bcl-XL, an antiapoptotic Bcl-2 protein, bound to the InsP₃R to increase its sensitivity to InsP₃ and enhance spontaneous Ca²⁺ signalling (White et al., 2005; Li et al., 2007). The present study tested the hypothesis that the structurally related family members Bcl-2 and Mcl-1 had similar effects on InsP₃R-dependent Ca²⁺ handling. Bcl-2 or Mcl-1 were stably transfected into wild type DT40 cells endogenously expressing all three InsP₃R isoforms, or into DT40 cells genetically deficient in InsP₃R (DT40-InsP₃R-KO). The effect of Bcl-2 and Mcl-1 on ER store content and InsP₃-R-dependent Ca²⁺ release was monitored directly in permeabilized cells loaded with the low affinity indicator mag-fura-2. In the DT40-WT background, the steady-state [Ca²⁺]ER was significantly lower in Bcl-2 and Mcl-1 expressing cells; 30.3 ± 1 μM and 28.8 ± 1 μM respectively (mean ± SEM), compared to vector only expressing cells (35.9 ± 3 μM; n ≥ 36). The lowering of steady-state [Ca²⁺]ER by Bcl-2 and Mcl-1 was partially reversed by application of heparin (100 μg/ml), an InsP₃ antagonist, during store loading. In addition, the filling state of the ER stores in DT40-InsP₃R-KO cells expressing Bcl-2 (55.6 ± 2 μM) and Mcl-1 (52.7 ± 2 μM) was not significantly different from DT40-InsP₃R-KO controls (59 ± 2 μM; n ≥ 69). These data suggest that InsP₃R_Rs are required for Bcl-2 and Mcl-1 to regulate store content. The effect of Bcl-2 and Mcl-1 on InsP₃-mediated Ca²⁺ release was determined by recording [Ca²⁺]ER in response to 0.1 or 10 μM InsP₃ and examining the first order derivative of the release phase (d[Ca²⁺]ER/dt) as a function of [Ca²⁺]ER. Cells expressing Bcl-2 and Mcl-1 displayed faster Ca²⁺ release in response to 0.1 μM InsP₃ but not 10 μM. Thus, Bcl-2 and Mcl-1 decrease the apparent sensitivity of InsP₃R-dependent Ca²⁺ release to low levels of InsP₃. Fura-2 imaging experiments were carried out to assess the effect of Bcl-2 and Mcl-1 expression on whole cell Ca²⁺ signalling. In the absence of stimulation, intact DT40-WT cells display spontaneous InsP₃R-dependent Ca²⁺ oscillations. In cells expressing empty vector the mean oscillation frequency was 3.45 ± 0.1 mHz; however, oscillation frequency was significantly higher in both Bcl-2 (6.18 ± 0.2 mHz) and Mcl-1 (8.19 ± 0.3 mHz) expressing cells (P < 0.001, Mann-Whitney test, n ≥ 313). Taken together, these data demonstrate that Bcl-2 and Mcl-1 regulate [Ca²⁺]ER handling and InsP₃R-dependent Ca²⁺ signalling in ways that are qualitatively similar to Bcl-XL.


Oral Communications

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Isoflavone induced activation of endothelial nitric oxide synthase is associated with cellular ROS production: is F-actin pulling the strings?

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The risk of cardiovascular disease is increased in postmenopausal women and growing concerns over the use of hormone replacement therapy (HRT) have prompted a search for alternative therapy. We have shown that dietary phytoestrogens (genistein, daidzein and its metabolite equol) modulate endothelium dependent relaxation in vitro and lower blood pressure in vivo (Mahn et al., 2005). More recently, we reported that physiological concentrations of equol (100nM) rapidly (2min) activate eNOS via phosphorylation of Akt and ERK1/2 (Joy et al., 2006). In the present study, we have investigated whether equol (100nM) induced eNOS activation is associated with (i) increased production of reactive oxygen species (ROS), (ii) eNOS dissociation from caveolin-1 and (iii) changes in the F-actin cytoskeleton.

Human umbilical vein endothelial cells (HUVEC) were incubated in Krebs Henseleit buffer containing lucigenin (5μM), vehicle (DMSO) or equol (100nM) in the presence or absence of NADPH oxidase inhibitors apocynin (10 μM) and diphenylionium (1 μM) or the mitochondrial complex-I inhibitor rotenone (5 μM). HUVEC were also serum deprived for 4h before treatment (2min) with vehicle (DMSO) or equol (100nM) and confocal immunofluorescent staining for eNOS (475ex 515em). Mitochondrial superoxide production was detected in serum deprived HUVEC loaded with Mitosox dye for 30min. Cells were then treated for 20min with vehicle (DMSO), equol (100nM) or the mitochondrial complex-III inhibitor antimycin-A (100ng ml⁻¹). Similarly, Phalloidin was used to visualize F-actin by confocal fluorescence microscopy using wavelengths (nm) 560ex 625em for Mitosox or Phalloidin, and 375ex 450em for nuclear Hoechst staining.

Inhibition of NADPH oxidase and mitochondrial complex-I abrogated equol stimulated ERK1/2, Akt and eNOS phosphorylation (n=4 cultures, p<0.05 Student’s t-test). Equol increased mitochondrial superoxide production, with basal fluorescence (96 ± 5 arbitrary units, mean ± SEM, n=4 cultures) elevated by ~50% (141 ± 7, n=4 cultures, p<0.01 Student’s t-test) and stimulated ROS production was attenuated by inhibitors of NADPH oxidase and rotenone (n=4 cultures, p<0.05 Student’s t-test). Immunostaining for F-actin and eNOS revealed alterations in their intracellular distribution. eNOS was found to translocate from the membrane to the cytosol and was accompanied by


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the appearance of F-actin stress fibers, highlighting that equol may alter intracellular eNOS trafficking. In summary, equol stimulated mitochondrial ROS production may be important for activation of eNOS, with equol induced alterations in the F-actin cytoskeleton modulating mitochondrial ROS production (Felty et al., 2005) and subsequent eNOS activation.


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**C70**

**Involvement of nitric oxide, superoxide anion and heme oxygenase-1 in cytoprotective actions of white and red wine polyphenols in atherosclerosis**

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Previous evidence suggests that the content of polyphenols in alcoholic beverages are responsible for their beneficial effects in cardiovascular diseases such as atherosclerosis (1) characterised by increased oxidative stress (2). The aim of the present study was to examine the effects of wine polyphenols on the production reactive oxygen radicals and the expression of antioxidant enzymes in vascular tissue in atherosclerosis.

ApoE-deficient mice, that spontaneously develop atherosclerosis were fed a control diet or diets containing dealcoholized white (DWW) or red wine (DRW) (25 ml wine/ kg body weight) for 20 weeks. The presence of nitrotyrosine compounds in aortic tissue was decreased in mice treated with DRW for 20 weeks (76%, n=6).

The production of O2.- was increased in groups treated with DWW and DRW for 12 weeks (83 ± 10, 122 ± 10 and 125 ± 18 light units/mg protein/min for control, DWW- and DRW-treated mice, respectively, n=8, S.E.). The presence of nitrotyrosine compounds in aortic tissue was decreased in mice treated with DRW for 20 weeks (76%, n=6).

Tox-1 expression was increased in HUVEC incubated with 1% and 3% DRW for 8 h (197 and 684% for 1% and 3% DRW, respectively, n=3). The present findings suggest that physiological levels of endogenous NO and O2.- production may be involved in the protection afforded by wine polyphenols against the progression of atherosclerosis via their ability to upregulate expression of the antioxidant enzyme HO-1.


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**C71**

**Effects of cigarette smoke extracts on endothelial migration are independent of oxidative stress**

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Endothelial damage is an early step in atherosclerotic lesion formation, which is enhanced by pro-atherogenic inhibition of endothelial repair. Previous in vitro studies have suggested a role for increased production of oxygen free radicals in endothelial damage repair. Since cigarette smoke extracts induce oxidative stress, we have examined the potential role of additional smoke-induced oxidative stress in mediating inhibition of endothelial repair in a migration (scratch wound) assay. *Methods*. Confluent monolayers of human umbilical vein endothelial cells (HUVEC) were scratched with a pipette tip, creating a ~ 800 μm wound. Migration of HUVEC across the wound was assessed over 20 hours by image capture and computer analysis of wound width using the IncuCyte platform (Essen Instruments). Cigarette smoke total particulate matter (TPM) from University of Kentucky 3R4F reference cigarettes was trapped on a Cambridge filter pad, eluted in DMSO, and added to cells immediately after wounding. Free radical production was examined by loading cells with the non-fluorescent indicator dyes 2',7' dichlorofluorescein (DCFH) or dihydroethidine (DHE) 4 hours after wounding and measuring free radical generated fluorescent dye products by fluorescence microscopy. In experiments with ascorbic acid, cells were incubated with the antioxidant both for 5 hours prior to and during exposure to TPM. *Results*. Contrary to previous reports, fluorescence emitted from cells loaded with DCFH or DHE was not greater at the wound edge when compared to cells located away from the wound. (Table 1). Incubation of cells with the antioxidant L-ascorbic acid alone (200 μM) had no effect on endothelial migration in the endothelial wound repair assay and was also without effect on the ability of TPM to inhibit endothelial repair in this assay (Table 2). *Conclusion*. Our data suggest that oxidative stress is unimportant in endothelial wound repair in vitro.
and may argue against a role for oxidative stress in the inhibition of endothelial wound repair caused by cigarette smoke extracts.

Table 1. Comparison of fluorescence at and away from the wound edge in cells loaded with DCFH and DHE. *P<0.05 vs wound edge. All data are mean±s.e.m. n=24 in all cases.

<table>
<thead>
<tr>
<th>Time</th>
<th>DCFH fluorescence (arbitrary units)</th>
<th>DHE fluorescence (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound</td>
<td>3.7±0.4</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Away from wound</td>
<td>2.9±0.3</td>
<td>2.1±0.2</td>
</tr>
</tbody>
</table>

Table 2. Effects of TPM on endothelial wound recovery. All data are mean±s.e.m. *P<0.05 vs control. **P<0.05 vs TPM alone.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage area of wound/10 min</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td></td>
<td>0.0±0.1</td>
</tr>
<tr>
<td>2mM Ca²⁺-sensitive acid</td>
<td>69.3±2.9</td>
<td>11</td>
</tr>
<tr>
<td>2μM 6-mercaptopurine</td>
<td>69.3±2.9</td>
<td>10</td>
</tr>
<tr>
<td>2μM 6-mercaptopurine + 10mM Ca²⁺-sensitive acid</td>
<td>35.2±4.0</td>
<td>12</td>
</tr>
<tr>
<td>4μM TPM</td>
<td>7.5±1.7</td>
<td>12</td>
</tr>
<tr>
<td>4μM 6-mercaptopurine + 10mM Ca²⁺-sensitive acid</td>
<td>48.1±2.7</td>
<td>18</td>
</tr>
</tbody>
</table>

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2- and 3-dimentional imaging of ureteric precapillary pericytes: morphology, Ca²⁺ signalling and function

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It is well known that the control of local blood flow in capillaries is controlled by the "precapillary sphincters" (PS) although their nature and the mechanism controlling their function remain controversial. Precapillary pericytes are good candidates for regulating blood flow and perform sphincter function. However, little is known about the mechanisms controlling Ca²⁺ signalling and their contractile activity in situ. We used confocal imaging of in situ rat ureteric microvessels (17 vessels from 15 rats) loaded with the Ca²⁺-sensitive indicator Fluo-4 in order to investigate morphology, Ca²⁺ signalling and mechanical activity of precapillary pericytes. The effects of central (Phenylephrine, PhE) and local (endothelin-1, ET-1) factors as well as caffeine on Ca²⁺ signalling and contraction of precapillary pericytes and their effect on the diameter of the vascular wall of the precapillary branches of the ureteric microvessels (i.d.<10μm) have been investigated.

The ureteric microvascular tree consists of 3 generations of microvessels. The last (third) generation of the microvessel has a monolayer of smooth muscle coat in its proximal part followed by a coat of precapillary pericytes running circumferentially around the endothelium in its distal part. Third order branches give off side branches of microvessels which have only a coat of precapillary pericytes (pericytic microvessels). Precapillary pericytes formed an asymmetrical coat with a thick body located on one side of the vessel giving 2-3 fingers like processes which tightly wrapped around the endothelium. Each precapillary pericyte occupied a length of 10.10±0.48 μm of the vessel (n=15). Live staining of nuclei with propidium iodide revealed that pericytic nuclei were curved and were located in the body of the pericyte. Ureteric microvascular pericytes were totally resistant to caffeine (10mM) and PhE (10-100μM) but readily responded to ET-1 (10nM). Precapillary pericytes responded with a single spike-like Ca²⁺ transient resistant to ryanodine and removal of [Ca]o but which was abolished by SERCA pump inhibitor cyclopiazonic acid (20μM) or inhibitor of IP₃, R 2-APB (50μM). The Ca²⁺ transient induced by ET-1 consisted of an initial fast component followed by a slowly decaying sustained component producing local constriction of the microvessel which lasted for 2-3 minutes. Transmitted light imaging revealed that contraction of precapillary pericytes produced complete closure of the microvessel blocking the flow of red blood cells. The data obtained suggest that precapillary pericytes are likely to act as local sphincters controlling capillary blood flow by responding to local factors.

This work was supported by the British Heart Foundation. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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Mechanisms underlying cell toxicity of IMD-0354, an inhibitor of IkB kinase, in isolated rat ventricular myocytes

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Tumour necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β), have been implicated in Ca²⁺ dysregulation and depressed contractility in ventricular tissue (Duncan et al., 2007). These cytokines stimulate several signalling pathways, one of which results in activation of IkB kinases, phosphorylation of IkB and subsequent translocation of nuclear factor-κB (NF-κB) to the nucleus to modulate gene transcription. Inhibition of NF-κB translocation with IMD-0354 (IC50 ~250 nM) during ischaemia/reperfusion injury reduces infarct size and improves functional recovery (Onai et al., 2004). Our aim was to investigate the role of NF-κB signalling in altered Ca²⁺ regulation induced by TNF-α and IL-1β in ventricular myocytes. Rats (~250g) were sacrificed humanely and ventricular myocytes prepared using a standard collagenase/protease dispersion technique. Cells were loaded with fura-2 AM, superfused with Tyrode solution (30 °C) and stimulated electrically (1 Hz). Data are mean ± SEM and statistical comparisons made with paired or unpaired t-tests as appropriate or Friedman Repeated Measures ANOVA on ranks.

In contrast to Onai et al. (2004), our initial experiments showed IMD-0354 to be toxic; exposure to 0.1 μM IMD-0354 led to an initial increase in contractility (by 60 ± 13%, n=11; P<0.001) followed by failure of both contractions and Ca²⁺ transients (Fig. 1A) before the cell entered rigor, reminiscent of complete metabolic inhibition (Lancaster & Harrison, 1998). Time from application to rigor was dose-dependent; 335±28, 417±39, 499±32, 751±78 s at 1.0, 0.3, 0.1 and 0.03 μM IMD-0354, respectively. The initial increase in contractility was associated with an increase in myofilament sensitivity (P<0.05, n=11) assessed from plots of cell length vs fura-2 fluorescence (Spurgeon et al., 1992). During the phase of contractile failure myofilament
sensitivity was significantly decreased (P<0.05, n=11), time to peak contraction accelerated (P=0.002), time to half relaxation reduced (P=0.005, Fig. 1B), consistent with an intracellular acidosis.

DMEM and Tyrode supplemented with foetal calf serum (FCS) protected cells from IMD-0354 toxicity (contractility was stable after >38 min exposure to 0.1 μM IMD-0354 with FCS present). Time to the development of a rigor contracture was not delayed significantly by cyclosporin A (P=0.214).

These data illustrate that IMD-0354 is toxic at concentrations 10-fold below the IC50 for IκB kinase inhibition unless superfusion solutions contained FCS. This may suggest that IMD-0354 toxicity does not result from blockade of NF-κB activity.


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Ca2+ efflux via the sarcolemmal Ca2+ ATPase occurs only in the t-tubules of rat ventricular myocytes

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Ca2+ influx into cardiac ventricular myocytes, via the L-type Ca2+ current, appears to occur mainly across the t-tubule membrane. Ca2+ efflux, via Na+/Ca2+ exchange (NCX), also appears to occur predominantly, but not exclusively, across the t-tubule membrane (1). However the location of the sarcolemmal Ca2+ ATPase is unknown. We have therefore investigated the distribution of Ca2+ efflux via the Ca2+ ATPase between the t-tubule and surface membranes.

Ventricular myocytes were isolated from the hearts of male Wistar rats, and detubulated as described previously (2). Intracellular Ca2+ was recorded using fluo-3 in conjunction with confocal microscopy during electrical stimulation at 0.5 Hz. Following a train of stimuli, 20 mM caffeine was used to release Ca2+ from the sarcoplasmic reticulum (SR); this was repeated in the presence of 10 mM NiCl2, to inhibit NCX, or following 8-10 minutes incubation with 20 μM carboxyeosin, to inhibit the sarcolemmal Ca2+ ATPase, in control and detubulated myocytes. The proportion of Ca2+ removed from the cytoplasm by different pathways was calculated from the rate constants of decline of the electrically stimulated Ca2+ transient, and those obtained in the presence of caffeine, as described previously (3).


This work was supported by the British Heart Foundation.

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FTY720, a sphingosine 1-phosphate analogue, prevents ischemic/reperfusion-induced cardiac arrhythmias in an ex vivo rat heart model via activation of p21-activated kinase/protein kinase Akt signaling

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Recent studies demonstrated a role of Sphingosine-1-phosphate (S1P) in protection against the stress of ischemia/reperfusion injury (I/RI). In experiments reported here, we have investigated the signaling through the S1P cascade by FTY720, a sphingolipid drug candidate displaying structural similarity to S1P, underlying the S1P cardio-protective effect. In ex vivo rat heart and isolated sino-atrial node models, FTY720 significantly prevented I/RI induced arrhythmic events including premature ventricular beats, VT and sinus bradycardia as well as A-V conduction block. Real-time PCR and Western blot analysis demonstrated the expression of the S1P receptor transcript pools and corresponding proteins including S1P1, S1P2 and S1P3 in tissues dissected from sino-atrial node, atrium and ventricle. FTY 720 (25 nM) significantly blunted the depression of the levels of phospho-Pak1 and phospho-Akt with ischemia and with reperfusion. There was a significant increase in phospho-Pak1 levels by 35%, 199%, 205% after 5, 10 and 15 mins of treatment with 25 nM FTY720 compared with control non-treated myocytes. However, there was no significant difference in the levels non-phospho-Pak1 expression between non-treated and FTY720 treated. Phospho-Akt levels were increased by 44%, 63%, and 61% after 5, 10 and 15 min of treatment with 25 nM FTY720 respectively. Our data provide the first evidence that FTY720 prevents the arrhythmias induced by I/RI, and indicate its potential significance as an important and new agent protecting against ischemia/reperfusion induced arrhythmias. The cardio-protective effect of FTY720 is likely to involve activation of signalling through the Pak1 and Akt cascade.

We thank Dr James Tellez for his support. The project was supported by The Wellcome Trust (ML), The British Heart Foundation (ML, EJC) and National Institute of Health grants RO1 HL 64035 and PO1 HL 62426 (Project 1) (RJS).

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Diurnal variation in excitation-contraction coupling in rat ventricular myocytes: sensitivity to b-adrenergic stimulation

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Many pathological cardiovascular events show morning prevalence, possibly reflecting diurnal changes in cardiac haemodynamics, metabolism and sympathetic activity. Whilst around 10% of rat cardiac gene expression displays diurnal variations, little is known of whether this impacts on cardiac excitation-contraction (E-C) coupling. We have, therefore, set out to determine whether cardiac E-C coupling shows diurnal variation and if this influences the arrhythmia threshold of ventricular tissue to sympathetic stimulation.
Single ventricular myocytes were isolated from hearts excised at two opposing timepoints, ZT3 and ZT15, where ZT0 refers to “lights on”. [Ca^{2+}]_i was measured using Fura-2, cell shortening with a cell-edge detection system and L-type Ca^{2+} current density using the whole cell, patch clamp technique. The data show that basal percent cell shortening and systolic [Ca^{2+}]_i was significantly higher in ZT3 than ZT15 myocytes; with a 3% cell shortening of 12.4±0.3 in ZT3 myocytes (n=209) vs 11.0±0.2 (n=216) in ZT15 myocytes (S.E.M; P<0.05), and a peak systolic [Ca^{2+}]_i of 422±12nM (n=166) in ZT3 vs 341±9nM (n=176) in ZT15 myocytes (S.E.M; students t-test, P<0.01). The SR Ca^{2+} content revealed by the application of 20mM caffeine was significantly higher in ZT3 myocytes, with a peak Ca^{2+} of 672.8±20.5nM (n=71) vs 550.9±12.9 (n=97) in ZT15 myocytes (S.E.M; students t-test, P<0.001). We looked at β-adrenergic stimulation with isoprotenerol (ISO) to simulate sympathetic activity. We found no significant difference in EC_{50} of ISO-stimulation of systolic Ca^{2+}, but a significant reduction in the steady-state response at concentrations >3nM in ZT15 myocytes, with a maximum systolic [Ca^{2+}]_i, recorded in 100nM ISO, of 2.330±402nM vs 3nM ISO, of 2,330±402nM in ZT3 myocytes vs. 1,384±109nM (n=9) in ZT15 myocytes (S.E.M; students t-test, P<0.01). A similar diurnal variation was shown in the response of the L-type Ca^{2+} current to ISO, with a maximal percentage increase in current density of 67.0%±8.5% (n=15) in ZT3 myocytes compared to 37.4%±5.2 (n=16) in ZT15 myocytes, (S.E.M; students t-test, P<0.01.) In addition to any diurnal variation in the arrhythmia threshold to ISO. The percentage of ZT3 myocytes that developed arrhythmias was significantly greater than ZT15 myocytes, with a peak Ca^{2+} of 43.3±12nM (n=198) in ZT3 vs 34.8±9.5nM in ZT15 (n=201) (S.E.M; student t-test, P<0.01). Our data shows the existence of diurnal variation in E-C coupling, at rest and in response to β-adrenergic stimulation. The data also show diurnal variation in the threshold for arrhythmogenesis to sympathetic stimulation.

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The b2-adrenoceptor agonist, clenbuterol, decreases force in isolated intact mouse fast and slow skeletal muscle fibres

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Clenbuterol is a β2-adrenoceptor agonist, structurally similar to adrenaline, used in the treatment of asthma. Although its primary action is bronchodilation, there is growing evidence that it, like other β2-adrenoceptor agonists, may also have anabolic effects when used for prolonged periods (Lynch & Ryall, 2008). However, the mechanism(s) underlying the anabolic effects of β2-adrenoceptor agonists is still poorly understood. It is also uncertain whether β2-adrenoceptor agonists have any direct effects on muscle contraction. Therefore, the primary aim of this study was to investigate the effects of clenbuterol on the contractile properties of isolated intact mouse fast and slow twitch skeletal muscle fibres. All the experiments were performed at 20±0.1°C on small muscle fibre bundles isolated from either the extensor digitorum longus (fast twitch muscle) or soleus (slow twitch muscle) of CD-1 mice 46±1.6 (n=9; S.E.M) days old. The mice were humanely killed and all the experiments conformed to the Animals (Scientific Procedures) Act 1986. The fibre bundles were mounted horizontally between a force transducer and a servomotor and were continuously perfused with mammalian Ringer’s solution. Twitch and tetanic contractions were then recorded in Ringer’s solution with or without any added clenbuterol. In another experiment, the fibre bundles were treated for 1hr with the standard Ringer’s solution or the standard Ringer’s solution with clenbuterol. Proteins isolated from these bundles were then immunoblotted for the levels of phosphorylated AKT, extracellular signal regulated kinases 1&2 (ERK1&2) and phospholamban.

At all concentrations investigated (100nM - 250μM), clenbuterol decreased twitch and tetanic contractions in both fibre types. Used at concentrations <150μM, its effects were completely reversible. However, above this concentration they were not. For example, 50μM clenbuterol reversibly decreased twitch tension to 72±4% and tetanic tension to 23±8% (n=4; S.E.M) of controls in slow twitch fibres. The corresponding values in the fast twitch fibres were 81±6 and 55±4% (n=5; S.E.M), respectively, of controls. In addition to its effects on force, clenbuterol also increased the phosphorylation of ERK 1&2 and the dephosphorylation of AKT in both fibre types. In contrast, it increased the phosphorylation of phospholamban in slow twitch fibres but decreased it in fast twitch fibres. From these results we suggest that clenbuterol decreases force production in mammalian fast- and slow-twitch skeletal muscles by regulating the phosphorylation of phospholamban. Lynch GS & Ryall JC (2008). Role of β-adrenoceptor signalling in skeletal muscle: Implications for muscle wasting and disease. Physiol Reviews

This research was funded by the University of East Anglia. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
increasing transcription of cytoprotective proteins though activation of redox-sensitive transcription factors such as Nuclear Factor kB (NF-kB) and Activator protein-1 (AP-1). ROS may play a crucial role in ageing and increased mitochondrial superoxide generation is implicated in this process. We previously showed aberrant DNA binding activity of NF-kB and AP-1 in muscles of old mice compared with adult both at rest and following contractile activity. Mice lacking Cu, Zn superoxide dismutase (SOD1; Sod1−/− mice) show accelerated loss of skeletal muscle mass and function (1). The aim of this study was to examine ROS in isolated skeletal muscle fibres from adult Sod1−/− mice and the effect of a lack of SOD1 on the adaptive responses in muscle following contractile activity.

Adult C57/BL6 wild type (WT) mice and Sod1−/− mice were anaesthetised (65mg/100g pentobarbitone sodium) and the hind limbs subjected to an isometric contraction protocol (2). Immediately following the contractions mice were killed according to UK legislation. Single muscle fibres were isolated from the flexor digitorum brevis (FDB) and intracellular superoxide activity monitored using dihydroethidium (DHE) at rest and after electrically stimulated contractions (0.5 sec every 5 sec at 50 Hz for 15 min). Apocynin (an inhibitor of NAD(P)H oxidases) was used to determine the potential source of ROS. Gastrocnemius muscles were analysed for NF-kB and AP-1 DNA binding activity by Electrophoresis Mobility Shift Assay and for components of the NF-kB activation pathway by western blotting. Data indicated aberrant DNA binding activity of AP-1 and NF-kB in muscles of Sod1−/− mice, similar to that seen in muscles of old WT mice (2) and identified p50 and p65 as components of the NF-kB complex. Phosphorylated IkBα was increased by ~60% in muscles of Sod1−/− mice. Electrical stimulated contractions of FDB fibres from WT mice induced a significant increase in ethidium fluorescence that was abolished by apocynin treatment. There were no differences in ethidium fluorescence in quiescent muscle fibres from 6 month Sod1−/− mice compared with fibres from WT mice. These findings indicate that a lack of SOD1 causes attenuation of adaptive responses of skeletal muscle to contractile activity analogous to that seen in muscle from aged mice, but this is not associated with increased cytosolic superoxide activity measured by oxidation of DHE.


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PBS after which they were incubated in fresh media, containing 11.1mM D-glucose, 2mM L-glutamine, in the absence or presence of IL-6 (50pg/ml) added. After 24h incubation, a media aliquot was removed and used for quantization of insulin, D-glucose, glutamate, urea and nitrites; while the cells were used for the measurement of glutathione metabolism, AMPK/AMPK-P e iNOS expression. After the 24h incubation, the cells were stimulated acutely for 40 min in the presence of 1.1mmol/l glucose followed by 20 min in the presence of 16.7mM glucose and 10mM alanine, when an aliquot of the incubation medium was removed and analyzed for acute insulin secretion. At lest three different experiments were made (*P ≤ 0.05). IL-6 incubations increase insulin secretion over 38% (1379 ± 162ug/mg protein/24h against 994 ± 151ug/mg protein/24h from the control group). Moreover, IL-6 not only increase the chronic insulin secretion but also induced changes in the basal and acute stimulated levels. Basal levels of insulin secretion were increased by almost 100% in the presence of IL-6 (4.8±2ug/mg protein/20min from the control group to 9.6±3.2ug/mg protein/20min with IL-6) indicating an improvement on b-cell sensitivity. AMPK levels were decreased by 75% with a concomitant increased expression of AMPK-P by 84%. We also found a raised iNOS expression together with an intensified nitric oxide production, as measured by nitrite release (0.34 ± 0.12umol nitrite/mg protein/24h from the control groups to 3.59±0.86umol nitrite/mg protein/24h with IL-6 incubation). Both enzyme activities have been suggested as mediators for the increased insulin secretion. IL-6 did not induce redox changes as measured by the glutathione metabolism. Those results indicate that IL-6 can exert positive effects on the b-cell metabolism, protection and function. IL-6 may act as a communication factor between skeletal muscle cells and pancreatic b-cells after exercise, so elevating insulin secretion to achieve optimal concentrations for glucose uptake and metabolism by contracting muscle.

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**Hypoxia triggers oxidative-nitrative stress in the human brain**


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Oxidative stress may be responsible for the reduction in systemic nitric oxide (NO) bioavailability and impaired neurovascular reactivity recently observed during acute exposure to inspiratory hypoxia (Bailey et al., 2009). However, the brain’s contribution to these systemic changes in redox-homeostasis remains unknown.

Therefore, the present study determined the trans-cerebral exchange kinetics of oxidative-nitrative stress biomarkers in response to hypoxia. We hypothesised that hypoxia would increase the net cerebral output of free radicals that would inactivate NO as indicated by a decrease in the net uptake or consumption of nitrite (NO2-) and increased output of 3-nitrotyrosine (3-NT).

Ten healthy males aged 27 (mean) ± 4 (SD) years were examined in normoxia and following 9h passive exposure to hypoxia (12.9%O2). Global cerebral blood flow (CBF) was measured using the Kety-Schmidt technique with paired samples obtained from the radial artery and internal jugular vein. Global cerebral plasma flow (CPF) was determined as CBF x (1-haematocrit). The serum concentration of spin-trapped α-phenyl-tert-butyl-nitrone (PBN)-adducts was assessed via X-band electron paramagnetic resonance spectroscopy. Plasma NO2- was determined by ozone-based chemiluminescence using modified triiodide reagent and 3-NT via ELISA. Trans-cerebral net exchange was calculated as the arterio-jugular venous concentration difference x CPF. Data were analysed with a two-way repeated measures ANOVA and post-hoc Bonferroni-corrected paired samples t-tests.

Despite a marked reduction in PaO2 (107 ± 6 to 46 ± 3 mmHg, P < 0.05), the cerebral metabolic rate for O2 was preserved (2.43 ± 0.54 in normoxia vs. 2.49 ± 0.34 in hypoxia, P > 0.05) due to an increase in global CBF (85 ± 15 in normoxia vs. (PaCO2-corrected) 123 ± 24 mL/100g/min, P < 0.05). Hypoxia increased the net cerebral output of PBN-adducts identified as lipid-derived alkoxyl radicals (-73 ± 192 vs. -360 ± 253 AU/g/min, P < 0.05). This was associated with an attenuation in the net uptake of NO2- (126.4 ± 93.9 vs. 16.0 ± 46.7 nmol/g/min, P < 0.05) and increased output of 3-NT (-3.1 ± 9.0 vs. -10.7 ± 18.2 nmol/g/min, P < 0.05). These findings provide the first direct evidence for increased oxidative-nitrative stress in the hypoxic human brain. The regional loss of NO2- likely reflects the combined effects of NO “consumption” to support the observed increase in CBF to preserve cerebral O2 delivery and NO “loss” subsequent to oxidative inactivation by superoxide.


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Effects of systemic inflammation on arterial hypoxaemia and the alveolar-arterial oxygenation gradient during acute inspiratory hypoxia in healthy humans

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Systemic inflammation is associated with mild arterial hypoxaemia by compromising oxygen (O2) diffusion (Preas II et al, 2001) and may therefore augment the widening of the alveolar-arterial oxygen (Aa) gradient observed upon exposure to hypoxia. In the current study, we tested the hypothesis that lipopolysaccharide (LPS) infusion, a human experimental model of systemic inflammation, would further compound arterial hypoxaemia and increase the Aa gradient during acute exposure to inspiratory hypoxia in healthy humans. Twenty four healthy male volunteers aged 25 (mean) ± 4 (SD) years old were enrolled in the study after ethical approval and informed consent. In a double-blind fashion, subjects were randomised to

1) hypoxia for 12 hours (12.9% O2) and a four-hour intravenous infusion of saline from 4-8 hours, n = 11
2) hypoxia for 12 hours (12.9% O2) and a four-hour intravenous infusion of LPS from 4-8 hours (total dose of 0.3 ng/kg), n = 13

Arterial blood samples were obtained and the Aa gradient was calculated at 0, 4, 9 and 12 hours as FIO2(pAtm – pH2O) - (PaCO2/RQ) + (1-RQ / RQ) - PaO2, where FIO2 denotes the inspired fraction of O2, pAtm is the prevailing atmospheric pressure, pH2O is the partial pressure of water, PaCO2 is arterial PCO2, PaO2 is arterial PO2, and the respiratory quotient (RQ) is assumed to be 0.8.

Inspiratory hypoxia reduced PaO2 and oxygen saturation and induced a hyperventilatory response with decreases in PaCO2 (P < 0.0001 for all, ANOVA), whereas the Aa gradient increased (P < 0.01, ANOVA). LPS infusion induced systemic neutrocytosis, increased the core temperature (P < 0.01 for both, random mixed model) and potentiated the effect of inspiratory hypoxia on the Aa gradient (P < 0.01, random mixed model; Figure 1), but affected neither the severity of arterial hypoxaemia nor the hyperventilatory response (both NS, random mixed model). In conclusion, the present findings indicate that systemic inflammation may augment the effects of acute inspiratory hypoxia on the pulmonary circulation.

Haemostatic response to inspiratory hypoxia and physical exercise; interpretive implications of plasma volume shifts

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During physiological stress, plasma is shifted to the extravascular space resulting in a concentration of diffusible blood constituents. This haemoconcentration is important to most haemostasis factors because they are too large to pass through endothelial pores with plasma. Research has established that blood is hyper-coagulable after exercise in normoxia, as seen by a shortening of activated partial thromboplastin time (aPTT). However results for prothrombin time (PT) & thrombin time (TT) remain equivocal. In hypoxia, studies have shown both activation & suppression of coagulation. Reasons for these differences have been attributed to the use of varying protocols & study populations. Both hypoxia & exercise independently contract plasma volume (PV), yet interpretive implications of this remain unexplored. The purpose of the present study was to evaluate & compare the independent effects of hypoxia & exercise on coagulation times with & without correction of PV. 18 healthy males were recruited & administered 6 hours passive exposure to normobaric hypoxia (Fraction of inspired oxygen [FIO2] 12%). After exposure, they performed an incremental cycling test to exhaustion. Blood was sampled at three time points: FIO2 21% [Normoxia rest], after 6 hours exposure [Hypoxia rest] & post hypoxic exercise [Hypoxia exercise]. It was then tested for plasma levels of Fibrinogen (FB), PT, TT & aPTT. Comparing all results to normoxia rest, in uncorrected data (PV-) there were no significant differences in any marker at hypoxia rest. However, FB was significantly increased and aPTT significantly shortened at hypoxia exercise. PT and TT remained unchanged. Upon correction for PV (PV+) the changes in FB and aPTT were abrogated & there were significant elongations of PT & TT at hypoxia exercise. FB & aPTT remained unchanged. These data suggest blood coagulation is affected by PV. The question of whether or not to correct for plasma volume remains a clinically important concept that deserves consideration.
Oxygen uptake kinetics and cardiac output during cycling in females with type 2 diabetes mellitus

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The dynamic response of oxygen uptake during submaximal cycle exercise appears to be slowed in subjects with type 2 diabetes mellitus compared to non-diabetic controls. This slowed VO2 response may be due to slowed responses of cardiac output, limb blood flow and/or oxygen conductance, although human data are lacking. This human study examined the effect of type 2 diabetes mellitus on cardiac output and the dynamic response of VO2 during three intensities of cycling.

Nine middle-aged type 2 diabetic and nine healthy non-diabetic females were recruited for the study. Initially, the ventilatory threshold (VT) and peak VO2 were determined using a maximal graded cycle test. Then subjects completed a series of constant-load exercise bouts (7 min long) on separate days, during which the dynamic response of VO2 was assessed three times, and cardiac output once, at each of three intensities (50%VT, 80%VT & 50%(peakVO2–VT)). Cardiac output was recorded by a closed circuit inert gas rebreathing technique at rest and at 300th & 220th sec of the bout. Dynamic response parameters (e.g., time constant) of the VO2 response were determined by fitting a monoeponential function to the VO2 data. Ethical approval was granted by the Trinity College Dublin, Faculty Research Ethics Committee. Results were analyzed using a one-way ANOVA and are shown as mean±SD.

The time constant was significantly larger (P<0.05) in the diabetic group compared with the non-diabetic control group at 50% VT (43.9±14.6 s diabetics, 29.3±11.7 s non-diabetic controls) and 80% VT (47±7.8 s diabetics, 35.4±6.3 s non-diabetic controls); but not at 50% [peak VO2–VT] (47.7±10.1 s diabetics, 47.4±6.5 s heavy controls). Cardiac output responses during exercise (both at 30 and 220 s) were not different between the two groups.

The results confirm that type 2 diabetes mellitus slows the dynamic response of VO2 during light and moderate exercise in females; but that this is probably not related to a slowed cardiac output.


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Analysis of Timed Up and Go (TUG) test in young and elderly humans and in elderly fallers

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The TUG test is widely used in the clinical assessment of postural stability – with some health authorities asking for an annual assessment for all patients over 65. The subject has to stand up from a chair, walk round an object 3 metres in front of the chair and sit again. Clinically, the test has both high sensitivity and high selectivity with TUG times of greater than 14 seconds being associated with a high risk of falling for older adults living in the community (Shumway-Cook et al., 2000 – but note recent dissent from the Tromso study: Thrane, 2007). Our study compared 34 young subjects (mean 28 SD 10 years) 43 elderly subjects (72 SD 5) and 20 elderly fallers (75 SD 4, who self-reported an average of 1.8 trips in the previous year). The TUG test was completed in a laboratory setting and was measured by CODA mpx30 (Charnwood Dynamics, UK) 3D motion analysis. The test was undertaken five times by each subject with data averaged from the last three.

TUG times were progressively slower in the elderly fallers (12.3s SEM 1.0) compared to the elderly (9.2s SE 0.3) and the young subjects (7.7s SEM 0.2). These changes were related mainly to changes in the stride length rather than cadence. A regression of TUG times against the ABC balance confidence scale showed an equal increase in TUG times with decreasing confidence. However, elderly fallers showed an equal increase in TUG times with decreasing confidence of both the elderly and elderly fallers but accounting for only a minor part of variance of TUG times (R 0.27 and 0.34 respectively). Young subjects showed only very small variance in confidence.

Regression of the sit to stand transition (S2S) at the start of the TUG test showed a high correlation with both the elderly and elderly fallers but accounting for only a minor part of variance of TUG times (R 0.73 and 0.75 respectively). However elderly fallers were distinguished by choosing a wider stance during standing (18.1cm SEM 1.4 fallers, 14.5cm SEM 0.5 elderly, 14.3cm SEM 0.2 healthy).
Fibre size and oxidative capacity of the external anal and urethral sphincters

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The external urethral (EUS) and anal (EAS) sphincters are two striated muscles which play a fundamental role in the maintenance of urinary and faecal continence. Despite their common embryological origin and synchronised electrical activity in vivo, these muscles display different contractile properties. The aim of this study was to explain the findings from in vitro functional experiments by examining some of the structural characteristics of the EAS and EUS.

The fatigue indices of sphincters from eight female Wistar rats (200-250g) were measured. The EAS and EUS were mounted as ring preparations in tissue baths containing Tyrode’s solution maintained at 37°C and bubbled with 100% O₂. Tubocurarine (10⁻⁴M) was added to the bath solution to ensure nerve independent muscle contraction. The fatigue stimulation protocol consisted of 50 200ms trains at 50Hz which were 4 seconds apart. Data are expressed as mean ± SEM and statistically analysed with a students t-test.

The EAS was much more susceptible to fatigue than the EUS. At the end of the fatigue protocol the final contraction of the EAS had fatigued to 41.86 ± 2.73% of the initial contractile force. Conversely, the contractions of the EUS were relatively unaffected by this particular stimulation protocol, the final contraction of the EUS was 94.93 ± 3.09% of the first, p<0.001 (n=8). The absolute force produced by the muscle per cross sectional area or the specific force was measured. At 50Hz the specific force of the EAS was twice that of the EUS, 4.7 ± 0.6 mN.mm⁻² vs. 2.4 ± 0.3 mN.mm⁻², p=0.014. This suggested that the EAS is composed of larger muscle fibres than the EUS. In order to determine the average area of the striated muscle fibres in the EAS and EUS, the sphincters from three animals were oriented so that the muscle fibres were cut transversely. These sections were then incubated with antibodies against laminin (1:500) to highlight the diameter of each fibre. The areas of 450 fibres were measured form each sphincter muscle. The cross sectional area of fibres in the EAS was on average 376 ± 5 μm² while in the urethra it was 187 ± 4 μm² (P<0.0001). The smaller specific force and fibre size of the urethra coupled with its fatigue resistance indicates that it has a higher oxidative capacity than the anal sphincter. This was confirmed by succinate dehydrogenase (SDH) histochemistry. In conclusion, the voluntary muscle of urinary continence in the rat is composed of small, fatigue resistant muscle fibres in comparison to the more powerful but fatigable voluntary sphincter of the anal canal.

This work was supported by the Health Research Board and University College Dublin.

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Cholecystokinin participates in the LPS-induced hypophagia through corticotrophin-releasing-factor neuron activation

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Administration of lipopolysaccharide (LPS) decreases food intake. However, the mechanisms underlying this hypophagic effect are not well established. LPS increases peripheral production of cytokines that in turn induce cholecystokinin (CCK) secretion, a satiety-related peptide. CCK induces an activation of brainstem neurons that show reciprocal projections with hypothalamic neurons. Corticotrophin-releasing-factor (CRF) and alpha-melanocyte stimulating hormone (α-MSH) are neuropeptides with anorexigenic properties. This study aimed to investigate the effects of LPS on food intake, CRF and α-MSH neuron activation in rats pretreated with devazepide (CCK-1 receptor antagonist). Adult male Wistar rats (220–300g) were singly housed in metabolic cages with free access to chow offered in a metal container, to verify food consumption. On the day of the experiment the chow was removed and rats (n=7-11) were weighed and pretreated with devazepide (1mg/Kg, ip) or vehicle (0.5/0.5/9, tween80/DMSO/saline0.9%), rats (n=7-11) were weighed and pretreated with devazepide (1mg/Kg, ip) or vehicle (0.5/0.5/9, tween80/DMSO/saline0.9%) 30 minutes before LPS (100μg/Kg, ip) or saline (0.1ml/100g bw, ip) injection. Thereafter, food was offered for 2h (18:00-20:00h). Another set of rats (n=5-7) was pretreated with devazepide or vehicle followed by LPS or saline injection and 4h after they were anaesthetized with trisbromoethanol (1ml/100g bw, ip) and transcardially perfused with 4% paraformaldehyde for brain collection. In rats saline injected, devazepide (3.85±0.9 g/100g; p<0.01) increased food intake compared to group pretreated with vehicle (2.89±0.4 g/100g). Food intake was decreased after LPS in vehicle (1.1±0.6 g/100g; p<0.001) pretreated animals, which was reversed by devazepide (2.2±0.9; p=0.06) pretreatment. Devazepide and vehicle pretreatment in saline rats resulted in similar immunoreactivity to Fos/α-MSH (devazepide; 0.5±0.5 and vehicle 0.7±0.9) in the ARC and Fos/CRF in the medial (PaPM; devazepide 4.2±2.4 and vehicle 4.3±1.1) and posterior parvocellular subdivisions of the PVN (PaPO; devazepide 3.2±1.6 and vehicle 3.8±1.4). In the group pretreated with vehicle, LPS treatment increased Fos/CRF double labeled neurons in the PaPM (22.9±6.4; p<0.001) and PaPO (9.1±1.6; p<0.001). No change in the Fos/α-MSH double labeled neurons in the ARC was observed after LPS stimulus (devazepide 0.3±0.7 and vehicle 0.3±0.7). Pretreatment with devazepide decreased the LPS-induced Fos/CRF double labeling in the PaPM (9.5±3.6; p<0.001) and PaPO (4.8±5.5; p<0.001). The present data suggest that CCK participates in the hypophagic effect during endotoxemia through CCK-1 receptor. This effect is likely to be mediated by an activation of CRF neurons in the PVN but not α-MSH in the ARC.

Desensitization of hypophagic effects in the endotoxin tolerance is associated with a decrease of STAT-3 phosphorylation not mediated by changes in SOCS-3 mRNA expression in the arcuate nucleus

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Desensitization of hormone response and hypophagic effect to repeated exposure to endotoxin has been described. Leptin, produced by adipose tissue, reduces food intake and boosts energy expenditure via activation of JAK/STAT signaling pathway in hypothalamic neurons, via a phosphorylation of STAT-3, which in turn stimulates SOCS-3 (an intracellular molecule that triggers a negative feedback loop) gene expression in the arcuate nucleus (ARC). We evaluate the effects of exogenous leptin administration in rats under single or repeated exposure to LPS on food intake and p-STAT-3 expression in the ARC. Male Wistar rats (250-300g) received single or repeated injections of LPS (100μg/Kg ip) or saline (0.15M NaCl, 1ml/kg), between 0400h-0500 PM, during 6 days. Two hours after the last injection, rats received intracerebroventricular (icv) injection of leptin (10μg) or vehicle (saline). The anaesthetic for cannulation was trisbromoethanol (1ml/100g bw, i.p.). Food intake was determined during 24h (n=8). Another set of rats, subjected to the same protocol, were anaesthetized (as above) and transcardially perfused with 4% formaldehyde. After 20 minutes the brains were collected for p-STAT-3 immunolabeling by immunohistochemistry (n=6). A third set of rats were treated with single or repeated LPS and two hours after the brains were collected by decapitation for determination of SOCS-3 mRNA expression by real time PCR (n=7). ANOVA one or two way, followed by Student-Newman-Keuls, was used to analyze the data (mean±SEM). Single LPS decreased food intake and body weight(p<0.05), compared to control rats. In turn, repeated LPS did not change the food intake and body weight. Icv leptin reduced(p<0.05) the food intake and body weight in 6 saline treated rats, but no changes in these parameters were observed in animals under single or repeated LPS. We observed an enhancement(p<0.05) of p-STAT-3 expressing neurons in the ARC after a single LPS followed by vehicle treatment compared to control (64.5±19.4 vs 31.1±19.8), with no changes in p-
Diet high in saturated fat impairs the hypothalamic neuropeptide and melanocortin-4 receptor expression

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The hypothalamic neuropeptide neurons play the pivotal role in energy balance. The arcuate nucleus (ARC) neurons respond to peripheral hormones and nutrients through neuropeptide Y/agouti-related peptide (NPY/AgRP) and pro-opiomelanocortin/cocaine-amphethamine regulated transcript (POMC/CART). These neurons project to the paraventricular nucleus (PVN) and lateral area of the hypothalamus (LHA) where activate the second order of neurons producing e.g. orexins and melanin-concentrating hormone (MCH). The aim of this study was to investigate the effect of feeding with different fats on neuropeptide expression in the ARC and LHA, and melanocortin receptor-4 (MC4R) in the PVN. Three groups of rats were fed six weeks a low-fat diet (10% energy from fat), next three a high-fat diet (40% energy from fat) prepared with the same fat; lard - source of saturated fatty acids, sunflower oil - source of n-6 polyunsaturated fatty acids (PUFA), or fish oil rich in n-3 PUFA (8 rats/group). Throughout the experiment body weight and food intake were monitored. Before decapitation and brain removal rats were i.v. anaesthetized with ketamine (20 mg/kg) plus xylazine (10 mg/kg) and blood was drawn by heart puncture. Plasma level of glucose, leptin and insulin were measured. The ARC, PVN and LHA were punctured from frozen brain sections. NPY, galanin-like peptide (GALP), POMC, pre-orexin, MCH, MC4R expression was measured by RT-PCR. The whole hypothalamus was assayed for NPY and POMC expression by in situ hybridization (4 rats/group).

The lard diets fed rats gained the highest body weight and ate the most energy vs. rats fed the PUFA diets. The NPY expression in the ARC and compact zone of the dorsomedial nucleus in obese high-lard fed rats was lower than in low-lard, and high-PUFA fed rats. The GALP expression increased by about 50% and 40% in the ARC in high-sunflower oil and high-fish oil groups, respectively vs. rats fed the corresponding low-fat diet. POMC expression in the ARC was increased in both high-PUFA fed rats vs. that fed the corresponding low-PUFA and high-lard diets. The POMC expression in the whole hypothalamus was increased, and the pre-orexin and MCH mRNA transcript levels in the LHA were decreased exclusively in high-fish oil fed rats. The expression of MC4R in the PVN was lower in high-lard than in low-lard fed rats. The high-fat fed rats had a significantly increased leptin, insulin and glucose levels independently of the kind of dietary fat.

Summarizing, high ingestion of fish oil originate PUFA favoured anorexigenic POMC expression in the hypothalamus and down-regulated orexigenic peptides in the LHA. The high-saturated feeding failed to up-regulate POMC expression and down-regulated MC4R expression in the PVN. Our study indicates that hyperphagia caused by diet high in saturated fat may impair melanocortin transmission in the hypothalamus.

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ng/ml). Furthermore, TMX blocked the LH secretion in the afternoon of proestrous (F(1,82) = 39.28 p<0.05; Duncan p<0.05; vehicle: 28.66 ±/−7.13 ng/ml; TMX: 4.74 ±/−0.5 ng/ml) the injection of leptin did not restore the plasma LH concentration in the animals treated with TMX. Conclusion: Our results indicate that the central action of leptin on LH secretion depends on estrogen modulation.

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**Effects of a glucokinase activator on glucolipotoxic and cytokine-induced beta cell dysfunction and cell integrity**

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A pathological feature of both type 1 and type 2 diabetes is loss of pancreatic beta cell mass through increased apoptosis. The molecular mechanisms mediating increased apoptosis are unknown but new evidence links the beta cell glucose sensor “glucokinase” to beta cell survival. The proapoptotic protein BAD is localised with glucokinase in beta cells potentially linking glucose homeostasis with beta cell functional integrity.1. Exposure of islets to proinflammatory cytokines or glucolipotoxic conditions activate beta cell apoptotic pathways with concomitant decreases in beta cell insulin secretory function. Glucokinase has been implicated as a mediator of beta cell dysfunction in both cytokine and glucolipotoxic conditions2,3. Molecular compounds which bind to an allosteric activation site on glucokinase have recently been developed. GKA50 is a potent activator of glucokinase which increases enzyme activity with enhanced insulin secretion in vitro and in vivo. Given the probable role of glucokinase in the regulation of beta cell survival/apoptosis we have used GKA50 to elucidate the effects of increased glucokinase activation on glucolipotoxic and cytokine induced beta cell integrity and insulin secretion (using the rat-derived beta cell line BRIN-BD11). Apoptosis was induced by culturing cells in the presence of a lethal proinflammatory cytokine cocktail (IFNγ, IL-1β, TNFα) or chronic high glucose and lipid levels (25mM glucose, 100μM palmitate) for 24hr with or without GKA50. A thorough analysis of cell viability and apoptosis was performed. Cell apoptosis was examined by DNA fragmentation, LDH release and Trypan blue staining. Cell viability was determined by WST and neutral red assays and mitochondrial membrane potential. Chronic and acute stimulated insulin secretion was measured by ELISA. Significant cell death and impairment of insulin secretion occurred in proinflammatory or glucolipotoxic experimental conditions (p<0.05). When viability was examined after exposure to either cytokines or glucolipotoxic conditions those cells treated with GKA50 were protected from loss of membrane integrity as cells treated with GKA50 had a significantly reduced level of LDH release following incubation in glucolipotoxic conditions (p<0.05). However, DNA fragmentation which was enhanced by pro-inflammatory cytokine or glucolipotoxic conditions, or suppressed levels of insulin secretion, were not altered by GKA50 addition. Glucokinase is a critical enzyme in beta cell physiology and its activity has important implications for metabolism and insulin secretion. However an increase in glucokinase activation does not alter DNA fragmentation responses or insulin secretion after incubation in either glucolipotoxic or pro-inflammatory cytokine conditions. However our results support the hypothesis that the detrimental effects of cytokines or glucolipotoxicity occurs via a post-glycolytic event.


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**b-secretase modifies insulin signalling and GLUT4 translocation in skeletal muscle cell lines**

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β-secretase (β-site APP Cleaving Enzyme 1 (BACE1)) is a key player in the development and progression of Alzheimer’s disease (AD). Sequential cleavage of amyloid precursor protein by β- and γ-secretase produces β-amyloid peptides, the major component of plaques in AD patient brains. BACE1 has been validated as a therapeutic target for AD as BACE1 knockout (KO) mice have low β-amyloid levels. BACE1 KO mice also exhibit a lower body weight and better glucose disposal than their wild type littermates. Using C2C12 (mouse) and L6 (rat) cultured myoblasts we show that pharmacological inhibition of BACE1 enhances insulin signalling, as measured by protein kinase B phosphorylation (p-PKB). Treatment of C2C12 cells with 5 nM insulin increased p-PKB levels by 6.07 ± 1.27 fold (P < 0.001, n = 10), compared to un-stimulated cells. A 24-hour pre-treatment with 250 nM BACE1 inhibitor (1) enhanced insulin-stimulated p-PKB levels, 10.27 ± 1.52 fold (P < 0.001, unpaired Student’s t-test, mean ± SEM; n = 10) compared to control cells. Comparison of insulin-stimulated p-PKB levels in the presence and absence of the BACE1 inhibitor showed that BACE1 inhibition significantly enhanced insulin stimulated p-PKB levels (P < 0.05). L6 myoblasts were more insulin sensitive, with 0.3 nM insulin increasing p-PKB levels by 3.18 ± 0.55 fold (P < 0.01, n = 4) compared to control cells. L6 myoblasts pre-treated with BACE1 inhibitor increased p-PKB by 5.15 ± 0.79 fold in response to insulin, (P < 0.001 versus control; n = 4) and a significant enhancement in insulin sensitivity (P < 0.05). In cultured L6-GLUT4myc cells, treatment with BACE1 inhibitor per se increased translocation of the glucose transporter (GLUT4; 1.53 ± 0.08 fold; P < 0.001, n = 5). Insulin (100 nM) increased GLUT4 translocation by 1.40 ± 0.11 fold (P < 0.01, n = 5) in untreated
cells and by 1.82 ± 0.20 fold (P < 0.05, n = 5) in cells pre-treated with BACE1 inhibitor. Thus, BACE1 inhibition increases GLUT4 translocation supporting the notion that BACE1 reduction enhances glucose uptake. However, at least part of this effect in these muscle cells appears insulin-independent. Raised 5’ AMP activated protein kinase (AMPK) activity increases GLUT4 translocation independently of insulin (2). Treatment of C2C12 and L6 cells with BACE1 inhibitor increased AMPK phosphorylation (1.66 ± 0.43 fold; P = 0.06, n = 8 and 1.72 ± 0.39 fold; P < 0.05, n = 11 respectively) and phosphorylation of its substrate, acetyl CoA carboxylase (ACC; 1.72 ± 0.17 fold; P < 0.01, n = 8 and 1.33 ± 0.21 fold; P = 0.06, n = 11 respectively), indicative of enhanced AMPK activity. Thus, we propose that skeletal muscle BACE1 levels modify glucose uptake in an insulin and AMPK dependent manner.


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Protein Kinase D Modulates Aldosterone-induced ENaC Activity in Renal Cortical Collecting Duct Cells through the Regulation of Subcellular Trafficking and MR-dependent Gene Expression

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Aldosterone treatment stimulates the phosphorylation and activation of Protein Kinase D 1 (PKD1), in murine cortical collecting duct cells (M1CCD), through the transactivation of the epidermal growth factor receptor (EGFR) [1]. PKD1 belongs to a family of serine/threonine kinases known to be important modulators of subcellular trafficking, through the regulation of vesicle fission from the Golgi [2]. The epithelial sodium channel (ENaC) is a major effector of aldosterone action in the kidney and plays a crucial role in the maintenance of whole body sodium homeostasis. Active ENaC is believed to be a heterotrimer composed of one each of the alpha, beta and gamma subunits. ENaC activity is dynamically regulated by aldosterone through multiple mechanisms. The nuclear mineralocorticoid receptor (MR) in complex with aldosterone behaves as a ligand-dependent transcription factor which stimulates the tissue-specific upregulation of ENaC subunit expression. In the distal nephron, the ENaC alpha subunit is under the transcriptional control of ligand-bound MR, while beta and gamma are constitutively expressed [3].

Using M1CCD cells where endogenous PKD1 was stably knocked down, we examined the role PKD1 plays in the aldosterone-mediated regulation of ENaC activity via transcription and/or trafficking of pre-expressed ENaC subunits, over an extended period of aldosterone treatment. The amiloride-sensitive, trans-epithelial current (I_{TE}) was measured in wild-type (WT) and PKD1 suppressed M1CCD cells, grown to confluency on semi-permeable supports. A detectable rise in I_{TE} was observed in WT cells within 2h of aldosterone treatment, an effect which was maximal after 24h. The induction of I_{TE} by aldosterone was inhibited in PKD1 suppressed cells. Using immunocytochemistry and laser scanning confocal microscopy, we observed a stark increase in ENaC alpha expression in WT cells treated with aldosterone for 24h, an effect which was absent in the PKD1 suppressed cells. Moreover, using a specific plasma membrane marker, we observed an aldosterone-mediated induction of apical membrane insertion of the constitutively expressed ENaC beta subunit in WT cells. Aldosterone treatment failed to affect the subcellular localization of ENaC beta in PKD1 suppressed cells. Overall, PKD1 plays a central role in the aldosterone-mediated regulation of ENaC activity, through both transcriptional control and subcellular trafficking.


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In-utero nutritional programming of kidney development and its long term impact on renal oxidative stress following juvenile obesity

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Maternal nutrient restriction, coincident with early fetal kidney development, can increase glucocorticoid action in the newborn kidney1. Fetal adaptations in the kidney may also determine the long term consequences following juvenile obesity and the onset of hypertension. One important mechanism implicated in both obesity and renal disease is enhanced oxidative stress, leading to glomerulosclerosis. Two key regulators of this process are the angiogenic hypoxia inducible factor (HIF)-1α and the pro-apoptotic receptor Fas. The present study, therefore, examined how the early in-utero diet results in renal adaptations to oxidative stress induced by juvenile obesity. Pregnant sheep (n=26) were randomly assigned to a normal (7 MJ/day) or nutrient restricted diet (NR, 3.5 MJ/day), from 30-80 days of gestation (term = 147 days) and fed to requirements at all other times. After weaning, the NR (NR-O, n=11) and obese (O, n=7) offspring were reared in an obesogenic environment to promote fat deposition. The lean group (L, n=8) remained
Cardiovascular Response to Air-jet Stress Differs in Borderline Hypertensive Rats with Respect to Wistar Rats-Sequence Analysis of Spontaneous Baroreflex

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Cardiovascular morbidity and mortality can be triggered by emotional stress: it can cause development of arterial hypertension. Development of hypertension is also dependent on genetic background. Borderline hypertensive rats (BHR) with respect to Wistar rats (WR) develop larger cardiovascular response induced by juvenile obesity in NR offspring, with persistent changes in markers of oxidation. We have, therefore, shown, for the first time in this model of fetal programming, that increased systemic and renal oxidative stress alters the gene regulation of angiogenic and pro-apoptotic genes such as HIF-1α and Fas. These differential adaptations may delay the onset of glomerulosclerosis2 without necessarily improving vascular function.


This study was supported by the British Heart Foundation

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Six male CD rats (Charles River), kept under a 12h:12h light-dark cycle at 24±0.5°C ambient temperature were used. Under general anaesthesia (Diazepam, 5mg/Kg intramuscular; Ketamine-HCl, 100mg/Kg, intraperitoneal), animals were implanted with electrodes for EEG and EKG recording, a thermistor to measure hypothalamic temperature (Thy), a microinjection guide cannula to target VMMR. One week after surgery, rats were randomly microinjected, on different days, with 100nl of the following: i) GABA-A agonist muscimol (1mM); ii) GABA-A antagonist bicuculline (1mM); iii) 0.9% saline. Preliminary analysis of data from muscimol injection was carried out during wake in two rats. HRV was analyzed within both the time (R-R mean: R-R standard deviation (STD-RR); root mean square successive difference (RMSSD)) and frequency domains (High (HF, 0.6–2.4 Hz) and Low Frequency (LF, 0.06–0.6 Hz) bands). Effects of injections were separated into six temporal blocks according to the dynamics of changes in core temperature following VMMR inhibition (3). At the end of the experiment, animals were sacrificed and histological control was carried out. Table 1 shows changes in HRV parameters and Thy (% of baseline levels, mean ± SEM) during the six temporal blocks. The LF band, expression of CV sympathetic drive, was clearly reduced following VMMR neurons inhibition. Changes in HRV parameters appeared to be specifically due to VMMR neuron inhibition and not to changes in Thy. In conclusion, VMMR neurons seem to participate in maintaining tonic sympathetic CV outflow.

Table 1

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Oral Communications

Renal excretory responses to a volume load in conscious Wistar rats with heart hypertrophy

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Heart hypertrophy is considered to be an initial indicator of a progression into heart failure which is accompanied by an activation of the autonomic nervous system to maintain cardiac output. We reported (Flanagan et al., 2008) in anaesthetised rats that an acute volume expansion decreased renal sympathetic nerve activity (RSNA) and that this reflex was absent in rats with heart hypertrophy after isoprenaline/caffeine or thyroxine administration. At the kidney, this raised level of RSNA would cause fluid retention exacerbating the load on the heart. This study examined whether in conscious rats the ability to excrete a volume load was altered in any way in heart hypertrophy. A further objective was to examine how these responses were altered following denervation of the kidneys.

Two groups of Wistar rats (n=6) were used, one intact and the other subjected to a bilateral renal denervation 1 week previously. The animals were anaesthetised (3% isoflurane in O2) and each kidney in turn was exposed via a flank incision, all fat and connective tissue stripped from around the renal artery which was then bathed with 10% phenol in ethanol for 1 min, rinsed with saline and then muscle and skin sutured. Tail vein blood samples were obtained under brief isoflurane anaesthesia and a 24h urine collection taken. On day 1 at 10.00 am, animals received an oral gavage of tap water (2ml/100g body weight) and urine samples were collected every 2h for 6h. Thereafter, the rats received isoprenaline (5mg/kg) every 72 h for 2 weeks and drinking water containing caffeine (61.5mg/kg). At week 1 and 2, tail vein sampling, oral gavage and urine collection was repeated. Creatinine clearance (glomerular filtration rate, GFR), urine volume (UV) and absolute sodium excretion (UNaV) were determined. Data, means ± S.E.M. were taken as significant when P<0.05 (ANOVA).

The intact rats had a basal GFR of 1.73±0.25ml/min/kg, UNaV of 0.52±0.1μmol/min/kg and UV of 22.8±3.08μl/min/kg which were unchanged at weeks 1 and 2. The water load in intact rats caused cumulative increases in UV, from 3.5±0.22ml at 2h to 5.3±0.21ml 6h, and in UNaV from 1.98±0.26μmol/min/kg at 2h to 16.4±8.37μmol/min/kg at 6h. At week 2 the magnitude of the cumulative increases in UV and UNaV were reduced (both P<0.05) by some 60%. In the rats subjected to bilateral renal denervation, the water load resulted in a cumulative rise in UV, from 3.26±0.30ml at 2h to 5.96±0.22ml at 6h, and UNaV from 1.6±0.25μmol/min/kg over 2h to 7.11±0.74μmol/min/kg at 6h, responses similar to those obtained in the intact rats. The magnitude of the increases in UV and UNaV after the water load were unchanged after the 2 weeks of isoprenaline/caffeine treatment in the renally denervated group which was different from the intact group (P<0.05).

These findings demonstrated that in the isoprenaline/caffeine model of heart hypertrophy there was a blunted ability to excrete a volume load, which was most likely due to a lack of suppression of RSNA as the excretory response was unchanged following renal denervation. Exactly how the cardiopulmonary receptor sensitivity changes in heart hypertrophy remains to be explored.


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Midbrain control of micturition in the rat

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In most socialised animals voiding can be suppressed, even when the bladder is full, until the individual is in a safe and socially acceptable environment. In the rat, stimulation of bladder afferents during filling activates a spino-midbrain-spinal micturition reflex pathway that relays in the periaqueductal grey (PAG) (DeGroat, 2006). We have investigated whether the synaptic relay in the PAG could be a site of tonic inhibitory control over micturition.

Urethane anaesthetised (1.5g/Kg i.p.) male Sprague-Dawley rats were instrumented to record femoral arterial pressure, heart rate and tracheal airflow. A cannula inserted through the dome was used to measure intravesicular pressure and for infusion of saline (6ml h-1) to induce periodic (0.6±0.05 min-1, mean ± S.E.M.) increases in bladder pressure, accompanied by expulsion of urine through the urethra.

The GABA agonist muscimol (250pmol in 50nl) microinjected into the caudal ventrolateral PAG (vPAG, P-8.8 Paxinos & Watson, 1986, n=3), but not at other sites in the PAG (n=16) completely suppressed the periodic contractions. Microinjection of the GABAA antagonist bicuculline (1pmol in 50nl) into the caudal vPAG (n=13) evoked an increase in the frequency (from 0.7±0.1 to 2.3±0.4min-1, P < 0.05, Student’s paired t-test) and threshold (14.5±1.0 to 20.6±2.0 mmHg, P < 0.05) of contractions that lasted 1106±253s (range 263s-2911s). At 8/13 (68.5%) sites this effect was accompanied by a pressor response (mean arterial pressure 86.9±4.6 to 123.9±8.5 mmHg, P < 0.01), which lasted 1680±253s (range 780s-2718s), tachypnoea (158.8±5.6 to 323.1±45.9 breaths min-1, P < 0.05), exophthalmus and pupillary dilatation. At the remaining sites (n=5) there were no accompanying cardio-respiratory changes.

In contrast, bicuculline microinjected at more dorsal sites in the caudal PAG (n=14) evoked a long lasting (>60min) irreversible increase in bladder pressure (16.2±0.8 to 25.9±1.9mmHg, P < 0.01) on top of which frequent (3.1±0.5 min-1) low amplitude contractions were superimposed. These changes in bladder activity were accompanied by a sustained pressor response (88.1±3.1 to 139.1±5.6mmHg, P < 0.01), tachycardia (438.4±12.6 to 547.4±41.0 beats min-1, P < 0.01), increases in respiratory amplitude (128.1±16.7 to 316.7±44.3, arbitrary units P < 0.01) and respiratory rate (164.7±5.1 to 318.6±22.3 breaths min-1, P < 0.01) and pronounced signs of autonomic activation (exophthalmus and pupillary dilatation).

The results suggest i) the functional integrity of the caudal vPAG is necessary for reflex micturition to occur in response to bladder filling and ii) the synaptic relays in this region are under tonic GABAergic control. In addition, the dorsal PAG, whilst not essential for execution of the reflex, can facilitate contractile activity of the bladder in concert with intense sympatho-activation.

Reduced synaptic inhibition underlies respiratory apneas in a mouse model of Rett syndrome (RTT)

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RTT is a neurodevelopmental disorder caused by mutations in the X-linked gene that encodes the transcription factor methyl-CpG-binding protein 2 (Mecp2) (1). Included in the phenotype are respiratory disorders that include frequent apneas and periodic breathing that are most prevalent in young females (2). Heterozygous female mice (Mecp2+/−) with deletion of the 3rd and 4th exons of Mecp2 mimic these respiratory disturbances but the central neuronal mechanisms have not been fully determined. We have hypothesized that insufficient GABA synaptic inhibition underlies these respiratory disorders and augmenting GABA in awake animals reduces their incidence. Studies were carried out in B6.129P2(C) tm1.1Bird (3) heterozygous females (Mecp2+/-) and wild type littermates (Mecp2+/+) using an in situ preparation to record phrenic, central vagal (cVN), hypoglossal (HN) and abdominal (ABD) nerve activity (4). Animals were anaesthetized deeply with 5% halothane and once they failed to respond to noxious pinching of a paw and the tail were decerebrated. Apneas (TE ≥ 1.0 s) were more frequent in Mecp2+/- (168.2 ± 29.1/hr) (SEM) (n=9) than Mecp2+/+ mice (56.8 ± 12.9/hr) (n=13) (p= 0.005, Student t test). Apneas were characterized by prolonged postsynaptic activity in cVN (as observed previously in male Mecp2−/− mice (5)) that terminated before the end of phrenic apnea. In addition, during the apnea there was a hypoglossal nerve discharge that occurred at the time a phrenic burst was anticipated from the respiratory cycle length of preceding bursts. This continued throughout the duration of the apnea (Fig). In some apneas the abdominal nerve exhibited a sustained burst.

1-[2-[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711), 5 – 10 µM in the perfusate was used to block GABA reuptake. Apneas were reduced from 152.3 ± 12.1 to 115.3 ± 16.0/hr (p=0.005, Student t test). Apneas that remained after NO-711 were characterized by a shorter duration, lower activity in HN and absence of activity in ABD, consistent with reduced excitability of the network or increased synaptic inhibition. The results suggest that apnea in this mouse model of RTT results from, in part, insufficient GABA mediated synaptic inhibition in the ponto-medullary respiratory network leading to a loss of rhythmic phrenic bursts.

Figure: Apnea in Mecp2+/- mouse. Traces are from above: integrated HN, raw HN, integrated cVN, raw cVN, integrated phrenic and raw phrenic nerve activity.


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National Institutes of Health NS057815 (JFP)
University of Bristol Benjamin Meaker Visiting Professorship (JMB)

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Knockdown of SERCA-2 in human airway smooth muscle from healthy subjects recapitulates a phenotype associated with asthma


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Cytosolic [Ca2+]i plays a critical role in the function of airway smooth muscle (ASM) and the sarco/endoplasmic reticulum [Ca2+] ATPase (SERCA) is an important mechanism for regulating cytosolic [Ca2+]. ASM from asthmatics is characterised by increased proliferative and migratory responses, and we have reported that ASM cells obtained from asthmatic volunteers exhibit abnormal Ca2+ homeostasis arising from reduced SERCA-2 expression (Mahn et al, 2007). The aim of this study was to determine whether knockdown of SERCA-2 in ASM cells from healthy subjects could reproduce features of the pro-asth-
Ca2+ uptake, was increased from 109 ± 60 P
nated using Fura PE3; proliferation was assessed by 3[H]-thymi-
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way wall remodeling in asthma
A. Gourine1
60P
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medulla
Chemosensitive astrocytes in the rostral ventrolateral
in arterial PCO2 and pH, which results in the activation of
in long-term plasticity and NMDAR-mediated synaptic trans-

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Chemosensitive astrocytes in the rostral ventrolateral medulla
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ATP is released from the chemosensitive regions located on the
ventral surface of the medulla oblongata in response to changes
in arterial PCO2 and pH, which results in the activation of
the respiratory network (1, 2). In vitro studies using brainstem slices
have shown that increases in PCO2 or [H+] results in the gener-
ation of intracellular calcium waves ([Ca2+]i) in ventral surface
astrocytes and this effect can be mimicked by the application of
ATP. In the present study, we used an in vivo preparation to
detect astrocytic calcium responses in ventral surface astro-
cytes induced by respiratory acidosis. Methods: Six male
Sprague Dawley rats were used in accordance with the UK Ani-
imals (Scientific Procedures) Act 1986 and associated guide-
lines. Animals were anaesthetised with a mixture of ketamine
(60 mg kg⁻¹ I.M.) and medetomidine (250 μg kg⁻¹ I.M.) and
received bilateral microinjections into the rostroventrolateral medulla (2μl each side) of an adenosine vector encoding a Ca2+-
sensitive protein Case12 (3) under control of an enhanced GFP
promoter. Anaesthesia was reversed with atipemazole (250 mg
kg⁻¹ I.M.) and post-operative care was taken. Seven days after
injections, animals were anaesthetised with propofol (30 mg
kg⁻¹ h⁻¹, i.v.), paralysed with gallamine triethiodide (10 mg
kg⁻¹, i.v.; then 1–2 mg kg⁻¹ h⁻¹, i.v.) and artificially ventilated.
The adequacy of anaesthesia during neuromuscular blockade
was assessed by monitoring heart rate and blood pressure sta-
bility. The ventral surface of the medulla was exposed and con-
tinuously perfused with HBSS (pH 7.4) and phrenic nerve activ-
ity was recorded. GFP expression in the ventral surface of the
medulla was visualized using a fluorescence microscope. Case12
decay fluorescence was used as an indicator of [Ca2⁺], specifically
in astroglia. At the end of recordings, animals were intracardially
perfused with 4% paraformaldehyde and the brainstems were
subsequently processed for immunohistochemical detection
of Phox2B and GFP. Results: Lowering ventral surface pH from
7.4 to 7.2 increased phrenic nerve activity and resulted in pro-
found elevations in [Ca2⁺], in surface astrocytes. Immunohis-
stochemical analysis of the injected areas revealed strong GFP
expression in GFP-expressing astrocytes located in the mar-
ginal area of the ventral medullary surface. These astrocytes
were often seen surrounding blood vessels and in close contact
with chemosensitive Phox2B-immunoreactive neurons of the
retrotrapezoid nucleus (RTN). Conclusion: Medullary astrocytes
surrounding ventral penetrating arteries could play an impor-
tant role in integrating chemosensory information by sensing
physiological changes in arterial PCO2 and pH. We suggest that
these astrocytes shape the chemosensory responses of the RTN
neurons and the central respiratory network as a whole.


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C105

Synaptic NMDA Receptor Subunit Composition and Plasticity
in Principal Cells and Interneurons in Hippocampus
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N-methyl-D-aspartate receptors (NMDARs) have a pivotal role
in long-term plasticity and NMDAR-mediated synaptic trans-
mission is subject to plasticity although the mechanisms involved are little understood. Subunit composition determines the properties of NMDARs and there is evidence for subtype differences between receptors at different subcellular locations and between different neuronal cell types. NR2D-containing NMDARs are predominantly expressed at extrasynaptic locations and our previous data suggests that extrasynaptic NR2D-containing NMDARs relocate to the synapse during NMDAR-LTP. Expression of the NR2D subunit has also been demonstrated in inhibitory interneurons in hippocampus. Hippocampal slices were prepared from male Wistar rats (3-5 weeks old) and whole-cell patch-clamp recordings were performed to compare NMDAR-mediated synaptic transmission in granule cells (GCs) and interneurons (INs) in dentate gyrus. NMDAR-EPSCs were evoked by stimulation of the medial perforant path, at a test frequency of 0.033 Hz, at 34°C. Decay kinetics were significantly slower for NMDAR-EPSCs recorded in interneurons, with a weighted decay time constant (τD) of 85 ± 8 ms (n = 21), compared to τD of 32 ± 1 ms in granule cells (n = 17, p < 0.001). NMDAR-EPSC amplitude and 10-90% rise time was similar between granule cells and interneurons (amplitude 28 ± 3 pA and 24 ± 5 pA in GCs and INs, respectively, 10-90% rise time 4.7 ± 0.3 ms and 5 ± 0.9 ms in GCs and INs, respectively). The NR2D-prefering antagonist UBP141, inhibited NMDAR-EPSCs in INs by 27 ± 4 % of control amplitude (n = 7) but had no effect on currents recorded in GCs (99 ± 3 % of control, n = 4). Ifenprodil had similar effects on NMDAR-EPSC amplitude in both cell types, inhibiting EPSCs by 28 ± 6 % of control in GCs (n = 5) and by 35 ± 9 % of control in INs (n = 7). Our findings suggest that different NMDAR subtypes are expressed at perforant path synapses on principal cells and interneurons, and this variation may be associated with differences in spike timing and synaptic plasticity.

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C107

Deletion of TASK1 and TASK3 channels disrupts firing but not glucose or pH responses in orexin neurons

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Neurones in the hypothalamus that express the peptide hypocretin/orexin are essential for the regulation of sleep-wake transitions, appetite, and metabolism (Hara et al., 2001; Adamantidis et al., 2007). They sense changes in extracellular glucose and pH that occur within the physiological range, such that an increase in glucose is inhibitory and a decrease in pH is excitatory (Burdakov et al., 2006; Williams et al., 2007). It has been demonstrated that both glucose and pH modulate the excitability of orexin cells by regulating a leak K+ current, most likely mediated by channels of the family of two-pore domain K+ channels. Specifically, pharmacological data suggested that TASK1 and/or TASK3 channels were responsible for the glucose- and pH-sensing properties of orexin neurones (Burdakov et al., 2006; Williams et al., 2007).

To test this hypothesis, we performed whole-cell recordings in vitro from identified GFP-labelled orexin cells from mice that lack TASK1 and TASK3 channels (KO mice). Animal procedures
were carried in accordance with the Animals (Scientific Procedures) Act 1986 (UK).

We found that intrinsic excitability was reduced in KO mice: compared to wild-type orexin cells, the relationship between firing rate and current in KO neurons was significantly lower at high current levels (P < 0.01 for current ≥ 12 pA/pF). The action potential threshold was more positive in KO cells (KO, -18.5 ± 0.3 mV; wild-type, -21.4 ± 0.2 mV; P < 0.001, n > 150 spikes in each group), as was the after-hyperpolarization potential (KO, -40.7 ± 0.3 mV; wild-type, -47.3 ± 0.3 mV; P < 0.001, n > 150 spikes in each group). Spontaneous firing rates were not significantly different between groups (KO, 9.4 ± 2.6 Hz; wild-type, 13.8 ± 2.1 Hz; P = 0.21, n = 7 cells for each group). Moreover, when the extracellular glucose concentration was increased from 1 to 5 mM, all KO orexin cells tested (n = 10) were hyperpolarized in a manner indistinguishable from wild-type cells.

The HPTS ratio was calibrated using a single-point normalization to the fluorescence of the patch-pipette. Slices were maintained in HEPES-buffered Ringer’s solution.

The somatic membrane potential (SPM) and calcium indicators in the patch-pipette to pH 7.6 did not significantly alkalize the soma. This is not consistent with the pH gradient resulting from simple dialysis with the patch-pipette. We considered several other artefacts that might underlie the apparent pH gradient including non-linearities in the PMT gain, chromatic aberration and anoxia. To overcome chromatic aberration we produced pH and calcium images from serial confocal sections using only in focus structures. These images showed little systematic relationship between regional pH and either the depth in slice or Ca^{2+} level. However, regional pH and the rate of regulation was related to distance from the soma.

Our study suggests a heterogeneity of resting pH and evoked pH shifts in cerebellar Purkinje neurons. It is consistent with most of the functional pH regulation occurring within the dendritic tree rather than at the soma. This could result in the uncoupling of excitability of soma and dendritic arbor and may explain the different reversal potentials reported for GABA_A receptors in different cellular regions.

We thank the MRC for financial support.

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Long-lasting pH gradients are induced by electrical activity in rat cerebellar Purkinje neurons

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Intracellular pH (pH_i) is a powerful modulator of cell excitability (Xiong et al., 2000) yet there is no information on the resting pH_i in dendritic regions. Understanding how dendritic pH_i is regulated is important since epilepsy and some forms of ataxia appear to involve pH shifts. We have used fluorescence confocal imaging to investigate regional resting pH_i in Purkinje neurons.

Purkinje neurons, in cerebellar slices, were whole-cell patch clamped using pipettes containing a standard solution (Willoughby & Schwiening, 2002) with the fluorescent pH indicator 8-hydroxyperpyrene-1,3,6-trisulphonic acid (HPTS, 125 μM) and the calcium indicator Fura Red (62.5 μM) at pH 7.3. They were illuminated with alternating 405 nm and 488 nm light and pH-sensitive emission was collected at 505-550 nm and calcium-sensitive emission at 510-700 nm using a Leica SP5 confocal microscope (40x 0.8 NA water immersion objective). The HPTS ratio was calibrated in vitro (Willoughby et al., 1998) using a single-point normalization to the fluorescence of the patch-pipette. Slices were maintained in HEPES-buffered Ringer’s solution.

The soma was always more acidic (pH 6.98±0.06 mean±SD) than the dendrites (pH 7.27±0.14 mean±SD at ~120 μm from the soma, n=7) or the patch-pipette (pH 7.3) ~3 mins after the last depolarization (1 s to ~+20 mV). Further increasing the pH of the patch-pipette to pH 7.6 did not significantly alkalize the soma. This is not consistent with the pH gradient resulting from simple dialysis with the patch-pipette. We considered several other artefacts that might underlie the apparent pH gradient including non-linearities in the PMT gain, chromatic aberration and anoxia. To overcome chromatic aberration we produced pH and calcium images from serial confocal sections using only in focus structures. These images showed little systematic relationship between regional pH and either the depth in slice or Ca^{2+} level. However, regional pH and the rate of regulation was related to distance from the soma.

Our study suggests a heterogeneity of resting pH and evoked pH shifts in cerebellar Purkinje neurons. It is consistent with most of the functional pH regulation occurring within the dendritic tree rather than at the soma. This could result in the uncoupling of excitability of soma and dendritic arbor and may explain the different reversal potentials reported for GABA_A receptors in different cellular regions.

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Activity-dependent long-term potentiation produced by exogenous nitric oxide during NMDA receptor blockade in hippocampal slices

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Nitric oxide (NO) is a freely diffusible intercellular signalling molecule in most brain areas and has been widely implicated in synaptic plasticity and other phenomena. NO generation is linked to NMDA receptor activation and physiological NO signal transduction is achieved through guanylyl cyclase activation and cGMP accumulation (Garthwaite, 2008). In the hippocampus, NO has long been implicated as a retrograde messenger in NMDA receptor-dependent long-term potentia-
tion (LTP), but its precise role remains unsettled. If NO were acting purely presynaptically and if presynaptic changes contribute to LTP, it is predicted that exogenous NO should partially overcome the inhibitory effect of NMDA receptor blockade on LTP. To test this hypothesis, NMDA receptor-dependent LTP was studied at Schaffer collateral-CA1 synapses using field potential recording in hippocampal slices of male mice aged 6-8 weeks. LTP was induced using a 1-s, 100-Hz tetanus. Values of potentiation given below refer to mean field EPSP slopes 55-60 μm/s following the tetanus, normalised to baseline responses (100%) ± SEM. As expected, the NMDA antagonist, D-APS (50 μM), reversibly blocked LTP (110 ± 6% following tetanus during NMDA blockade; 169 ± 14% following a second tetanus after D-APS washout; n = 5). When present during tetanic stimulation in the presence of D-APS, the NO donor PAPA/NONOate produced a long-lasting potentiation (152 ± 9%; n = 5), as predicted by the retrograde messenger hypothesis. This NO-induced potentiation was, however, slow to develop (20 min to plateau) and, unexpectedly, it did not occlude subsequent LTP, which was, instead, substantially enhanced (266 ± 9%; n = 5). Both the NO-induced potentiation and the subsequent enhancement of LTP were dependent on exogenous NO concentration (maximal at 3 μM PAPA/NONOate) and on there being a coincident tetanic stimulation (115 ± 4% during NMDA blockade with exogenous NO but no tetanus; 190 ± 11% following a subsequent tetanus after washout of D-APS and PAPA/NONOate; n = 5). Both effects were also blocked by the guanylyl cyclase antagonist, ODQ (10 μM; 114 ± 7% following tetanus during NMDA blockade and with exogenous NO; 159 ± 10% following subsequent tetanus; n = 5), implying that they both involve the generation of cGMP and so could be mechanistically linked. It is concluded that the apparent rescue of LTP by exogenous NO paired with a tetanus when NMDA receptors are blocked cannot simply be explained by the NO compensating for a missing component of normal LTP. Instead, the results show that exogenous NO, in an activity-dependent manner, evokes a long-term enhancement of synaptic transmission of similar amplitude to that normally produced during LTP, but that is additive to normal LTP.


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*Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.*

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**Increased expression of transforming growth factor-β1 alters synaptic structure in vivo and regulates neuronal activity in vitro**

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Transforming growth factor-beta 1 (TGF-β1) is a multifunctional injury-related cytokine that orchestrates key events of development, disease and repair (Gomes et al. 2005). Secreted by both neurons and glial cells in the central nervous system (CNS), increased expression of TGF-β1 is associated with neurological diseases and brain trauma (Buckwalter et al. 2004). To date, its role in the regulation of synaptic structures and transmission in the mammalian CNS remains unclear. To investigate the effects of chronic over-secretion of TGF-β1 on the structural and functional properties of neural cells, we used transgenic mice (T64) that over-express active form of TGF-β1 in hippocampal and cortical astrocytes. Immunohistochemical analysis demonstrated that in comparison with wild-type (WT) littermate controls, the number of cells in T64 mice expressing glial fibrillary acidic protein (GFAP, astrocyte marker), and CD11b (microglia marker) increased significantly in the hippocampus and cortex. Notably, large immuno-clusters of synaptophysin (presynaptic protein) and calbindin-D28K (CaBP, calcium binding protein) positive immunoreactivity of neurons were, respectively, decreased in the CA3 region and the dentate gyrus through to the mossy fiber terminals. Decrease in CaBP and increase in GFAP protein levels in the T64 model were confirmed. Cultured primary astrocytes from T64 mice show higher rate in proliferation and more elongated cell bodies and processes when compared with WT controls. To examine the effect of TGF-β1 alone in the regulation of neuronal functions, we cultured hippocampal neurons from WT rat embryos in the presence or absence of TGF-β1 (4.0 ng/ml, 7-12 days). Voltage-clamp recordings in these cultured neurons revealed that TGF-β1 significantly increased the amplitudes of voltage-gated K+ currents, Na+ currents and glutamate-induced currents (p<0.05, Student’s t-test) in treated cells. Taken together, our results suggest that chronically increased expression of TGF-β1 may activate glia and induce alterations in synaptic structures of surrounding neuronal populations. Further, TGF-β1 alone appears, at least in culture condition, to increase neuronal activity.

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*Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.*
Motor Unit Size-Dependent Intrinsic Withdrawal of Neuromuscular Synapses During Postnatal Development of Mouse Muscle

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At birth every muscle fibre has functional synaptic inputs from multiple motor neurons, all but one of which are eliminated. Synapse elimination is driven by competition between synapses co-innervating a muscle fibre (Betz et al., 1980). However, an unresolved question is whether synapse elimination can occur in the absence of competition. Some evidence suggests that ‘intrinsic withdrawal’ of synapses continues at some neuromuscular junctions (NMJs) after partial denervation at birth (Fladby and Jansen; 1988), leaving some endplates uninnervated.

We have re-examined the question of ‘intrinsic withdrawal’ by directly measuring motor unit (MU) size in transgenic mice expressing yellow fluorescent protein in motor neurons (thy1.2:YFP). We used confocal microscopy to determine MU size in the absence of competition. Some evidence suggests that synapse elimination can occur in the absence of competition. We found that the average MU size was significantly smaller in control mice (54 ± 11, n = 29, mean ± SD) than in neonates (137 ± 63, n = 10, p<0.001, Tukey HSD) and in AwPD (103 ± 29, n = 10, p<0.001, Tukey HSD) though the difference between neonates and AwPD only approached significance (p=0.058, Tukey HSD). While the smallest MU size in those two groups was approximately the same, there were several MUs that were larger in the neonates than the maximum MU size in the AwPD, suggesting that only motor units above a certain size may lose synapses in the absence of competition.

The present data suggest that there is an intrinsic limit to the number of synapses a motor neuron can sustain and that this number may decline as axons and muscle fibres grow. We have been modifying a computational model of synapse elimination to examine the effect of growth on MU size (Rasmussen & Willshaw, 1993).


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The effect of TNF-a treatment on skeletal muscle myokine production

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The role of skeletal muscle in locomotive function is well defined; however recent research has indicated a role as an immunogenic organ. During systemic inflammation, muscle is exposed to pro-inflammatory factors and Tumor necrosis factor alpha (TNF-α) has been implicated as a key initiator of the early inflammatory response. Studies have indicated that muscle responds rapidly to stress by the increased production of myokines and stress or Heat Shock Proteins (HSPs) and data suggest that these proteins may be released as signalling proteins to other cells (1, 2, 3). We hypothesise that elevated levels of TNF-α initiates the production and release of myokines and HSPs from skeletal muscle.

C2C12 myotubes were cultured & differentiated in vitro (4). Myotubes were treated with TNF-α in culture media (25ng/ml) for 3 or 6 hours; culture media and cell lysates were harvested and analysed for the presence of cytokines using Luminex multiplex (20-plex) bead analysis. RNA was isolated using TRI reagent/Rneasy clean up extraction method and reverse transcribed to single strand cDNA. Gene expression was analysed using a murine cytokine qPCRarray assay. Western blotting was used to quantify levels of HSPs in the lysate and media following TNF-α exposure (4). Cell viability was assessed by trypan blue exclusion and LIVE/DEAD staining.

qPCR array analysis of cell lysates showed greater than four-fold up-regulation of complement component 3, MCP-1, CCL5, CXCL1, CXCL5, CXCL9 and CXCL10. Luminex multiplex bead analysis showed significant (ANOVA, P-value ≤ 0.05, n=6) levels of IL-6 and RANTES located in the media at 3 hours (288pg/ml ± 70.30; 7200pg/ml ± 626.64 respectively) and 6 hours (553pg/ml ± 147.63; 2122pg/ml ± 307) following TNF-α exposure. Western blot analysis, showed significant up-regulation of intracellular levels of HSP60 and HSP70, which peaked at 3 hours following treatment and significant levels of HSP60 were detected in the culture media (ANOVA, P-value ≤ 0.05, n=6). Furthermore there was no significant loss of cell viability for up to 8 hours following TNF-α exposure.

C2C12 myotubes showed significant up-regulation of myokine expression in response to TNF-α treatment and increased release of myokines. These data further support the theory that muscle can release cytokines in response to stress, thus acting as an immunogenic organ. The specific release of HSP60 supports the suggestion that heat shock proteins can be released into the extracellular environment in response to stress, and we hypothesise that they may function as danger signals for the immune system(5).


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C113

Real-time measurement of intracellular reactive oxygen and nitrogen species in single isolated mature skeletal muscle fibres in mice overexpressing nNOS

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Introduction: Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are continuously produced by skeletal muscle. Their generation is augmented during contractile activity and may play an important role in signaling adaptive responses and/or mediate some degenerative processes [1]. Purpose: To elucidate the roles of ROS and RNS in detail, we examined their intracellular activities using fluorescence microscopy in single isolated mature skeletal muscle fibres from mice overexpressing neuronal nitric oxide (nNOS) and wild type control mice both at rest and following a period of contractile activity. Methods: Genetically modified mice overexpressing nNOS (type I NOS) [2] and wild type (C57Bl/6) mice were used in this study. Each group consisted of 9 mice aged 22 ± 1 months. Mice were killed by cervical dislocation and the flexor digitorum brevis muscles were dissected. Muscles were incubated in collagenase to isolate single muscle fibres. Fibres were then plated on culture dishes which had been previously coated with collagen and cultured for 24 h [3, 4]. Contractile activity was induced by electrical stimulation [3, 4]. Fibres were loaded with 4-Amino-5-Methylamino-2’,7’-Difluorofluorescein Diacetate (DAF-FM DA), Dihydroethidium (DHE) or 2’,7’-Dichlorofluorescein Diacetate (DCFH DA), fluorescent probes for the assessment of Nitric Oxide (NO·), Superoxide (O2−) and a general probe for ROS respectively. Unpaired Student’s t-test for single comparisons, significance p<0.05. Results: At rest the nNOS mice had a higher rate of increase in DAF-FM fluorescence compared with the control group implying a higher NO· generation (p<0.05). However, no significant differences were observed between the stimulated fibres from the two groups. Ethidium fluorescence from DHE-loaded fibres increased in both groups indicating a potential increase in the rate of O2− generation in the sarcoplasm after contractions (p<0.05) with no difference between the rates in wild type and transgenic mice. Fibres from nNOS overexpressors showed a decrease in ethidium fluorescence compared with the fibres from control mice at rest (p<0.05). Following DCFH loading, no differences were seen in DCF fluorescence between the groups. Discussion: Contractile activity increased ROS and RNS in fibres from both wild type and nNOS overexpressing mice. The mice overexpressing nNOS appeared to generate more NO· at rest compared with those from control mice. However following contractile activity the fluorescence from fibres did not differ between the groups. These data indicate that overexpression of nNOS can increase NO bioavailability in skeletal muscle fibres and reduce the availability of O2−.


Palomero J et al. (2008). Antioxid Redox Signal. 10, 1463–1474


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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C114

Effects of genetic variation on activity of citrate synthase and levels of signalling proteins in skeletal muscles of mice

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Identifying genetic factors modulating skeletal muscle characteristics can provide an insight into health and disease. A study of inbred strains of mice is a starting point of such an effort. Genetic variations among strains often result in differences in muscle size and exercise performance, but the cellular mechanisms behind these differences are not clear. The aim of the study was to investigate activity of citrate synthase (CS), a marker for mitochondrial oxidative capacity, and levels of protein kinase B (PKB) and extracellular signal-regulated kinase 1 and 2 (MAPK(erk1/2)), as two growth regulators, in skeletal muscle fibres in mice overexpressing nNOS (type I NOS) [2] and wild type (C57Bl/6) mice. The quadriceps muscle of 14 week old male mice was dissected after sacrifice and snap frozen. 40-70 mg of the muscle were homogenised and centrifugated. The supernatants were taken and the protein concentration was measured using the Bradford assay. The CS activity was assessed by the Western blotting. One way ANOVA showed that CS activity (n=9 per strain) was strain dependent (P<0.0001) with A/J mice showing lower values (P<0.01, Tukey’s test) than the other strains. The difference between the A/J and PWD/PhJ mice was particular marked
The ANOVA also showed that the Strain factor had a significant effect on the level of PKB (n=5 per strain, ANOVA, P<0.01). Muscles from mice of the PWD/Phj strain had lower levels of PKB than muscles in the A/J, BALB/cByJ or DBA/2J mice (P<0.01, Tukey’s test). There were no significant differences in MAPK/Erk1/2 between the strains. Results of the study show that the genetic variations influence CS activity and levels of some growth regulators in muscles of mice. This might have a direct effect on muscle morphology and function. Thus studies of inbred mice can improve our understanding of the underlying mechanisms of variation in muscular function.


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**C115**

**ERα-mediated rapid signals are requested for estradiol-induced skeletal muscle differentiation**

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17β-estradiol (E2) mediates a wide variety of complex biological processes. While E2 effects on the reproductive system are quite well established, less is known about how it affects the physiology of other tissues. Skeletal muscle expresses both estrogen receptor isoforms (ERα and ERβ). In addition, female rat muscles show fewer histopathological changes after repeated eccentric contractions than male muscles; ovariectomized female rats exhibit higher indexes of exercise-induced muscle membrane damage which disappear in after estradiol treatment. The mechanisms underlying the role played by E2 and remain still elusive. In actively proliferating rat myoblast cells (L6) (grown in 10% serum), E2 rapidly increased the glucose transporter type 4 (Glut-4) translocation at the cell membranes; successively, the increase of well known differentiation markers of myogenesis (i.e., myogenin and myosin heavy chain, MHC) was evidenced by Western blot analysis at 6 and 24 h, respectively, after E2 stimulation. Seven days after E2 treatment the alignment and fusion of L6 myoblasts into multinucleated myotubes were visible. Next we compared the effect of E2 and IGF-I. Both hormones influenced the three muscle differentiation markers considered, even if the E2 efficacy was less than IGF-I. In addition, E2 effects were completely prevented by the pure ER inhibitor. The contribution of both nuclear and extra-nuclear action mechanisms on E2-induced modification of differentiation markers was thus evaluated by pre-treating cells with cycloheximide, actinomycin, and the palmitoylation inhibitor 2-Br, that prevents the membrane starting signals of both receptor isoforms. These data indicate that both E2-dependent rapid signals and nuclear action are required for E2-induced L6 differentiation. In particular, ERα-dependent AKT activation is necessary to control the first step of myogenic differentiation. Moreover, both receptors mediate the E2-induced activation of p38 which, in turn, affects the expression of myogenin and MHC. The contribution of ERα in activating the previously identified E2-dependent effects has been evaluated by using both ERα and ERβ selective agonists (PPT and DPN, respectively) and by reducing ERα with specific siRNAs. As expected, E2 was unable to induce AKT phosphorylation, whereas the E2-dependent activation of p38 was still present, in ERα-depleted cells. Intriguingly, E2 was not able any more to induce the Glut-4 translocation and the increase of myogenin and MHC level in ERα-depleted cells, thus confirming the pivotal role of ERα in the L6 differentiation. All together these data indicate that E2, like other extra-cellular growth factors, modulates specific cell signals affecting the skeletal muscle development providing the basis of gender-related physiological differences in skeletal muscle recovery after damage.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**C117**

**Effects of exogenously induced fever and hyperthermia on endocrine functions and behavior in the pre-pubertal rat**

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Hyperthermia may cause pathological changes in various organs including the endocrine system and brain. In this study, we examined effects of exogenously induced fever and hyperthermia on adrenal and thyroid functions and behaviors in pre-pubertal male Sprague-Dawley rats. Three groups of 30-day old rats were used. Body temperature was increased to 39∞C (fever; Group I) and 41∞C (hyperthermia; Group II) in a hyperthermia induction chamber for 30 min. The rats in the Group III served as control (36 ∞C). Temperature of the laboratory was 21∞C. Core temperature of the animals was monitored by using a rectal probe throughout the experiments. All animals received saline (0.2 ml x 4 times, ip) and were decapitated 48 h after the experiments (day 32). Blood samples were collected. Serum cortisol, fT3, fT4 and dehydroepiandrosterone (DHEA) levels were determined by chemiluminescence assay. Pituitary and...
adrenal glands were dissected out and processed for histopathological examination. To assess the activity and anxiety of the animals, the open field test and elevated-0-maze test, respectively, were used in all groups 24h before (day 29) and after (day 31) hyperthermia induction. Experiments were approved by the local ethics committee. Results were statistically analyzed by using One-Way Analysis of Variance followed by LSD test.

Serum cortisol levels (3.22±0.9) were significantly reduced in the fever (1.3±0.9) and hyperthermia (1.09±0.7) induced groups (p<0.01). Serum levels of thyroid hormones did not significantly differ among the groups. DHEA values were below the limit of detection in all groups. Histopathological examination revealed that fever caused hyperemia in the pituitary and adrenal glands. However, mild degeneration was observed in both glands in the hyperthermia group. Progression time in the open field test was significantly decreased and anxiety test scores increased in animals exposed to 39°C compared to the control values (p<0.01). These parameters were more pronounced in the hyperthermia group (p<0.01). In conclusion, hyperthermia induced stress may cause delayed reduction in serum cortisol levels which may be associated with behavioral abnormalities in pre-pubertal male rats.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

C118

Involvement of both AMPK and mTOR pathways in the arcuate nucleus of rat hypothalamus during ambient hypoxia

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Acute environmental hypoxia is known to promote reduction of body weight and marked decrease in food intake, as a result of hypoxia per se. This alteration in feeding behavior is known as altitude–induced anorexia but the molecular mechanisms underlying such alterations remain unclear. AMP-activated protein kinase (AMPK) functions as a major regulator of cellular metabolism and recent data demonstrated that together with the mammalian target of rapamycin (mTOR), AMPK plays a critical role in regulating food intake. Decreased AMPK activity in the hypothalamus reduces food intake and body weight, whereas mTOR activation is required for the appetite-suppressing response to a variety of anorexigenic signals. We previously reported that hypothalamic AMPK activity was reduced by hypoxic stimulus concomitant to hypophagia in rats, response which could be linked to the transient hyperglycemia observed at the same time (Simler et al). Here we determine whether this reduction occurs specifically in the arcuate nucleus of the hypothalamus and if the mTOR signaling could contribute to the mechanisms of altitude-induced anorexia.

Adult male wistar rats were either submitted to normobaric hypoxia (10% O2) during 2 or 6 h, or maintained in normoxia. At the end of experimental conditioning, animals were anesthetized with pentobarbital (70 mg/kg body weight) and brains were quickly removed. The arcuate nucleus and the paraventricular nucleus (PVN) were extracted from 1mm slices with a punch needle. Activity of the AMPK pathway was assessed by measuring the phosphorylation levels of both AMPK and acetyl-CoA-carboxylase (ACC). Activity of the mTOR pathway was examined by the phosphorylation level of p70 S6 kinase (p70 S6K) and the S6 ribosomal protein.

Results: The AMPK pathway in the arcuate nucleus was progressively affected during hypoxia, as a result of diminished levels of both AMPK and ACC. This confirmed what we previously reported on the whole hypothalamus after 6h of hypoxia. Conversely, in the arcuate nucleus, the activity of the p70 S6K and of its target S6 ribosomal protein were markedly enhanced after exposure to hypoxia affording for an increased activity of the mTOR pathway. A cross-regulation between AMPK and mTOR seemed to proceed to control food intake in the arcuate nucleus, which is consistent with anorexigenic signals led by ambient hypoxia. Such modifications were not observed in the PVN, suggesting a specific response of the AMPK and mTOR pathways within the hypothalamus during environmental hypoxia. Icv injections of the AMPK activator AICAR and of an inhibitor of mTOR (rapamycin) will provide further knowledge of the molecular mechanisms that drive this hypoxia-induced anorexia.

Conclusion: These findings provide support for the hypothesis that AMPK and mTOR interact in the arcuate nucleus to regulate feeding during acute environmental hypoxia.


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Growth Hormone is present in Human Retinal Ganglion Cells and Correlates with Cell Survival

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Locally synthesized growth hormone (GH) may act as a survival factor in a number of tissues (Sanders & Harvey, 2008). Experimental studies with chick retinal ganglion cells (RGCs) suggest that GH, synthesized within the developing retina, may have autocrine/paracrine roles in the regulation of the waves of cell death characteristic of RGC differentiation (Sanders et al. 2008). There is also evidence that endogenous GH may have a similar neuroprotective function in the adult rat retina, however, there is no information concerning the possible presence or action of GH in the human retina. In this study we show, for the first time, that GH is present in the human retina and that the local expression of retinal GH correlates with RGC cell survival. GH-immunoreactivity, identical in size to that in the pituitary gland (22kDa), was detected by Western blotting as a single band in extracts of cadaver retinas (obtained with ethical approval).
Using tissue sections from eyes collected post-mortem (with ethical approval), this immunoreactivity was largely confined to large, rounded cells in the GLC (ganglion cell layer) of the neural retina and co-localized in RGCs, labeled by a synuclein antibody. GH receptor (GHR) immunoreactivity was similarly located in the GCL and 35% of these cells were both GH- and GHR- positive. None of the cells that had GH immunoreactivity were apoptotic, as determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). In summary, these results demonstrate the presence of GH and its receptor in RGCs of the human retina in which GH expression correlates with cell survival. These results are consistent with our earlier in vivo and in vitro experimental studies in chick embryos that show autocrine or paracrine actions of GH in RGCs that promote cell survival during development and suggest that GH is a novel neurotrophic factor for human RGCs.


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Recovery from exercise in humans: dependence on vagal activity

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Arrhythmic event risk is augmented with low vagal activity. Exercise training can reduce this risk by increasing vagal activity. The reduction in vagal activity immediately following exercise may increase the risk of cardiac arrhythmias and sudden cardiac death. To date the effect of baseline vagal activity on the recovery of cardiac autonomic activity up to 1 hour post-exercise has not been examined. 28 healthy, untrained volunteers (10 females, 21.0 ± 3.1 years) performed a 20 min hard exercise (age: 25.3 ± 6.46 cm, weight 68.8 ± 14.5 kg) giving consent to participate. At the first visit all the steps of the protocol were explained and participants were shown how to attach a Polar S810i HR monitor (Polar Electro OY, Kempele, Finland) for RR-interval recording. Par-
participants were instructed to make two separate RR-interval recordings; at the start of a revision session at home and at the start of the written exam. The revision session was recorded at the same time of day as the scheduled exam (MSc in Sports Science). The HR data was analysed post hoc using Kubios HRV analysis software, (University of Kupio, Finland). Following the removal of any artefacts, the data were divided into 12, five min epochs. Results from all epochs were averaged. Three indices of vagal activity were calculated: the root mean square of successive differences (RMSSD), natural logarithm of high frequency power (frequency domain) (lnHF), and standard deviation of instantaneous beat to beat variability (SD1). Paired sample t-tests were used to compare the differences between the days, level of significance (p value: 0.05). HR during exam was 9.7 beats per minute (bpm) higher compared with revision (82.6±9.0 bpm Vs 72.9±9.8 bpm) (p value: 0.002). There was a significant decrease in all three indices of vagal activity during exam: RMSSD, lnHF, SD1 (with p values: 0.01; 0.03; 0.017, respectively). These results suggest that there is a significant reduction in cardiac vagal activity under conditions of emotional stress and cognitive stress compared with the response to cognitive challenge alone. Moreover, this suggest that HRV can be used to assess changes in the ANS with mental stress.

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The protective effect of vagus nerve stimulation against ventricular fibrillation is preserved during muscarinic receptor blockade

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Classically the effect of vagus nerve stimulation is considered to be entirely mediated via muscarinic receptor activation. We have previously shown that direct vagus nerve stimulation (VNS) reduces the slope of action potential duration restitution whilst simultaneously protecting the heart against ventricular fibrillation (VF) initiation in the absence of background sympathetic activity (Ng et al. 2007). We have extended this finding to show that this effect is mediated via nitric oxide (NO) (Brack et al. 2007). In this study our aim was to determine if the protective effects of VNS are dependent on muscarinic receptor activation. Adult male New Zealand rabbits (2.9±0.1Kg, n=9) were used. The isolated heart preparation with intact autonomic nerves was obtained under propofol anesthesia (1 mg/kg, i.v.) as previously described (Ng et al, 2001). The cervical vagus nerves were stimulated at 9.6±1.6Hz, 7.2±1.1V. Ventricular effective refractory period (ERP) was measured with the single extrastimulus method following a 20-beat drive train (300ms cycle length) whilst ventricular fibrillation threshold (VFT) was determined as the minimum current required to induce sustained VF with rapid pacing (30 stimulul x 30ms). ERP and VFT were studied at baseline and during VNS, before and during perfusion with 0.1μM atropine. Data are mean±SEM, whilst statistical analysis was performed using 2-factor repeated measures ANOVA with Bonferroni post tests, P<0.05 was considered significant. During control, VNS decreased heart rate from 147.8±6.5 to 89.9±6.4 bpm (P<0.001) whilst significantly increasing both ERP and VFT from 130.0±6.7 to 147.8±6.7 ms (P<0.001) and from 2.2±0.3 to 5.3±0.5mA (P<0.001) respectively. During perfusion with atropine, the effects of VNS on heart rate (143.1±9.8 to 142.2±9.3 bpm, P>0.05) and ERP (137.8±5.7 to 133.3±4.1 ms, P>0.05) were lost whilst the increase in VFT was preserved where VNS increased VFT from 2.0±0.3 to 4.7±0.7mA (P<0.001). These data suggest an acetylcholine / muscarinic receptor independent mechanism underlying the vagal protection of the heart against VF. The context of these findings needs further exploration in relation to the possibility of alternative neurotransmitters and the vagus-NO pathway.


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Action potential, sodium and gap junction channels in rat pulmonary vein myocytes

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The pulmonary veins (PV) are important in the initiation and maintenance of atrial fibrillation, which may be related to slow excitation-conduction in the PV (Nattel, 2002; Aldhoon et al. 2009). In this study, the essential factors for action potential conduction in the PV have been investigated with single myocytes isolated from rat left atrium (LA) and four PVs (left superior LSPV, left inferior LIPV, right superior RSPV and right inferior RIPV). Whole cell current clamp and voltage clamp were used to record action potential (AP) and the fast Na⁺ current (I Na). Single cell real-time PCR was employed to measure the abundance of mRNAs for Na⁺, 1.5 and gap junction channel (Cx43), and the expression of Cx43 protein on cell membrane was located by immunofluorescence. The results showed that: [i] the maximum velocity of AP phase 0 upstroke was significantly faster (62±10.5 V/s) in LA myocytes than in all PV myocytes (Fig. 1 A and D); [ii] I Na density was remarkably higher (16.4±2.9 pA/pF) in all PV myocytes (apart from RSPV myocytes) than in LA myocytes (Fig. 1 B, C and E); [iii] the relative abundance of Na⁺, 1.5 mRNA in the LSPV and LIPV myocytes was 1.7 times higher than in LA myocytes (Fig. 1F), whereas
no difference in the Cx43 mRNA abundance between LA and PV myocytes was observed; and [iv] the majority of Cx43 expression in LA myocytes was located at the cell-ends in the intercalated disc, whereas the Cx43 expression in PV myocytes was distributed at both cell-ends and in the lateral cell membrane. It is surprising and interesting that \( I_{Na} \) density and the abundance of Nav1.5 mRNA in PV myocytes are inconsistent with the depolarization velocity of AP as compared with those in LA myocytes. In general, the conduction velocity is mainly associated with \( Na^+ \) and gap junction channels as well as their functional balance, and \( I_{Na} \) is the large current that has sufficient reserve or redundancy for the fast depolarization. Therefore, it is concluded that the difference in the cellular distribution of Cx43 protein in PV myocytes is predominant to slow excitation-conduction as a result of anisotropic properties of the tissue more susceptible to atrial fibrillation.

**Figure 1. Action potential, \( I_{Na} \) and Nav1.5 mRNA in LA and PV myocytes**

A, recordings of action potentials from LA and PV myocytes. B and C, \( I_{Na} \) traces from a LA and LIPV myocytes during voltage clamp. D, maximum velocity of phase 0 upstroke of AP in the various myocytes (n=10~19). E, peak density of \( I_{Na} \) (n=11~15). F, relative abundance of Nav1.5 mRNA (n=7). Significantly different * from LA myocytes and # from LSPV myocytes (P < 0.05, one-way ANOVA).


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mination of ventricular rhythm during atrial tachyarrhythmias. Since both properties progressively hinder AV conduction in presence of high atrial rates, they contribute to protect the heart from dangerous high frequency ventricular rhythms.


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Corticosteroid-independent nuclear localization of mineralocorticoid receptor in mouse cardiac myocytes

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Aldosterone plays a key role in extracellular volume homeostasis by promoting sodium reabsorption in the kidney distal tubule. The accepted model for aldosterone action involves the binding of the hormone to the mineralocorticoid receptor (MR) in the cytoplasm and subsequent translocation of the aldosterone-MR complex to the nucleus, where it acts as a transcription factor. MR is also expressed in non-epithelial tissues such as the heart, where its physiological role is unclear. However, inappropriate MR activation leads to the development of cardiac fibrosis and renovascular disease. To improve our understanding of MR functional roles in the heart, we investigated the subcellular localization of the receptor in mouse left ventricle and in immortalized cardiac myocytes in the absence or presence of corticosteroids. Tissues were obtained from adrenalectomized (ADX) 8-week-old C57BL/6 mice. Preparative surgery was performed on mice anesthetized by intraperitoneal administration of 50 mg/kg ketamine and 1 mg/kg medetomidine. After the procedure, anesthesia was reversed with 1 mg/kg atipamezole. Mice were kept for four days after ADX with free access to saline prior to harvesting the tissue. The procedures were approved by the local ethics committee and accorded with current national legislation. Three independent experiments with 3 mice in each group were performed. MR localization in the left ventricle was studied by cellular fractionation (1). MR subcellular localization was further tested by transient transfection of wild type or mutant MR in HL-1 cells derived from adult mouse cardiac myocytes (2). MR localization in HL-1 cells was studied by indirect immuno-fluorescence (3) and cellular fractionation (1). Three independent experiments were performed for each condition tested. Our results show that both endogenous MR in the left ventricle and transfected MR in HL-1 cells are detected exclusively as nuclear chromatin-bound factors. Depletion of corticosteroids by ADX in mice or by culturing HL-1 cells with charcoal-stripped serum or serum-free medium did not affect MR chromatin binding. Deletion of nuclear localization signal 0 (NLS0) (1) rendered mutants exclusively cytoplasmic, while mutations in NLS1 partially redistributed MR to the cytoplasm. Taken together, our results suggest that MR is constitutively nuclear in mouse cardiac myocytes, independently of corticosteroids. The subcellular localization of the naïve receptor is mainly determined by NLS0. This stands in sharp contrast to the behaviour of MR in other cell types, where it appears evenly distributed over the nucleus and the cytoplasm. The differential behaviour of MR in cardiac myocytes might have important consequences for its function, both in genomic and non-genomic pathways.


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Volumetric analysis in Xenopus laevis oocytes expressing the rat g-aminobutyric acid transporter GAT1 and the not functional mutant Q291N

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Water transport across membranes is a fundamental process for cells. Currently accepted water transport pathways are the lipid bilayer itself and specialized proteins, the aquaporins. Recently it has been shown that several cotransporters, such as SLGT1 and GAT1 mediate a water influx across membranes (Loo et al., 1999; Lapointe et al., 2002). Some observations support the idea that local osmotic gradients built up immediately after cotransport activity are fully responsible for the cell swelling (Lapointe et al., 2002). An alternative hypothesis suggests a direct coupling of water, ion and solute (Zeuthen et al., 1996). γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in mammalian brain and the GABA transporter GAT-1, belonging to the neurotransmitter sodium symporter family (NSS), is an integral membrane protein responsible for the reuptake of GABA from the synaptic cleft.

Glutamine 291 is a strictly conserved residue in all members of the NSS family. It has been previously published that Q291 mutants cannot transport GABA or give rise to currents even though they are targeted to the plasma membrane (Mari et al., 2006).
In order to better understand water transport in cotransporters we verified whether the not functional mutant GAT1 Q291N is able to mediate water transport.

To this aim, we performed six independent volumetric analysis experiments in hypotonic conditions ($\Delta$Osm = 167) using Xenopus laevis oocytes expressing the wild type (wt) and the mutated protein (Dorr et al., 2007).

The expression of GAT1 on oocyte surface was confirmed by radiolabelled aminoacid transport experiments.

In each experiment volumetric variations were measured in three groups of 7-14 oocytes (GAT1, GAT1 Q291N and control oocytes) and the mean permeability factors (Pf) of each group were calculated. The Pf of GAT1 expressing oocytes ranged from $1.41 \times 10^{-3}$ to $7.74 \times 10^{-3}$ cm/s (mean ± S.E.); the Pf of Q291N ranged from $1.33 \times 10^{-3}$ to $6.44 \times 10^{-3}$ cm/s to $1.75 \times 10^{-3}$ to $7.27 \times 10^{-3}$ cm/s (mean ± S.E.); the Pf of control oocytes ranged from $7.30 \times 10^{-4}$ to $4.57 \times 10^{-3}$ cm/s to $1.46 \times 10^{-3}$ to $5.63 \times 10^{-3}$ cm/s (mean ± S.E.).

As expected, in five experiments out of six the Pf of GAT1 was higher than the Pf of control oocytes (p<0.05 Student’s t-test). Interestingly, in five experiments out of six also the Pf of GAT1 Q291N was significantly higher than control Pf value (p<0.05). Our experiments thus showed that both wt protein and mutant mediate a water influx across Xenopus oocyte membrane.

In conclusion though at the moment we cannot choose for one of the above described hypothesis regarding water transport through cotransporters, our results indicate that, under particular conditions, a water influx occurs both when the cotransport mechanism can take place (GAT1 wt) and when it cannot (GAT1 Q291N).


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The adaptor protein PDZK1 interacts with the glutamate transporter EAAC1 and regulates its surface expression


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The glutamate transporter EAAC1/EAAT3 is expressed in the central nervous system (CNS), where it mediates the uptake of the neurotransmitter from the synaptic cleft. It is also expressed in non neuronal tissues, in particular intestine and kidney, where it represents the main pathway of aminoacid reabsorption [1]. Its surface expression is strictly regulated by interaction with accessory protein, e.g. PDZ proteins (from the name of the first proteins discovered PSD95, DLG and ZO1). These proteins contain multiple protein-protein interaction domains, that bring together and localize transporters, channels, enzymes and receptors in specific plasma membrane domains [2].

In the C-terminal of EAAC1 there is a class I PDZ proteins target sequence (-SQF), whose removal induced internalization of the transporter in endocytotic compartments (Fig.1). Therefore, PDZ proteins play a key role in regulating EAAC1 expression in the plasma membrane, but none of the EAAC1 interacting partners have been so far identified. Aim of this work was to identify the PDZ proteins interacting with EAAC1 and to verify whether they play a role in the EAAC1 localization at plasma membrane.

In epithelia, possible candidate are members of the NHERF (Na‘/H’ Exchanger Regulatory Factor) family, PDZ proteins that organize the apical domain of the plasma membrane [3]. The interaction between PDZK1/NHERF3 and EAAC1 was proved by means of several experiments. By yeast two hybrid system assay [4], we found that the C-terminal tail of EAAC1 (last 40 or 26 aminoacids) directly interacted with PDZK1, and the interaction required the first and second PDZK1 PDZ domain (n=3). Affinity chromatography [5] confirmed that PDZK1 is retained by the C-terminal tail of wild type EAAC1 (WT) (n=4).

Co-immunoprecipitation experiments demonstrated the binding of PDZK1 to wild type transporter (WT GFP-EAAC1), but not to a mutant transporter lacking the PDZ target sequence (ΔTSQF GFP-EAAC1) (n=3) (Fig.2). Similar experiments performed in intestinal tissues confirmed the EAAC1/PDZK1 interaction in vivo (n=3).

In agreement with these results we found co-localization of PDZK1 with WT EAAC1 in over-expressing system (MDCK) as well as in native tissues (n=3).

PDZK1 not only co-localized but also functionally interacted with the transporter, because the presence of mycPDZK1 caused a 1.34±0.13 fold increase (T-test, p≤0.05) in the WT EAAC1 surface activity, without affecting ΔTSQF activity. Thus, PDZK1 interacts with EAAC1 in vitro e in vivo, regulating its localization and function at plasma membrane. Further studies are needed to clarify the molecular mechanism by which PDZK1 controls the membrane localization of EAAC1.
Immunofluorescence. MDCK cells were stably transfected with the indicated GFP-EAAC1 mutant transporters, fixed and analyzed by confocal microscopy. WT EAAC1 is mainly expressed at the plasma membrane, whereas ΔTSQF mutant is expressed in an endocytotic compartment. Scale bar, 10 μm

Co-immunoprecipitation. Lysates of MDCK cells stably expressing EAAC1 wild type or ΔTSQF were immunoprecipitated with an anti-PDZK1 antibody; the immunocomplexes were resolved by 9% SDS-PAGE and immunostained with an anti-GFP antibody. 10% of the cell lysate used in the immunoprecipitation assay was probed (Lysate). PDZK1 antibody was able to co-immunoprecipitate only WT EAAC1 from cell lysates.


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The Importance of Flavonone B-Ring Catechol in Suppression of Intracellular Fenton Reactions

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Quercetin, like dehydroascorbate (DHA) is transported into cells via the passive glucose transporters, GLUT1 and 4(1). We have demonstrated that quercetin, like ascorbate, acts as an electron donor to the erythrocyte transmembrane oxidoreductase, DcytB(3;4). Ascorbate also interacts with Fe2+/Fe3+ and H2O2 to produce very reactive OH radicals. In contrast to ascorbate, quercetin is a known suppressant of the Fenton reaction (2). We have compared the iron chelating efficacy of several flavonones to determine which hydroxyl groups suppress intracellular free OH radical formation. After overnight loading of Madin Darby canine kidney cells with 20μM ferrous sulphate, the cells were washed free of adherent extracellular Fe2+ with 300μM desferrioximine, an impermeant iron chelator which suppresses extracellular Fenton reactions, then exposed to varying concentrations of permeable polyphenols, 100μM H2O2 and the luminescent OH radical sensor L-012. The intracellular luminescence was assayed in a 96 well luminescence plate reader. The I.C.50 of quercetin and luteolin-induced inhibitions of intracellular iron-dependent luminescence was 3.4±0.9 and 3.7±0.8 μM respectively and with 3,5-dihydroxyflavone and chrysin was 9.4±1.5 μM and 18.1±14 μM (S.E.M, 2 experiments, with each compound, 6 concentrations and 3 replicates).

The differences between the efficacies of the flavonone suppression of the Fenton reaction shows the key importance of the 3'-4' dihydroxylated B ring as an Fe2+ chelator, present in both quercetin and luteolin.

Since human ingestion of the natural product quercetin, even in large quantities, is non-toxic (5), it may be a useful therapeutic agent against oxidative stress induced by iron overload, as in thalassemia and pre-eclampsia.


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Transport of the photodynamic therapy agent 5-aminolevulinic acid by the amino acid transporter PAT1 (SLC36A1) and the dipeptide transporter PepT1 (SLC15A1)

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5-Aminolevulinic acid (ALA) is a pro-drug used in photodynamic therapy, fluorescence diagnosis and fluorescent-guided resection which produces selective accumulation of protoporphyrin IX in tumour tissue (1). Relatively high oral doses of ALA are used to obtain protoporphyrin IX accumulation in colonic tumours as there is also accumulation in normal gastrointestinal mucosa. Due to the structural similarity of ALA and GABA, or PepT1-expressing oocytes (-60mV, Na+ free, pH 5.5). ALA port was measured by two-electrode voltage-clamp in PAT1-expressing oocytes. [3H]ALA and [3H]amino acid uptake (10-100μM, 0.5-5mCi.ml⁻¹, Na+ free, pH 5.5 buffer) were measured across the apical membrane of human intestinal Caco-2 cell monolayers (5min, 37°C) grown on permeable filters and in PAT1- or PepT1-expressing X. laevis oocytes (40min, 22°C), as described previously (2). Data are mean ± SEM (n) with ANOVA plus Tukey post-test. In Caco-2 cell monolayers, ALA (10mM) inhibited apical uptake of several PAT1 substrates (e.g. GABA) but not that of other amino acids (e.g. methionine). In PAT1-expressing oocytes, [3H]ALA uptake was significantly greater than that in water-injected oocytes at pH 5.5 (p<0.001) and pH 6.5 (p<0.01) but not at pH 7.4 (p>0.05). PAT1-mediated [3H]ALA uptake was reduced (p<0.001) by 91.6 ± 2.7% (n=20) and 96.4 ± 1.7% (n=20) by GABA and 5-hydroxy-tryptophan [OH-Trp, a PAT1 inhibitor (3)], respectively (both 20mM). ALA is known to be a PepT1 substrate (4). In PepT1-expressing oocytes, [3H]ALA uptake was induced by the PepT1 inhibitor 4-aminomethylbenzoic acid [AMBA, 30mM (5)] by 81.8 ± 1.7% (n=20) but not at pH 7.4 (p>0.05). PAT1-mediated [3H]ALA uptake was significantly reduced (p<0.001) by 91.6 ± 2.7% (n=20) and 96.4 ± 1.7% (n=20) by GABA and 5-hydroxy-tryptophan [OH-Trp, a PAT1 inhibitor (3)], respectively (both 20mM). ALA is known to be a PepT1 substrate (4). In PepT1-expressing oocytes, [3H]ALA uptake was induced by the PepT1 inhibitor 4-aminomethylbenzoic acid [AMBA, 30mM (5)] by 81.8 ± 1.7% (n=20). Rheogenic transport was measured by two-electrode voltage-clamp in PAT1- or PepT1-expressing oocytes (-60mV, Na+ free, pH 5.5). ALA induced current consistent with H+/ALA symport with a Kₘ of 1.6 ± 0.9 mM for PepT1 (n=4) and 10.4 ± 5.6 mM for PAT1 (n=4). In Caco-2 cells, OH-Trp and AMBA significantly (p<0.001) inhibited apical ALA uptake, in an additive manner. The relative expression of PAT1 and PepT1 will determine ALA uptake in normal mucosa and gastrointestinal tumours and, in turn, influence both ALA bioavailability and tumour-specific accumulation of protoporphyrin IX.

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Megalin binds to NHERF1 and NHERF2 scaffold proteins

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Albumin endocytosis in the renal proximal tubule is regulated by a number of transmembrane and accessory proteins including the scavenger receptor megalin. Previously we have demonstrated an essential role for the scaffold proteins NHERF1 and NHERF2 in albumin uptake. NHERF1 and NHERF2 are PDZ domain containing proteins that interact with specific sequences that form a PDZ binding domain (S/TXXΦ) in the C-terminus of proteins. Interestingly, megalin contains a functional PDZ binding domain (SDV), however the interaction with the scaffold proteins NHERF1 and NHERF2 has not been investigated.

In this study we investigated if there is an interaction between megalin and NHERF1 and NHERF2, and then characterized the specific domains required for this interaction. Firstly, we investigated if the proteins co-localize in a proximal tubule cell model, the opossum kidney (OK) cell line. Confocal analysis of OK cells demonstrated that the distribution of megalin was predominantly apical with some cytosolic localization. Importantly, NHERF1 had a strong apical localization which overlapped with megalin. Further, as previously described (Hryciw et al, 2006) NHERF2 was predominantly cytosolic, and this protein co-localized with megalin in this region. This indicated that the proteins had an overlap in distribution in proximal tubule cells. Further, immunoprecipitation experiments were performed using anti-megalin, anti-NHERF1 and anti-NHERF2 antibodies that were incubated with rat kidney lysate. The immunoprecipitates were analysed by Western blot analysis using the anti-NHERF1 and anti-NHERF2 and anti-megalin antibodies, respectively. These studies clearly indicated that megalin bound to NHERF1 and NHERF2 in vivo. To determine which domains in NHERF1 and NHERF2 were required for this interaction, GST fusion proteins were generated as described previously (Hryciw et al 2006; Lee et al 2007). These fusion proteins included the full length NHERF1 proteins as well as their 2 PDZ domains (PDZ1 and PDZ2) and C-terminal ezrin binding domain. Incubation with rat kidney lysate and analysis by Western blot analysis indicated that megalin bound to PDZ2 of NHERF1 and PDZ2 and the C-terminal ezrin binding domain of NHERF2. Finally, we used fusion proteins to determine if the C-terminus of megalin was the site of interaction with NHERF1 and NHERF2. GST-pull down experiments and rat kidney lysate supported this. Therefore, we have described for the first time an interaction between megalin and the scaffold proteins NHERF1 and NHERF2. As the NHERF proteins have been shown to be required for the formation of macromolecular complexes in other cell...
systems, as well as binding to NHE3 and CIC-5 that are essential transmembrane proteins required for endocytosis, further investigation should determine if the complex is required for albumin endocytosis in proximal tubule cells.


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Prolyl Hydroxylase Inhibition Attenuates Colonic Epithelial Secretory Function

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BACKGROUND: Inflammation of the gastrointestinal tract due to inflammatory bowel diseases and ischemic colitis is commonly associated with hypoxia and altered epithelial transport. In hypoxia, key O2 sensors called prolyl hydroxylases (PHDs) are inactivated, thereby allowing transcription factors such as hypoxia inducible factor (HIF) and nuclear factor κB (NFκB) to be activated. While recent studies have shown that inhibition of PHDs is protective in murine colitis, little is known of their role in regulating intestinal epithelial transport function. AIM: To investigate the role of PHDs in regulation of epithelial secretory function. METHODS: The pan specific inhibitor dimethyl-oxalylglycine (DMOG) was used to inhibit hydroxylase activity. Transepithelial Cl ion secretion, the primary driving force for fluid secretion in the intestine, was measured as changes in short circuit current (Isc) across voltage clamped monolayers of T84 cells grown on permeable supports. Results are expressed as mean ± standard error of the mean for a series of n experiments. Statistical analyses were made by one way ANOVA using Tukey multiple comparisons test. p values ≤ 0.05 were considered to be significant. RESULTS: Pre-treatment of T84 cells with DMOG (1mM, 24 hrs) significantly attenuated Cl ion secretion in response to the cAMP and Ca²⁺ dependent secretagogues, forskolin (FSK) and carbachol (CCh), respectively. Responses to CCh and FSK were 20.2 ± 2.6% (n = 16; p < 0.001) and 38.6 ± 6.7% (n = 11; p < 0.001) of those in control cells, respectively. Transepithelial resistance was not altered by DMOG treatment indicating that it did not exert toxic effects. The effects of DMOG on secretory responses were apparent after 3 hours and maximal at 18 hours, and this was concurrent with the cellular accumulation of HIF, which was apparent at 3 hours and sustained up to 24 hours. Secretagogue-induced basolateral K⁺ and apical Cl⁻ conductances were not altered by DMOG. In contrast, Na⁺/K⁺ ATPase activity was significantly reduced to 27.6 ± 8.1% (n = 7; p ≤ 0.01) by DMOG. Western blot analysis of transport proteins revealed that DMOG also decreased expression of both the catalytic subunit of the Na⁺/K⁺ ATPase pump and the NKCC1 cotransporter. CONCLUSIONS: These studies demonstrate a novel role for PHDs in regulating intestinal epithelial secretory function. Our data suggest that by virtue of their ability to modulate transport protein expression, PHDs are likely to be important regulators of intestinal fluid and electrolyte transport in hypoxic conditions.

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Farnesoid X receptor activation attenuates colonic epithelial secretory function

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At pathophysiological (mM) concentrations, bile acids acutely stimulate chloride and fluid secretion across colonic epithelial cells, leading to the onset of diarrhoea. However, our previous studies show that at physiological (μM) concentrations, the predominant colonic bile acid, deoxycholic acid (DCA) chronically inhibits colonic Cl⁻ secretion. However, the underlying mechanisms remain unknown.

The Farnesoid X receptor (FXR) is a nuclear hormone receptor that is activated by bile acids. FXR is involved in the regulation of bile-acid biosynthesis and FXR agonists have received much research interest in treatment of diseases associated with bile acid malabsorption. However, to date a role for the FXR in regulating epithelial transport processes has not been reported. To investigate the role of FXR in regulating colonic epithelial Cl⁻ secretion.

Cl⁻ secretory responses to the Ca²⁺-dependent agonist, carbachol (CCh; 100 μM) or the cAMP-dependent agonist, forskolin (FSK; 10 μM), were measured as changes in short circuit current (Isc) across voltage-clamped monolayers of T84 colonic epithelial cells. Immunocytochemistry and laser scanning confocal microscopy were used to examine the subcellular localization of FXR. Results are expressed as mean ± SEM and statistical analyses were made by one way ANOVA and Student Newman Keul’s post-hoc test.

FXR expression was confirmed in T84 cells by western blotting. Treatment with the FXR agonist, GW4064 (2μM for 24 hrs) induced FXR translocation from the cytosol to the nucleus. GW4064 pretreatment attenuated subsequent secretory responses to CCh and FSK to 54.8 ± 4% and 72.2 ± 3% of those in control cells, respectively (p≤0.001, n=14). The effects of GW4064 were concentration-dependent, with antiserum effects apparent at 0.1 μM. As previously reported, pretreatment of T84 cells with DCA (50μM for 24 hrs) also attenuated secretory responses to CCh and FSK. However, the antiserum effects of GW4064 and DCA were additive and effects of GW4064, but not DCA, were inhibited by an FXR antagonist, guggulsterone (5μM), suggesting independent mechanisms of action. Furthermore, CCh-stimulated Na⁺/K⁺ ATPase activ-
Cystic Fibrosis and Lipoxins


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Contribution of Rho kinase to calcium-contraction coupling in airway smooth muscle

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We investigated theoretically and experimentally the role of Rho kinase (RhoK) in Ca²⁺-contraction coupling in rat airways. Isometric contraction was measured on tracheal, extra- and intrapulmonary bronchial rings, in response to carbachol and 50 mM external KCl (n=6 to 8). [Ca²⁺]i recorded in freshly isolated tracheal myocytes using Indo-1 (n=19 to 25). Values are expressed as mean ± SEM. Statistical comparisons were done using a Student’s t test. Theoretical modeling consisted in a four-state model of the contractile apparatus coupled with a model of Ca²⁺-dependent MLCK activation and RhoK-dependent MLCP inactivation. Analysis of the time course of contraction to carbachol (0.3 and 10 μM) showed that force development occurred in two phases: (i) a short-time, Hill-shaped contraction obtained within 90 s, (ii) followed by a maintained or an additional delayed contraction. Hill fitting of the first phase showed that the short-time maximal contraction (stFmax) of tracheal rings to 10 μM carbachol was 77.8±2.6% of total maximal force, and the time to obtain half-stFmax was 17.4±1.6 s. Values of similar range were obtained in bronchial rings. [Ca²⁺]i responses to 10 μM acetylcholine (ACH) consisted in a fast peak followed by a plateau and, in 42% of the cells, superimposed Ca²⁺ oscillations. Exposure to the RhoK inhibitor Y27632 (10 μM) did not alter the resting [Ca²⁺]i, nor the parameters of the ACh-induced Ca²⁺ response. Whatever the concentration of carbachol and the location along the airway tree, Y27632 did not modify the basal tension but decreased the amplitude of the short-time response, without altering the additional delayed contraction. Calyculin A (MLCP inhibitor) increased the basal tension, and abolished the effect of RhoK inhibition on carbachol-induced contraction. Stimulation by 50 mM external KCl solution in the presence of atropine induced a short-time contraction followed by a sustained tension, both depending of

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the presence of external Ca^{2+}. As with carbachol-induced contraction, Y27632 decreased the amplitude of the short-time response, without altering the delayed tension. KN93 (CaMKII inhibitor) and DIDS (Ca^{2+}-activated Cl^- channels) had no influence on the effect of RhoK inhibition. We conclude that Ca^{2+}-dependent but CaMKII-independent RhoK activation contributes to the early phase of the contractile response via MLCP inhibition. On these bases, we implemented our previously published model of Ca^{2+}-contraction coupling (1). The model explains the time course of the short-time contraction and the role of RhoK by Ca^{2+}-dependent activation of MLCK and RhoK, which inactivates MLCP. Oscillatory and non-oscillatory [Ca^{2+}]_i responses result in a non-oscillatory contraction which amplitude is encoded by the plateau value and oscillation frequency.


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Estrogen effects in human airway epithelial cells

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While gender differences incidence and severity of asthma are clinically recognized, the physiological role of sex steroids is less clear, with cyclical exacerbations (pre-menstrual asthma) occurring in late luteal phase when estrogen levels are low, while high sex steroid levels (e.g. pregnancy) are not always associated with worsening of symptoms. Similar to endothelium in vasculature, airway epithelium is a source of bronchodilatory agents such as NO. Using human bronchial rings and epithelial cells derived from such samples (obtained from surgery), we tested the hypothesis that estrogens facilitate bronchodilation by enhancing NO production in airway epithelium, resulting in reduced intracellular Ca2+ ([Ca2+]i) in airway smooth muscle. Values are means with SE. Acute exposure to 1 nM 17β-estradiol (E2) relaxed epithelium-intact bronchial rings contracted with 1 μM ACh to a significantly greater extent (72 ± 9% of max. force) than epithelium-denuded rings (42 ± 8%; p<0.05, n=4, t-test). E2-induced relaxation was blunted by the NO scavenger PTIO (48 ± 9%). In isolated human airway epithelial cells (n=20 per group) loaded with the fluorescent NO indicator DAF-2, 1 nM E2 increased fluorescence levels (28 ± 3 arbitrary units (au) with 5 ± 2 au baseline) to that induced by 1 μM ACh (32 ± 7 au) or 50 μM ATP (41 ± 4 au). In the presence of E2, ACh effects on DAF-2 fluorescence were enhanced (69 ± 5 au), while PTIO substantially blunted these effects (22 ± 6 au). The estrogen receptor (ER) specific agonists THC (ERα) and DPN (ERα) both induced comparable NO production (31 ± 6 au vs. 38 ± 6 au). Neither ACh nor E2 affected fluorescence of the NO-insensitive dye DAF-4. Western blot analyses of eNOS phosphorylation at Ser1177 showed an ~5-fold increase with E2 exposure. Finally, in Transwell plates where human epithelial cells and airway smooth muscle cells were cocultured, exposure of epithelial cells to 1 nM E2 induced 58 ± 7% reductions in ACh-induced elevation of [Ca2+]i, in airway smooth muscle cells. Overall, these studies indicate a prominent role for airway epithelium and epithelium-derived NO in the bronchodilatory effects of estrogens in humans. Carey, M.A., et al. Trends Endocrinol Metab 2007; 18: 308-13. Chambliss, K.L., and Shaul, P.W. Endocr Rev 2002; 23: 665-86. Supported by NIH grants HL088029 and HL090595, and the Flight Attendants Medical Research Institute (FAMRI).

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Regulation of spontaneous activity in interstitial cells of Cajal of the rabbit urethra by ATP


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Interstitial cells of Cajal (ICC) in the urethra are considered to be putative pacemaker cells which are involved in the generation of spontaneous myogenic tone (Sergeant et al., 2000). Urethral ICC are spontaneously active and the frequency of this activity is modulated by several neurotransmitters, including noradrenaline and nitric oxide, (Sergeant et al., 2002 & 2006). ATP is also considered to be an important neurotransmitter in the urethra, therefore experiments were performed to test if spontaneous activity in urethral ICC was also regulated by ATP. New Zealand white rabbits were humanely killed and ICC were freshly dispersed from the urethra as described previously (Sergeant et al., 2000). ICC were then plated and loaded with Fluo-4 AM for study of cytosolic Ca2+ signals using a spinning disk confocal microscope. STICs were recorded at ~60 mV using the amphotericin B perforated patch technique. ATP (10 μM) increased the frequency of STICs in urethral ICC from 9 ± 1.6 to 26 ± 3.7 min-1 (p<0.05). These effects were mimicked by the P2Y receptor agonist 2-methylthio ADP (2-MeSADP, 1 μM), which increased STIC frequency from 7.25 ± 0.5 to 26.25 ± 3.2 min-1, n=8 (p<0.05). The broad spectrum purinergic receptor antagonist suramin (100 μM) reduced the mean frequency of ATP evoked inward currents from 17.9 ± 2.8 to 1.3 ± 0.66 min-1 (n = 9, p<0.05) and those evoked by 2-MeSADP from 31 ± 6.1 to 4.2 ± 2.1 min-1 (n = 6, p<0.05). Similar results were achieved with the selective P2Y1 receptor antagonist, MRS2500 (100 nM) which reduced the mean frequency of ATP evoked inward currents from to 17 ± 4.5 min-1 to 6.2 ± 2.5 min-1 (n=8, p<0.05) and 2-MeSADP induced currents from 25.8 ± 4.3 to 7 ± 1.8 min-1 (n=6, p<0.05). Johnston et al., (2005) showed that STICs in urethral ICC are associated with global Ca2+ oscillations therefore we investigated if this activity was also modulated by ATP and 2-MeSADP. Application of ATP (10 μM) significantly increased the frequency of Ca2+ waves from 9 ± 1.4 to 29.1 ± 4.5 min-1 (n = 14, p<0.05) and 2-MeSADP (1 μM) caused an increase from 7.3 ± 1.6 to 17.1 ± 2.8 min-1 (n=10, p<0.05). The frequency of the ATP induced Ca2+ oscillations was reduced from 27 ± 8.7 min-1 to 6.6 ± 2.1

in either WT or KO mice. No differences were detected in the mRNA levels of TASK-2, TASK-5, TWIK-2, Kγ1.5 or Kγ2.1 between WT (N=3) and KO (N=3) mice. PA isolated from KO mice failed to develop intrinsic tone and displayed unaltered vasoconstrictor responses. The absence of TASK-1 in PA SMC did not affect I(K,IN) and was not compensated by up-regulation of other K+ channel subunits. The results question the importance of TASK-1 in mouse PA and highlight species differences in the resting K+ conductance.

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min⁻¹ by suramin (n=7, p<0.05) and similar results were achieved with MR52500, which decreased the mean frequency of ATP induced Ca²⁺ oscillations from 28.1 ± 4.2 to 10.3 ± 3.9 min⁻¹ (n=6, p<0.05).

These data demonstrate that spontaneous activity in urethral ICC is modulated by ATP and that this effect is likely to be mediated by P2Y receptors.


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Vasomotor responses to reactive oxygen species in rat mesenteric arteries

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Reactive oxygen species (ROS) are important mediators of vascular tone and play a key role in cardiovascular disease. We showed previously that LY83583, a generator of superoxide anion, caused a superoxide dismutase (SOD)-inhibitable constriction of rat pulmonary arteries that was directed primarily via Rho-kinase-mediated Ca²⁺-sensitization (Knock et al., 2009).

In the present study we investigated the vasomotor responses to LY83583 and hydrogen peroxide (H₂O₂) in intact and alpha-toxin permeabilised U46619 (100 nM)-pre-constricted rat mesenteric arteries (MA), mounted on a wire myograph. In intact MA, LY83583 was applied in the presence of L-NAME (1mM) in order to eliminate superoxide scavenging of NO. Statistical analysis was by Student t-test.

Both LY83583 (10µM) and H₂O₂ (100µM) strongly relaxed U46619 pre-constricted intact MA (LY83583: 86 ± 6% relaxation at 15min, n=10; H₂O₂: 94 ± 1% relaxation at 5min, n=2), and the former was partially prevented by combined pre-incubation of SOD and catalase (40 ± 14% relaxation at 15min, n=9, P<0.01 vs. LY83583 alone). In contrast, when MA were pre-constricted with 30 mM KCl, LY83583 caused further sustained constriction and no relaxation (119 ± 15% enhancement at 5 min, n=4, P<0.05).

In alpha-toxin permeabilized MA (pre-constricted with 100nM U46619 at pCa 6.4), LY83583 caused modest but concentration-dependent relaxation (10µM: 24 ± 3% relaxation, n=15, P<0.01 vs. vehicle; 30µM: 60 ± 5% relaxation, n=12, P<0.01 vs. DMSO vehicle). LY83583-induced relaxation was prevented by the Rho-kinase inhibitor Y27632 (10µM, n=6, LY83583 relaxation not significant vs. vehicle), but was insensitive to blockade by either the antioxidant tempol (LY83583: 37 ± 6% relaxation, n=6, P=0.05 vs. vehicle) or SOD (LY83583: 36 ± 3% relaxation, n=7, P<0.05 vs. vehicle). The underlying pCa 6.4 constriction was insensitive to LY83583 or vehicle. H₂O₂ however, and in contrast to its effect in non-permeabilized MA, caused constriction at pCa 6.4 both in the absence (43 ± 7% enhancement at 15min, n=5, P<0.01 vs. time control) and presence of U46619 (17 ± 3% enhancement at 15min, n=4, P<0.001 vs. time controls).

In summary, LY83583 either relaxes or constricts MA, depending on the nature of the pre-constriction. LY83583-generated superoxide more strongly relaxes intact compared to permeabilized U46619 pre-constricted MA, whereas exogenous H₂O₂ relaxes intact but constricts alpha-toxin permeabilized MA. ROS therefore contribute to multiple constriction/relaxation pathways in MA. LY83583-induced Rho-kinase dependent Ca²⁺-desensitization in MA does not appear to be mediated via ROS.


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Automated calcium spark detection algorithm for linescan images containing Poisson noise

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The physiological significance of calcium sparks in cardiac, skeletal and smooth muscle cells has received a great deal of attention in recent years. Such studies rely upon the accurate detection and analysis of brief, localised release events in order to quantify changes in spark frequency and properties under different conditions. In order to adequately characterise the time course of sparks, images are often obtained using confocal laser scanning microscopy in linescan mode. The manual analysis of this data is time consuming and prone to user bias, because the small size of many events and relatively low signal-to-noise ratio make it difficult to discern small amplitude sparks from noise artifacts.

The main source of noise in these images is photon noise, which follows a Poisson distribution and entails that the noise variance is larger when the background fluorescence is increased. Previously published automated spark detection algorithms are limited in at least one of two related ways: (1) sparks are assumed to occur from a constant baseline, and (2) the noise is assumed to be Gaussian, with a fixed variance throughout the image. These algorithms are therefore inappropriate for reliably identifying sparks in images containing multiple cells with differing baseline fluorescence levels, or occurring on top of global calcium elevations. Both situations arise in images of calcium signaling events in the smooth muscle of retinal arteriolar segments, as described by Curtis et al. (2004).

We have developed new software that overcomes these issues by using a wavelet-based variance stabilisation technique to automatically adapt spark detection to changes in baseline fluorescence. In addition to facilitating the analysis of images for which no automated algorithm currently exists, preliminary tests indicate that the use of a more accurate noise model means that our algorithm can also offer improved detection accuracy in any linescan containing sparks, particularly at low signal-to-noise ratios.


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Human mesenchimal stem cells as a novel approach for radiation-induced vascular malfunction therapy

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Therapeutic effect of mesenchymal human stem cells transplantation (MSCT) has been evaluated in a whole-body irradiated (6 Gy) rats. Experimental design of the study comprised large conductance Ca2+-dependent K+ channels (BKCa) activity measurements in aortic smooth muscle cells using patch clamp technique in whole-cell modification, non-invasive systolic arterial blood pressure measurement and simultaneous measurement of contractile force and [Ca2+]i.

Bone marrow was aspirated in heparin from the sternum of healthy volunteers after informed consent (with 1%lidocaine as a local anaesthetic). Mesenchymal stem cells (MSC) were separated using negative selection procedure with monoclonal antibodies (Human RosetteSep Mesenchymal Stem Cell Enrichment Cocktail, StemCell Inc.). The isolated MSC after Ficoll-Hypaque centrifugation were resuspended in MesenCult medium (StemCell Inc.) supplemented with appropriate Mesenchymal Stem Cell Stimulatory Supplements and cultivated 20 - 32 days in the same medium with additional recombinant human growth factors: SDF-1a, EGF, and PDGF-AA (CHO-grade). After two passages MSC were transplanted intravenously to irradiated rats on the 7th day of post-irradiation in a single dose of 16-20x106 cells per rat.

Whole-body irradiation produced a decrease of BKCa activity in aortic myocytes. This was paralleled by a reduction of the NO-dependent ACh-induced vascular relaxation and arterial hypertension development. Thus, the vasorelaxing force of BKCa was diminished in irradiated myocytes. Simultaneous measurements of contractile force and [Ca2+]i showed that myofilament Ca2+ sensitivity defined as the ratio of force change to [Ca2+]i significantly increased following irradiation.

MSCT effectively restored outward currents (from 13±1 to 24±1 pA/pF, P<0.05, n=12) mainly due to paxillin-sensitive BKCa component, and led to an increase in amplitude of maximal ACh-induced endothelium-dependent relaxation in irradiated vascular tissues from 43±3% to 87±6% (P<0.05, n=12). MSCT normalized myofilament Ca2+ sensitivity (from 0.068±0.007 to 0.030±0.004 mN/nM, P<0.05, n=12) and arterial blood pressure from 152±4 to 121±2 mm Hg (P<0.05, n=12).

The data obtained suggest that MSC demonstrate a clearly expressed therapeutic potential to normalize vascular abnormalities induced by ionized irradiation and appear to be worthwhile therapeutic approach in case of vascular malfunction induced by ionizing irradiation.

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Lesions of the commissural nucleus of tractus solitarii increase osmotic-induced activation of paraventricular and supraoptic hypothalamic nuclei

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Neurons in the magnocellular division of paraventricular nucleus (PVN) and supraoptic nucleus (SON) of hypothalamus are activated by osmotic stimulus increasing the release of oxytocin and vasopressin, hormones that induce natriuresis, antidiuresis and vasoconstriction (McCann et al, 2003). PVN and SON receive important ascending neural connections from the nucleus of tractus solitarii (NTS) (Tribollet et al, 1985). Previously we have demonstrated that rats with lesions in the commissural subdivision of the NTS (commNTS) increased water intake, natriuresis and arterial pressure after intragastric 2 M NaCl load. Considering that the increase in arterial pressure in commNTS-lesioned rats is dependent on vasopressin, in the present study we investigated the effects of commNTS lesions on c-fos expression in the PVN and SON produced by intragastric hypertonic sodium load.

Male Holtzman rats (300-320 g, n = 3-4/group) were anesthetized with ketamine (80 mg/kg of body weight, ip) combined with xylazine (7 mg/kg of body weight, ip) and submitted to electrolytic (1 mA x 10 s) or sham lesion of the commNTS. Fifteen days after lesions, rats received 2 ml of 2 M NaCl intragastrically, which increases plasma osmolality in 4% (Pereira et al, 2002) or 2 ml of 0.15 M NaCl. Two hours after sodium load, rats were deeply anesthetized with pentobarbital (50 mg/kg of body weight, ip), perfused with 4% paraformaldehyde and brains were removed. Immunohistochemistry for c-fos expression was performed in brain slices using DAB staining. Data are expressed as means ± SEM and analyzed by one-way ANOVA.

In sham rats, intragastric 2 M NaCl induced c-fos expression in magnocellular PVN (70 ± 12 vs. 0.15 M NaCl: 8 ± 2 positive cells/section – each 150 μm bilaterally, p <0.05), parvocellular PVN (27 ± 4 vs. 0.15 M NaCl: 5 ± 1 positive cells/section, p <0.05) and SON (191 ± 12 vs. 0.15 M NaCl: 2 ± 1, positive cells/section, p <0.05). CommNTS lesions increased c-fos expression induced by intragastric 2 M NaCl in magnocellular and parvocellular PVN (139 ± 22 and 40 ± 27 positive cells/section, respectively, p <0.05 vs. sham) and SON (270 ± 11 positive cells/section, p < 0.05 vs. sham). Double labeling immunofluorescence demonstrated that a significant number of c-fos positive cells in PVN and SON in both commNTS- and sham-lesioned rats were oxytocinergic or vasopressinergic.

The results show increased number of PVN and SON neurons activated by osmotic stimulus in commNTS-lesioned rats, which may have a correlation with the increased natriuresis and arterial pressure in commNTS-lesioned rats after hypertonic sodium load.

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**PC3**

**Effects of exogenous or endogenous hydrogen peroxide centrally on the pressor response to central cholinergic activation**


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Recent results from our laboratory demonstrated that intracerebroventricular (icv) injection of hydrogen peroxide (H2O2), a reactive oxygen species, reduced water intake and pressor responses induced by icv injection of angiotensin II (Lauar et al., 2008). Thus, in the present study we investigated the effects of icv injection of H2O2 or ATZ (3-amino-1,2,4-triazole, a catalase inhibitor) on the pressor responses induced by icv injection of the cholinergic agonist carbachol.

Male Holtzman rats (280-320 g, n=8/group) were anesthetized with ketamine (80 mg/kg of body weight) combine with xylazine (7 mg/kg of body weight) and had stainless steel cannulas implanted in the lateral ventricle (LV). Mean arterial pressure (MAP) and heart rate (HR) were continuously recorded and H2O2 (5 μmol/1 μl) or PBS (vehicle, 1 μl) was injected into the LV 1 min before the injection of carbachol (4 nmol/1 μl) or saline was injected into the LV 10 min before the injection of carbachol.

The previous icv injection of H2O2 or ATZ reduced the pressor responses produced by icv injection of carbachol (12 ± 4 and 13 ± 4 mmHg, respectively, vs. vehicle or saline: 25 ± 4 and 30 ± 4 mmHg, respectively). No significant change on HR was produced by carbachol alone (-13 ± 13 and -31 ± 15 bpm, respectively) or combined with H2O2 or ATZ (-3 ± 9 and -8 ± 17 bpm), compared with vehicle or saline (-2 ± 16 and 13 ± 13 bpm, respectively).

The results show that central injections of H2O2 or ATZ reduced the pressor response induced by icv injection of carbachol, suggesting that exogenous or endogenous H2O2 may inhibit central pressor mechanisms activated by central cholinergic activation.


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**PC4**

**Macrophage inhibitory migration factor in the paraventricular nucleus of hypothalamus attenuates hyperosmotic-evoked sympathoexcitation in the rat**

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Systemic hyperosmotic stimulation (HS) evokes increases in sympathetic nerve activity (SNA) mediated by activation of angiotensin II type 1 (AT1) receptors in the hypothalamic paraventricular nucleus (PVN), as described by Chen and Toney (2001). Recently, macrophage inhibitory migration factor (MIF) can antagonize the hypertension evoked by angiotensin II acting at the level of the PVN (Li et al., 2006). This inhibitory effect of MIF is due to its intrinsic thiol-protein oxidoreductase (TPOR) activity (Sun et al., 2007). In this study, we evaluated the effect of virally mediated over expression of either MIF or C60SMIF (which lacks TPOR activity) in the PVN on the sympathoexcitation induced by hyperosmolality (HS). Under deep halothane anaesthesia, male Wistar rats (65-85 g) were decorticated to make insentient and perfused intra-arterially (Antunes et al., 2006). HS was induced by raising perfusate osmolality from 290 to 380 mOsmol for 40 s. Adeno associated viruses employed for over expression of MIF (1.0x10⁸), C60SMIF (1.0x10⁸) or eGFP (8.3 x 10⁸) were injected bilaterally (500 nl/side) into the PVN as was saline as a control. Seven to 10 days later the HS-induced sympathoexcitation in both the saline and eGFP groups (increases of 27 ± 4% and 25 ± 4%, respectively) was not observed in the MIF group (4 ± 5%). Conversely, the HS induced SNA response was potentiated (45 ± 6%) in the C60SMIF group. When MIF injections were located outside the PVN a response similar to control was observed. Immunohistochemical analysis demonstrated that MIF expression in PVN was restricted to neurones some of which were immunopositive for vasopressin. We propose that MIF acting within the PVN is a major counter regulator of HS-induced sympathoexcitation, which is dependent on its TPOR activity. Further, enhancing TPOR activity may have therapeutic potential in restricting salt-induced hypertension.


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PC5

Chronic dehydration switches the control of sympathetic activity from forebrain to hindbrain in the rat

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An increase in plasma osmolality induced by dehydration causes an increase in sympathetic nerve activity (SNA). The central nervous mechanisms underlying the increase in SNA after dehydration are not fully established. Here, we investigated the sequential effects of systemic administration of Losartan (20 μM, angiotensin II type 1 (AT1) receptor antagonist), brain transections and chemical inhibition of commissural nucleus tractus solitarii (cNTS) on ongoing thoracic SNA after chronic dehydration (DH; 3 days of water deprivation).

Subsequent to DH, experiments were performed in the in situ working heart-brainstem preparation of rat (Antunes et al., 2006). Under deep halothane anaesthesia (assessed by an absence of a limb withdrawal reflex to noxious pinching), the rat was transected below the diaphragm, decorticated to make insentient and perfused with oxygenated Ringer’s solution via the descending aorta. Perfusion pressure, heart rate, phrenic nerve activity and thoracic SNA were recorded. Data are expressed as mean ± SEM.

In euhydrated (EH) rats (290 mOsmol perfusate), systemic application of Losartan and subsequent pre-collicular transection (to remove the hypothalamus) reduced SNA by -20±3% and -44±2% respectively (n=5; P<0.05). In contrast, in DH rats (340 mOsmol perfusate, n=6) Losartan, subsequent pre-collicular and then pontine transections failed to reduce SNA (-33±8% and -12±4%; P=0.753, P=0.995 and P=0.372, ANOVA). However, transection at the medulla-spinal cord junction reduced SNA by -70±8%. In intact DH, but not EH rats, reversible inactivation of cNTS using isoguvacine, (a GABAA receptor agonist; 100 mM, 100 nl), reduced significantly baseline SNA (-33±7% in DH; P<0.01 vs 3±8% in EH). Since it was demonstrated that dehydration increases FosB staining in the cNTS (Li et al, 2007), we chronically blocked AP1 transcription factor activity in the cNTS using a viral vector expressing a FosB dominant negative (Ad-CMV-IRESEGFP-dnFosB; 2.9x10⁶ pfu/ml) 5-7 days prior to 3 day water deprivation. In these animals, inactivation of the cNTS was ineffective (-6.9±8.7% vs. -49±8%, control rats with Ad-CMV-eGFP in NTS; P<0.05, Student’s t test).

In addition, in DH rats in which AP1 was blocked in the cNTS, SNA was now decreased after pre-collicular transection (-45±8%), a response that was similar to that seen in control EH rats (-35±6%).

These data indicate that in the EH rat baseline SNA is dependent on both the hypothalamus and AT1 receptors. Following chronic dehydration, the regulation of SNA transfers to the medulla oblongata, particularly the cNTS. This plasticity seems to be mediated via activation of the AP1 transcription factor; if AP1 transcription factor activity is blocked in NTS during dehydration, then control of sympathetic activity reverts back to forebrain regions.

Antunes, VR; Yao, ST; Pickering, AE; Murphy, D; Paton, JFR. J. Physiol, 576(2): 569-583, 2006.


Supported by CNPq, Capes, Royal Society and BHF.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC6

Reactive Oxygen Species Mediates Acute Vascular Contraction Responses In Rat Mid Cerebral Artery

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Intro: Pressure-dependent myogenic responses play a significant role in modulating autoregulation of resistance arteries, especially in cerebral circulation [1]. Increasing evidence suggests a potential physiological role for NADPH-oxidase and reactive oxygen species (ROS) in pressure-dependent myogenic tone [2]. There may also be potential link between ROS and actin cytoskeletal dynamics [3]. In this study, we investigated the potential role of NADPH-oxidase/ROS and actin polymerization in acute pressure-dependent vascular contraction.

Method: Adult male Sprague-Dawley rats (12 weeks, 250–300g) were asphyxiated using CO2 and euthanized by cervical dislocation. Pressure-dependent responses were recorded in isolated mid cerebral arteries (MCA; Diameter 150 ± 7.81 μm) using pressure myography. Pressure-dependent responses were studied by pressure-step (40 to 80 to 120 mmHg). Confocal microscopy was used to visualize staining of F-actin using phalloidin-FITC (1 μM) in pressure-fixed vessel. Statistics analysis was measured by two-way ANOVA for repeated measure and values presented as mean ± SEM. Results & Discussion: Pressure-dependent myogenic tone in MCA control was 11.46 ± 3.05, 18.72 ± 2.88 and 23.75 ± 2.61 % at 40, 80 and 120mmHg, respectively (n=14). Pressure-dependent myogenic tone was significantly reduced following NAC, DPI and Cyto D incubation. The antioxidant N-acetylcysteine (NAC, 10mM, n=4) reduced myogenic tone to 7.72 ± 1.31, 7.44 ± 2.81, 8.06 ± 1.44 % at 40, 80 and 120mmHg respectively. The NADPH-oxidase inhibitor diphenyleneiodonium (DPI, 10μM, n=3) reduced myogenic tone to 5.93 ± 1.53, 2.00 ± 2.33, -1.15 ± 2.25 % at 40, 80 and 120mmHg respectively. DPI and Cyto D reduced myogenic tone to 9.52 ± 4.32, 3.79 ± 0.67, 0.45 ± 1.78 % at 40, 80 and 120mmHg, respectively. Likewise, the actin polymerization inhibitor Cytochalasin D (Cyto D, 5μM, n=4) reduced myogenic tone to 9.52 ± 4.32, 3.79 ± 0.67, 0.45 ± 1.78 % at 40, 80 and 120mmHg, respectively. Time-control experiments showed sustained pressure-dependent myogenic tone. NAC and DPI did not have any effect on high-K+ PSS (60mM) constriction. The confocal study showed that there was a marked increase of actin polymerization in vascular smooth muscle.
(VSM) at 120mmHg compared to 40mmHg. The effect of Cyto D greatly reduced the fluorescence measured at 120 mmHg. This further confirmed a dynamic actin polymerization contribution to the pressure-dependent myogenic tone mechanism in the cerebral arteries. Conclusion: These data suggest that the activation of NADPH oxidase/ROS/actin polymerization as part of the acute pressure-dependent myogenic constriction mechanism.


Ministry of Higher Education (Malaysia), SIPBS

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**PC7**

**Effect of sex and maturation on sympathetic innervation density of the rat caudal ventral artery (CVA) – a role in thermoregulation?**


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Cutaneous sympathetic vasoconstrictor nerves are tonically active; changes in their activity regulate skin blood flow and are important in thermoregulation. Studies in humans have shown that female reproductive hormones have substantial influences on thermoregulatory responses (1) and that before the menopause, the vasoconstrictor influences of sympathetic nerve fibres are greater in females than males (2). Sympathetic nerve activity to skeletal muscle vasculature increases with age in both women and men, but is lower in women than men in the 20–29 year age range (3). Whether the same is true of cutaneous sympathetic vasoconstrictor nerves is not known, nor whether any differences between males and females exist pre-puberty. Further, it is not known whether there are differences in sympathetic nerve innervation density that might contribute to sex-related differences in thermoregulation. Thus, we have investigated the effect of sex and maturation on sympathetic nerve innervation density of the CVA of the tail, the main thermoregulatory organ in the rat.

Four groups (n=6) of Wistar rats were used: juvenile male and female (4-weeks) and sexually mature (12-weeks) male and females. Post-menopausally, sympathetic nerve activity is higher in females than males (3), so raising the question as to how the menopause affects sympathetic innervation density.


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**PC8**

**Do NADPH oxidase (Nox)-derived reactive oxygen species (ROS) modulate muscle vasodilator responses to acute systemic hypoxia or adenosine?**


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Two major sources of ROS in vascular tissue are xanthine oxidase ( XO), which generates ROS from the metabolites of adenosine and Nox1. The vasodilatation that occurs in skeletal muscle during acute systemic hypoxia is largely mediated by adenosine and nitric oxide (NO)2. During acute systemic hypoxia, ROS levels increase in skeletal muscle, and the hypoxia-induced muscle vasodilatation is modulated by XO-derived ROS which may act directly, or by interacting with NO3: O2. Decreases NO bioavailability by combining with NO to form peroxynitrite. Nox activity is increased in chronic conditions such as hypertension4, but Nox expression was also increased by acute hypoxia in pulmonary artery5. It is not known whether ROS generated acutely from Nox can modulate skeletal muscle dilatation.

Thus, in anaesthetised male Wistar rats (Alfaxan: 3-6ml.kg-1 hr-1 iv), arterial blood pressure (ABP) and femoral blood flow (FBF) were recorded; femoral vascular conductance (PVC) was computed (FBF/ABP). Responses evoked by 5-minute periods of hypoxia (breathing 8% O2) and adenosine infusion (1.2mg.kg-1 hr-1 iv) were recorded before and after, the Nox inhibitor apocynin (13mg.kg-1 iv, Group 1), the NO synthase inhibitor L-NAME (10mg.kg-1 iv, so as to maximize ROS levels) followed by apocynin (Group 2) and L-NAME followed by a second Nox inhibitor DPI (Group 3). The muscle vasodilatation was analysed as the
change in the integral of FVC (IntFVC) in arbitrary conductance units (CU). Comparisons were made by ANOVA, P<0.05 being considered significant.

Acute hypoxia induced a fall in ABP (from 103±3 to 50±4mmHg), and muscle vasodilatation (IntFVC increased from 2.8±0.4CU by 2.6±0.4CU). In Group 1, apocynin had no effect on the hypoxia-induced increase in IntFVC. As expected, in Group 2, L-NAME increased baseline ABP (to 130±1mmHg) and decreased IntFVC (to 1.6±0.2CU) reflecting removal of the effect of tonic NO release, and the hypoxia-induced increase in IntFVC was attenuated (1.2±0.2CU), but apocynin had no effect on baseline (1.7±0.2CU) or on the hypoxia-induced dilatation (1.4±0.2CU). Similarly, in Group 3, DPI had no effect on the hypoxia-induced increase in IntFVC after L-NAME. Moreover, adenosine infusion induced similar dilator responses to hypoxia, but they were not affected by apocynin or DPI.

The pharmacological inhibitors of Nox currently available are not fully selective and when used individually, may have led to overestimation of the role of Nox. The lack of effect of apocynin or DPI in the present study leads us to propose that Nox-derived ROS do not modulate the vasodilatation induced in skeletal muscle by acute systemic hypoxia, even when the inhibition of NO synthase prevents the scavenging of ROS by NO. It may be that a chronic condition, such as chronic hypoxia, is required to increase NOx activity in systemic tissues.

Contribution of NPY Y1 and NPY Y2 receptors to sympathetic vasoconstriction in diabetic rat tail artery at two time points

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Vascular dysfunction is a common consequence of diabetes mellitus. Alterations in sensitivity/responsiveness to neurotransmitters (noradrenaline (NA), ATP and NPY) may underlie functional abnormalities of diabetic blood vessels (Speirs et al., 2006). Previous studies have reported considerable variation in such alterations, potentially due to sampling at different time-points after induction of diabetes. In this study, contributions of NPY Y1 and NPY Y2 receptors to sympathetic vasoconstriction were explored in tail artery of diabetic rats at 20 and 40 weeks of age.

Tail arteries were excised from diabetic (60 mg.kg-1 streptozotocin, i.p. injection at 8 weeks) and control Sprague-Dawley rats at 20 and 40 weeks of age. Receptor expression was determined at mRNA level using RT-PCR (normalized to β-actin). Iso- metric contractions were recorded from proximal sections (3-5 mm, endothelium-denuded) in response to electrical stimuli (5 impulses, 1ms duration at 20Hz) delivered every 90 seconds. NPY (100nM) potentiated responses in all groups (20 week control vs diabetic: 39±10%, mean ± S.E., n=8; vs 62±11%, n=6; 40 week control vs diabetic: 56±4%, n=8 vs 69±8%, n=5; P<0.01 in each case, unpaired Student’s t-test). No differences were detected between time-points. NPY Y1, specific antagonist BIBP3226 (1μM) reduced responses in all groups (20 week control vs diabetic: 40±7% vs 37±6%, n=8 and P<0.01 in each case; 40 week control vs diabetic: 32±3 % vs 26±3%, n=5 and P<0.05 in each case). No differences were detected between groups. NPY Y2 agonist PYY3-36 potentiated responses in 20 week old diabetic artery (P<0.01, 42±5%, n=9) relative to control (1±2%, n=5). However, this effect was not observed in 40 week old diabetic artery (12±3%, n=5) relative to control (4±%, n=5). NPY Y2 specific antagonist BIIE0246 significantly reduced responses in 20 week old diabetic artery (P=0.01, 32±6.10%, n=9) relative to control (3.4±6.25%, n=8). Similar to Y2 agonist, this was not apparent in 40 week old diabetic artery (5±1%, n=5) relative to control (3±1%, n=5).

NPY Y1 receptor expression increased (P<0.05; 3.63±0.07, n=5) in diabetic arteries at 20 weeks relative control (1.28±0.06, n=5). However, no change in NPY Y1 receptor expression (1.19±0.02, n=6) was observed in diabetic artery at 40 weeks relative to control (0.82±0.06, n=5). NPY Y1 receptor expression was elevated in 20 week old diabetic artery (P<0.01, 3.38±0.1, n=5) relative to control (1.22±0.04, n=5). In contrast, there was a reduction in expression (P<0.01, 0.36±0.02, n=5) in 40 week old diabetic artery relative to its control (1.09±0.01, n=5). These data indicate that enhanced NPY Y1 and Y2 receptor expression and its associated contribution to vasoconstriction in a model of Type 1 diabetes has a temporal aspect to its involvement in peripheral vascular dysfunction.


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mice (both type 1 diabetes models); expressional studies were supplemented with functional studies in rat tail arteries. Tail arteries were excised from diabetic (60 mg.kg-1 streptozotocin, i.p. injection at 8 weeks) and control Sprague-Dawley rats at 20 weeks and Akita mice and controls at 10 weeks. Receptor expression was determined at mRNA and protein level using RT-PCR and Western blotting (normalized to β-actin mRNA and β-actin respectively). Isometric contractions were recorded from proximal sections of rat tail arteries (3-5 mm length, endothelium-denuded). Agonist concentrationresponse curves were expressed as a % of contraction induced by 60 mM KCl.

Maximal contraction induced by NA was greater in arteries of diabetics (279±17%, mean ± S.E., n=20, P<0.001; 2-way ANOVA, Bonferroni post-hoc tests) relative to arteries of age-matched control rats (n=20, 179±12%). α1a-AR expression was not increased in arteries of diabetic rats relative to controls at mRNA (1.16±0.16 vs. 1.01±0.05; n=5 each, unpaired students t-test) or protein level (1.17±0.31 vs. 0.79±0.15; n=5 each). Maximal contraction produced β,γ-methylene ATP was greater in arteries of diabetics (P<0.001; 103±12%, n=20) relative to age-matched control rats (69.9±11%; n=20). Parallel increases in P2X1 receptor expression were observed at both mRNA (P<0.01; 103±9%; n=20) and protein level (P<0.01; 0.48±0.04) relative to age-matched controls (1.22±0.37; 0.24±0.01; n=5).

Similarly, P2X1 receptor mRNA expression was increased in tail arteries of diabetic mice compared to age-matched control mice (P<0.05; 0.93±0.15 vs. 0.49±0.18; n=5 each) however in contrast to the absence of expressional changes in tail arteries of diabetic rats, α1a-adrenoceptor mRNA expression was also increased (P<0.05; 1.54±0.26; n=5) in tail arteries of diabetic mice relative to age-matched control mice (0.94±0.06; n=5).

These data indicate that augmented expression of P2X1 receptors, but not of α1a-AR, may contribute to changes in peripheral vessel function in Type 1 diabetes; contractile studies in Akita diabetic mice are awaited to confirm the contribution of the observed augmented expression of P2X1 receptors and α1a-AR to altered function in diabetes.


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PC11

Conduction Propagation Dysfunction with No Apparent Changes in Cellular Electrical Action Potentials in Human Atria: A Simulation Study

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Aim: Mutations in the SCN5A gene encoding for the α subunit of the cardiac sodium channel, ÍNa, might result in dysfunction of cardiac excitation wave propagation. In this study we computationally evaluated the functional impacts on atrial excitation of a recently identified mutation SCN5A-W1191X in patients with Brugada syndrome and complication of atrial fibrillation (AF) [1]. By using biophysical simulations, we investigated (a) the effects of the mutation on atrial electrical action potentials (AP) at the cellular level; (b) the effects of the mutation on propagation of atrial excitation waves on spatially extended virtual human atrial tissues.

Methods: The Courtemanche et al. [2] model of human atrial cell was modified to incorporate experimental data of Shin et al. [1] on the mutation-induced loss-of-function in ÍNa, which resulted in a reduction in the maximal channel conductance. The modified model was implemented to simulate APs under control and mutation conditions. Characteristics of APs for both conditions were computed that include the resting potential, AP amplitude (APA), maximum upstroke velocity (dV/dtmax), AP duration at 50 % repolarisation (APD50) and at 90 % repolarisation (APD90) and over shoot (OS). Single cell model was then incorporated into an one-dimensional reaction diffusion partial differential equation model of human atrial strand, using which the conduction velocity (CV) was determined by pacing the 1D strand with standard S1-S2 stimulus protocols.

Results: The SCN5A-W1191X mutation resulted in no significant change in either APD50 or APD90. However, it reduced substantially the dV/dtmax, which reduced from 217.081 mV/ms in Control to 109.5 mV/ms under mutant conditions. Consequentially the computed OS was also decreased from 24.75 mV in Control to 8.5 mV in mutation. In the 1D simulations, the measured CV for solitary excitation waves was reduced from 0.27 mm/ms in Control to 0.20 mm/ms in mutation conditions.

Conclusions: SCN5A-W1191X does not alter the human atrial AP morphology significantly, but decrease its excitability resulting in a dramatic slowing of CV. Reduction of CV shortens the wavelength of atrial excitation waves, allowing 2D and 3D substrates to sustain re-entrant waves, which is pro-arrhythmic.

Summary of Results

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SCN5A-mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>resting potential (mV)</td>
<td>-60.5</td>
<td>-60.0</td>
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<tr>
<td>APD50 (ms)</td>
<td>31.14</td>
<td>31.12</td>
</tr>
<tr>
<td>APD90 (ms)</td>
<td>21.6</td>
<td>21.14</td>
</tr>
<tr>
<td>CV (m/s)</td>
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<td>4.3</td>
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<td>APD90 (% CV)</td>
<td>10.4</td>
<td>(9.6±1.1)</td>
</tr>
<tr>
<td>overshoot (mV)</td>
<td>3.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

A. AP profiles under Control (solid line) and SCN5A mutant (dashed line) conditions. APD90 is not affected and overshoot is reduced. The maximum upstroke velocity is dramatically reduced under mutant conditions. B. CV restitutions under Control (solid line) and SCN5A mutant (dashed line) conditions. CV under mutant conditions was reduced due to mutation.


Glutamatergic synapses in rodent midbrain dopaminergic neurones show an increase in the ratio of AMPA receptor (AMPAR)-mediated excitatory postsynaptic currents (EPSCs) to NMDA receptor (NMDAR)-mediated EPSCs following a single dose of amphetamine administered 2–24 hours prior to EPSC recordings (Saal et al., 2003; Faleiro et al., 2004). This form of synaptic plasticity may contribute to behavioural adaptations seen in response to amphetamine, and may reflect changes in synaptic AMPAR expression, NMDAR expression, or both. Previously we have shown that synaptic NMDARs in rat substantia nigra dopaminergic neurones contain both NR2B and NR2D subunits (Brothwell et al., 2008). We have used whole-cell patch-clamp recordings to determine whether or not the sensitivity of NMDARs to the NR2B-preferring antagonist, ifenprodil, is changed in dopaminergic neurones following a single dose of amphetamine. Rats aged ~postnatal day (P)6 or ~P13 were given a single intra-peritoneal injection of amphetamine (2.5 mg kg\(^{-1}\)) or saline control, and the locomotor response for 45–60 minutes following the injection was computed (number of infrared beam breaks); locomotor activity was significantly greater (P<0.05; t-test) in rats injected with amphetamine compared with naïve rats (Table 1). In the second set of experiments NMDAR-EPSCs were pharmacologically isolated and the effect of ifenprodil (10 μM) was determined. There was no significant difference in the inhibition of NMDAR-EPSCs by ifenprodil (10 μM) in rats aged ~P13 injected with amphetamine compared with naïve rats (Table 1). There were significant differences in the EPSC / NMDAR-EPSC ratio, as determined by a single dose of amphetamine (P<0.05; ANOVA) in rats aged ~P14 injected with amphetamine. Rats aged ~postnatal day (P)6 or ~P13 were given a single intra-peritoneal injection of amphetamine (2.5 mg kg\(^{-1}\)) or saline control, and the locomotor response for 45–60 minutes following the injection was computed (number of infrared beam breaks); locomotor activity was significantly greater (P<0.05; t-test) in rats injected with amphetamine compared with naïve rats (Table 1). In the second set of experiments NMDAR-EPSCs were pharmacologically isolated and the effect of ifenprodil (10 μM) was determined. There was no significant difference in the inhibition of NMDAR-EPSCs by ifenprodil in rats injected with amphetamine (Table 1). These results suggest that the change in the AMPAR-EPSC / NMDAR-EPSC ratio is developmentally sensitive, and that the proportion of synaptic NR2B-containing NMDARs is not altered by a single dose of amphetamine.
previous observations which identified an anti-oxidative effect of EPA (Lonergan et al., 2004). These data demonstrate that the EPA derivative, DPA, like EPA itself, possesses anti-oxidative properties and that this is likely to be a consequence of the modulatory effect of the fatty acids on microglial activation.


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PC14

The lipopolysaccharide-induced increase in pro-inflammatory cytokine production is exaggerated in mixed glia prepared from mice deficient in CD200

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CD200 is a cell-membrane protein expressed on several cells including neurons and endothelia. Its cognate receptor, CD200R, is primarily expressed on cells of the myeloid lineage, including microglia. It is believed that engagement of CD200 with its receptor can lead to immunosuppression, thus restraining myeloid cells from tissue-damaging activation. Recent evidence from this laboratory has indicated that there is an age-related decline in CD200 expression in the hippocampus and it is suggested that this decrease could contribute to the enhanced pro-inflammatory profile observed in the brain of aged rats.

The action of the inflammatory stimulus lipopolysaccharide (LPS) was investigated in mixed glia prepared from neonatal C57Bl/6 wild-type (WT) and CD200-/- mice. Mixed glia were incubated in the presence or absence of LPS (1 μg/ml) for 24 hours and following this, the supernatant was collected for analysis of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α by ELISA and analysis of mRNA expression of these cytokines by Q-PCR. LPS significantly increased IL-1β, IL-6 and TNF-α at mRNA and protein levels in glia prepared from WT and CD200-/- animals (p < 0.05; ANOVA, n=6). However LPS increased production of pro-inflammatory cytokines to a greater extent in mixed glial cultures prepared from CD200-/- mice compared with WT mice and the differences were statistically significant at both mRNA and protein levels (p < 0.05; ANOVA).

These findings, which indicate that mixed glial prepared from CD200-/- mice were more responsive to LPS than glia prepared from WT mice and are consistent with our previous observation that interaction of CD200 with its receptor contributes to the maintenance of microglia in a quiescent state.

This work was supported by a grant obtained from the EU.

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PC15

Downregulation of CD200 is coupled with the inflammatory phenotype in the CNS of mice with experimental autoimmune encephalomyelitis

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Microglial activation has been identified as one factor which contributes to the deterioration in function associated with experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis. Microglial activation is characterized by an increase in the expression of cell surface markers such as CD40 and an increase in the production of proinflammatory cytokines such as interleukin 1β (IL-1β). Recent evidence has indicated that microglial activation is modulated by the interaction between CD200 which is expressed on neurons and its cognate receptor, CD200 receptor, which is expressed on microglia (Lyons et al., 2007). The objectives of this study were to establish whether any change in microglial activation in the spinal cord of mice with EAE was associated with a downregulation of CD200 and to assess whether similar changes were observed in hippocampus.

EAE was induced in C57 mice by injection of myelin oligodendrocyte glycoprotein (MOG), pertussis toxin (PT) and complete Freund’s adjuvant (CFA), and 48 hours later by an additional injection of PT. Clinical symptoms were observed over 21 days (Reinke et al., 2007) and symptoms consistent with the onset of EAE were observed after 10 days and these progressed to hindlimb weakness thereafter. At the end of the 21 day period, mice were sacrificed and spinal cord and hippocampus were removed. Tissue was prepared for analysis of CD40 and CD200 mRNA by QPCR and for analysis of pro-inflammatory cytokines by ELISA.

We observed an increase in the expression CD40 mRNA in the hippocampus and the spinal cord of mice with EAE compared with control mice (p<0.05, ANOVA, n=6). We further report an increase in IL-1β protein in the hippocampus and spinal cord of mice with EAE mice (p<0.05, ANOVA, n=6). The increase in CD40 mRNA was associated with a decrease in CD200 mRNA and protein in the spinal cord of EAE mice (p<0.05, ANOVA, n=6) providing further evidence of an inverse correlation between microglial activation and expression of CD200.

The data highlight the importance of the CD200 receptor-ligand interaction in the inflammatory phenotype associated with the symptoms of EAE.
Modulation of synaptic transmission by tumor necrosis factor-alpha after hypoxic exposure in rat hippocampal slices

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The brain is highly dependent on oxygen supply in order to maintain synaptic function and conductivity. Although an hypoxic event may lead to hippocampal neuronal death, an early neuroprotective response to this insult is the activation of adenosine1 receptors (A1R). The role of pro-inflammatory components in an ischaemic/hypoxic episode is still controversial, although deleterious effects of pro-inflammatory cytokines in the area of injury are well documented. Recent evidence suggest a role for tumor necrosis factor-alpha (TNF-α) in protecting neuronal cells against hyperexcitability induced by hypoxic insults [1]. In the present study we have investigated the modulatory actions of TNF-α on synaptic transmission during and after hypoxia. We have also investigated the role of A1R in this effect. Hippocampal slices (350 μm) were obtained from P21 male Wistar rats that were humanely killed under anesthesia (4% isoflurane, by inhalation). fEPSPs were elicited from the Shaffer collateral pathway in the CA1 region every 30 s. Long-term potentiation (LTP) was induced with 3 trains of 100 stimuli applied at 100Hz every 30 s. Statistical analysis was performed using Mann-Whitney test. All results are represented as mean±S.E.M. All fEPSP slope measurements are presented as a percentage of baseline recordings. Following a 2 hr hypoxic exposure with N2 perfusion (PO2 37±2 mmHg above the slice; 30.8±10.4% compared to controls), the fEPSP returned to control levels. Inhibition of A1Rs (8-cyclopentyl-1,3-dipropylxanthine; DPCPX; 200 nM) significantly reversed the hypoxia-induced synaptic depression (85.7±4.3%, versus controls; n=5, p<0.05) but impaired the maintenance of LTP after re-oxygenation (96.4±10.6% versus 143.8±8.2% in controls, 1 hr post tetanus; n=5; p<0.005). DPCPX also attenuated the hypoxia-induced depression of the pharmacological isolated NMDA fEPSP (40.5±5.2% versus 61.2±2.7%, n=5 p<0.05, 30 min after hypoxic exposure). 30 min TNF-α treatment (3ng/ml) during hypoxic exposure attenuated the recovery of the fEPSP after re-oxygenation (61.0±7.2% versus 86.7±6.6%; n=5; p<0.05). LTP induced after re-oxygenation was not affected by pre-treatment with TNF-α during hypoxia (138.0±8.3%, compared to controls, n=5). Liquid Chromatography and Mass Spectrometric analysis for cAMP was carried out in hippocampal slice homogenates to investigate if A1Rs were activated after TNF-α treatment (3ng/ml) and re-oxygenation. Unexpectedly, an increase in cAMP was observed in TNF-α treated slices (20.6±2.3nM versus 11.4±1.5 nM controls; n=4; p< 0.05), an effect reversed by the p38 mitogen-activated protein kinase inhibitor (SB203580; 1 μM) (8.3±1.4 nM; n=4; p<0.05). These data show that TNF-α can modulate synaptic transmission after a hypoxic exposure, an effect that does not seem to involve A1R activation during re-oxygenation.

References:

PC17

Calcium signalling during sperm-female tract interaction

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The events during migration of human sperm through the female reproductive tract remain almost completely uncharacterised. Not only are sperm known to be sensitive to products of the female tract but animal data suggests the female tract alters gene expression after exposure to sperm (1), implying two-way communication. We have used single-cell fluorescence imaging to observe calcium signalling in both human sperm and human reproductive tract cells upon initial contact and during sperm adhesion and release. Explants and primary cell lines were prepared from donated human reproductive tract tissue removed during surgery (2). An immortalised oviductal cell line (OE E6/E7) has been used as an internal standard (3). Human sperm were harvested via a modified swim-up technique. Briefly, sperm were selected by their ability to migrate through a viscous medium (~ 140 centipoise) into sEBSS media, then washed and resuspended in sEBSS media containing 0.3% FBS (charcoal stripped) at a concentration of 1 x106 cells per ml. Sperm were incubated for at least 3 hours at 37°C 6% CO2 before use. To investigate whether there was rapid cell signalling occurring in human female tract cells upon exposure to sperm, tract cells were labelled with 7.6 μM Calcium Green-1, Am for 1h at 37°C 6% CO2 to monitor intracellular calcium levels. Sperm were labelled with 5 μM Syto64, a red fluorescent nuclear dye, to allow tracking of sperm movement and contact with cells. Raw intensity values were imported into Microsoft Excel and normalised. Values are percentage change in fluorescence ± S.E.M.
Initial data with tract cells show that explants demonstrated transient responses of 9±5% (n=3) and an average peak duration of ~20s. Primary tract culture responses were 15±10% (isthmus, n=3), 7±4% (ampulla, n=3), both with similar peak durations of ~30s. OE E6/E7 cells had larger responses of 22±10% (n=6) and generated transients with an average peak duration of ~35s. Human sperm bound to and interacted with cells from all tract zones. Sperm swimming patterns appear to be modified when in the presence of reproductive tract cells. We then investigated whether calcium signals occurred within sperm whilst in contact with tract cells. Sperm were loaded with 7.6 μM Calcium Green-1, AM for 1h at 37°C 6% CO2. Detailed observations of signals in attached cells and responses to progesterone exposure are currently being examined. Preliminary evidence suggests that certain calcium signals may relate to observed motility changes. Research into these processes may lead to a better understanding of the basic reproductive biology, which will allow the development of rational treatments and diagnostics.

All procedures were in accordance with Local Ethics Committee guidelines and patient and donors provided informed consent. Georgiou AS et al. (2007) J Proteome Res. 6(12), 4656-66.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC18**

**Electrical events and mechanisms of [Ca2+], mobilisation induced by P2X receptor stimulation in rat renal resistance artery myocytes**

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Apart from being released as a co-transmitter from sympathetic nerve terminals, interleukin ATP acting upon P2Y and P2X purinoceptors has been shown to be an important paracrine regulator of renal preglomerular microvascular function [1]. Indeed, inactivation of P2 receptors in renal resistance blood vessels inhibits autoregulatory behaviour [2]. Stimulation of P2X purinoceptors (P2X-Rs) in renal vascular smooth muscle cells (RVSMSCs) increases [Ca2+], thus triggering the myocyte contraction [3]. In this study we related the dynamics of [Ca2+]i changes induced by selective P2X-R stimulation to corresponding changes in the cell membrane potential and the kinetics of P2X-R mediated cationic current (I_{P2X}), and analysed the mechanisms of purinergic [Ca2+]i mobilisation in RVSMSCs at sub-cellular level. Experiments were conducted on single RVSMSCs freshly isolated from arcuate and interlobular arteries dissected from rat kidney [4]. Changes of [Ca2+]i in RVSMSCs loaded with Fluo-4 were visualised using fast x-y confocal imaging. Electrical recordings were performed using perforated-patch technique. Data are presented as mean±S.E.M. RT-PCR analysis conducted on 500 RVSMCs collected under the microscope with glass micropipette confirmed the expression of genes encoding P2X1-R, P2X4-R, P2Y1-R and P2Y2-R. The purity of phenotype of SMCs collected for RT-PCR analysis was confirmed by expression of the genes encoding SMC marker (SM-MHC) but not the markers for fibroblasts and endothelial cells (CD34), neurons (PGP9.5) and pericytes (NG2). Selective stimulation of P2X-Rs with 10 μM α,β-methylene adenosine 5‘-triphosphate (AMP-CPP) evoked [Ca2+]i transient initiated by sub-plasmalemmal [Ca2+]i, upstroke (SPCUI) [5]. Combination of confocal Ca2+ imaging with electrical recordings revealed that: (1) peak of both the AMP-CPP – induced action potential and I_{P2X} preceded the peak of [Ca2+]i transient; (2) kinetics of I_{P2X} was consistent with predominant contribution of P2X1-Rs; (3) the amplitude of the [Ca2+]i transient detected under voltage-clamp (V_c=-60 mV) was reduced by about 50% in comparison to that observed under current-clamp. These observations suggest that both Ca2+ entry through voltage-gated Ca2+ channels (VGCCs) and Ca2+ release from intracellular stores also contribute to AMP-CPP – induced [Ca2+]i mobilisation. This was confirmed with selective pharmacological agents. AMP-CPP – induced [Ca2+]i transient was attenuated by 56.9±3.3% (n=26) following depletion of intracellular Ca2+ stores by 10-min incubation with 10 μM cyclopiazonic acid and by 34.0±3.3% (n=14) following block of VGCCs with 5 μM nicardipine. The [Ca2+]i transient remaining in the presence of both drugs had a peak amplitude 31.4±4.3% of that in control (n=16) and resulted from direct Ca2+ entry through P2X-Rs.


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**PC19**

**Downregulation of ACh secretion in reinnervated mouse neuromuscular junctions involving PKC activity and voltage-dependent K+ channels**

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On early stages of adult skeletal muscle reinnervation multiple axonal inputs per muscle fiber exist. These excessive inputs need to be eliminated until only one endplate per fiber remains. Synaptic elimination includes decrease of synaptic activity and subsequent retraction of the silent terminals [1]. Previously we have shown that Ca2+ entering the terminal through L-type Ca2+ channels triggers the release of intracellular Ca2+ from ryanodine stores, which in turn leads to depression of ACh release.
in reinnervated neuromuscular junctions [2]. Ca$^{2+}$-dependent protein kinase C which is known (along with other substrates) to modulate the activity of voltage-dependent K$^{+}$-channels (Kv) was considered as a possible target for this Ca$^{2+}$ signal.

Study of electrical activity of regenerating motor synapses was performed 11 days after the mechanical crushing (nembulal (50 mg/kg) i.p. for general and 0.5% lidocaine hydrochloride s.c. for local anesthesia) of n. peroneus communis, supplying m. extensor digitorum longus in adult mice. Spontaneous and evoked synaptic transmission and its sensitivity to Kv-channels and Ca$^{2+}$-dependent protein kinase C modulation was examined. Mann-Whitney test was performed for statistical analysis. All data are presented as mean±S.E.M. Blockade of protein kinase C, with chelerytrine (4 μM) and bisindolylmaleimide I (1 μM) showed a significant increase in quantal content (QC) of EPSPs, up to 58±7% and 66±9% respectively (n=71, p<0.05). This increase was very similar to effects of L-type Ca$^{2+}$-channels blocker nifedipine (10 μM): it caused rapid 60±5% elevation of QC (n=60). But nifedipine applied after chelerytrine was not able to affect amplitude or QC of EPSP’s. QC was 12±1 in control, 18±2 under influence of chelerytrine (p<0.05) but 17±2 under influence of both chelerytrine and nifedipine (n=65).

Upregulation of QC caused by blocking the ryanodine receptors with ryanodine (5 μM) was prevented by adding chelerytrine to the bath solution: 8±1 in control, 15±1 after incubation with ryanodine (p<0.05), 10±1 with both ryanodine and chelerytrine (n=67). These results suggest that activation of protein kinase C may be triggered by Ca$^{2+}$ influx from the intracellular ryanodine stores, which in turn is activated by Ca$^{2+}$ entering the terminal through L-type Ca$^{2+}$-channels. Application of Kv-channels blocker 4-aminopyridine (6 μM) was found to greatly increase QC of EPSP’s in newly formed synapses: from 19±2 in control to 30±2 (p<0.05, n=63). Pre-incubation with chelerytrine prevented further enhancement of synaptic transmission by 4-aminopyridine. EPSP’s QC reached 17±2 in control, 30±2 after incubation with chelerytrine (p<0.05) and 30±2 with both chelerytrine and 4-aminopyridine (n=70). The data obtained demonstrate that increased activity of Kv-channels controlled by PKC may be the mechanism of Ca$^{2+}$-dependent inactivation in newly formed synapses.


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PC20

Insulin protects against oxidant-induced impairment of Ca$^{2+}$ homeostasis and plasma membrane Ca$^{2+}$.ATPase (PMCA) in pancreatic acinar cells

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Pancreatitis is an inflammatory disease of the exocrine pancreas, characterised by auto-digestion of the pancreas and necrotic cell death. Oxidative stress and impairment of intracellular calcium ([Ca$^{2+}$]) homeostasis has been implicated in this disease. Previous studies have shown that oxidative stress impairs hormone-evoked Ca$^{2+}$ signalling and induces an irreversible increase in [Ca$^{2+}$]i (Ca$^{2+}$ overload) (Bruce et al., 2007). This oxidant-induced Ca$^{2+}$ overload response coincided with inhibition of the PMCA and mitochondrial depolarisation. Moreover, this oxidant-induced PMCA inhibition could occur without impairment of mitochondrial Ca$^{2+}$ handling or ATP depletion and was attenuated by inhibitors of the mitochondrial permeability transition pore (Baggaley et al., 2008).

Several studies have demonstrated that insulin can activate pro-survival pathways and protect from pancreatic cell injury. Therefore the aim of the current study was to test the effects of insulin on oxidant-mediated impairment of Ca$^{2+}$ homeostasis and inhibition of the PMCA. Pancreatic acinar cells were isolated from Sprague-Dawley rats by collagenase digestion. The effect of hydrogen peroxide (H$_2$O$_2$) on resting [Ca$^{2+}$]i was tested on fura-2-loaded cells treated with or without insulin (1-10nM). In addition, we utilised an in situ Ca$^{2+}$ clearance assay in which the PMCA activity was pharmacologically isolated. A paired experimental design was used to directly compare clearance phases in which H$_2$O$_2$ was applied during the second clearance phase and the rate normalised to the first clearance phase. All data are presented as mean values ± standard error.

Insulin pre-treatment (1nM) had no effect on the H$_2$O$_2$ induced increase in resting [Ca$^{2+}$]i (50nM: 0.47 ± 0.14 ratio units, n = 3 control and 0.40 ± 0.15 ratio units, n = 4 insulin treated). However, 1nM insulin caused apparent shift in the proportion of cells that recovered following H$_2$O$_2$ treatment (100μM: 36% of total cells, n = 5 control and 56% of total cells, n = 6 insulin treated) and an increase in the magnitude of recovery from the H$_2$O$_2$-mediated Ca$^{2+}$ overload response (50μM: 27.13 ± 0.93%, n = 3 control and 57.21 ± 9.13%, n = 4 insulin treated). Furthermore, H$_2$O$_2$ inhibited the PMCA in a concentration-dependent manner (500μM: rate = 18.44 ± 4.75%, n = 6 compared to control: rate = 91.70 ± 12.54%, n = 8). This inhibition was attenuated in cells treated with 1nM insulin (500μM: rate = 44.53 ± 9.13%, n = 4). In summary, these data suggest that insulin could protect against oxidant-induced Ca$^{2+}$ overload and oxidant-inhibited inhibition of the PMCA. This may have important implications for the prevention of necrotic cell death associated with pancreatitis. Future work will involve elucidating the molecular mechanisms of this insulin-mediated protection of the oxidant-inhibited inhibition of the PMCA.

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Inhibition of skeletal muscle differentiation by tumour necrosis factor-α is reversed by the omega-3 polyunsaturated fatty acid eicosapentaenoic acid: a mechanism associated with inhibition of nuclear factor-κB and upregulation of peroxisome proliferator activated receptor γ

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Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid with anti-inflammatory and anti-cachetic (1, 2) properties which we reported to be protective against the damaging effects of TNF-α during skeletal muscle differentiation (3). Inflammatory cytokines such as TNF-α may contribute to muscle wasting through inhibition of myogenic differentiation via a nuclear factor-κB (NF-κB)-dependent pathway (4). Thus, we hypothesised that EPA may exert its actions downstream of TNF-α through NF-κB-mediated effects on gene transcription. C2C12 (or C2C12 stably transfected with an NF-κB reporter construct) myoblasts were differentiated by culture in growth medium containing 2% horse serum. EPA (50 μM) was added at the start of differentiation and myotube formation allowed to progress for up to 48 hours in the presence or absence of TNF-α (20ng/ml). At various time-points, whole cell lysates were prepared for measurement of NF-κB activation by luminescence. In parallel, total RNA was extracted and cDNA synthesised for quantitative real-time PCR transcriptional analysis of peroxisome proliferator activated receptor (PPARγ), a downstream target for TNF-α. In response to TNF-α treatment, NF-κB activation was significantly (p<0.05) enhanced. However, EPA treatment significantly (p<0.05) attenuated this TNF-α-mediated activation of NF-κB at all time-points up to 24 hours after treatment. Furthermore, whereas TNF-α treatment alone downregulated PPARγ expression, normalised against β-actin, by 2-fold (p<0.05), EPA co-treatment reversed this inhibition, increasing PPARγ expression by 2-fold (p<0.05) at 12 hours. In summary, activation of NF-κB by TNF-α inhibits skeletal muscle differentiation but is blocked by administration of EPA and this is associated with upregulation of PPARγ expression. These data support a protective role for EPA, particularly where pathologically high levels of TNF-α may be present and support further work to elucidate its mechanisms of action.


Thanks to Ramon Langen for the C2C12 stably transfected with an NF-κB reporter construct

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The effect of increased fructose and/or salt intake on maternal liver and plasma lipids in rats

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The prevalence of non-alcoholic fatty liver disease (NAFLD) in the developed world has markedly increased. NAFLD is a multifactorial disease but a specific causal link has been made to increased fructose consumption (1). A major concern for women living in a contemporary western society is excess nutrient availability, especially salt and simple sugars (such as fructose). The present study has therefore examined the effect of increased maternal salt and fructose intake on maternal metabolism and growth. 32 virgin Sprague Dawley rats (~180g) were randomly divided into dietary groups; 1) control diet (CD, n=8) fed purified chow and tap water, 2) salt diet (SD, n=8) fed purified chow containing 4% NaCl and tap water, 3) fructose diet (FD, n=8) fed purified chow and 10% fructose in tap water and 4) fructose & salt diet (FSD, n=8), fed 4% NaCl purified chow with 10% fructose in tap water. Animals were fed ad libitum for 28 days prior to conception, mated and maintained on experimental diets until day 20 of gestation, whereupon they were euthanized. Blood samples were taken 14 days prior to conception and at day 20 of gestation for analysis of protein, fat and carbohydrate metabolism using an auto analyser (RX-IMOLA, Randox). Prior to feeding the experimental diets, there was no significant difference between the body weights of dams (220±10g). During pregnancy all animals gained a similar amount of weight (115±6g). Prior to pregnancy, fructose intake increased triglyceride (P<0.001) in FD (1.06±0.08mmol/l) and FSD (0.91±0.11mmol/l) when compared to CD (0.72±0.08mmol/l). NEFA was also increased (P<0.001) in FD (0.67±0.04 mmol/l) when compared to CD (0.55±0.04 mmol/l). Maternal liver weight was specifically increased 20-25% (P<0.001) by the consumption of fructose, FD (15.22±0.32g), FSD (14.64±0.32g) vs. CD (12.17±0.37g). In addition, a significant redistribution of adipose tissue in the dams consuming excess fructose was observed; relative to CD, the gonadal depot in FD was reduced (FD, 4.91±0.51g vs. CD, 7.15±0.59) but perirenal depot increased (FD, 2.96±0.11g vs. CD, 1.98±0.13). All data was analysed as a 2x2 factorial design with fructose+salt+fructose* salt included (general analysis of variance). Data is shown as estimated marginal means ± SEM for plasma samples and organ weights (CD: n=8, SD: n=8, FD: n=8, FSD: n=8). The data indicate that increased maternal intake of fructose in water and of salt in diet has marked effects on the dam’s metabolism. The excess energy intake as a result of increased simple sugar consumption leads to significant alterations in

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lipid metabolism, hepatomegaly and a shift in adipose tissue distribution. Intra-hepatic triglyceride concentrations in the enlarged livers and the possible consequences of these changes in maternal tissues on later growth and metabolism of adult offspring are currently being investigated.

Metabolic disturbances in non-alcoholic fatty liver disease. Christopher D. Byrne, Rasaa Olufadi, Kimberley d. Bruce,Felino R. Cagampang and Mohamed H. Ahmed Clinical Science (2009) 116, 539–564 (Printed in Great Britain)

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PC23

Exercise-induced stress differentially alters expression of nuclear receptor genes in mouse skeletal muscle and heart

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The transcriptional peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC1α) is described as the master regulator of skeletal and cardiac muscle metabolism, mediating the beneficial effects of exercise (1). Its expression increases with oxidative stressors. PGC1α has been shown to regulate muscle peroxisome proliferator-activated receptor beta (PPARβ) and estrogen related-receptor alpha (ERRα) expressions. We have previously described an increase in muscle PPARβ expression with exercise training (2). It is still unclear if PGC1α has a redundant or a counteractive role with PPARβ and ERRα in response to metabolic challenges. We investigated these genes expression in both skeletal and cardiac muscles after exhaustive aerobic exercises. Heat shock protein 70/72 (HSP70/72) expression is used as a biomarker of the cellular response to stress induced by exercise loads. Two groups of FVB mice performed an exhaustive run on a treadmill at critical (CS; n=10; speed: 23.7 ± 3.4 m.min⁻¹; duration: 55 ± 28 min) or peak speed (PS; n=10; speed: 32.3 ± 4.5 m.min⁻¹; duration: 37 ± 30 min), whereas one group of mice remained at rest (Control; n=10). Mouse performances were individually determined by pre-test evaluations (3). Mice were sacrificed 2 hours after the end of exercise. One-way ANOVA test was used for statistical analysis. Exercise induced a significant 12 fold increase in muscle HSP70/72 expression after exercising at CS, whereas the increase after exercising at PS was not as strong. We reported here that in contrast to PGC1α, which was up-regulated, PPARβ and ERRα expressions were down-regulated in skeletal muscle in response to exercise. Our findings provided evidence to support the notion that PGC1α on one hand, and PPARβ and ERRα on the other hand, have distinct roles in the regulation of muscle adaptation to exercise, which may depend on the magnitude of the exercise-induced stress. In cardiac muscle, the increase in HSP70/72 expression was not significant and PPARβ and ERRα expressions remained unchanged. However, PGC1α was increased and the magnitude was significantly higher after CS than after PS suggesting that cardiac response to exercise is subtle and different from skeletal muscle. It is questionable if the decrease in PPARβ expression shown with these forced exercises is beneficial or harmful as such a down-regulation is not observed after voluntary exercise. Whether these responses are necessary to induce subsequent adaptations observed with chronic exercise remains to be elucidated. In heart, the preservation of PPARβ and ERRα expression may be a protection. Our results showed that exercise loads (intensity and duration) should be considered while recommending exercises for prevention or treatment of metabolic diseases.


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PC24

Exercise for a precocious detection of impairment in mdx mice model

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The mdx mouse with essential dystrophin deficiency is an established animal model of Duchenne’s muscular dystrophy (DMD) in human. However, hindlimb muscle of mdx mice does not exhibit severe and progressive muscle weakness before 15 month, which is late compared to DMD patients, excepted for the diaphragm that is severely affected earlier by the disease (Dupont-Versteegden, 1996). More over respiratory function seems to be decreased at muscle level, mdx muscle mitochondria had only 60% of maximal respiration activities of control mice skeletal muscle mitochondria (Kuznetsov et al., 1998). However how the diaphragm and these less efficient mitochondrion affect the whole body performance (running velocity and maximal oxygen consumption) in young and older mice was not established. As mice are animals with mostly anaerobic displacements and mdx mice are not severely impaired before 15 months, we hypothesized that running performance should not be greatly affected conversely to maximal oxygen consumption in mice between 5 and 9 months.

Six mdx mice of 5 months and 6 mdx mice of 9 months of age were tested on a treadmill inserted in a metabolic chamber (Columbus Instrument). Exercise protocols were performed to determine maximal (Vpeak) and critical velocity (CS) as well as VO2max (Billat et al. 2005, Ferreira et al., 2007) and the corresponding velocity (vVO2max) Results are compared with 7 control mice aged 5 and 9 months.
Results showed that at 5 months VO2max of mdx mice was not different from control (49.38 ± 5.48 vs 48.14 ± 3.24 ml.kg-0.75.min-1, p = 0.639). However mdx mice performed higher Vpeak (26.20 ± 2.26 vs 19.86 ± 2.27, p = 0.001) and vVO2max was higher (25.5 ± 2.26 vs 15.57 ± 3.21, p < 0.001). Surprisingly VO2max did not decrease between 5 and 9 months old mdx mice. Even more the decreased of other parameters of performance between 5 and 9 months (vVO2max, CS, Vpeak) were more severe in mdx than in control mice (Figure 1).

These results indicate that all parameters of performance in mdx mice were affected differently and physiological speeds decreased before the hindlimb muscle impairment. The metabolism of running mdx mice differed markedly from control and this can explain beneficial effects of exercise previously demonstrated (Kaczor et al., 2007). Exercise allowed to underline deficiency precociously (9 months) compared with other in vivo parameters used in the literature (around 15 months). Further analyses are in progress to determine the respective part of the diaphragm and skeletal muscle energetics impairment responsible for this severe decrease of performance in mdx mice. Thus exercise can be a useful tool for pathological models, included in the mdx model.

Figure 1: Change in performance parameters (percentage) of control (n = 7) and mdx (n = 6) mice at 9 months compared with 5 months. VO2max: maximal oxygen consumption. Vpeak: maximal velocity reached. vVO2max: velocity associated with VO2max. CS: critical speed.


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Twinfilin-1 mRNA in isolated adult rat cardiomyocytes
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It has recently been discovered that the expression profile of microRNA changes in the diseased heart (1, 2). Some of the dysregulated microRNAs have been shown to affect the expression of important cardiomyocyte genes, resulting in pathophysiology characteristic of the disease (3). It is likely that there are similar effects of other microRNAs that are dysregulated in heart disease. It has recently become possible to study the effects of specific microRNAs on cardiomyocyte physiology by transfecting adult rat cardiomyocytes with microRNA mimics (1). Fluorophore-tagged mimics have been used as a positive control to indicate successful transfection, although a positive control for functional activity of transfected microRNA would enhance these studies. Analysis of microRNA gene targeting has shown that miR-1 destabilises the mRNA of several target genes, with the actin-binding protein twinfilin-1 (Twf-1; PTK9) being the most strongly affected (4). Twf-1 mRNA down-regulation would therefore be a good indicator of miR-1 function. Twf-1 mRNA is known to be present in mouse heart (5), but its presence in adult rat cardiomyocytes has not been reported. We have detected Twf-1 mRNA in isolated adult rat cardiomyocytes using quantitative real-time PCR (qPCR) on an Applied Biosystems 3700 machine. Adult male Sprague-Dawley rats were anaesthetised by intraperitoneal injection of pentobarbital (100 mg/kg). Hearts were removed and perfused with Kreb’s buffer containing collagenase and hyaluronidase to isolate cardiomyocytes, which were seeded onto laminin-treated cover slips (8 mm diameter) and maintained in culture medium. The number of cells on each cover slip was estimated by counting cells in 6 fields of view. The TaqMan® Gene Expression Cells-to-CT™ Kit (Applied Biosystems) was used for cell lysis and reverse transcription. A Twf-1 TaqMan® assay (Rn01407564_g1, Applied Biosystems) was used for qPCR. Control reactions without reverse transcription (RT- ) were used to estimate the maximum number of cardiomyocytes per sample that could be fully lysed using the Cells-to-CT™ protocol, as indicated by the elimination of genomic DNA by DNase I treatment. RT- assays from 1400 or more viable cardiomyocytes (n=4) produced amplification products, indicating incomplete cell lysis. RT- assays were negative (below amplification threshold after 40 cycles) in lysates from 1000 or fewer viable cardiomyocytes (n=4), while assays from these lysates gave amplification products after reverse transcription (RT+). Within-sample ΔCt values for Twf-1 vs glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Rn01775763_g1, Applied Biosystems) were 3.7, 3.4 and 2.4 for 3 separate samples. These results establish the principle that quantitation of Twf-1 mRNA relative to an endogenous control gene such as GAPDH could be used as a functional assay for microRNA transfection in adult rat cardiomyocytes, using miR-1 as a positive control.


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A physiological mode of heart perfusion in vivo and in vitro
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Langendorff mode of heart perfusion is widely used in biomedical research. It is a constant retrograde perfusion with unnatural pressure in four chambers, which causes abnormal heart conditions. For example, rabbit heart rate in vivo is 205~220/min at rest, whereas the heart rate of Langendorff perfused rabbit heart is 120~150/min (Döring & Dehnert, 1988). Furthermore, the preload of left ventricle in Langendorff perfused heart is highly increased as the aortic valve is unable to work as normal, which may cause acute congestive heart failure. To investigate arrhythmia in the isolated hearts from rabbit and rat, we have developed a physiological mode of heart perfusion in vitro that simulated the working heart in vivo as shown in Figure 1. In this system, the regular flow through the heart was maintained, and each chamber was automatically controlled and filled with changing volume and pressure as usual. As a result, normal heart rate in vivo could be kept in vitro for more than 24 hours. The pressure in each chamber was adjustable and controllable, which was useful to study those arrhythmia associated with stretch and/or pressure. To investigate the role of autonomic nerve system in atrial fibrillation, the system was also used to carry out the experiments in vivo with intact autonomic innervation of the heart perfused physiologically with Tyrode solution as shown in Figure 2. Based on characteristics of the system, this mode is valuable to functional studies on arrhythmia that is a systemic disorder at organ level and highly related to the pressure in heart chamber.

**Figure 1. A physiological mode of heart perfusion in vivo**
Pressure transducer (PT), pulmonary artery (PA), aorta (A), superior vena cava (SVC), left atrial appendage (LAA). Pulmonary veins and inferior vena cava were tied.

Figure 2. Physiological heart perfusion in vivo with intact autonomic innervation

In accordance with the Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985), anaesthesia was induced and maintained with Hypnoval (1 mg/kg, I.V.) and pentobarbitone (2 mg/5 min, I.V.) before and during dissection of the vagus nerves, and an overdose of pentobarbitone (60 mg, I.V.) was given before opening the thoracic cavity (André Ng et al. 2001). The descending aorta and inferior vena cava were connected, whereas SVC was tied.


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Age-related differences in response to chemical hypoxia of isolated perfused cardiomyocytes

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Aim: The heart undergoes stages of postnatal development during which it responds differently to cardiac insults. Whether such age-related differences are due to developmental changes in single heart cells is not presently known. In this study we investigated the effect of chemical hypoxia on freshly isolated cardiomyocytes from different postnatal ages of the rat heart.

Methods: Cardiomyocytes were enzymatically isolated from 14, 21 day and adult rat hearts. Isolated cardiomyocytes were perfused with HEPES buffer (1 mM Ca$^{2+}$) and field stimulated at 0.2 Hz in a chamber under an inverted microscope at 34°C.

Chemical hypoxia was induced by switching the perfusion buffer to one containing 2.5 mM NaCN and no glucose. Cardiomyocytes were viewed on a monitor and the effects of metabolic inhibition were noted. The times taken for the cells to cease contraction and to enter a state of rigor were recorded. Concentrations of ATP were measured in myocardial biopsies from the same aged rats, using HPLC. Data expressed as mean ± standard error.

Results: The lengths of time for cardiomyocytes to both cease contraction and enter rigor were significantly shorter for cardiomyocytes from 21 day old rats compared to all other age groups (p<0.0001, ANOVA, n=30). Times taken to cease contraction were (in minutes) 12±2, 6±2, 17±3 for 14, 21 and adults respectively. This trend was mirrored in the times taken to enter rigor for all ages. Since going into rigor would depend on availability of myocardial ATP, we measured the concentration of ATP in myocardial biopsies from all age groups. Mean ATP levels in freshly excised hearts were 14.4±1.8, 12.5±1.6, 17.1±0.8 nmol/mg protein in 14, 21 days and adult respectively. ATP was significantly lower at 21 days compared to adults (p=0.024, unpaired t test, n=5).

Conclusion: This work demonstrates that the age-related response to chemical hypoxia is likely to be due to differences in the basal levels of myocardial ATP.

This work is supported by the BHF.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Differences between functional properties of atrium and pulmonary vein region

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The pulmonary vein sleeves (PVS) play an important role in atrial fibrillation (AF) (Nattel, 2002). However, the functional properties of the PVS region have not been well characterized (Aldhooon et al. 2009). In this study, a set of monophasic action potential (MAP) and modified bipolar electrodes and three force transducers were used to simultaneously record MAP and extracellular potentials and mechanical contraction at left and right atriums and PVS region of rabbit heart perfused physiologically in vitro, when the electrical stimulation was applied every ten sinus rhythms with decreasing delay from 200 ms to 0 ms between the tenth depolarization time of right atrium action potential and the stimulation as shown in Figure 1. The results showed that: [i] the mechanical relative refractory period was significantly longer (38±7 ms, 53±9 ms, n=8) at left and right atriums than at the PVS region; [ii] the mechanical absolute refractory period was remarkably longer (12±3 ms, 16±5 ms,
The purpose of the present investigation was to assess the influence of dietary nitrate on resting blood pressure in healthy normotensive humans. Nine healthy males (mean ± SD, age 26 ± 7 years, body mass 81.6 ± 6.2 kg) volunteered to participate in this ethically-approved study. In random order, the subjects consumed 500 mL of either beetroot juice (BTJ, containing 11.2 ± 0.6 mM of nitrate) or blackcurrant squash (as a placebo, PLC) per day for six consecutive days. The conditions were separated by a washout period of 7 days. Resting blood pressure was measured using an automated blood pressure machine and blood samples were drawn to determine plasma [nitrite] on days 4, 5 and 6 of each condition. Data were analysed using repeated-measures ANOVA.

On days 4-6, plasma [nitrite] was significantly increased by beetroot juice consumption but was unchanged following placebo consumption (BTJ: 160 ± 80 vs. PLC: 80 ± 60 μM; P<0.05). Beetroot juice consumption led to a significant reduction in systolic blood pressure on day 4 (BTJ: 124 ± 2 vs. PLC: 132 ± 5 mmHg; P<0.01) with the difference remaining significant on days 5 and 6. Diastolic blood pressure was not significantly affected by the intervention on day 4 (BTJ: 71 ± 9 vs. PLC: 71 ± 7 mmHg) or on subsequent days. These data extend the earlier report of Webb et al. (2008) by showing that increased dietary nitrate consumption (in the form of 500 mL/day of beetroot juice) results in a sustained reduction in systolic blood pressure in healthy normotensive humans. We suggest that dietary nitrate reduces blood pressure by increasing nitric oxide availability and enhancing vasodilatation in the microcirculation (Lundberg & Govoni, 2004). Increased dietary nitrate consumption might be considered a natural strategy to maintain or enhance cardiovascular health. Lundberg JO, Govoni M (2004). Inorganic nitrate is a possible source for systemic generation of nitric oxide. Free Radic Biol Med 37, 395-400.


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The trapezius muscle is activated submaximally for prolonged periods during tasks of daily living such as bag carrying, but it remains to be established if this task causes central fatigue. It has been reported that VA of trapezius can be measured using electrical stimulation (ES) of the motor nerve; however this technique provides no information regarding the changes occurring at a cortical level following a fatiguing task. Transcranial magnetic stimulation (TMS) of the motor cortex might provide this information, and the aim of this study was to identify if TMS can be used to assess cortical VA of trapezius. With ethical approval and written informed consent eleven healthy subjects were recruited. Electromyographic recordings were made from the right trapezius, ES was delivered to the right accessory nerve and TMS applied to the left motor cortex. Subjects sat on a chair with their feet suspended, and a force transducer was positioned over their right shoulder. Maximum voluntary contractions (MVC) of the right trapezius were carried out to identify target forces of 10, 30, 50, 70, 90 and 100% MVC. Subjects performed contractions to these target forces in a random order during which ES or TMS was applied. For ES, superimposed twitch (SIT) size decreased linearly as contraction strength increased (mean $r^2=0.75$, $p<0.05$ (n=10)). For TMS this relationship was polynomial across all contraction strengths (mean $r^2=0.67$, $p<0.05$ (n=8)). VA during MVCs of the trapezius was 81.0 (12.9)% when measured with ES (n=8) and 78.1 (13.3)% when measured with TMS (n=8). SITs evoked by TMS were significantly larger than by ES at 10–90% MVC ($p<0.05$). Maximally evoked direct motor responses to ES (M-waves) were significantly larger than motor evoked potentials (MEPs) at contraction strengths ≤90% MVC ($p<0.05$). There was a polynomial relationship between contraction strength and both M-wave ($r^2=0.89$) and MEP ($r^2=0.99$) amplitudes with ES and TMS, respectively. The non-linearity of the SITs to TMS at contraction strengths of <70% MVC may reflect lowered levels of corticospinal excitability, as supported by smaller MEP amplitudes, at these strengths. A similar result has been found using TMS in other muscle groups [3,4]. TMS can be used to assess cortical VA of the trapezius and this technique could be useful to assess the changes that occur at a cortical level as a result of central fatigue [5].


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Assessment of the physiological loads and subjective discomforts of respirator at constant work load exercise under prolonged condition

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Literatures indicated the respirators are required and in use in only 20–30% of the work phases [4]. It is postulated that work wearing respirator at constant load under prolonged condition may cause additional physiological strain due to restriction of heat loss from the respiratory system [1,3] and the anaerobic respiration. This study aimed to evaluate the effects of wearing respirators at constant work load under prolonged conditions. Eight male and eight female physically fit university students [3] voluntarily participated in the study, which was approved by the IRB of CMU. A full-face respirator and a control quarter-face mask were employed. The evaluated independent variables included gender, work time (10 and 50 min) and respirator type. Besides, for the prolonged exercise condition, the data during initial 10 minute (D1) and last 10 minute (D5) were also analyzed. The evaluated dependent variables were summarized. Subjective rate of perceptual exertion (RPE) were measured with a 10 point Borg scale questionnaire. All subjects carried out the basic aerobic data measurement and 4 experiments at 3 different days with at least one day off between the experiment days. On the first day the maximal oxygen consumptions were measured using a revised protocol [2]. The total 4 experiment conditions were completely randomized. The physiological variables were analyzed by repeated measure ANOVAs. The subjective RPE was analyzed using Wilcoxon test. The ANOVAs and some descriptive statistics are summarized. Results indicated that working for constant load under prolonged condition significantly increased minute ventilation ($p<0.001$), oxygen consumption ($p<0.001$), working pulse ($p<0.05$), metabolic rate ($p<0.001$) and total RPE ($p<0.001$). While comparing the D1 with D5, the similar trends were found more significantly especially for female subjects. The oxygen consumption were found significantly increased from 1.09±0.04 (D1) to 1.86±0.04 liter/min (D5). It is suggested that the work-rest ratio should be reconsidered under constant load prolonged work and micro break may be helpful which need further study.


Regulation of Post-Exercise Haemodynamics Following Hyperoxia in Man: Role of Adrenergic Vasoconstriction

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Hyperoxic exercise attenuates post-exercise vasodilatation independent of reduced NO (New et al, 2008) but coincident with a diminished bioavailability of atrial natriuretic peptide (New et al, 2009). The present study investigated the influence of adrenaline (AD) and nor-adrenaline (NA) on post-exercise haemodynamics. 9 males, mean arterial pressure (MAP) = 106 ± 5 mmHg (50 ± 10 yr), not on medication, were studied following 30-minutes of cycle exercise at 70% maximal oxygen consumption in hyperoxia (50% O2) and normoxia (21% O2). Subjects were followed post-exercise for 2-hours.

Hyperoxic exercise blunted post-exercise haemodynamics by attenuating the reductions (from normoxic baseline) in SVR (21 ± 20.7 vs. 38 ± 19.3 %), stroke volume (SV) = 11 ± 8.2 vs. 19 ± 5.5 % and 11 ± 8.9 vs. 15 ± 9.6, P<0.05 vs. normoxic exercise at post, (P1 and P2 respectively) and MAP (3 ± 4 [elevation] vs. 0.5 ± 5 mmHg, 3 ± 3 vs. 6 ± 4 mmHg, 3 ± 3 vs. 4 ± 3 mmHg, P<0.05 vs. normoxic exercise at post, (P1 and P2 respectively) (Paired samples T-tests). AD and NA concentrations were invariant between conditions P>0.05, Paired samples T-tests; Table 1).

These results indicate that an augmented adrenergic response is not a principle governor of the attenuated vasodilatation. Whether changes in alpha- or beta-receptor sensitivity play a role requires further investigation, although, it appears that the deleterious influence of hyperoxia on post-exercise haemodynamics has non-adrenergic origins.

Table 1. Systemic Venous Concentration of AD and NA

<table>
<thead>
<tr>
<th>FIO2</th>
<th>AD</th>
<th>NA</th>
<th>AD</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>normoxic</td>
<td>1.74±0.05</td>
<td>1.34±0.09</td>
<td>0.001±0.1</td>
<td>2.15±0.6</td>
</tr>
<tr>
<td>hyperoxic</td>
<td>0.52±0.06</td>
<td>2.07±0.35</td>
<td>0.014±0.08</td>
<td>3.15±0.20</td>
</tr>
</tbody>
</table>

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PC34

Muscle deoxygenation changes with repeated Wingate power tests

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This study examined the changes in muscle deoxygenation (HbHb) following repeated 30s Wingate power tests to determine the muscle power-deoxygenation relationship. It was hypothesized that tissue HbHb would correlate with changes in anaerobic power, reflecting the energy contribution following repeated Wingate trials. Muscle HbHb was used to reflect O2 utilization, and total blood volume (Thb) was calculated from the oxy-Hb and HbHb signal (HbO2 + HbHb). Eleven competitive male cyclists (mean ± SD age, height, mass =21.8±7.6 yr; 177.5±5.7 cm; 73.7±10.4 kg) volunteered to perform three 30s anaerobic Wingate power tests (Wn1, Wn2, Wn3) with 60s rest between trials on a Monark cycle ergometer. The resistance was set at 90 kg. Tissue oxygenation was monitored continuously from the right vastus lateralis muscle using dual wavelength near infrared spectroscopy (NIRS). Mean (n=11) maximal power (Watts) was significantly reduced with each consecutive Wingate, from Wn1 (598±135W), to Wn2 (475±74W), to Wn3 (446±97W), with the fatigue index = 40-44%. Muscle HbHb closely tracked these power changes but did not reach peak muscle HbHb until 20s after the start of each Wn test. As well, muscle HbHb was reduced and remained below baseline during the recovery period between Wingate tests. Average 30s power was significantly (ANOVA; p<0.05) reduced from Wn1 (474±79W) to Wn2 (377±62W) to Wn3 (338±61W), as was HbHb for Wn1 (0.27±0.26OD), Wn2 (0.17±0.26OD), and Wn3 (0.10±0.25OD). A significant correlation was found between anaerobic power vs. HbHb for Wn1 (r= -0.79) and Wn2 (r= -0.78), but not for Wn3 (r= -0.29). Significant correlations were also found for Thb change vs. anaerobic power (r=0.72–0.87). These results suggest that 1) NIRS can track changes in muscle HbHb and Thb during anaerobic power testing and that decreases in muscle power correlated with muscle HbHb and Thb; and 2) the muscle began to deoxygenate at the onset of maximal exercise during the Wingate tests sug-relating to...
Thirst perception in dehydrated sickle cell disease patients in steady state

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Liberal fluid intake is one of the key management strategies in sickle cell anaemia (SCA) patients in steady state, but less work has been done on the patient’s desire to drink water. Using the Visual Analogue Scale (Thompson et al, 1986), we studied thirst perception (TP) in twenty euhydrated SCA patients and twenty eight control (HbA) subjects, as well as during dehydration in thirteen SCA patients and nine control HbA subjects. Consent was obtained from all subjects and the protocol approved by the Ethics Committee of the University of Benin Teaching Hospital (UBTH), and Sickle Cell Center, Benin City, Nigeria. During euhydration, TP was significantly higher in male SCA patients compared to the HbA subjects (8.67 ± 0.23 cm, n = 11 vs. 3.12 ± 0.74 cm, n = 16, p<0.05). In females, TP in SCA patients was lower than in HbA subjects, but this was not statistically significant (3.20 ± 0.55 cm, n = 9, vs. 4.33 ± 1.0 cm, n = 12). Thus, male SCA patients, but not female patients are in a state of relative dehydration. After 13 hours of dehydration, TP was reduced in both male (7.88 ± 1.10 cm, n = 5 vs. 6.46 ± 1.8 cm, n = 5, ns) and significantly in female (9.3 ± 0.33 cm, n = 4 vs. 3.64 ± 0.80 cm, n = 8, p<0.05). Thus, while dehydration increased TP in HbA subjects, in marked contrast, it reduced TP in SCA patients. Fluid intakes after dehydration in SCA patients (male: 760 ± 150 ml and female: 513 ± 40 ml), were not significantly different from the control HbA subjects in both male (720 ± 195 ml) and female (623 ± 24 ml). It can be concluded that female SCA patients do not have normal response to dehydration with regards to TP after a period of dehydration. Since dehydration stimulates the release of vasoactive hormones like vasopressin, this may explain why female patients are less prone to crisis (Stringer et al., 2005) than their male counterparts.

We thank the Management of UBTH and the Sickle Cell Center, Benin City, Nigeria, for allowing us access to the patients, and to all our subjects for their cooperation.

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Poster Communications

Mucosal stimulation enhances spontaneous electrical activity in the rabbit urethra through activation of M3 receptors

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Although urethral tone is spontaneous, it can be modulated by inhibitory nitrenergic and excitatory noradrenergic and cholinergic nerves (Thornbury et al., 1992). The purpose of this study was to identify which acetylcholine receptor subtype was responsible for increasing tone in the proximal rabbit urethra and examine how their stimulation affected spontaneous electrical activity.

Rabbits were humanely killed with pentobarbitone (I.V.) and the proximal 3 cm of the urethra was removed. The urothelium was removed before the preparation was pinned out on a silicon rubber base and superfused with Krebs solution at 35-37°C. For isometric tension recording experiments, circular strips of urethra were adjusted to a resting tension of 5 mN and superfused with Krebs solution at 35-37°C containing 100 μM NO-ARG and 1 μM guanethidine.

When contractions were elicited by transmural nerve stimulation (0.5 Hz – 4 Hz, 50 V, 0.3 ms pulse width, 60 s duration) via electrodes placed at either end of the organ bath, frequency dependent increases in tone were observed and these were blocked by tetrodotoxin (1 μM, n=4). The peak amplitude of the neurogenic contractions evoked by stimulating at 4 Hz was significantly reduced from 19.1±4.6 mN to 7.2±2.3 mN (p<0.05, mean±SEM, paired t-test) by atropine (1 μM), suggesting that muscarinic receptors were involved in this response. Methoctramine (1 μM, M2 blocker) had little effect on these responses (n=6). However, the M1/M3 muscarinic receptor antagonist 4-DAMP (100 nM) significantly reduced the neurogenic contractions evoked by 2 Hz and 4 Hz from 1.2±0.3 and 2.3±0.4 mN to 0.4±0.2 and 1.0±0.4 mN (p<0.05, n=6) respectively, suggesting that acetylcholine mediates its effects on tone via activation of M1 or M3 receptors.

When the proximal urethra was impaled with sharp microelectrodes, regular spontaneous slow waves which had spikes superimposed upon a plateau were recorded as demonstrated previously (Bradley et al., 2004). Carbachol increased the frequency of the slow waves from 3.1±0.3 min⁻¹ (n=9) to 3.3±0.8 min⁻¹ (n=3), 5.9±0.5 (n=6), 6.8±0.8 (n=4), 12.3±2.2 (n=4) min⁻¹ in response to 10 nM, 100 nM, 300 nM and 1 μM carbachol respectively. To examine if this effect was via stimulation of M1/M3 receptors, we examined the effects of 300 nM carbachol on electrical activity before and during incubation of the tissue...
Ca\textsuperscript{2+} channel-induced contraction in basilar artery is mediated by metabotropic Ca\textsuperscript{2+} release from sarcoplasmic reticulum

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We have described in rat basilar artery that L type Ca\textsuperscript{2+} channel activation can activate Ca\textsuperscript{2+} release from sarcoplasmic reticulum (SR) in the absence of extracellular Ca\textsuperscript{2+} through a metabotropic pathway (mechanism denoted as Calcium-Channel Induced Calcium Release, CCICR) (Del Valle-Rodríguez et al., 2003). The calcium-release mechanism depends on the conformational change of L-type Ca\textsuperscript{2+} channels and the downstream activation of the G protein/phospholipase C (PLC) cascade, leading to synthesis of InsP\textsubscript{3} and Ca\textsuperscript{2+} release from the SR. Because previous results were obtained in isolated myocytes bathed in free Ca\textsuperscript{2+} medium, the aim of this work was to study the functional role of CCICR in physiological conditions (i.e. in arteries bathed in medium containing Ca\textsuperscript{2+}). Experiments were done in rat cerebral arteries obtained from anaesthetized (sodium pentobarbital, 50 mg/Kg, ip.) animals of weight 250-300 g (30 rats and 100 arterial rings). Isometric force and cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) were measured in rat basilar arterial rings and in intact arteries. Ca\textsuperscript{2+} channel activation produced an initial rapid rise in cytosolic Ca\textsuperscript{2+} and a second plateau phase that was maintained until the end of the stimulus. Concomitant with this change in [Ca\textsuperscript{2+}], a reduction of the arterial diameter was detected. Both signals were transient when Ca\textsuperscript{2+} was eliminated from the extracellular medium. Cyclopiazonic acid, ryanodine and U73122, inhibitors of SR Ca\textsuperscript{2+} pump, ryanodine receptors and PLC respectively, reduced the maintained phase of contraction whereas the transient component was not significantly affected. Our results suggest that in physiological conditions Ca\textsuperscript{2+} channel-induced contraction of the basilar artery can be mediated by both Ca\textsuperscript{2+} influx from the extracellular medium (ionotropic function of Ca\textsuperscript{2+} channel) and Ca\textsuperscript{2+} release from the SR through a metabotropic pathway.

Identification of a novel pH-gating mutation in the Kir1.1 (ROMK) channel using a yeast genetic complementation assay

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Gating of inwardly rectifying potassium (Kir) channels by intracellular pH is important for many aspects of channel physiology and pathophysiology. In mammalian Kir1.1 (ROMK) an intrasubunit H-bond between a lysine in TM1 (K80) and a backbone carbonyl group in TM2 (A177) at the helix bundle crossing is thought to control the kinetics of the gating motion of the TM helices in response to pH and PIP2. However, the exact pH gating mechanism is not fully understood and further studies are needed to determine which other residues are important for Kir1.1 pH sensitivity.

In this study, we exploited a yeast genetic complementation assay to screen for gain of function (GoF) mutations in Kir1.1 which might alter pH gating, given that the intracellular pH in yeast is highly acidic and predicted to inhibit Kir1.1 function. However, the screening of a random-mutated Kir1.1 library in SGY1528 K\textsuperscript{+} uptake deficient yeast yielded only two mutants (K80M and K80I) which were already known to decrease pH sensitivity. We therefore used a chimeric channel (30C) which contained the transmembrane domain of Kir1.1 and the cytoplasmic domain of Kir4.1. This chimera showed no complementation in yeast even with the mutation K80M. However, upon screening of a randomly mutated library a large number of novel gain of function (GoF) mutations were found. Positive GoF mutant clones were verified and sequenced. These mutations were found to occur in many regions of the Kir1.1 transmembrane domain, including known gating sensitive regions and those that may interact with PIP2, with only one mutation identified in the Kir4.1 section of the 30C chimera. Because these mutations might permit channel function in yeast by reducing pH sensitivity these GoF mutations were made individually in the Kir1.1 channel and their pH-sensitivity measured electrophysiologically.

Many mutants had only a modest effect on Kir1.1 pH-sensitivity. However, one novel mutant in TM2 had a dramatic effect reducing pH sensitivity to a similar extent to the K80M mutation. In addition, the whole cell currents of each GoF mutation...
were recorded in neutral conditions and correlated with the screening results. These results showed the validity of this yeast genetic complementation assay to screen for pH-gating mutations in K+ channels. Further electrophysiological studies on these mutations would help to understand the mechanism by which these mutations affect pH-gating.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC41**

Comparative study of quercetin-filled liposomes, free quercetin and 'empty' liposomes regenerative effects on BKCa channels activity in rat aortic smooth muscles cells under ionized irradiation

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It is well known that exposure to excess levels of radiation leads to vascular contractile anomalies due to depression of the endothelium-dependent vasodilatation and increase of the vasoconstriction. These effects of radiation realized through in a large conductance Ca2+-activated K+ channels (BKCa) activity in vascular tissue. Both 'empty' phosphatidilcholine liposomes (PCL) and free quercetin (Q) are known to repair radiation-induced vascular tone abnormalities, but Q is rather toxic and low soluble agent. So, the goal of this study was to compare effects of low toxic and high soluble phosphatidilcholine liposomes filled with quercetin (PCL-Q) and PCL, Q as well its simple mixture (PCL+Q) on functional activity of BKCa channels in rat thoracic aorta smooth muscle cells (SMCs) after ionizing irradiation.

SMCs of thoracic aorta were obtained from anesthetized intact male Wistar rats (200-250 g) (100% survival rate (dose for LD50, 10 Gy)). The effects of whole body γ-irradiation (6 Gy) and quercetin-filled PCL on BKCa channels functional activity were studied using patch-clamp technique in whole-cell configuration. BKCa current density (paxilline-sensitive current) in control SMCs were 25±2 pA/pF at +70 mV (n=20). Ionizing irradiation decreased BKCa current density in SMCs to 13±1 pA/pF (n=15, P<0.05) and 5±1 pA/pF (n=17, P<0.05) on the 9th and 30th days, respectively.

PCL-Q (3.4 µg/ml by quercetin and 124.0 µg/ml by lecithin), being added to the external solution, effectively increased BKCa current density in post-irradiated SMCs – to 24±3 pA/pF and 17±2 pA/pF (n=7, P<0.05) on the 9th and 30th days, respectively. The total effect PCL-Q appears to be the result of additive action of Q and PCL. The ratios of therapeutic effectiveness of PCL-Q, Q and PCL, being expressed quantitatively, may be presented as 1.0:0.6:0.4. The substances studied was without effect on the BKCa channels activity in control rat aorta SMCs. Experimentally obtained for PCL-Q and theoretically expected for Q+PCL dose-response curves demonstrate good coincidence. I/V curves obtained on the 9th and 30th days of post irradiation using simple mixture of PCL and Q in comparison with PCL-Q, confirm an additive effect PCL-Q constituents, i.e. the effects of PCL and Q simply summarize.

The data obtained in this study clearly indicate that the ability of PCL-Q to restore BKCa channel activity damaged following irradiation can be realized due to additive action of liposomal form of lecithin itself and Q encapsulated into lipid vesicles. Encapsulation of flavonoids appears to be worthwhile therapeuetic approach in case of ionizing irradiation accident as well in patients which underwent external radiotherapy.

This study was supported by a Physiological Society 'Centre of Excellence Award Scheme'.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC42**

Molecular mechanisms behind the acute oxygen sensing of potassium channels in primary human pulmonary artery smooth muscle cells

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Acute hypoxic inhibition of potassium (K+) channels is a critical step in regulatory processes designed to link lowering of oxygen levels to cellular responses. In the pulmonary circulation the vascular tone in response to hypoxia is determined in part by oxygen-sensitive ion channels but the molecular mechanism behind is still unknown.

Methods: Primary smooth muscle cells were isolated from human pulmonary arteries (hPASMC) from patients (n = 20) undergoing lung surgery for lung cancer without a history of pulmonary vascular disease or arterial hypoxemia as described earlier (Olschewski et al 2006). The study protocol for tissue donation was approved by the “Institutional Review Board” of the Medical University of Graz in accordance with national law and with guidelines on Good Clinical Practice/International Conference on Harmonization. Written informed consent was obtained from each individual patient. Hypoxia-induced changes in ion conductance and in intracellular calcium homeostasis of hPASMC were investigated by switching the perfusing medium from normoxia to moderate hypoxia (pO2 of 25-35 mmHg). The whole-cell patch-clamp technique was used as previously described (1). Changes in intracellular calcium were measured by using fura-2am loaded hPASMC. Immunofluorescence of phosphorylated tyrosine kinase in normoxia and after 30 minutes of hypoxia was performed using phosphospecific antibody. Intergroup differences were assessed by a factorial analysis of variance with post hoc analysis with Fisher’s least significant difference test.

Results: Voltage-activated (Kv), calcium-sensitive (KCa) and non-inactivating TASK-1 K+ currents were reversibly inhibited by moderate hypoxia. The reduction of Kv current measured at +50 mV was 43±3% (n=8, p<0.01) and 56±5% for KCa (n=7, **Posters**
p<0.01). TASK-1 current was blocked to 51±3% (n = 22, p<0.001) at 0 mV. PP2, a selective inhibitor of Src tyrosine kinases abolished the effect of hypoxia (n=25), whereas PP3 (n=16), Ro-31-8220 and Gö 6983 (PKC inhibitors, n=18), KT 5720 (PKA inhibitor, n=15), Y-27632 (Rho-kinase inhibitor, n=16) or compound C (AMP kinase inhibitor, n=20) failed to attenuate the hypoxia-induced reduction of the currents. Hypoxic challenges induced a transient increase in intracellular calcium concentration (n=50). The effect of hypoxia was abolished when hPASMC were preincubated with PP2 (n=40). In hPASMC a redistribution of phosphorylated tyrosine kinase fluorescence to the plasma membrane was observed under hypoxic conditions, whereas it was localized in the cytosol in normoxic cells (n=3). Conclusion: Our data indicate that the tyrosine kinase (src) pathway is required for the acute response to hypoxia of K+ channels and intracellular calcium homeostasis in primary human PASMC.


The study was supported by European Union and by the Medical University of Graz.

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Nerve injury induces down-regulation of M channel expression in peripheral sensory neurons

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Neuropathic pain is a severe health problem, which is further complicated by the lack of effective therapy. Search for new treatments has recently focused on ion channel candidates, since expression and activity of many ion channels relevant to pain signalling have been found altered following nerve injury. There is compelling evidence for the role of non-inactivating, subthreshold Kv7 (KCNQ, 'M type') potassium channels in pain transmission, as the membrane excitability of small nociceptive DRG neurons increases following pharmacological or receptor-induced inhibition of M current (Passmore et al, 2003; Linley et al, 2008). Immunohistochemistry in adult rat DRG revealed that Kv7.2 protein is expressed within subpopulations of CGRP-, IB4- and TRPV1-positive neurons. Behavioural tests demonstrated that acute in vivo inhibition of M channels expressed in nociceptive neuronal terminals of rat hindpaw induced by the intraplantar injection of specific Kv7 blocker, XE991 (10 nmol/site), resulted in spontaneous nocifensive behaviour not observed in rats injected with vehicle. We next studied a possible role of M channels in the development of chronic pain following peripheral nerve injury. To this end we investigated the expression of KCNQ2 in the DRG and sciatic nerve neuroma of Partial Sciatic Nerve Ligation (PSNL) operated rats. Under general anesthesia with 2% isoflurane, the nerve was exposed, partially ligated and sectioned. Real-time RT-PCR experiments revealed that the level of KCNQ2 mRNA within PSNL injured rat L4 and L5 DRG was significantly decreased 30 days following nerve injury in comparison to sham operated rats (mean±s.e.m, 0.23±0.05 to 0.09±0.03 relative to housekeeping gene U6, n=6, P = 0.03 using students t-test). We also studied the expression of the Repressor Element 1-Silencing Transcription factor (REST), a transcription factor that can bind to KCNQ DNA in vitro and reduce M channel expression in cultured DRG neurons (Ooi et al, 2009). REST mRNA expression was increased in the L4 and L5 DRG of PSNL injured rats in comparison to sham REST expression (0.008±0.002 to 0.019±0.004 relative to U6, n = 6, P = 0.07). In addition KCNQ2 mRNA was decreased in the ipsilateral nerve neuroma of PSNL injured rats in comparison to sham sciatic nerve KCNQ2 mRNA expression (1x10^-4±0.4x10^-4 to 1x10^-5±0.3x10^-5 relative to U6, n = 6, P = 0.04). Preliminary immunohistochemical analysis of PSNL rat L4 DRG showed a decrease in Kv7.2 protein expression 15 days following injury in comparison to sham operated rats. In sum our data suggest that transcriptional down-regulation of M channel expression in nociceptive sensory neurons can contribute to increased excitability of peripheral nerves induced by nerve injury.


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ATP independent translocation of STIM1 and formation of STIM1-Orai1 complexes


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Stromal interacting molecule one (STIM1) is currently known to be a calcium sensor protein located in the endoplasmic reticulum (ER). Depletion of the ER calcium store triggers translocation of STIM1 into subplasmalemmal puncta where it activates calcium channels and initiates store operated calcium entry (SOCE). We show that inhibition of ATP production induced a slow calcium leak from the ER that was followed by formation of subplasmalemmal STIM1 puncta. Depletion of cytosolic ATP also initiated the loss of phosphatidylinositol 4,5-bisphosphate (P(4,5)P2) from the plasma membrane. Although STIM1 puncta formed by inhibition of ATP synthesis co-localised with clusters of Orai1 channels these complexes were inefficient to facilitate calcium influx. Restoration of ER calcium levels in the absence of ATP permitted STIM1 re-translocation from subplasmalemmal puncta to the ER. Therefore we suggest that dynamic re-arrangement of STIM1 and the formation of STIM1-Orai1 complexes is an ATP independent process that can occur under conditions of P(4,5)P2 depletion.

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**PC46**

Neuromuscular fatigue in exhausting exercise at VO2max: influence of two modalities  
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It is commonly admitted that time-to-exhaustion corresponds to about six minutes for an exercise performed at an intensity allowing eliciting 100% of the VO2max. Although many works have tried to identify the limiting factors in time-to-exhaustion at VO2max by investigating energetic or metabolic parameters, the determinants underlying this performance key factor remain unclear. The purpose of this study will be to address this question through a neuromuscular approach. Our hypothesis will be that a neuromuscular invariant could explain exercise breakout in subjects performing a maximal exercise up to volitional exhaustion.

11 physically trained subjects got involved in this study. Each of them had to perform in a random order 2 pedalling exercises designed to elicit 100% of their individual VO2max, separated by 1 week recovery. Each exercise was lead up to exhaustion. The first one was performed at a constant power corresponding to the maximal aerobic power (MAP). During the second one, external power output initially corresponded to the MAP, but was secondarily adjusted so that VO2 remains maximal despite decreased load.

Neuromuscular testings were performed before and immediately after each exercise in order to assess neuromuscular fatigue generated by each condition.

Power output during exercise performed at a variable load was modelized in regard to MAP, power at individual anaerobic threshold and time-to-exhaustion at MAP.

Time-to-exhaustion significantly increased in variable load condition. Neuromuscular fatigue was not significantly different between the two conditions. Constant neuromuscular fatigue between the two conditions suggests that this parameter could play a key role in volitional exhaustion during maximal exercise.

It seems possible to dramatically increase time-to-exhaustion at VO2max. This result could be of interest in field performance training.

VO2max could be elicited at a submaximal external power output, what could be an important result in field rehabilitation. Further investigation is required to better understand underlying mechanisms in fatigue during maximal metabolic load.

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**PC47**

Effect of equal-volume creatine supplementation with different dosing frequency on muscle hypertrophy and strength in young adults  
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Creatine (Cr) supplementation during resistance training (RT) increases muscle mass and strength. However, it is unknown whether the volume and frequency of Cr ingestion will influence the adaptations from RT. This study determined the effects of equal-volume Cr supplementation with different dosing frequency on muscle hypertrophy and strength. Young healthy adults (N=22; 11 male, 11 female; 21±3 years) were randomized to supplement with Cr during 2 days/week of RT (Cr2, 0.15g/kg-1, N=11, 6 male, 5 female) or 3 days/week of RT (Cr3, 0.10g/kg-1, N=11, 6 male, 5 female). Creatine was consumed in equal amounts immediately before and immediately after each RT session. The RT program consisted of 2 sets (Cr2) or 3 sets (Cr3) of 10 repetitions (10RM) to muscle fatigue for 9 whole-body exercises. Prior to and following Cr supplementation and RT, measurements were taken for muscle hypertrophy (elbow and knee flexors and extensors; ultrasound), and muscle strength (1-repetition maximum leg press and chest press). Repeated measures ANOVA (mean ± SD) showed creatine supplementation increased muscle hypertrophy and strength over time (p<0.05). There was a greater change in leg press strength in the Cr2 group (Cr2: 223±93→362±220kg, 62% vs. Cr3: 168±54→238±112kg, 42%, p=0.05). There were no differences between groups for changes in muscle size of the elbow flexors (Cr2: 2.9±0.9cm→3.2±1.0cm, 10% ; Cr3: 2.7±0.6→3.0±0.6cm, 11%), elbow extensors (Cr2: 3.4±1.2→4.3±0.7cm, 26%; Cr3: 3.0±1.2→3.7±0.9cm, 23%), knee flexors (Cr2: 5.0±1.0→5.4±0.7cm, 8%; Cr3: 4.7±1.1→5.4±0.8cm, 15%), and knee extensors (Cr2: 4.3±1.0→4.7±1.4cm, 9%; Cr3: 3.7±0.7→3.9±0.7cm, 5%), or chest press strength (Cr2: 109±66→128±73kg, 17%; Cr3: 77±49→85±42kg, 10%). These results suggest that the volume of Cr ingestion may be more important than the frequency of Cr ingestion for improving lower-body muscle strength in young healthy adults.

RIVALUS Inc., Canada.

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Effects of β₂-agonists on force in anoxic rat EDL muscle. The role of the Na⁺/K⁺-pumps

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Fatiguing stimulation of isolated rat skeletal muscle leads to depolarization of the cellular membrane and a considerable loss of force (1) suggesting excitability of the membrane as an important mediator of the fatigue. Na⁺/K⁺-pump activity increases dramatically in response to β₂-agonist addition, improving restoration of the Na⁺, K⁺ homeostasis and the contractile performance of a working muscle. Reduced availability of oxygen to the muscles has been shown to increase the rate of fatigue in isolated muscle (2) and isolated diaphragm muscle strips (3). The present study examines the effect of β₂-agonists on force in anoxic rat EDL muscle and the role of the Na⁺/K⁺-pumps in mediating this effect.

EDL muscles were prepared from 4 wk old Wistar rats and mounted on holders for isometric contractions. Muscles were stimulated intermittently at 40 Hz for 15 min (10 sec on, 30 sec off, 10 V, 0.2 ms pulses) during anoxic conditions (95% N₂, 5% CO₂) and force recovery was followed during reoxygenation (95% O₂, 5% CO₂) for up to 240 min. β₂-agonists (salbutamol (10⁻⁵ M) or salmeterol (10⁻⁶ M)) were added either 15 min prior to the anoxic stimulation protocol or during recovery. Intracellular Na⁺ content was measured at the end of the reoxygenation period. The statistical significance of any difference between the control group and the treated group was ascertained using Student’s t-test and the post hoc Bonferroni test. Stimulation during anoxia leads to rapid force decline and only partly recovery during the following reoxygenation period. Addition of salbutamol or salmeterol prior to the anoxic stimulation protocol improves force by 32% (P<0.001, n=7) of initial force. Addition of salbutamol to control muscles reaches 27% (P<0.05, n=7) and 10% (P<0.001, n=7) respectively, within the first 2-3 min of the fatiguing protocol, hereafter force declines rapidly in all groups. In the following reoxygenation period force in the control muscles decreases 27 ± 3% of initial force, whereas force in salbutamol treated muscles increases up to 42 ± 5% (P<0.05, n=7) of initial force. Addition of salbutamol to control muscles at 150 min in the reoxygenation period improves force by 102 ± 16% (P<0.001, n=12). Muscles treated with a β₂-agonist also showed decreased intracellular Na⁺ content indicating that the Na⁺/K⁺-pumps are involved. Inhibition of the Na⁺/K⁺-pumps by ouabain or 2-deoxyglucose abolishes the positive effect of salbutamol on force. No detectable increase in the release of an intracellular enzyme (LDH) is observed during this anoxic protocol indicating that the observed force loss is not due to loss of cellular integrity.

The main findings are that force during and following an anoxic period can be improved by addition of β₂-agonists (eg. salbutamol or salmeterol) most likely due to a stimulating effect on the Na⁺/K⁺-pumps.

Mikkelsen et al., Am J Physiol Regul Integr Comp Physiol 290, R265-R272, 2006
van der Heijden et al., Am J Physiol 276, L474-L480, 1999

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Relationship between changes in force and linear dimensions of Rectus Femoris muscle in man using ultrasound imaging

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BACKGROUND
Ultrasound imaging (USI) is used increasingly for assessing muscle activity during contraction in research and clinical practice [1]. The relationship between changes in cross-sectional shape and linear dimensions that occur during contraction varies in different muscles and cannot be assumed. Both linear and curvilinear relationships have been found for increases in muscle thickness with force, e.g. in calf [2] and abdominal [3,4] muscles.

METHODS
In 14 healthy males, aged 22-27 years (mean 24.8), isometric force of quadriceps was measured using a Biodex dynamometer with the knee flexed to 90 degrees, during maximal voluntary contractions (MVC) and randomly at 10, 20, 30, 50 and 75% of MVC, held for 3 seconds each. An ultrasound scanner (Aquilla, ESAOTE; 6 MHz linear transducer) was used to image RF at mid-thigh at rest and during contractions. Two muscle dimensions (depth or thickness and width) were measured offline. Pearson’s correlation was used to examine the relationship between change in muscle dimension (normalized as percentage) and force. Differences in mean dimensions between force levels were examined by repeated measures analysis of variance and paired samples T-test with Bonferroni correction (p<0.008).

RESULTS
As force increased, there was a curvilinear decrease in RF width (r=-0.92; p<0.01), with decreases in width greatest at low forces, <30% MVC (Fig 1). Resting width (mean=4.41cm, SD=0.07) was significantly different to that at all contraction levels (p<0.05). Significant differences were also found between contraction levels (p<0.008), except between 20% - 30% MVC, and 75% - MVC (p>0.008). At MVC, width was 3.27cm (SD=0.47), approximately 25% smaller than at rest. Thickness of RF increased with contraction but only changed minimally (p>0.05), from a mean of 2.34cm (SD=0.32) at rest to 2.57cm (SD=0.3) at MVC.

CONCLUSIONS
Dynamic assessment of RF in young males can be made by measuring decreases in width on USI scans to reflect increases in force, which are most evident at low forces. Muscle thickness cannot be used as a measure of contractile activity of RF.
These findings are relevant to healthy young males and larger numbers are needed to calculate predictive regression equations. The relationship also warrants investigation in healthy males and females of different ages and habitual activity, as well as populations with muscle dysfunction.


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PC50

Effects of hydrogen peroxide on rat sternohyoid muscle endurance

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Obstructive sleep apnoea (OSA) is characterized by recurrent collapse of the upper airway during sleep, subjecting the patient to recurrent bouts of intermittent hypoxia. Chronic intermittent hypoxia (CIH) has been shown to induce upper airway muscle dysfunction, perhaps via the production of reactive oxygen species. First, we examined the effect of hydrogen peroxide (H2O2) on contractile properties of the rat sternohyoid muscle at varying temperatures. Second, we investigated the effect of H2O2 on the expression of iron chelator, deferoxamine (DFX) on skeletal muscle contraction. DFX prevents the generation of hydroxyl radicals from H2O2.

Adult male Wistar rats were anaesthetized with 5% isoflurane and killed by spinal transection. Sternohyoid muscle strips were mounted isometrically in water-jacketed tissue baths at 27°C or 35°C and either bubbled with a hyperoxic (95% O2/5% CO2) or anoxic (95% N2/5% CO2) gas mixture. Studies were conducted under control conditions (no drug), in the presence of 1mM H2O2 or 1mM H2O2 plus 1mM DFX. Strips were set to optimal length and force-frequency relationship was assessed by stimulating the muscle every two minutes at 10, 20, 30, 40, 60, 80 and 100 Hz for 300ms. Fatigue was induced by repeated tetanic contractions (40Hz, 300ms duration) every 2 seconds for 2 minutes.

At 27°C, H2O2 had no significant effect on muscle force under hyperoxic (22±3 vs. 17±2 N/cm², mean±SEM, control n=5 vs. H2O2 n=5 at 100 Hz, P>0.05, ANOVA) or hypoxic conditions. H2O2-treated muscle strips exhibited increased fatigue under hyperoxic conditions (63±3% vs. 39±3%, control n=6 vs. H2O2 n=6 at 2 min, P<0.05, ANOVA). Similar results were seen in hypoxia (63±7% vs. 25±2%, control n=7 vs. H2O2 n=5 at 2 min, P<0.05, ANOVA). At a more physiological temperature of 35°C, H2O2 again did not alter sternohyoid contractile force under hyperoxic or hypoxic conditions. Fatigue was increased in H2O2-treated muscle strips compared to control strips in hyperoxia (98±5% vs. 57±9%, control n=6 vs. H2O2 n=7 at 2 min, P<0.05, ANOVA) and hypoxia (60±7% vs. 15±6%, control n=7 vs. H2O2 n=6 at 2 min, P<0.05, ANOVA). Co-incubation of H2O2 with DFX significantly improved the fatigue index of the muscle strips compared to H2O2 alone in hypoxia (83±3% vs. 57±9%, H2O2 plus DFX n=6 vs. H2O2 n=7 at 2 min P<0.05, ANOVA) but this improvement did not reach statistical significance in hypoxia. In summary, H2O2 increased fatigability of a pharyngeal dilator muscle but failed to show any effect on muscle force at both 27°C and 35°C. Muscle strips co-incubated with H2O2 and DFX showed a marked improvement in muscle endurance partially reversing the effects of H2O2 alone. DFX prevents the formation of hydroxyl radicals from H2O2 and therefore the effects of H2O2 on muscle fatigue are presumably partially attributed to H2O2 itself and partially to an effect of hydroxyl radicals.


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Dihydrotestosterone (DHT) modulates force production in isolated mouse skeletal muscles by regulating the phosphorylation of myosin light chains by ERK 1&2

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In a recent study we showed that treating isolated intact mouse fast- and slow-twitch skeletal muscle fibres with physiological levels of dihydrotestosterone (DHT), for one hour, leads to an increase in maximum isometric tension (Po) in the fast twitch fibres and a decrease in Po in the slow twitch fibres (Mutungi, 2008). However, in that study the molecular mechanisms underlying these effects were not investigated. Therefore, the primary aim of this study was to investigate the molecular mechanisms underlying the effects of DHT on Po in mouse skeletal muscle fibre bundles.

All the experiments were performed at 20°C using small muscle fibre bundles isolated from either the extensor digitorum longus (edl, a mainly fast twitch muscle) or soleus (a predominantly slow twitch muscle) of adult female CD-1 mice 49±4 (n=8; S.E.M) days. The mice were humanely killed and all the experiments conformed to the Animals (Scientific Procedures) Act 1986. The fibre bundles were treated for 1hr with either 630 μg/ml DHT dissolved in absolute ethanol (experimental) or the vehicle only (6.3 μL/100ml; controls). In another experiment, the bundles were pre-treated for 30 minutes with inhibitors of either the epidermal growth factor receptor (EGFR) (100nM AG1478) or the androgen receptor (AR) (3μM flutamide and 1μM cyproterone acetate) before treatment with DHT plus the inhibitor for 1hr. At the end of each experiment, the bundles were processed for immunoblotting and the changes in the phosphorylation of extracellular signal regulated kinases 1&2 (pERK1&2) and myosin light chains (pMLCs) were probed using antibodies against the phosphorylated proteins.
The findings show that treating the fibre bundles with DHT led to a 2-3 fold increase in the phosphorylation of ERK 1&2 in both fibre types and MLC in the fast twitch fibres only. Moreover, this increase was blocked by AG1478 but not by flutamide and cyproterone acetate. From these results we suggest that DHT modulates force production in mammalian skeletal muscles by regulating the phosphorylation of MLCs by ERK1&2. Mutungi G. (2008). The effects of androstane (a synthetic DHT) on maximal isometric force in intact mouse skeletal muscle fibres. Proc Physiol Soc 11, C56.

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**Posters:**

**PC52**

**Interaction of D9-tetrahydrocannabinol and Buprenorphine with Multidrug Resistance Proteins in Human Placenta**

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Drug misuse by pregnant women is a leading preventable cause of fetal and neonatal morbidity and mortality. It has been suggested that in both the UK and the USA 10-16% of pregnant women use illicit drugs. The level of exposure of any one fetus is likely to be determined by the capacity of the placenta to act as a barrier. The multi drug resistance proteins (MDRPs) P-glycoprotein (P-gp) and breast cancer resistance protein (BRCP) are located on the maternal facing (microvillus) plasma membrane of the placenta. In this location, these proteins may act to prevent xenobiotics in maternal plasma reaching the fetus at toxic concentrations. The aim of the present study was to determine whether two commonly used substances i.e. Δ9-tetrahydrocannabinol (THC) and buprenorphine (BUP) interact with P-gp and/or BRCP in human placental tissue.

Term placentas were collected (with ethics committee approval and informed consent) within 30 mins of delivery. Small villous fragments were dissected and used to measure accumulation of [3H]-vinblastine (a P-gp substrate) and [3H]-mitoxantrone (a BRCP substrate) in the presence or absence of 20μM THC or BUP. Accumulation at 10 mins (initial rate) and 120 mins (equilibrium) was measured in all cases.

BUP had no significant effect on either vinblastine (n=5 placentas) or mitoxantrone (n=3) accumulation at 10 or 120 mins. THC caused a significant (p<0.05 Wilcoxon signed rank test, n=5) decrease in accumulation of vinblastine at 120 mins (50%) and there was a trend towards reduced accumulation (37%) at 10min (P=0.06 n=5). THC had no significant effect on mitoxantrone accumulation although there was a trend towards increased accumulation at both time points (10 mins 21% and 120mins 38% p=0.06 n=4).

The reduced accumulation i.e. increased efflux of vinblastine in the presence of THC suggests stimulation of P-gp activity by this component of cannabis. These data are consistent with previous observations in insect cell membranes containing human P-gp where THC caused increased P-gp ATPase activity1. Extrapolating these in vitro data, we postulate that THC stimulation of placental P-gp activity would have a marked effect on the exposure of the fetus to other drugs which are substrates of this MDRP, taken by women using cannabis in pregnancy.


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**PC53**

**Vascular reactivity of early pregnancy human placental chorionic plate arteries**

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**Background:** We have previously demonstrated that placental chorionic plate arteries (CPAs) at term exhibit a biphasic (transient contraction followed by maintained relaxation) response on exposure to histamine [1]. Here we wished to determine if early pregnancy CPAs displayed similar vascular reactivity.

**Method:** Placentas (N=5) were obtained following elective medical or surgical termination of pregnancy. Gestational age was estimated from date of last menstrual period and confirmed by ultrasound dating. Biopsies were placed into ice-cold HCO3-buffered physiologic salt solution (PSS). Arteries were dissected from the chorionic plate, mounted onto a wire myograph, normalised at 0.9 of Ls,1kPa and equilibrated (37°C; 20 mins; 5%O2/5%CO2 7% O2). Contraction was assessed with 120mM potassium solution (KPSS) and the thromboxane-mimetic U46619 (10^-10 to 2x10^-6M). Histamine hydrochloride (HIS; 10^-6M) was added to pre-contracted vessels (EC80 dose of the thromboxane-mimetic U46619 for 30 mins) for 60 mins. Experiments with HIS were also performed in the presence of indomethacin (I; 10^-5M) and indomethacin plus Nω-nitro-L-arginine, (IN; both 10^-5M). Arterial relaxation to donated nitric oxide (NO) was assessed using sodium nitroprusside (SNP; 10^-5M). All data are expressed as median (range).

**Results:** Normalised luminal internal diameters were 486 (210-1008) μm (n=20 arteries). Maximal arterial contraction to KPSS and U46619 was 2.0 (0.4-5.0) kPa and 3.2 (1.2-6.8) kPa (n=20 arteries from N=5 placentas) respectively. HIS induced a biphasic response in pre-contracted CPAs; a transient contraction followed by maintained relaxation to 95 (23-95) % of EC50 U46619-induced contraction. Contraction and relaxation to HIS alone were not significantly affected by pre-incubation with I or IN (P<0.05; Friedman’s test followed by Dunn’s post hoc
Expression of an electrically silent voltage-gated potassium channel in the human placenta

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Potassium (K) channels are important for normal placental function. Activity and expression of a number of K channel isoforms has been reported in syncytiotrophoblastic and vascular tissues [1,2]. mRNA expression of \( K_{v}9.3 \), a voltage-gated (\( K_{v} \)) channel linked to tissue responses to oxygenation [3], has been demonstrated in whole placental homogenate by RT-PCR. However, the channel’s electrophysiological properties and the lack of a human isoform specific antibody have hampered further investigations. We aimed to produce an antibody to human \( K_{v}9.3 \), with a view to examining protein expression and localisation in human placental tissues.

Term placentas were collected from uncomplicated singleton pregnancies or from women whose pregnancies were affected by fetal growth restriction. An affinity-purified polyclonal antibody was raised in rabbit to residues 131-147 of the human isoform of \( K_{v}9.3 \). Channel expression in samples of placental homogenate was confirmed by Western blotting. Protein expression and localisation was investigated using standard immunohistochemical techniques. Scored data were assessed using Mann-Whitney U-test with a threshold of \( P < 0.05 \) indicative of statistical significance.

\( K_{v}9.3 \) expression was confirmed by Western blotting. A single discrete band was observed at \( \sim 61 \text{kDa} \); signal was ablated by antibody pre-absorption (10X excess of blocking peptide) and incubation with pre-immune serum. Immunohistochemical staining was assessed by three independent researchers using an arbitrary scoring system, blinded to the identity of the tissue. Strong staining was observed in syncytiotrophoblast, particularly localised to the microvillous membrane. Endothelial cells within intermediate and stem villus and chorionic plate blood vessels stained positively for \( K_{v}9.3 \), but staining was absent in capillary loops of terminal villi. Vascular smooth muscle cell staining was only apparent in larger diameter vessels. Expression was not significantly altered in tissue from women with fetal growth restriction.

Expression of \( K_{v}9.3 \) suggests human placental tissues may respond to oxygenation fluctuations via alterations in cell membrane potential. This may have important implications for syncytiotrophoblast cell turnover, nutrient transport and for blood flow in fetoplacental blood vessels.


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Intermittent Hypoxia Impairs Rat Upper Airway Muscle Function: Protective Effects of a Superoxide Scavenger

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Upper airway muscle dysfunction is implicated in obstructive sleep apnoea, a debilitating respiratory disorder associated with cardiovascular and neurocognitive morbidities. Hypoxia-reoxygenation is a central feature of the disorder due to recurrent apnoea. We wished to characterize the effects of hypoxia (H), intermittent hypoxia (IH) and hypoxia-reoxygenation (HR) on pharyngeal dilator muscle function in adult male rats. As reactive oxygen species are implicated in skeletal muscle dysfunction, we hypothesized that antioxidant treatment would ameliorate the deleterious effects of (intermittent) hypoxia.

Rats were killed humanely, under 5% isoflurane, by cervical spinal cord transection. Isometric contractile properties of isolated sternohyoid muscle strips were examined at 35°C under control (95%O2/5%CO2) or test conditions i.e. [H = 95%N2/5%CO2; IH = 3 cycles of 5 min control/5 min H; or HR = 15 min H/15 min control] in vitro in standard physiological salt solution with or without 10mM Tempol (a superoxide scavenger). All muscle strips were set to optimum length (i.e. length producing maximum isometric twitch force). After an equilibration period, peak tetanic force at 100Hz was determined under control conditions before drug or gas treatment. Next, muscles were randomly assigned to groups. After a 30 min incubation period, force-frequency relationship was determined by electrical field stimulation with stimulus frequencies ranging 10-100Hz. Forces in all trials were expressed relative to peak force measured at the beginning of each experiment (% of initial).

All gas treatments caused significant decreases in sternohyoid muscle force compared to control (e.g. 89±9 vs. 28±4*, 61±7 and 60±8*, mean±SEM at 100Hz, % of initial, control (n=7) vs. H (n=8), IH (n=9) and HR (n=8), *p<0.01 ANOVA). Likewise, muscle performance during repeated stimulation (40Hz, 300msec, every 2 sec for 150 sec), performed after force-frequency trials, was significantly impaired by all gas treatments. Antioxidant treatment with Tempol partly ameliorated the decline in muscle force in all groups (e.g. 86±9 vs. 44±7*, 70±5 and 69±13, mean±SEM at 100Hz, % of initial, control (n=7) vs. H (n=8), IH (n=9) and HR (n=8), *p<0.05 ANOVA). Additionally, Tempol rescued sternohyoid forces during the fatigue trial in IH and HR treated muscles such that they were not different from control values.

Our results indicate that acute hypoxia and hypoxia/reoxygenation impairs pharyngeal dilator muscle function. Antioxidant treatment ameliorates these effects suggesting that reactive oxygen species-dependent mechanisms are implicated. As upper airway muscle dysfunction is implicated in OSA, we speculate that antioxidant supplementation might prove useful as an adjunct therapy in the treatment of the disorder.

Supported by the Health Research Board, Ireland (RP/2006/140).

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC57

Effects of Hypoxia on Geniohyoid Muscle Force and Endurance in Young Lean and Old Obese Male Rats

E. Lucking and K.D. O’Halloran

University College Dublin, Dublin, Ireland

Age, obesity and male sex are independent risk factors for the development of obstructive sleep apnoea (OSA). Upper airway dilator muscle fatigue is implicated in the pathophysiology of OSA. As antioxidant capacity decreases with age, we speculated that old obese rats would show decreased endurance and hypoxic tolerance.

Young (2-3 month old), lean (268±10g), male Wistar rats (n=14) and old (19-20 months), obese (822±41g), male Wistar rats (n=14) were used in this study. Rats were killed humanely, under 5% isoflurane, by high cervical spinal cord transection. Isometric contractile properties of isolated geniohyoid muscles were examined at 35°C under control (95%O2/5%CO2) or hypoxic (95%N2/5%CO2) conditions. Force-frequency relationship was determined at stimulus frequencies ranging 10-100Hz. Curve-fitting analysis was employed allowing us to determine the values for min, max, slope and EF50 (i.e. stimulus frequency producing 50% of peak force).
Fatigue was assessed by repeated stimulation of the muscle (40Hz, 300ms, every 2 sec for 3 min). Two-way analysis of variance (with Bonferroni correction) was employed to test for statistically significant effects of age and hypoxia.

Geniohyoid muscle maximum force was 6.9±1.5 and 4.2±0.6 N/cm², mean±SEM, control (n=7) and hypoxia (n=7). Corresponding values in young rats were 4.0±0.9 and 1.2±0.3 N/cm² (p>0.05 ANOVA). The EF₅₀ values were left-shifted in old compared to young animals (p<0.01 in hypoxia alone). Geniohyoid 3min fatigue index in old rats was 48±4% and 7±1%, [% of initial force, control (n=7) and hypoxia (n=7)]. Corresponding values in young rats were 78±6% and 24±4%. There was a statistically significant effect of age (p<0.001) and hypoxia (p<0.01), but no significant interaction. Force potentiation in the early phase of the fatigue trial was observed in all groups but was significantly greater in old compared to young animals in hypoxia (186±5% vs. 146±8%, % of initial force). The right lung was removed and flash frozen for later analysis.

This study illustrates that age (and/or) obesity causes plasticity in an upper airway dilator muscle. Though intrinsic fatigability of the geniohyoid decreases significantly with age both in hypoxia and hypoxia, it should be noted that the muscles from old obese rats generated more force under hypoxic conditions than young animals, and this was largely maintained during repeated stimulation. We conclude that increased pharyngeal collapsibility associated with age and obesity relates more to neurogenic than myogenic impairment.

Supported by the Health Research Board, Ireland (RP/2006/140).

*Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.*

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**PC58**

**VEGF family members can inhibit angiogenesis in the hypoxic lung**

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We have recently shown, for the first time, that angiogenesis occurs in the pulmonary circulation in response to chronic hypoxia, a common complication of chronic lung diseases. Much is known about the role of members of the vascular endothelial growth factor (VEGF) family in mediating neovascularisation in the systemic circulation. However, to date their role in hypoxia-induced pulmonary angiogenesis remains less clear. Male specific pathogen free Sprague Dawley rats (n = 7-8 per group) were exposed to normoxia or chronic hypoxia (10% O₂) for 1, 3, 7, 14 and 21 days. Following the exposure period, the rats were deeply anaesthetised (70mg/kg-1 sodium pentobarbitone (i.p.)) and killed by exsanguination via the femoral vessels. The right lung was removed and flash frozen for later analysis of mRNA by real-time PCR. To examine the interactions of specific VEGF ligands in vitro, human pulmonary microvascular endothelial cells were grown under sterile cell culture conditions until confluent. A single wound was created in the cell monolayer by scraping with a sterile pipette tip, the cells treated with vehicle or recombinant proteins (PlGF, VEGF A, VEGF B) and the wound assessed 24 hours later. The percentage reduction in wound width was then calculated.

VEGF A mRNA expression in the hypoxic lung was not altered at any time point examined in vivo (over a 3 week interval). VEGF B and PlGF mRNA expression was significantly increased at 7 and 14 days, with PlGF remaining augmented following 21 days exposure to chronic hypoxia. VEGF A (8ng/ml) augmented the mean (±SEM) rate of wound healing over vehicle alone. A low concentration of PlGF (40ng/ml) potentiated the rate of VEGF A induced wound healing. However, a higher concentration of PlGF protein (160ng/ml) did not alter the rate of VEGF A induced wound healing. VEGF B (20ng/ml) inhibited the actions of VEGF A.

These results suggest that the interaction between VEGF ligands is complex and is critically concentration dependent. Thus, further in vivo experiments are required to fully elucidate the role of members of the VEGF family in hypoxic pulmonary angiogenesis.

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**PC59**

**Oxidant/antioxidant status in hypoxic rats after submission to deep hypothermia**

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The use of deep hypothermia as a protective factor in hypoxia has been controversial. (Matthew et al., 2002; Riess et al., 2004). The purpose of this study was to describe the blood acid-base parameters and the profile related to oxidative stress, malondialdehyde, nitric oxide and glutathione in plasma, in deep hypothermic Sprague Dawley rats (21.5±1°C breathing room air or hypoxic air (10% O₂ in N₂), compared with normothermic
animals also breathing room air or hypoxic air. Rats were anaesthetized I.P. (intraperitoneally) with sodium pentobarbitone (60mg/Kg b.w.) and maintained with respiratory aid. The animals were humanely killed with an I.P. overdose of anaesthetic. Data were analyzed by the two-way ANOVA using the Student-Newman-Keuls test to identify significant differences (p<0.05).

Hypoxia exposure results in an oxidative stress with an increase in malondialdehyde, nitric oxide and a decrease in glutathione; however, if hypothermia is previously applied the situation is reversed with a stabilization of the malondialdehyde and an increase in the antioxidant glutathione (Table 1).

On the other hand, the determination of pH, Pco₂, and the ratio [OH⁻/H⁺] discarded a respiratory imbalance but showed a mild metabolic acidosis in the hypothermic groups (Table 2).

We proposed a metabolic acidosis as a mechanism which explains this protection since it helps to keep the intracellular reducing power, preserving glutathione and avoiding intracellular alkalinisation.

Table 1. Plasma oxidative stress-indicators

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>NHT</th>
<th>HT</th>
<th>NHT</th>
<th>NHT vs. HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (μmol/g)</td>
<td>2.00±0.1</td>
<td>3.87±0.37</td>
<td>4.66±0.71</td>
<td>4.79±0.71</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>NOc (μmol)</td>
<td>60±30±</td>
<td>23±32±2</td>
<td>23±30±2</td>
<td>23±32±2</td>
<td>ns</td>
</tr>
<tr>
<td>O2Hb(%)</td>
<td>20±33±5</td>
<td>18±31±2</td>
<td>28±31±2</td>
<td>24±37±2</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM

Table 2. Acid base parameters

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>NHT</th>
<th>HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCO₂ (mmHg)</td>
<td>65±6±5</td>
<td>65±6±5</td>
<td>65±6±5</td>
</tr>
<tr>
<td>pH</td>
<td>7.35±0.05</td>
<td>7.30±0.03</td>
<td>7.30±0.04</td>
</tr>
<tr>
<td>Pco₂ (mmHg)</td>
<td>26±13±4</td>
<td>20±13±4</td>
<td>26±13±4</td>
</tr>
<tr>
<td>[HCO₃⁻]</td>
<td>16±6±3</td>
<td>16±6±3</td>
<td>16±6±3</td>
</tr>
<tr>
<td>Sae (mEq/L)</td>
<td>2±1±1</td>
<td>2±1±1</td>
<td>2±1±1</td>
</tr>
</tbody>
</table>


This work was supported by research grant PI 081389

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC60

The superoxide scavenger Tempol improves pharyngeal dilator muscle function in old obese male rats

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Age, obesity and male sex are major risk factors for the development of obstructive sleep apnoea/hypopnoea syndrome (OSAS). Pharyngeal dilator muscle dysfunction is implicated in the pathophysiology of OSAS. We wished to examine the effects of acute hypoxia on contractile properties of an upper airway dilator muscle in old obese male rats. Furthermore, we wished to test the hypothesis that Tempol, a superoxide scavenger, would protect against hypoxia-induced impairment of pharyngeal dilator muscle function.

Old (18-20 month), obese (902±33g), male rats were killed by cervical spinal cord transection under 5% isoflurane. Sternohyoid (SH) muscles were dissected and removed for study. Isometric contractile and endurance properties were examined in physiological salt solution at 35°C under either hyperoxic (95%O₂/5%CO₂) or hypoxic (95%N₂/5%CO₂) conditions in vitro. Experiments were carried out in the absence (control) or presence of Tempol (10mM). Muscles were set to optimum length (i.e. length producing maximum isometric force). Force was measured in response to electrical field stimulation at stimulus frequencies ranging from 10-100Hz and was expressed as a function of muscle cross-sectional area (i.e. specific force). We also examined muscle performance in response to repeated stimulation (40Hz, 300ms, every 2 seconds for 2 minutes).

Hypoxia was associated with significant decreases in SH muscle force (peak force was 27±2 vs. 18±1, mean±SEM N/cm², hyperoxia (n=8) vs. hypoxia (n=8), p<0.05 ANOVA). Tempol rescued force in hypoxia-treated muscle strips (peak force = 24±2 N/cm², (n=8), p<0.05 vs. hypoxia). Interestingly, the positive inotropic effect of Tempol was also observed in hyperoxia [32±2 N/cm², (n=8), p<0.05 vs. hypoxia] suggesting that the inotropic effect was not dependent on the bath PO₂, and was more likely related to a beneficial effect of scavenging of free radicals produced by endogenous metabolism and muscle contraction. We observed improved muscle performance during the fatigue trial under both hyperoxic and hypoxic conditions, owing largely to increased force potentiation during the early phase of the trial in Tempol-treated muscles [e.g. 8±1 vs. 15±2 N/cm², hypoxia (n=8) vs. hypoxia+Tempol (n=8) at 20s of the 2min trial, p<0.05].

In summary, the main finding of this study was that Tempol has a significant positive inotropic effect on SH muscle in old obese male rats. Pharmacotherapy is considered a viable clinical option in the treatment of OSAS and agents that improve pharyngeal dilator muscle function might prove useful, since these muscles play a pivotal role in the maintenance of pharyngeal airway calibre. We conclude that antioxidant treatment may be beneficial as an adjunct therapy in the treatment of OSAS.

Supported by the Health Research Board (Ireland)

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PC61

Respiratory muscle plasticity following chronic intermittent hypoxia in male but not female Wistar rats

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Upper airway muscle dysfunction is implicated in obstructive sleep apnoea – a debilitating respiratory disorder associated with cardiovascular and neurocognitive impairments. Chronic
intermittent hypoxia (CIH), a central feature of sleep apnoea results in oxidative stress/injury and tissue dysfunction. We wished to characterise the effects of CIH on ventilation and pharyngeal dilator muscle function in male and female rats. We tested the hypothesis that sex differences would exist in the effects of CIH on the respiratory system.

Adult Wistar rats were exposed to CIH (90s air/90s N₂ ; 5%O₂ at nadir) or sham treatment for 8 hours/day for 9 days. A subset of animals in both groups received chronic antioxidant treatment in the form of the superoxide scavenger Tempol (1mM in the drinking water). Following treatments, ventilation was assessed by whole body plethysmography. Subsequently, the animals were killed humanely and isometric contractile properties of the sternohyoid (SH) muscle were examined at 35°C under control (95%O₂/5%CO₂) or hypoxic (95%N₂/5%CO₂) conditions. SH peak force was decreased in CIH-treated male rats [23±1 vs. 16±1 N/cm², sham (n=8) vs. CIH (n=8), p<0.001; Student’s t test]. SH peak force was decreased in CIH-treated male rats [23±1 vs. 16±1 N/cm², sham (n=8) vs. CIH (n=8), p<0.001 ANOVA]. Chronic antioxidant treatment with Tempol ameliorated IH-induced muscle impairment. Conversely, in female rats, CIH treatment had no effect on ventilatory drive or SH peak force [21±1 vs. 20±1 N/cm², sham (n=8) vs. CIH (n=8), p>0.05 ANOVA].

The main finding of this study is that CIH causes functional plasticity in the respiratory system. We observed CIH-induced muscle dysfunction in male rats – an effect that was ameliorated by antioxidant treatment. Female rats were unaffected by CIH and female SH muscles showed greater hypoxic tolerance in vitro. This suggests a greater antioxidant capacity in females compared to males preventing hypoxic maladaptation both in vivo and in vitro. Our results may have relevance to obstructive sleep apnoea, which is more prevalent in men than pre-menopausal women.

Supported by the Health Research Board (Ireland).

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC62 Midbrain control of spino-olivary neurones: a role in passive coping behaviour?**

J.L. Leith, S. Koutsikou, R. Apps and B.M. Lumb

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Studies of descending control of spinal nociception have focused primarily on sensory and autonomic functions and have neglected investigation of the control of pain pathways that convey inputs to motor control centres, such as the olivo-cerebellar system. This is a critical gap in our understanding of pain processing given that noxious stimuli drive powerful motor responses, which have important consequences for survival.

The aim of the current study was to examine the effects of neuronal activation in the ventrolateral periaqueductal grey (vlPAG), which is a source of descending control and is implicated in coordinating passive coping behaviours, on responses of spino-olivary projection neurones in the dorsal horn to both innocuous and noxious peripheral stimulation.

Experiments were carried out in alphaxalone-anaesthetised (Alfaxan, 15-30mg.kg⁻¹.hr⁻¹, i.v.) male Wistar rats (280-300g; n=12). Extracellularly recorded antidromically activated (Lipski, 1981) spino-olivary neurones were characterised by their responses to peripheral stimulation and the effects of descending control following neuronal activation in the vlPAG were investigated. Application of low threshold (brush, tap, joint movement) and high threshold (noxious pinch) mechanical stimuli to receptive fields on the hindlimb revealed four classes of neurones (Class 1, n=1; Class 2, n=4; Class 3, n=3; Class 4, n=4; Menetrey et al, 1977). Responses of i) Class 2 and 3 neurones to noxious pinch (3.6N), ii) Class 1 and 2 neurones to innocuous pinch (0.6N) and iii) Class 4 neurones to joint movement were monitored before and after microinjection of an excitatory amino acid (50mM DL-homocysteic acid; 60-80nl) into the vlPAG at sites that evoked depressor responses.

Activation of the vlPAG produced a significant decrease in noxious pinch-evoked responses of Class 2 and 3 neurones (to 35.6%, P<0.05, and 1.2%, p<0.01, of control respectively; Kruskal-Wallis). In contrast, activation in the vlPAG increased the responses of Class 2 spino-olivary neurones to innocuous pinch (to 123.5%; n=2). Interestingly, responses of Class 4 spino-olivary neurones to joint movement were significantly increased (to 216%, p<0.001; Kruskal-Wallis) following activation of the vlPAG.

The data reveal differential descending control exerted by the vlPAG on responses of spino-olivary neurones to sensory stimuli of different behavioural significance, which could contribute to passive coping behaviours coordinated by the vlPAG.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

This work was supported by the BBSRC.
Vestibular and somatosensory influences on the position of balance during human standing
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Studies in which subjects balanced a ‘virtual body’ to exclude vestibular input suggest that proprioceptive input from the legs is sufficient to stand (Fitzpatrick et al., 1994) and that vestibular inputs normally play no part in controlling body sway (Fitzpatrick & McCloskey, 1994). However, there is no unique pattern of somatosensory input from the legs that signals the vertical alignment of the body. The implication that vestibular inputs are not involved with balance control is inconsistent with clinical experience, studies of reflex responses to balance perturbations (Allum & Pfaltz, 1985) and vestibular stimulation (Cathers et al., 2005). Thus, it seems that the vestibular system has a role in balance control but not one concerned with controlling the extent of body sway.

This study uses the virtual-body method to investigate the proprioceptive and vestibular contribution to human standing. Twelve healthy adults participated in this study that was approved by the Human Research Ethics Committee of the University of New South Wales. Balance was assessed under three conditions: (i) normal standing, (ii) splinted standing to prevent rotation of joints above the ankle and (iii) balancing a virtual body (inverted pendulum), which simulated standing but excluded vestibular and graviceptive inputs. In each condition, subjects were tested with and without additional weight attached to the body or pendulum. Sway, alignment angle and ankle torque were recorded.

While standing normally, the position of the centre of mass of the body was approximately over the centre of the perimeter of the feet. This was maintained when a 30 kg weight was fastened around the pelvis ($\Delta$ mean: 2.0 ± 4.4 mm). In contrast, adding 30 kg to the virtual body with vestibular input unavailable caused the centre of mass to shift back towards the ankles ($\Delta$ mean: 24.3 ± 1.5 mm; $P < 0.001$). With only somatosensory input available, subjects balanced a lightweight virtual body over the front of the feet, their matched virtual body over the centre of the feet and a heavyweight virtual body over the heels. These positions did not keep constant the centre of foot pressure, muscle force, ankle angle or the excursion of sway.

It is concluded that vestibular and graviceptive sensory input, although not used to control sway during standing, has a unique role in aligning the body’s centre of mass above the feet, a function that cannot be achieved by proprioceptive inputs alone.


NH&MRC Australia funded research.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Poster Communications

PC65

Descending modulation of cool- and cold-evoked spinal nociception by the periaqueductual grey
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The physiology of cold somatosensation has received much recent interest, and many studies have investigated the peripheral mechanisms of cool and cold detection. However little is known of the central processing of cool and cold-evoked responses, nor whether these responses may be modulated by descending control systems that have profound effects on the processing of other sensory modalities. In particular, descending control that originates from the midbrain periaqueductual grey (PAG) is an important determinant of the pain experience. The current study aimed to further investigate the responses of spinal dorsal horn neurones to cool and cold stimuli in the rat and to determine whether their activity may be modulated by descending control from the PAG.

Extracellular recordings were made from lumbar dorsal horn neurones with receptive fields on the hind limb in alphaxalone-anaesthetised (Alfaxon; 15-30mg.kg\(^{-1}\).hr\(^{-1}\), i.v.) male Wistar rats (280-300g; n=19). Cells were characterised according to their responses to low (brush, tap) and high (pinch) threshold mechanical stimulation applied to the receptive field of the cell and classified as class 1 (low threshold, n=3), class 2 (wide dynamic range, n=28) and class 3 (nociceptive-specific, n=6; Menetrey et al, 1977). Cells were then tested for responsiveness to thermal stimuli: acetone (‘cool’), ethyl chloride (‘cold’; both 1ml topically) and noxious heat (35°C water). In class 2 cells that responded to acetone and/or ethyl chloride, the effects of activation of the ventrolateral (VL)-PAG were tested. After 3 control responses to acetone or ethyl chloride (applied at 5min intervals), 60-80nl of DL-homocysteic acid (DLH; 50mM in physiological saline saturated with pontamine sky blue dye to mark injection sites) was pressure injected under stereotaxic guidance into the VL-PAG. Test responses to acetone or ethyl chloride were measured 10s after DLH injection and 3 further recovery responses to acetone or ethyl chloride were measured at 5min intervals.

Of the recorded units, 67% of class 1, 64% of class 2, and 33% of class 3 cells responded to acetone, and 100% of class 1, 68% of class 2, and 33% of class 3 cells responded to ethyl chloride. In class 2 cells, activation of the VL-PAG by DLH significantly reduced ethyl chloride-evoked responses to 17±7% of control (mean±S.E.M.; n=8; p<0.05, Kruskal-Wallis). However acetone-evoked responses in class 2 cells were not significantly reduced (92±36%; n=7; p>0.05, Kruskal-Wallis) by activation of the VL-PAG. The data show that cold-evoked responses in class 2 dorsal horn neurones, like other sensory modalities, can be modulated by descending control systems that originate in the PAG.


This work was supported by the BBSRC.

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PC66

Stimulation of the periaqueductal grey modulates cortical somatosensory evoked potentials elicited by an acute noxious stimulus
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The periaqueductal grey (PAG) plays a key role in descending control of spinal nociception, and therefore the pain experience. It is functionally divided into longitudinal columns (including the dorsolateral (DL) and ventrolateral (VL) PAG) which, when activated, produce anti-nociception and distinct behavioural responses, characterised as active and passive coping strategies respectively (Lovick and Bandler, 2005). The effects of PAG stimulation at the level of the spinal dorsal horn have been widely investigated, but concurrent changes in cortical activity are unknown. The aim of this study was to investigate whether PAG stimulation modulates the cortical response to an acute noxious stimulus, using somatosensory evoked potentials (SEPs) as the outcome measure. DL- and VL-PAG were stimulated independently to identify whether these regions, known to cause distinct behavioural responses, also differentially modulate the cortical response to an acute noxious stimulus.

Eight male Wistar rats (280-320g), anaesthetised with an i.v. infusion of alfaxalone (~25mg.kg\(^{-1}\).hr\(^{-1}\)) were studied. SEPs were recorded from four active dorsal electrodes, placed bilaterally over the primary somatosensory cortex (S1) and the vertex (Vx) (2.5mm caudal, 2.5mm lateral to bregma and 4.5mm caudal, 1mm lateral to bregma, respectively). Ground and reference electrodes were placed over left and right frontal sinuses respectively (10mm rostral, 1mm lateral to bregma). SEPs were evoked by noxious electrical tail stimulation, and amplitudes of a positive to negative waveform in the 10-30ms range were assessed before and after chemical stimulation of VL or DL-PAG using DL-homocysteic acid (DLH; 150nl; 50mM in physiological saline saturated with pontamine sky blue dye to mark injection sites).

DL-PAG stimulation significantly increased waveform amplitude recorded from both S1 and Vx (to peaks of 118.6±7.5% and 115.1±6.1% of baseline respectively, mean±S.E.M., both p<0.05, ANOVA + Tukey’s post-test, n=14, 8) with no significant difference between ipsilateral and contralateral recording electrodes (p>0.05, t-test, n=7, 4 for S1 and Vx respectively). Conversely VL-PAG stimulation did not significantly alter the waveform amplitude recorded from S1 (p>0.05, ANOVA + Tukey’s post-test, n=11), but significantly decreased the waveform amplitude recorded from Vx (75.0±6.3% of baseline, mean±S.E.M., p<0.01, ANOVA + Tukey’s post-test, n=6). These data show that stimulation of VL- and DL-PAG, to mimic activation occurring in response to environmental stressors,
differentially modulate cortical responses to acute noxious stimuli, which may in turn affect the behavioural response.


Funded by an MRC-GSK Case Studentship

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Oxytocin provokes increase of free intracellular Ca2+ levels in freshly isolated rat sensory neurons

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Although the cellular mechanisms remain unclear, available evidence indicates that beside its essential role in mammalian parturition and lactation the nonapeptide hormone oxytocin (OXT) plays an important role in nociceptive modulation (1). Evidence has accumulated that free intracellular calcium ([Ca2+]i) plays an important role in signal transduction to control a wide variety of cellular mechanisms including nociceptive transmission. Previous studies have also shown that OXT receptor is expressed in dorsal root ganglia (DRG) and in spinal dorsal horn. Hence, the present study was undertaken to investigate the possible effects of OXT on antinociception in freshly isolated rat dorsal root ganglion (DRG) neurons.

Isolated DRG neurons were plated on coated cover slips following mechanical isolation and responses to OXT was studied by monitoring changes in [Ca2+]i with a microscopic digital image analysis system in fura-2 loaded single neurons. Data were analyzed by using unpaired t test, with a 2-tailed P level of <0.05 defining statistical significance. The majority of the DRG neurons (small, medium and large) responded by increase in [Ca2+]i to extracellular application of OXT concentration-dependently. The mean 340/380 nm ratio was 0.80±0.04 (baseline, n=7), 0.92±0.06 (30nM OXT, P<0.05, n=7) and 0.81±0.04 (baseline, n=27), 1.29±0.07 (100nM OXT, P<0.001, n=27), respectively. The stimulatory effect of OXT (100nM) was persistent in Ca2+-free conditions (Baseline: 0.90±0.07 vs. 100nM OXT: 1.06±0.06, n=5).

We demonstrated first time that OXT evoked a robust increase of [Ca2+]i in freshly isolated rat DRG neurons. Although the physiological significance of the results obtained from this study remains to be elucidated, in agreement with previous studies our results indicates that OXT could modulate the somatosensory transmission (nociceptive or non-nociceptive) via inhibiting ATP receptor function (2) and/or enhancement of GABA effect currents in cultured rat sensory neurons.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Spinorphin Inhibits Membrane Depolarisation-Induced Intracellular Calcium Signals in Cultured Rat Dorsal Root Ganglion Neurons

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Spinorphin, an endogenous peptide with antagonist actions on enkephalin-degrading enzymes and P2X3 receptors, presents potential antinociceptive effects. It is known that the primary afferent sensory neurons are functionally heterogeneous and small sized subpopulation of cultured DRG neurons may serve as a cellular model for studying the peripheral nociception. The aim of the present study was to determine the effects of spinorphin on Ca2+ transients, evoked by high-K+ (30 mM), and whether there were differences in spinorphin effect among subpopulation of cultured rat dorsal root ganglion (DRG) neurons. Following enzymatic digestion and mechanical agitation the DRG neurons were cultured on coated coverslips and loaded with 5 µM Fura-2 AM. Standard fura-2 ratiometric technique was utilised for quantifying [Ca2+]i responses in individual DRG neurons using fluorescence imaging system consisting of CCD camera coupled to an inverted microscope with a 40x (1.30 NA) objective. All data were analyzed by using unpaired t test, P <.05 defining statistical significance. Spinorphin, inhibited the Ca2+ transients evoked with 30 mM K+ in concentration dependant manner in a subpopulation of sensory neurons. Spinorphin dose dependently inhibited the Hik+-induced [Ca2+]i responses (1.40±0.09 vs. 1.42±0.08, n=15, NS for 10 uM;1.45±0.09 vs. 0.91±0.08 for 100 uM spinorphin n=16, P<0.05; and 1.39±0.09 vs. 0.84±0.08 for 300 uM spinorphin n=20, P<0.05, respectively) only in small-diameter DRG neurones. Additionally, after application of spinorphin a significant percent of small-diameter nociceptive DRG neurones did not respond to stimulation by Hik+ (response rate after application of 10, 100 and 300 uM spinorphin: 95%, 55%, and 54%, respectively) while the percentage of the response was not significantly changed in large-diameter non-nociceptive DRG neurones. Results from this study indicates that spinorphin significantly inhibits calcium signalling, transient changes in free intracellular Ca2+ con-
Validity of student peer assessment

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The assessment of students by their peers has been posited as a useful means of class evaluation, giving students an insight into the marking process, in addition to the mark received. This is often appropriate in assessing group work in oral and poster presentation, and is particularly valuable if both product and process are assessed (Race et al., 2005). Van den Berg et al. (2006) point out that it is also useful in that it allows the students to work with colleagues in a way that they will do during their professional career. Wheater et al. (2005) demonstrated that results from peer assessment are reproducible and comparable with the equivalent staff results, helping to allay fears that students will ‘over-mark’ or attribute marks based on their personal feelings towards other students. The aim of this study was to compare the marks given by staff and students for the same pieces of work at this institution to see how similar they were.

The pieces of work involved were poster presentations in a second-year Physiology/Pharmacology module, and oral presentations given as part of a third-year Neuroscience Module. Results are expressed as mean ± standard error, and an unpaired t-test was used to compare data. Results were accepted as being statistically significant at the 95% level.

Staff marks are significantly higher than those given by students in both Neuroscience oral presentations, (n=50, 73.6±0.7 Vs 68.9±0.4), and Physiology/Pharmacology poster presentations, (n=32, 70±0.6 Vs 67.4±0.6). The results clearly indicate that advanced students tend to ‘under’ mark each other’s work in comparison to academic staff. Fears that students may be excessively lenient have proven unfounded. Similar trends have been shown in at least two other studies, both Heywood (2000) and Stefani (1994) found peer grading to be somewhat lower than staff grading. This merits further investigation to discover why this is the case, to enhance the learning process and facilitate any adjustments required in the guidance on attributing marks.


Poster Communications


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The Virtual Microscope in Histology Teaching: Evaluation of Effectiveness and Student Preference


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At the University of Bristol histology is taught alongside physiology and has traditionally been taught in practical classes using light microscopes (LM) and glass microscope slides of tissue specimens. As part of the ‘The Applied and Integrated Medical Sciences Centre for Excellence in Teaching and Learning’ (AIMS CETL) we have developed a virtual microscope (VM) to deliver digital scans of our collection of histological specimens via the internet. Users are able to navigate around the virtual slides at a range of magnifications on networked computers using a software application ‘Digital Slidebox’ (Slidepath, Dublin). The VM was introduced for histology teaching within the department from 2006.

Our previous study (MacMillan et al. 2008) on first year veterinary science students, with previous experience of the LM and VM, demonstrated that learning outcomes are not hindered by the use of the VM over the LM and may well be enhanced. In the current study we investigated the effectiveness of the LM vs VM in a group of first year BSc student volunteers with limited previous experience of LM and no experience of using the VM.

Student volunteers (n=27) were given a prior knowledge test based on identification of structures in two histological images taken from the virtual microscope (spinal cord and lung tissue). The students were then divided into two groups (n=13 and 14), with both groups undertaking self-guided tutorials on the spinal cord and lung however one group used the LM for the spinal cord tutorial and the VM for the lung tutorial and the second group did the reverse. On completion of the tutorials the students re-sat the prior knowledge tests and completed an opinion questionnaire.

The students’ test scores improved following the learning sessions and the improvement in scores was greater when the students had used the VM for the tutorial rather than the LM. The scores were tested for statistical significance using a one way analysis of variance and Tukey’s post comparison test. There was a significant improvement in the scores in the group doing the lung tutorial on the LM (p<0.05) and to a greater degree of confidence in the group doing the lung tutorial using the VM (P<0.01). There was also an increase in student scores following the tutorial on the spinal cord, the increase was significant for the group using the VM (P<0.01).

The questionnaire results show that the students considered the VM to greatly improve the opportunity for group learning and that it was more useful as a learning tool than the LM. In summary, the improvement in test scores following the tutorials provides evidence that the VM is a more effective learning tool than the LM. Questionnaire data demonstrates that the
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Final Year Undergraduate Teaching Projects Delivered via a Mobile Teaching Unit

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Final year projects that enable students to teach physiology to school age pupils develop communication skills that are of widespread value, especially for careers in education, medicine and communicating science to the general public.

Since 2007, we have offered final year projects that allow students to develop an A-level teaching session that takes place inside a Mobile Teaching Unit (MTU), a custom-built HGV lorry that expands into a classroom. The benefits of this arrangement are that teaching can be delivered in a self-contained unit that does not require resources from the school and allows university-level physiological recording equipment to be easily transported and set up on the school site.

Project students work in pairs and choose a physiological topic of interest that is relevant to their studies and maps onto the school curriculum. They then design a 1-hr teaching session using a combination of slides, videos, audioclips, demonstrations and practical activities undertaken by the school pupils.

Each project culminates in the MTU visiting a local school, where the project students deliver the teaching session to different pupil groups throughout the school day.

Each project must include experimental elements. These include collecting biometric data from the pupils, which the project students subsequently analyse and set within the context of relevant scientific literature. The undergraduates are also required to devise ways of quantitatively evaluating the teaching session in terms of pupils’ learning and enjoyment.

Two teaching sessions, one each on the respiratory and nervous systems, have been developed and evaluated to date. School pupils were supervised by the undergraduates in generating data to investigate respectively the correlation between height and forced vital capacity (FVC) (Ljustina-Pribic et al. 2001) and between age and reaction times (Wilkinson & Allison, 1989) in 16-18 year olds. Subsequent analysis revealed a positive correlation between height and FVC (r=0.755; p<0.01; n=46; Pearson’s correlation) and a negative correlation between age and auditory reaction times (r= -0.443; p<0.001; n=66; Spearman’s rank correlation). Additional reaction time data presented as mean ± SEM demonstrated that auditory reaction times were faster than visual reaction times by 0.04 ± 0.007 s (n=66) p<0.001.

Feedback collected from pupils and teachers demonstrates a high level of engagement in the MTU teaching sessions, with 91% of pupils stating that they enjoyed the sessions (n=111). School pupils also benefit academically, with 94% improving their scores in a physiology test delivered before and after the teaching session (n=119). The undergraduate students enjoy the challenge of designing, delivering and evaluating rigorous, ‘stand-alone’ teaching material in the MTU, and devising an appropriate related biometric research project.


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Validating the Human Patient Simulator (HPS) as an educational tool: A comparison of the responses to intravenous administration of adrenoceptor agonists with human data

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The Human Patient Simulator (HPS) (HPS 337; METI, Sarasota, Florida) is a high fidelity mannequin controlled by computer software. We have previously shown how the HPS can be used to demonstrate sympathetic and parasympathetic control of the cardiovascular system. (Maskell et al. 2008). The aim of this study was to compare the response of the HPS to the simulated intravenous administration of the adrenoceptor agonists, noradrenaline, adrenaline and isoprenaline with published human data (Allwood et. al. 1963) in order to validate the HPS as an educational tool for teaching cardiovascular physiology.

Baseline measurements were made of heart rate (HR), systolic (SBP), diastolic (DBP), mean arterial (MAP) blood pressure and peripheral vascular resistance (PVR). The responses were determined to the simulated intravenous administration of each of the three adrenoceptor agonists. They were infused at doses of 10mg/min for 15 minutes. Measurements of the variables were made at intervals of five seconds.

The infusion of noradrenaline resulted in appropriate increases in SBP, DBP, MAP and PVR but did not produce an appropriate decrease in HR.

The infusion of adrenaline resulted in appropriate changes in HR, SBP, MAP and PVR but did not produce an appropriate decrease in DBP.

The infusion of isoprenaline resulted in appropriate changes in HR and PVR but did not produce appropriate changes in SBP, DBP or MAP.

We conclude that the HPS is a potential tool for demonstrating responses to the adrenoceptor agonists noradrenaline, adrenaline and isoprenaline, but further work is required to improve the software pharmacodynamic conditions.
Validating the Human Patient Simulator (HPS) as an educational tool: determining the relationship between alveolar ventilation and the alveolar partial pressure of carbon dioxide

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The inversely proportional relationship between alveolar ventilation (VA) and the alveolar partial pressure of respiratory physiology and the practice of anaesthesia. PACO\textsubscript{2} = Vco\textsubscript{2} x K/VA where K is a constant and Vco\textsubscript{2} is the rate of production of carbon dioxide (equation 1). The Human Patient Simulator (HPS) was developed by anaesthetists for trainees to learn about the clinical practice of anaesthesia in a safe environment. The aim of this study was to determine how PACO\textsubscript{2} varies with alveolar ventilation in the HPS.

The manikin was intubated under complete neuromuscular blockade and connected to a mechanical ventilator (Siemens 900C Servo Ventilator) set to deliver 21\% Oxygen. The minute volume (VM) was adjusted by altering the respiratory rate and tidal volume and PACO\textsubscript{2} was recorded after 20 minutes. Physiological dead space was determined using the Bohr method and used to calculate alveolar ventilation (alveolar ventilation = [tidal volume – physiological dead space] x respiratory rate) (equation 2).

The relationship between alveolar ventilation and the PACO\textsubscript{2} in the HPS showed good correspondence with the theoretical relationship predicted by the alveolar ventilation equation (equation 1). The limitations of the study include the assumptions that the rate of carbon dioxide production remained constant throughout the experiment and that twenty minutes was sufficient to achieve a new steady state following a change in minute volume.

We conclude that the HPS is a valid educational tool for demonstrating the effects of hypoventilation and hyperventilation upon PACO\textsubscript{2} under conditions of external mechanical ventilation with 100\% neuromuscular blockade.

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Correlations between physical activity and academic performance amongst A-level students: An Undergraduate Ambassador Scheme research project

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The Undergraduate Ambassador Scheme (UAS) aims to improve achievement in science, technology, engineering and maths (STEM), increasing the proportion of school leavers studying STEM subjects in University. UAS students in our department also conduct a research project.

Obesity is a huge social and economic problem and is set to become the largest cause of ‘ill health’ ahead of malnutrition and infectious diseases in developed countries (Yusuf S, 2004). Contributory factors include relatively low food costs, increasingly sedentary leisure activities and, for school children, reduced emphasis on competitive ‘team’ sports in schools. The findings that physical fitness is correlated with academic achievement (Chomitz et al., 2009) and that taking time from academic activities to favour physical activity does not reduce achievement in science, technology, engineering and maths (STEM), increasing the proportion of school leavers studying STEM subjects in University. UAS students in our department also conduct a research project.

Participating A-level students were allocated numbers used in all data collection so UAS students remained blinded to the pupils’ identities. Using these numbers and an on-line questionnaire, data was gathered on average hours of sleep, history of recent and regular exercise, extracurricular activity and alcohol consumption as well as the student’s subjective assessment of their academic ability/confidence, personal motivation and contentment with body image. In a separate laboratory session, blood pressure, heart rate, body mass index, forced vital capacity and forced expiratory volume in one second were measured. Our measure of cardiovascular fitness (fitness) was recovery of heart rate over a one minute period immediately after intense exercise. Pupils’ academic grades (expressed as a score) were provided by the school, with pupils identified only by their allocated number.

Fitness was positively correlated with volunteered weekly hours of physical activity. Grades and weekly hours of physical activity were positively correlated for males (Pearson; p<0.05; R=0.73, R2 0.53), but negatively correlated for females (Pearson; p<0.05; R -0.43, R2 0.19). Interestingly, there was a difference in response of males and females to the questionnaire statement, ‘I exercise to loose weight’, with the median response being 6 for females and 0 for males (10 = agree completely) (Mann Whitney U; p<0.001). The negative correlation for the questionnaire response of females that they are ‘on top of their school work’ and fitness (Pearson; p<0.05, R -0.45), suggests that for females exercise is linked to body image. These data do not support a universal view that physical exercise and fitness correlate positively with academic achievement. Yusuf S et al. (2004). Lancet 364: 937–52.

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more accurate representation of the human data. This produced an improved correspondence between the HPS and human O$_2$-CO$_2$ diagrams. Subsequent refinement allowed demonstration of equivalent responses for acclimatized humans as well as appropriate changes in Oxygen saturation, arterial pH and heart rate. We conclude that the HPS response to hypoxia can be refined to increase its validity as an educational tool to illustrate altitude physiology.


Rahn H & Otis AB (1949). Man’s respiratory response to during and after acclimatization to high altitude. Am J Physiol 157, 445


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Using fast cyclic voltammetry, 5-HT increases can be detected in the nucleus tractus solitarius (NTS) in response to vagal afferent stimulation in anesthetized rats

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The NTS is richly innervated by 5-HT terminals (Steinbusch, 1981) originating centrally (Schaffar et al. 1988) and from vagal afferents (Nosjean et al. 1990). Vagal afferent activation of NTS neurons has been shown to be mediated by 5-HT$_3$ and 5-HT$_7$ receptors (Ramage & Villalón, 2008). This indicates vagal afferents cause the release of 5-HT within this nucleus. Experiments were carried out to determine if this release could be detected in real time in anesthetized rats using fast cyclic voltammetry. Sprague-Dawley rats (250-330g) were anesthetized with iso-flurane (5% in 100% oxygen) and maintained with either sodium pentobarbitone (60 mg kg$^{-1}$, i.v.) or α-chloralose (120 mg kg$^{-1}$, i.v.), neuromuscular blocked (α-bungarotoxin 150 μg kg$^{-1}$, i.v.) and artificially ventilated with O$_2$-enriched air. Depth of anaesthesia was assessed by the stability of BP and HR following a noxious stimulus and additional anaesthetic was given when necessary. NTS was exposed by removing the occipital bone. The left vagus nerve was exposed and tied distally to the stimulating site. A carbon fibre electrode (tip dia. 7-10 μm) was then inserted into the medial NTS and lowered until evoked potentials could be detected from vagal stimulation (10-400 μA, 0.2-1.0 ms, 10x threshold). The sites were confirmed histologically. The system was then switched to voltammetry. A modified form of fast differential scan voltammetry (Millar & Williams 1990) using trapezoidal (flat-top) scans limited to a +450 mV positive potential for the electrochemical detection of 5-HT was used. This waveform is highly selective, detecting 5-HT at levels of <10 nM. For dopamine or noradrenaline and ascorbate the detection thresholds are >100 nM and >1 μM, respectively. All values are means ± S.E.M. Responses were measured as the height of oxidation peaks.

Under barbiturates anaesthesia vagal stimulation (20 Hz) evoked an increase by 6±1 nM (n=5), a decrease by 11±4 nM (n=5) and one biphasic effect change in 5-HT levels, while under chloralose increases were mainly observed of 23±5 nM (n=14). In some cases there was no response at 20Hz, however at 50Hz an increase of 22±8 nM (n=7) was observed, while in others release could be observed at 5 and 10Hz of 29±9 nM (n=3) and 28±6 nM (n=5). Citalopram (100 μg kg$^{-1}$; i.v.; 20Hz) tended to cause a decrease in release from 20±7 to 15±5 nM. Increasing the dose had no further effect. Cadmium applied topically to the NTS (10$^{-3}$ M in 100μl; n=1) completely abolished release after 1st stimulus in a train of three. In one experiment using chloralose a decrease in 5-HT was observed.

The data indicates that vagal afferent stimulation can produce increases in the level of extracellular 5-HT and this seems to be an all or nothing response.


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5-HT$_7$ receptors play a role in the mediation of afferent transmission within the NTS in anesthetized rats

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Central 5-HT pathways acting via 5-HT$_{1A}$, 5-HT$_3$ and 5-HT$_7$ receptors play a critical role in the regulation of cardiovascular reflexes (Ramage & Villalón, 2008). 5-HT$_{1A}$ receptors are involved in the reflex regulation of parasympathetic (vagal) control of the heart and data indicates that the predominant location of these receptors is within the nucleus ambiguus. In contrast, 5-HT$_3$ receptors are involved in afferent processing within the nucleus tractus solitarius (NTS) where their activation involves the release of glutamate, probably to some extent from glia. However, the location(s) of 5-HT$_7$ receptors within the cardiovascular reflex pathways (Kellett et al. 2005) is unknown. The present study was carried out to investigate the effects of the 5-HT$_7$ receptor non-selective agonist 5-carboxamidotryptamine (5-CT) and the selective antagonist SB 258719 (forbes et al. 1998) applied ionophoretically on the ongoing and vagal-evoked activity of NTS neurons.
Central brain nuclei and the development of sympathoexcitation after myocardial infarction

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The central nervous system regulates cardiovascular homeostasis through the actions of sympathetic nerves. Sympathetic nerve activity is elevated in cardiovascular disease including myocardial infarction (MI). Sympathetic nerve activity is the output from brain nuclei that receive inputs from a multitude of peripheral sites. How the brain nuclei are involved in facilitating the sympathoexcitation occurring after MI and its progression to heart failure is unclear. Functional studies indicate that within the paraventricular nucleus of the hypothalamus (PVN), nitric oxide (NO) is responsible for a sympathoinhibitory action on sympathetic nerves and alteration of this may be responsible for the sympathoexcitation manifest in heart failure (Li & Patel, 2003). This investigation sought to correlate PVN neurochemical change after MI to identify which neurones are involved in the sympathoexcitation.

The University of Auckland Animal Ethical Committee approved all experiments. Male Wistar rats were anaesthetised with isoflurane (4% in 2.5 l/min O2). A left intercostal thoracotomy was performed to expose the heart, the pericardium was removed and the left coronary artery ligated. In the sham group the chest was opened, pericardium removed with no ligation of the coronary artery and in control no intervention was performed. Animals recovered for 3 weeks, when they were re-anesthetised (pentobarbitol 60mg/kg) and an echocardiograph performed to assess cardiac function. Following this animals were humanely killed (pentobarbital, 60mg/kg), perfused-fixed (4% paraformaldehyde) with removal of brain and spinal cord. Frozen sections (40 μm) were incubated in goat anti c-Fos followed by biotinylated donkey anti goat IgG then strepavidin Alexa Fluor 594. Sections were incubated in rabbit anti nNOS then goat anti rabbit Cy2. The tissue was examined under epifluorescence.

Fractional shortening, (measure of left ventricular function) for MI was less than half that of control and sham animals (MI: 19.5±0.9% SE, n=3, Sham: 48.5±5.7% SE n=5, Control: 49.3±6% SE n=4). Heart weight for MI was larger than that for control and sham (MI: 1.8±0.1g SE n=3, Sham: 1.4±0.1g SE n=5, 1.3±0.1g n=4). For sham and control, Fos immunoreactive (FOS-IR) neurones were localised to the parvocellular regions of the PVN. For the MI group, FOS-IR neurones were concentrated in the dorsal cap, a region involved in regulation of renal sympathetic nerve activity (Deering & Coote, 2000). Neuronal nitric oxide expression within the PVN was consistent across the groups.

These preliminary findings suggest 3-weeks after MI, neurones in the dorsal cap of the PVN appear to be preferentially activated. This may represent a state where neurones in the dorsal cap drive sympathetic activity but are still under the inhibitory influence of NO.


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Sexual intercourse is one of the most natural events in life, yet anecdotal it has also been associated with an increased risk of arrhythmia development, myocardial infarction or stroke. It has been suggested that elevations in sympathetic nerve activity (SNA) may mediate this increased cardiovascular risk. Thus an understanding of sympathetic outflow during coitus is liable to be important in recommending the safety of sexual activity in subjects with underlying cardiovascular disease. We have utilised a new telemetry technology to enable recordings of renal SNA, blood pressure and heart rate in male (n=5) and female rabbits (n=5) before, during and after mating. All procedures were approved by the University of Auckland Animal Ethics Committee. Rabbits underwent surgery to implant telemetry devices under isoflurane anaesthesia, at least 7 days prior to the experiment. Renal SNA was normalised to glomic blockade and the nasopharyngeal reflex response (0-100 normalised units, n.u.). ANOVA statistical analysis was performed (p<0.05). Sexual activity was associated with transient (8-14s) but extreme increases in renal SNA in both male (9±1 n.u. to 189±32 n.u.) and female (8±1 n.u. to 134±18 n.u.) rabbits. This increase was significantly greater than that observed during physical exertion immediately prior to mating (Male 27±6 n.u., Female 47±18 n.u.). Mean arterial pressure and heart rate also increased significantly during physical exertion in male (88±4 mmHg vs. 110±12 mmHg; 236±11 bpm vs. 312±18 bpm) and female rabbits (89±5 mmHg vs. 107±8 mmHg; 247±9 bpm vs. 307±31 bpm), with further increases observed during mating (Male 134±9 mmHg, 422±21 bpm; Female 128±11 mmHg, 366±15 bpm). These results show sexual activity in healthy adult rabbits to be associated with profound transient increases in sympathetic drive to the kidneys, as well as significant increases on the workload of the heart as indicated by increases in heart rate and arterial pressure. Our study supports the hypothesis that sexual activity in subjects with underlying cardiovascular disease could carry an elevated degree of risk associated with the extreme increase in SNA.

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Super oxide anion mediated endothelial dysfunction and the fate of nitrous oxide isoenzymes in the penis of long-term diabetic rat

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Oxygen and nitrogen derived free radicals and oxidant plays important role in the pathogenesis of diabetic endothelial dysfunction. Present study was aimed to analyze the role of super oxide (O2.) on endothelial impairment and its correlation with constitutive nitrous oxide synthetase isoenzymes in the penis of the long-term diabetic rat. Wistar albino rats (Rattus norvegicus) were randomly divided into two groups i.e. control (received 0.1M of citrate buffer) and diabetes (received single dose of Streptozotocin at 60 mg/kg in 0.01M citrate buffer through i.p.). At the end of 120th day, animals were sacrificed by overdose of anesthesia (thiopentone sodium 40mg/kg b.wt.) and immediately penile tissue were dissected collected and used for the various analyses like protein and mRNA expression of iNOS, nNOS, eNOS and MnSOD. Small pieces of tissues were used for histological and immunohistological analyses (INOS, nNOS, eNOS and MnSOD). In-situ detection O2.- using dihydroethedine was performed on fresh tissue sections. Results showed significant increase in the production of superoxide and significant decrease in the levels of NOS isoenzymes (iNOS - p<0.001, eNOS - p<0.001 and nNOS – p<0.001) and Mn SOD (p<0.001) in the penis of the diabetic animal. Levels of mRNA expression of NOS isoenzymes (INOS – p<0.001, eNOS – p<0.01 and nNOS – p<0.001) and MnSOD (p<0.001) were significantly deceased in diabetic rat penis. Histological observations showed increase in endothelial thickening of the diabetic rat penis. These observations indicates that the elevated levels of O2.- mediates endothelial dysfunction and this increased levels of O2.- might be due to the impaired mitochondrial antioxidant defense system or/and alterations in the electron transport system. This might down-regulate the mRNA expression of NOS isoenzymes or up-regulate the degradation of these enzymes. Thus understand the mitochondrial pathophysiological source of O2.- and mRNA expression of these enzymes under hyperglycemic condition would give more insight into ensuing erectile dysfunction.

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Anatomical and molecular mapping of rabbit free running Purkinje fibres


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The cardiac conduction system (CCS) consists of the sinoatrial and atrioventricular nodes, the bundle of His, the bundle branches and the Purkinje fibres (PF). The PF form the terminal portion of the ventricular conduction system and provide a rapid conduction pathway. They have been shown to have spontaneous diastolic depolarization, a more negative plateau potential than ventricular muscle and a susceptibility to the production of early afterdepolarizations. The PF have been linked to a number of ventricular arrhythmias including torsade de pointes arrhythmias associated with long-QT syndrome. The whole-mount immunohistochemical method employing midneurofilament as a marker of the CCS was used to map the anatomical distribution of the bundle branches and the free running left and right Purkinje fibres (LPF and RPF) in the rabbit heart. The left bundle branch (LBB) appeared as a ribbon like structure composed of many fine fibres (perhaps only one myocyte wide) running in parallel on the septal surface. The fine fibres formed denser strands before branching into a network of fine fascicles that covered the left ventricular free wall. The right bundle branch appeared to be narrower than LBB and ran over the septal surface. It had a number of fascicles branching from it along its length. These strands extended across the ventricle and formed a dense network on the right ventricular free wall.

Quantitative RT-PCR was used to analyse the expression of various transcripts in eight rabbits. ~30 transcripts for ion channels, connexins, Ca2+ handling proteins and cellular markers were investigated in the LPF, RPF, right atrium (RA) and left ventricle (LV). The results show that the PF have an ion channel expression profile distinct from that of the working myocardium. In the LPF and/or RPF, there was a significantly higher expression (versus LV) of Nav1.1, HCN1, HCN4 and NFM mRNAs and a significantly lower expression of Cav1.2, KvLQT1, ERG, KChIP2, SUR2, Cx45, RYR2, SERCA2a and NCX1 mRNAs. There was also a tendency for a higher expression of Kir3.1, TWIK-1 and Cx40 mRNAs and a lower expression of Kv1.4, Kir2.1, Kir6.2, RYR3 and NCX1 mRNAs in the PF. The expression in the free running PF was also distinct from that in the RA: in the LPF and/or RPF there was a significantly higher expression of NFM mRNA and a significantly lower expression of HCN4, Kir3.1, KChIP2, Cx45 and ANP mRNAs. There was also a tendency for a higher expression of Nav1.1, Kv4.2, Kv4.3, TWIK-1, Kir2.1 and minK mRNAs and a lower expression of Navβ1, Kir6.2, SUR2, Cx40 and SERCA2a mRNAs in the PF.

We have demonstrated that the free running PF form a complex asymmetrical network in the left and right ventricular chambers of the rabbit heart. We have also shown that the free running PF have a unique pattern of expression of ion channels, connexins, Ca2+ -handling proteins and cellular markers.
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Thyroxine induced heart hypertrophy: impact on cardiac function

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Cardiac hypertrophy occurs when there is increased workload on the heart and is recognised as the first stage in the progression into heart failure. Recently, this laboratory reported that cardiac function parameters were elevated in a rat model of hypertrophy induced using isoprenaline and caffeine and by chronic thyroxine administration (Flanagan et al. 2008). The aim of this study was to determine how different exposure times to thyroxine impacted on basal cardiac function parameters and on the ability of the heart to respond to a β-adrenergic challenge.

Groups of male Wistar rats (250-270g) were given normal diet tap water to drink (n=9) or received daily i.p. injections of thyroxine (1mg/kg) for 7 (n=9) or 14 (n=9) days. On the day of study, anaesthesia was induced using 1ml ip chloralose/urethane (16.5/250 mg/ml) and cannulae were inserted into a femoral artery and vein to measure mean arterial pressure (MAP) and heart rate (HR) and to infuse saline (0.9g/100ml NaCl) at 3ml/h, respectively. A micro-tip pressure transducer catheter was introduced into the left ventricle via the right carotid artery (Flanagan et al 2008) to allow computation of cardiac index (CI) and dP/dtmax. Following 1-2 h recovery, basal measurements were taken over a 3 min period; thereafter, isoprenaline, 0.75mg and dP/dtmax were raised. By contrast, a stimulatory challenge with isoprenaline, which transiently decreased MAP, increased HR by 14% and CI by 15% respectively. Administration of isoprenaline i.v. in the control rats transiently decreased MAP, increased HR by 14% and CI by 15% (both P<0.05), but minimally changed dP/dtmax. In the group given thyroxine for 14 days, isoprenaline i.v. caused significant reductions (both P<0.05) in CI and dP/dtmax of some 9 and 15% respectively.

Together, these data demonstrated that as the exposure to thyroxine was prolonged, heart size was increased and baseline cardiac function in terms of CI and dP/dtmax was raised. By contrast, a stimulatory challenge with isoprenaline, which enhanced cardiac function in normal rats, was blunted following the two weeks of thyroxine treatment and these parameters were decreased. The findings would suggest that in cardiac hypertrophy that although basal heart function was enhanced, the ability to respond to physiological challenges was impaired.


This work was in part supported by a Vacations Studentship awarded to F Lainis by the Health Research Board.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC87

Hypoxic Upregulation of the BMP Antagonist Gremlin Blocks BMP Signalling in Pulmonary Endothelial Cells

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Pulmonary hypertension (PH) is a common complication of chronic hypoxic lung diseases. Recently we reported that gremlin, a BMP antagonist, was selectively upregulated in hypoxic human pulmonary microvascular endothelial cells in vitro. Given the important role of bone morphogenetic proteins (BMP) in normal pulmonary vascular homeostasis, we postulated that upregulation of gremlin was an important pathogenetic mechanism contributing to the development of hypoxic PH. To examine BMP signalling in an in vivo mouse model of hypoxic PH, mice (male SPF C57BL/6) were exposed to environmental hypoxia (FiO2 0.10) and killed under anesthesia (sodium pentobarbitone, i.p.) for RNA or protein analysis. We demonstrated selective upregulation of gremlin in the hypoxic lung by RT-PCR (n=8) and immunohistochemistry (n=5). The gremlin target proteins BMP-2, -4 and -7 were basally expressed in the mouse lung (n=8) but hypoxia only caused upregulation of BMP-2 mRNA. However, BMP-2 protein was down-regulated in hypoxia (n=6), together with reduced Smad1/5/8 phosphorylation (n=6) and reduced expression of the BMP target gene, Id1 (n=8). Recombinant gremlin blocked BMP-2-stimulated wound healing in cultured human microvascular endothelial cells and also blocked BMP-2-induced Smad1/5/8 phosphorylation and Id1 expression in these cells. Conditioned medium from hypoxic endothelial cells also blocked BMP signalling and BMP stimulated endothelial wound healing. Finally, gremlin protein was upregulated in the pulmonary vascular endothelium of patients with IPAH (n=4) when compared with controls (n=4) as demonstrated by immunohistochemistry on lung sections. These findings demonstrate lung-selective upregulation of gremlin in pulmonary hypertension and suggest an autocrine role for gremlin in modifying BMP-induced endothelial responses. Gremlin may represent a previously unrecognized mechanism that plays an important role in the development of PH.

Health Research Board and UCD Ad Astra Scholarship

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Upregulation of ACh release in mouse synapses after activation of L-type Ca\textsuperscript{2+} channels is due to release of stored calcium

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It is well known that ACh secretion in mature mammalian motor synapses is triggered by Ca\textsuperscript{2+} influx into nerve terminals mainly through voltage-dependent Ca\textsuperscript{2+} channels of the P/Q-type [1]. Nerve terminals possess also Ca\textsuperscript{2+} channels of L-type - normally “silent”, but when activated, they provide additional Ca\textsuperscript{2+} influx which leads to increase of quantal content of single evoked end-plate potentials [2,3]. In many excitatory cells, Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels activates ryanodine receptors (RyRs) of calcium stores, thus elevating intracellular concentration of calcium. So, the aim of this study was to check the possible functional coupling between activation of L-type Ca\textsuperscript{2+} channels, subsequent release of stored calcium through RyRs and evoked mediator secretion. The cut in vitro neuromuscular preparation of mouse left hemidiaphragm and intracellular microelectrode recordings were used in order to analyse rhythmically (50Hz) evoked endplate potentials (EPPs) and spontaneous miniature endplate potentials. Statistical analysis was performed using a Mann-Whitney test; all data are presented as mean±S.E.M. 

Effects of tumour necrosis factor-a and glutamate pretreatment on glutamate-induced calcium influx in rat organotypic hippocampal cultures

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Glutamate-induced excitotoxicity contributes to neuronal damage during a cerebral ischaemic event such as stroke. Physiological levels of glutamate and proinflammatory cytokines such as tumour necrosis factor-\alpha (TNF-\alpha) play a role in the regulation of synaptic plasticity within the hippocampus. During a stroke pathophysiological levels may cause dysregulation of these processes, enhancing vulnerability of these cells to an ischaemic insult. Previous studies suggest that a mild transient ischaemic attack (TIA) within 72 h of a stroke may result in attenuation of its clinical severity (Castillo et al. (2003)). We have developed an in vitro model of TIA using organotypic hippocampal cultures (modified technique of Stoppini et al. (1991)). Hippocampal slices (400 \mu m) were cultured from male Wistar rats at post-natal day 7 (humanely killed - decapitated). At 6 days in vitro (DIV) cultures were pretreated for 30 min with 30 \mu M glutamate or 5 ng/ml TNF-\alpha. They were then placed in fresh media for 24 h (recovery period). As Ca\textsuperscript{2+} is a well-established mediator of glutamate-induced excitotoxicity, we investigated whether glutamate/TNF-\alpha preconditioning altered glutamate-induced Ca\textsuperscript{2+} influx. At 7 DIV pretreated cultures were loaded with a Ca\textsuperscript{2+} indicator dye, Fluo-4 (3 \mu M) and imaged with Zeiss laser scanning confocal microscope. Relative changes in fluorescence of Ca\textsuperscript{2+}-bound dye correlates with relative changes in [Ca\textsuperscript{2+}]i. After 20 s of baseline recording, 30 \mu M glutamate was applied and the Ca\textsuperscript{2+} response was recorded for 70 s. Data analysis was carried out with Zeiss Image Examiner software and statistics were compiled using one-way ANOVA followed by Bonferroni post-test. Results are expressed as mean±S.E.M. Pretreatment with 5 ng/ml TNF-\alpha/30 \mu M glutamate resulted in a reduction in glutamate-induced Ca\textsuperscript{2+} influx after 24 h (TNF-\alpha; 1293±28, n=3340, glutamate; 1088±23, n=2439, Vs. control; 2679±48, n=3524, p<0.001). Changes in baseline Ca\textsuperscript{2+} levels due to pretreatments were analysed using a ratiometric Ca\textsuperscript{2+} dye, Indo-1 (5 \mu M). Preliminary data suggest that TNF-\alpha/glutamate pretreatment significantly lowered baseline [Ca\textsuperscript{2+}]i compared to control. Thus, an acute mild TNF-\alpha/glutamate exposure may alter both basal [Ca\textsuperscript{2+}]i within the cell and its responsiveness to glutamate-induced Ca\textsuperscript{2+} influx. Pretreatment with 5 ng/ml TNF-\alpha in the presence of the mGluR5 metabotropic glutamate receptor antagonist, MPEP (10 \mu M), significantly enhanced TNF-\alpha’s preconditioning effect (1125±21, n=2298, p<0.05). The NMDA receptor antagonist, D-AP5 (100 \mu M) did not significantly alter the preconditioning effect of glutamate when co-applied (1072±27, n=1764, p>0.05). We conclude that TNF-\alpha’s preconditioning effects may be TNFR and
mGluR5 receptor mediated, whereas NMDAR activation during glutamate preconditioning may be less important.


Science Foundation Ireland.

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**PC90**

A novel method to measure the rate of glycolysis in single cells reveals fast tuning of astrocytic glycolysis by K+ but not by glutamate

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Neuronal activation demands metabolic energy that must be supplied within seconds. The study of the mechanisms catering for such demand has been hampered by lack of techniques capable of measuring metabolic flux with the required resolution. A Fluorescence Resonance Energy Transfer (FRET) - based microscopy method is presented here which offers much improved spatiotemporal resolution over existing methods like 2-deoxyglucose autoradiography and NMR spectroscopy. Applied to mouse astrocytes in culture and in brain slices, the method showed that glycolysis can be activated within seconds by physiological concentrations of extracellular K+. No activation was observed in response to glutamate, widely regarded as responsible for neuronal modulation of astrocytic metabolism. The effect of K+ on glucose metabolism was readily reversible, required Na+/K+ ATPase pump activation and was mediated by depolarization-induced alkalization (DIA), a phenomenon of hitherto unknown physiological significance. A rapid activation of astrocytic glycolysis was also observed in brain slices exposed to exogenous K+ and during electrical stimulation. These results expose a novel phenomenon linking synaptic activity to timely local production of lactate by astrocytes and ascribe astrocytic pH a central role in neurometabolic coupling and neurovascular coupling. This technique to measure glycolysis in single cells, in real time and reversibly, may also be used to investigate the transport and metabolism of glucose in other cell types.


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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC91**

Effects of Chronic and Acute Simvastatin Treatment on Synaptic Transmission in Hippocampal Slices from C57Black 6j Mice

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Statins are agents commonly used in the clinic to treat hypercholesterolemia, however little is known regarding their effects on neuronal function. Chronic treatment with simvastatin was shown to improve learning and memory in behavioural tasks (Li et al., 2006). The aim of this research was to investigate acute and chronic effects of simvastatin on synaptic transmission, paired pulse facilitation (PPF) and long-term potentiation (LTP) in the CA1 region of the hippocampus. Transverse hippocampal slices (400µm thick) were prepared from C57 black6j mice. EPSPs were recorded in the CA1 region. Paired pulse stimuli were applied at an interval of 50ms. Fibre volleys were evoked in the presence of DNQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione) by stimulating the alveus and recording in the CA1 cell body region. Baseline stimuli were applied at a frequency of 0.033 Hz, (0.1ms) and recordings made for 20min prior to application of simvastatin or LTP induction. LTP was induced using 100 or 200 Hz. 100 Hz protocol: Two stimulus trains at 100Hz for one second applied 30s apart. 200 Hz protocol: Two sets of ten trains of ten stimuli at 200Hz, 2s inter-train interval; 30s between sets. Control LTP (100Hz) measured at 60min in slices from 8week old animals (young) measured 169.0 ± 11.2% (n=13), while that induced using 200Hz measured 166.9 ± 6.5% (n=10). Acute application of simvastatin (35µM) caused a significant increase in the EPSP slope to 154.4 ± 12.3%, (p<0.05, n=12) measured at 35 min compared to vehicle (105.3 ± 3.6% n=4) whereas 10µM simvastatin did not increase the EPSP slope (118 ± 6% n=4) significantly. Simvastatin also decreased the PPF ratio (1.47 ± 0.06 to 1.30 ± 0.06; p<0.01, n=12) at 35 min. LTP (100 or 200Hz) measured from baseline prior to induction in the presence of simvastatin (35µM) was decreased significantly compared to control (133.2 ± 4.8% n=8 and 132.9 ± 10.9% respectively n=6). Following chronic simvastatin treatment (0.04%, in the diet for 6 months), LTP (100Hz) induced in slices from 18 month old animals measured 158.3 ± 6.9% (n=7) and was not significantly different to levels recorded in slices from 8 or 16 month old animals (177.8 ± 9.0%, n=7 and 175.2 ± 16%, n=8 respectively). In slices from young animals treated acutely with simvastatin (35µM), fibre volley amplitude increased to 137.3 ± 5.7% (n=9, p< 0.001) compared to vehicle control 105.3 ± 7.3% (n=4). Fibre volley amplitude recorded in slices from chronically treated animals increased.
Evidence that NK cells are present in the rat brain and have an age-related association with microglial activation

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Ageing is the most significant contributory factor in the pathogenesis of neurodegenerative diseases such as Alzheimer’s disease, and inflammatory changes in the brain contribute to the deficits associated with aging and neurodegenerative disorders. Fundamental to these changes is an increase in microglial activation, the immune cells of the brain, which are the primary source of pro-inflammatory cytokines. Microglial activation has been shown to be induced by IFN-γ (1), a macrophage-activating factor, but the cell source of IFN-γ in the central nervous system is not known. IFN-γ is produced by Natural Killer (NK) cells, which are traditionally found in the peripheral nervous system, and the finding that IFN-γ concentration is increased in the brain of aged rats suggests a possible infiltration of NK cells into the CNS.

In this study young (2-3 month old), middle-aged (14-15 month old) and aged (18-25 month old) male Wistar rats (n=6 per group) were anaesthetised (i.p.) with urethane, and intracardially perfused with saline. Dissociated cells were prepared from one half of the brain and assessed, using flow cytometry, for the presence of NK cells. Cortical and hippocampal tissue taken from the other half of the brain was assessed for IFN-γ mRNA, and expression of markers of microglial activation, MHCII, CD11b, TLR2 and TLR4.

The data demonstrate that NK cells are present in the rat brain and that the number of cells positive for NK cell markers, CD161a and NKp30, was significantly greater in brain tissue prepared from aged, compared with middle aged, rats (p<0.05; Student’s t-test). This finding was associated with a significant age-related increase in cortical and hippocampal mRNA expression of MHC II (p<0.05; 1-way ANOVA) and CD11b (p<0.001; Student’s t-test). These results were also coupled with age-related increases in hippocampal IFN-γ, TLR2 and TLR4 mRNA (p<0.05; Student’s t-test).

These findings provide the first evidence for the presence of NK cells in the aged rat brain, and suggest a possible role for NK cells in the activation of microglia during age-related neuroinflammation.

1. Butovsky, O., Bukshpan, S., Kunis, G., Jung, S., Schwartz, M. Microglia can be induced by IFN-gamma or IL-4 to express neural or dendritic-like markers. Mol. Cell. Neurosci., 2007; May 1: 17760122.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
channels allows us to suggest that activation of Ca\textsuperscript{2+} channels leads to slow and local elevation of Ca\textsuperscript{2+} concentration in nerve terminals. In contrary, similar efficacy of both Ca\textsuperscript{2+} buffers in preventing caffeine effects gives evidence that activation of ryanodine receptors is associated with tonic increase of intracellular Ca\textsuperscript{2+} level which influences high sensitive Ca\textsuperscript{2+} sensors, controlling mediator secretion.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC93A**

**In vitro evaluation of magnetic-fluorescent nanocomposites in mouse mixed glial culture**

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Magnetic-fluorescent nanocomposites enable the capabilities of both magnetic resonance imaging (MRI) and fluorescence-based technologies, such as confocal microscopy, to be exploited in single experiments\textsuperscript{1}. Here we report the in vitro evaluation of a novel Rhodamine B functionalised polyelectrolyte stabilised magnetic-fluorescent nanocomposite. Mixed glial cultures from neonatal mouse cortices were prepared, following decapitation in accordance with local ethical guidelines. Following mixed glia incubation in the presence of nanocomposite (5 \( \mu \text{M} \)) for 2 hours, nanocomposite uptake was assessed using confocal fluorescence microscopy and light microscopy. Live cell imaging data was acquired to investigate the method of nanocomposite internalization. Cell viability was measured in a mixed glia sample using the MTT assay, following incubation with nanocomposites (1, 5, 10, 20 \( \mu \text{M} \)) for 2, 18, 24, and 48 hours. The concentration of pro-inflammatory cytokines, IL-1\( \beta \) and IL-6, of mixed glia following a 2 hour incubation in the presence of the nanocomposites (1, 5, 10, 20 \( \mu \text{M} \)) was assessed using ELISA. MRI phantoms containing varying numbers of labelled cells in 0.5% Agarose were prepared. T2 and T2\textsuperscript{*} relaxation times of these phantoms were presented by mean and standard error. The student t test was used for statistical analysis and statistical difference was considered when \( p < 0.05 \).

Confocal and light microscopy confirmed the internalization of the nanocomposites. Live cell imaging data suggests both phagocytosis and endocytosis as methods of nanocomposite internalization. No reduction was seen in cell viability. There was no increase in the concentrations of IL-1\( \beta \) or IL-6 following a 2 hour incubation. The contribution to MR contrast was calculated using \( \gamma B \times (1/T2^*) – (1/T2) \) and was linear (Pearson Correlation, \( R = 0.9967, p<0.001 \)). TEM and confocal microscopy demonstrated the tendency of the nanocomposites to form linear chain-like assemblies following placement in a magnetic field.


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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC93B**

**Platelet L-Arginine-Nitric Oxide Pathway in Bipolar Disorder**

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The bipolar disorder (BD) is a humor disorder, according to DSM-IV (Diagnostic and Statistic Manual of Mental Disease). This disorder affects approximately 1.5% of the population and it is characterized by the presence of mania and depression episodes. Similarly to other psychiatric disorders, such as depression, anxiety and schizophrenia, the BD is an important cardiovascular risk factor. However, the exact mechanisms underlying this relationship remain unknown. Recent studies suggest the involvement of L-arginine-nitric oxide pathway on the pathophysiology of BD.

Nitric oxide (NO) is a lipophylic gas which is produced by different blood cells. L-arginine, it’s precursor, is transported to platelets by \( y^L \) carrier, and activates the enzyme NO synthase (NOS), which produces NO and L-citrulline. NO has many physiological functions, including: vasodilatation, neurotransmission and platelet aggregation inhibition. Despite intracellular concentration of L-arginine is above the Km of NOS, the extracellular transport of L-arginine is necessary for NO production. The aim of this study was to investigate L-arginine transport and NOS activity in platelet of bipolar patients.

Nine patients with BD and nine healthy volunteers were included in this study. Extracellular L-arginine transport into platelet was measured by kinetic methods, using crescent concentrations of \([^3\text{H}]\) L-arginine; and NOS activity was evaluated by the conversion of \([^3\text{H}]\) L-arginine on \([^3\text{H}]\) L-citrulline. Data were presented by mean and standard error. The student t test was used for statistical analysis and statistic difference was considered when \( p < 0.05 \).

Total L-arginine transport (pmol L-arginine/10\textsuperscript{6} cells/min) was similar in bipolar patients (\( V_{\text{max}} = 95 \pm 31 \)) and healthy controls (\( V_{\text{max}} = 86 \pm 19 \)). L-arginine transport via system \( y^L \) did not differ between bipolar patients (\( V_{\text{max}} y^L = 83 \pm 59 \)), compared with healthy controls (\( V_{\text{max}} y^L = 43 \pm 10 \)). NOS activity (pmol L-citrulline/10\textsuperscript{6} cells) was reduced on bipolar patients (0.059\( \pm \)0.018) compared with controls (1.138\( \pm \)0.027) (\( p = 0.04292 \)). These results suggest that NO production by NOS is reduced in bipolar patients. This lower production of NO can...
be contributing for the higher cardiovascular risk factor seen in patients with BD. Our next step is to determine platelet function, to see if platelet aggregation is increased in these patients. This would increase the incidence of thrombotic events in bipolar patients.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC93C

Control of spike timing by GABA(A) receptor-mediated inhibitory synaptic input during theta frequency oscillation in rat CA1 pyramidal neurons

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Extensive experimental evidence suggests that carefully controlled spike times relative to theta-frequency network oscillations play an important role in hippocampal memory processing. Although excitatory phase response properties have been well characterized, how inhibitory inputs could control spike timing is less well studied. Here we report the control of spike timing during theta oscillation by inhibitory input and characterize the spike timing characteristics in spike time response curves (STRC). Using whole-cell patch-clamp recordings from CA1 pyramidal cells in vitro and in vivo, we show that GABA(A) receptor-mediated IPSPs can not only delay but also advance the postsynaptic spike time depending on the timing of the inhibitory input relative to the oscillation. The maximum spike time delay was 23.2 ± 2.9 ms and the maximum spike time advancement was -4.1 ± 1.4 ms (n = 5). Dynamic clamp-simulated artificial IPSP mimicking GABergic input to the soma could also both delay and advance the spike time. The maximum spike time delay was 32.0 ± 2.1 ms and the maximum spike time advancement was -7.7 ± 0.8 ms (n = 8). The intrinsic mechanism underlying spike time advancement with IPSP is due to h-channels since the application of 10 μM ZD7288, an Ih channel blocker, completely abolished such advancement (n = 9). These results suggest that spike timing during theta-frequency oscillation can be bidirectionally controlled by inhibitory synaptic inputs and that the spike time advancement caused by IPSPs is due to h-channels intrinsic to the CA1 pyramidal neuronal membrane.

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PC94

Modulation of pancreatic alpha-cell function by leptin involves effects on electrical activity, calcium signalling and exocytosis

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Leptin inhibits insulin secretion by direct effects on the pancreatic beta-cell (Kieffer et al. 1997), and thus it can modulate glucose homeostasis. Although alpha-cells and glucagon secretion also play a critical function in the control of glycaemia, the effect of leptin on these islet cells has not been examined. In the present work, we have studied whether alpha-cells are directly regulated by this hormone. The existence of several leptin receptor isoforms (a-e subtypes) was observed by PCR in the glucagon-producing alpha-TC1-9 cell line (n=3). The b isofrom, the main one involved in leptin signalling, was demonstrated in alpha-TC1-9 cells by western blot (n=3) as well as in alpha-cells from OF1 mice and human by immunocytochemistry (n=3). The functional role of leptin was first analyzed by patch-clamp in both alpha-TC1-9 cells (n=8) and mouse alpha-cells (n=3). At 0.5 mM glucose, these cells exhibit electrical activity characterized by sodium and calcium action potentials (Gromada et al. 2004). In all the cells tested, leptin (6.25 nM) hyperpolarized the membrane potential, inhibiting the electrical activity induced by 0.5 mM glucose. Additionally, 6.25 nM leptin produced an inhibitory effect on the calcium signals in alpha-TC1-9 cells as well as in mouse and human alpha-cells (n=13, n=16, n=12, respectively). Similar effects on calcium signalling were observed at 0.625 nM leptin. Since alpha-cell exocytosis is calcium-dependent, we analyzed the effect of 6.25 nM leptin on glucagon secretion by radioimmunoassay. At this concentration, leptin reduced glucagon secretion at 0.5 mM glucose by almost 30% (n=8; p<0.05). However, in the presence of the PI3-kinase inhibitor wortmannin, leptin did not produce any effect, indicating that this pathway is involved. Our results indicate that leptin participates in glucose homeostasis not only via beta-cell actions but also through its inhibitory effect on alpha-cells.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Adaptation of CellProfiler software for high throughput measurement of cell size in phase contrast images

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Cell division and apoptosis are normal processes that are necessary for human health when kept in proper balance. Upsetting this balance can lead to a variety of diseases. Change in cell volume is a fundamental and probably essential feature of both cell division and apoptosis, so it is important to understand mechanisms of cell volume control. Particularly in the case of apoptosis, this mechanism is not well understood. Studying cell volume control requires an efficient method of measuring cell size in populations of cells. The method of choice is usually flow cytometry, which involves expensive equipment and high running costs that may tend to prohibit speculative pilot studies. We aimed to develop a cheap and easy method for measuring cell size that could generate pilot data to be followed up with more sophisticated methods. CellProfiler (1) is a software package that was designed for the automated analysis of fluorescence images, which feature high contrast between labelled cells and the background, enabling the software to identify cells in the image. On the other hand, phase contrast images tend not to have a high contrast between the cell interior and the background, but show an edge at the boundary of the cell. CellProfiler has an edge finder module that we have exploited in developing a pipeline that can automatically measure the sizes of cells in a large number of phase contrast images. The pipeline also incorporates upper and lower size cutoffs to rule out cell clusters and debris. Phase contrast images were taken of Jurkat cells in culture with a Nikon Diaphot inverted microscope fitted with a Nikon D-40 digital camera. Images were saved as jpg files for analysis by CellProfiler. The pipeline was validated against manual analysis using Scion Image software (Scion Corporation, Frederick, MD, USA). The jpg files were converted to tif files for Scion Image. In 5 images of well separated Jurkat cells in culture, manual analysis identified 236 cells with profile areas of (mean ± s.d.) 6608 ± 2466 pixels, while CellProfiler identified 185 cells with profile areas of 6190 ± 3175 pixels. Although CellProfiler missed some cells, this result indicates that it could be a useful tool for analysing changes in the size distribution of cell populations imaged with phase-contrast microscopy.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Table 1. ODC activity in spleen lymphocytes, μmol putrescine×mg⁻¹×min⁻¹

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* - P less than 0.05, n=6

Table 2. ODC activity in thymus lymphocytes, μmol putrescine×mg⁻¹×min⁻¹

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* - P less than 0.05, n=6


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Mesenchymal stem cells (MSCs) are multipotent progenitor cells which give rise to specialised skeletal cells, including chondrocytes, osteocytes and adipocytes. Under the appropriate conditions, these cells can be manipulated in vitro for the development of new tissue such as cartilage. Thus, MSCs pose as an ideal cell source for use in skeletal tissue engineering. When isolated from a donor source, the cells retain their multipotent potential in vitro and can differentiate along a specific lineage, depending on the local environment in which they reside. This study focused on elucidating the stimuli that enhance MSC differentiation along the chondrogenic route. Hypothesis signalling through hypoxia inducible factor-1α (HIF-1α) has been shown to promote chondrogenesis [1] and non-hypoxic stimulation of HIF-1α is currently under investigation. The endogenous cannabinoid system has recently been implicated in skeletal physiology [2]. Thus, the link between cannabinoids and chondrogenesis was examined in this study, including their possible role as non-hypoxic regulators of HIF-1α.

MSCs obtained from the femora and tibiae of 3 month old Wistar rats were cultured with chondrogenic growth factors (100nM dexamethasone, 100ng/ml, transforming growth factor-β, 50mM ascorbic acid-2-phosphate), Δ9-tetrahydrocannabinol (Δ9-THC, 5μM) or URB597 (1μM) for 3 weeks. URB597 inhibits the fatty acid amide hydrolase enzyme that degrades anandamide and functions in enhancing endocannabinoid tone. Cells were examined for proteoglycan deposition and expression of collagen II, as markers of chondrogenesis. Histological analysis of proteoglycans revealed a significant increase from 4.58±0.38 in control cells to 10.03±1.37 in cells treated with chondrogenic growth factors for 3 weeks (P<0.05, ANOVA, n=6). A similar increase was observed from 4.58±0.34 in control cells to 6.24±0.80 in Δ9-THC treated cells and to 6.70±0.38 in URB597 treated cells (P<0.05, ANOVA, n=6). Immunocytochemistry has shown that 3 week treatment with chondrogenic growth factors, Δ9-THC or URB597 induced an increase in collagen II immunoreactivity (n=6). The HIF-1α inhibitor, Vitexin (20μM), downregulated HIF-1α expression and attenuated the expression of collagen II, as assessed by immunocytochemistry (n=6). In conclusion, this study has demonstrated a regulation of MSC chondrogenesis by cannabinoids, under normoxic conditions via a pathway that involves, in part, HIF-1α. Optimisation of the in vitro conditions for chondrogenic differentiation of MSCs will have a significant impact in treating musculoskeletal defects in the ever advancing field of skeletal tissue engineering.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Calcium Handling in Superior Cervical Ganglia is enhanced in Prehypertensive Spontaneously Hypertensive rat

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Hypertension is associated with noradrenergic hyperactivity and oxidative stress(1). We hypothesized that Ca2+ signalling in postganglionic sympathetic neurons is enhanced in spontaneously hypertensive rats (SHR). To test this, we measured depolarisation ([K+]o) evoked Ca2+ influx in sympathetic neurons from hypertensive and normotensive Wistar-Kyoto (WKY) rats. Rats were humanely killed by an approved Home Office schedule 1 method. Superior cervical ganglia (SCG) were enzymatically isolated from age and gender matched SHR and WKY rats and plated onto poly-D-lysine/laminin coated 6 mm cover slips and cultured 2-3 days in N2 medium(2). Intracellular Ca2+ concentration ([Ca2+]i) was measured with ratio imaging using fura-2(3). Neurons were briefly perfused with Tyrode’s solution containing 100 mM KCl to induce membrane depolarization, and the resultant transient increase in [Ca2+]i was determined. In neonatal (4-7 days) rats, [Ca2+]i transients were significantly higher in the SHR (2.65 ± 0.11 μM, n=14) compared with the WKY rats (1.88 ± 0.12 μM, n=9; p<0.001, unpaired t-test). In young pre-hypertensive (4-5 weeks) rats, [Ca2+]i transients were higher in the SHR (male: 2.51 ± 0.34 μM, n=14; female: 1.98 ± 0.13 μM, n=23) compared with the gender matched WKY rats (male: 1.81 ± 0.30 μM, n=14, p<0.05; female: 1.66 ± 0.10 μM, n=24, p<0.07). In adult (16-18 weeks) rats the [Ca2+]i transients were also significantly increased in the SHR (male: 3.70 ± 0.21 μM, n=11; female: 2.93 ± 0.22 μM, n=13) compared with the gender matched adult WKY rats (male: 2.46 ± 0.17 μM, n=11, p<0.01, unpaired t-test; female: 1.83 ± 0.14μM, n=19, p<0.001, unpaired t-test). Interestingly, there were no gender differences in young SHR and WKY rats, whereas in adult SHR and WKY, [Ca2+]i transients were significantly higher in male than female (both p<0.05, unpaired t-test). These results demonstrate that increased Ca2+ transients were observed in postganglionic sympathetic neurons from newborn pre-hypertensive SHR when compared with the WKY. The overall responses are consistent with the hypothesis that enhanced Ca2+ signalling may contribute to the noradrenergic hyperactivity that precedes hypertension itself.


This work was supported by British Heart Foundation and Wellcome Trust.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC100

The role of nitric oxide in the diurnal variation in excitation-contraction coupling in ventricular myocytes

H.E. Collins and G. Rodrigo

Cardiovascular Sciences, University of Leicester, Leicester, UK

Over 10% of the rat cardiac genes exhibit diurnal variations, and cycling of metabolic genes link metabolic activity and oxygen consumption with peak power. We have demonstrated that key elements of E-C coupling in rat ventricular myocytes show strong diurnal rhythms, including their response to the non-specific β-adrenergic receptor agonist isoproterenol, which stimulates β1, 2 and 3-receptors. As β3-adrenergic receptor stimulation involves activation of nitric oxide (NO)-dependent pathways through coupling to nitric oxide synthase (NOS), we looked at the involvement of NOS in the diurnal variation of E-C coupling (Massion & Balligand, 2003; Seddon et al., 2007).

Adult male Wistar rats were housed with a 12 hour light/dark cycle. Hearts were excised from animals at two opposing time-points of ZT3 and ZT15, where ZT0 refers to “lights on”, and single left ventricular myocytes were isolated by enzymatic digestion. Measurements of [Ca2+]i, were made using Fura-2 and L-type Ca2+ channel density was determined electrophysiologically using the whole cell, patch clamp technique. We looked at the modulating effect of the non-specific NOS inhibitor L-NNA on systolic [Ca2+]i, and L-type Ca2+-current density at rest and during β-adrenergic stimulation with isoproterenol. L-NNA (500μM) had no significant effect on the basal systolic [Ca2+]i, and L-type Ca2+-current recorded from ZT3 or ZT15 myocytes in normal Tyrode. However, following stimulation with 5nM isoproterenol, L-NNA had a stimulatory effect on both systolic [Ca2+]i and L-type Ca2+-current density. This stimulation was greater in ZT15 compared to ZT3 myocytes, with an increase in systolic [Ca2+]i of 32.6 ± 7.5% (n=29) vs. 15.4 ± 2.6% (n=13) in ZT3 myocytes vs. 15.4 ± 2.6% (n=13) in ZT15 myocytes (S.E.M.; students t-test, P<0.05). However, we detected a significant 5-fold increase in nNOS mRNA levels in ZT15 over ZT3 myocytes (S.E.M; n=4, students t-test on ΔCt values, P<0.05). Our data shows that the diurnal variation in the sensitivity of E-C coupling of rat ventricular myocytes to stimulation with isoproterenol, and this appears to involve NOS.

H.E Collins has a Department of Cardiovascular Sciences studentship

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Mitochondrial Oxidative Responses To Increased Work Intensity In Rabbit Ventricular Myocytes Assessed By Intrinsic Fluorescence Methods

I.A. Ghouri, O.J. Kemi and G.L. Smith

Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK

Cardiomyocytes are intrinsically fluorescent and spectroscopic analysis of rabbit ventricular myocytes indicated that the majority of this fluorescence arises from the metabolic coenzymes nicotinamide adenine dinucleotide (NADH) in the reduced state and flavin adenine dinucleotide (FAD) in the oxidised state. The mitochondrial redox state of single cells was assessed using the mitochondrial inhibitors p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) and cyanide. Treatment with 2μM FCCP established a completely oxidised state, resulting in NADH fluorescence decreasing to a minimum and FAD fluorescence increasing to a maximum. Treatment with cyanide (2mM) established a completely reduced cellular state, generating maximal NADH and minimal FAD fluorescence. The mitochondrial redox state of single rod-shaped myocytes was examined with confocal microscopy. NADH was excited at 405nm and FAD at 488nm. From this, the average NADH redox state was calculated as 0.58±0.03 (n=22) and the average redox state of FAD was calculated as 0.17±0.01 (n=22). Similar experiments using 2-photon excitation fluorescence microscopy (exciting at 720 and 750nm) revealed a comparable value for NADH redox state of 0.57±0.04 (n=20), although FAD fluorescence could not be clearly detected with this method. Measurements of intrinsic fluorescence were then utilised in order to assess the mitochondrial redox response of cardiac cells to increased energy demand. Isolated cardiomyocytes were field stimulated and fractional shortening simultaneously recorded with epifluorescence measurements of NADH and FAD. Cells were paced at 0.5Hz and the stimulation frequency step increased to 1Hz, 2Hz and 3Hz in order to increase work intensity and energy demand. NADH was excited at 340nm and FAD was excited at 430nm. Step increasing stimulation frequency resulted in a decrease in NADH fluorescence and an increase in FAD fluorescence, indicating oxidation of the cell environment. The magnitude of response was dependent on stimulation frequency. The greatest response was obtained when pacing was increased from 0.5Hz to 3Hz, NADH fluorescence decreased by 11.16±1.42% and FAD increased by 11.74±1.34% (n=22). Reducing work intensity back to 0.5Hz pacing resulted in immediate recovery of metabolite fluorescence.

In conclusion, the majority of intrinsic fluorescence from isolated heart cells could be attributed to the metabolic coenzymes NADH and FAD. Metabolic inhibition enabled modulation of the oxidative status of these enzymes, allowing for calculation of relative redox state. Simultaneous measurements of redox status and fractional shortening in field-stimulated cells have demonstrated that rabbit ventricular myocytes become oxidised when work rates are increased, suggesting a transient mismatch between energy supply and demand.

Apolipoprotein E knockout (apoE−/−) mice fed a high-fat, Western-style diet for 6 months are grossly hypercholesterolaemic and develop atherosclerotic lesions in the brachiocephalic artery and aorta [1, 2]. In contrast to their male counterparts, female apoE−/− mice on high fat diet do not show evidence of occlusive coronary lesions or myocardial infarction. The aim of this work was to investigate whether high-fat diet alters the characteristics of Ca2+ transients of myocytes from these female mice during field stimulation at different frequencies. Female apoE−/− mice at 8 weeks old were fed either a high fat, Western-type diet (21% fat; 0.15% cholesterol) or were maintained on normal rodent diet for approximately 6 months. Cardiomyocytes were isolated as described previously [3]. Cardiomyocytes were also isolated from hearts of female wild type C57Bl/6 mice fed a standard rodent diet for comparison. Ventricular myocytes were superfused with normal Tyrode solution in a chamber on the stage of an inverted microscope. Single myocytes were chosen based on the criteria that they were rod-shaped with clear cross striations and well-defined edges, and were able to contract in response to field stimulation with no spontaneous contractions. Myocytes were loaded with 5 μM of the fluorescent indicator Fura-2. Cardiomyocytes were stimulated for 2 min at 0.2Hz followed by increasing the frequency of stimulation every 30 seconds to 0.5, 1 & 2 Hz and Ca2+ transients (Fura ratio) were measured using photometry. At 0.2Hz, wild type cardiomyocytes had larger transient amplitude, faster time to peak and time to decay compared to both groups of apoE−/− cardiomyocytes. The Ca2+ transient amplitude at 0.2 Hz was significantly smaller in apoE−/− cardiomyocytes fed high-fat diet compared to those fed normal diet (ratio: 0.41±0.03 vs. 0.65±0.08, p<0.05, t-test). Data shown are the mean ± S.E. for n=45 cells from 6 mice for apoE−/− high fat, n=24 cells from 3 mice for apoE−/− normal diet and n=16 cells from 3 C57Bl/6 wild-type mice. As stimulation frequency increased, the amplitude of the calcium transients decreased, with myocytes from apoE−/− high fat hearts continuing to show smaller transient amplitudes than myocytes from apoE−/− hearts fed a standard diet. Times to peak and time to decay of the calcium transients decreased as the stimulation rate increased, and was similar for both groups of cardiomyocytes. This work demonstrates that apoE−/− cardiomyocytes have altered calcium handling characteristics and that these alterations are accentuated by high fat diet feeding, indicating impor-
Temporal Characteristics of Phenotypic Alteration and its Functional Correlate in the STZ-induced Diabetic Rat Heart: Preliminary Observations

A. D’Souza, N.M. Woods and J. Singh

School of Forensic and Investigative Science, University of Central Lancashire, Preston, Lancashire, UK

The current article represents a set of preliminary observations in the investigation of the mechanisms underlying type-1 diabetic heart disease with primary reference to alterations in histological features of the myocardium and assessments of contractile function in streptozotocin (STZ)-treated type-1 diabetic male Wistar rats compared to age-matched controls. 6-8 weeks following STZ-administration (40 mg/kg), ventricular action potentials were measured in the isolated, spontaneously beating Langendorff perfused rat heart. Contraction and [Ca\(^{2+}\)]_i transients were measured in electrically stimulated ventricular myocytes by a Video Edge Detection system. At time points of 6-8 and 15 weeks, isolated ventricular tissue was processed for quantitative assessment of fibrosis, measurements of capillary density, myocyte diameter and myofiber to capillary ratio using routine staining techniques. Measurements of ventricular action potential indicated that heart rate (calculated in beats/minute) was significantly reduced (P<0.05, students t test) in the STZ-treated rats (174±13 bpm, n=6) compared to controls (238±12 bpm, n=5). The amplitude of contraction (as a percentage of resting cell length) was significantly greater (P<0.05, students independent samples t test) in STZ-induced diabetic myocytes (5.3±0.23% vs. 3.5±0.91%) compared to age matched controls (3.67±0.5%, n=33). The amplitude and time to peak of Ca\(^{2+}\) transients were not significantly altered (P>0.05, students t test) in the STZ treated myocytes whereas decay of the Ca\(^{2+}\) transient was significantly longer (P<0.05, students test) in STZ-induced diabetic myocytes (5.3±0.23% vs. 3.5±0.91%) compared to age matched controls (3.67±0.5%, n=33). The amplitude and time to peak of Ca\(^{2+}\) transients were not significantly altered (P>0.05, students t test) in myocytes from STZ-treated (67±3 milliseconds, n=16) compared to control rats (52.8±12 milliseconds, n=20). Visual analysis of stained sections did not reveal any major abnormalities at 6-8 weeks following STZ-administration in control vs. STZ-treated groups compared to the marked structural alterations evident in the 15 week STZ-treated right and left ventricle sections presenting as focal scarring, hypertrophied cardiomyocytes, myofibrillar loss, vacuolisation and large clusters of cells showing end stage apoptosis. Morphometric analysis undertaken in 25 fields for each parameter indicated a moderate reactive hypertrophy, reductions in capillary area fraction, significant reduction in capillary to myofiber ratio (1.03±0.76 vs. 0.74±0.5 ratio units) and a significant increment in collagen area fraction at 15 weeks of treatment (12.75±0.33%) compared to age-matched controls (5.14±1.83%) and 6-8 week STZ-treated rats (7.99±0.91%). (P<0.05, students t test). The results indicate that STZ-induced type 1 diabetes mellitus results in the development of a cardiomyopathic phenotype characterised by functional abnormalities manifesting as impaired Ca\(^{2+}\) homeostasis and contractility that appear to precede the development of histopathological changes.

This work was supported by the British Heart Foundation

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC104

Modified electrode placement changes wave amplitudes but not ST height on the 12 lead electrocardiograms of healthy subjects

J.P. Sheppard\(^1\), T. Barker\(^2\), T.H. Clutton-Brock\(^3\), M.P. Frenneaux\(^2\) and M.J. Parkes\(^1\)

\(^1\)School of Sport & Exercise Sciences, University of Birmingham, Birmingham, UK, \(^2\)Department of Cardiovascular Medicine, University of Birmingham, Birmingham, UK and \(^3\)Department of Anaesthesia and Intensive Care, University of Birmingham, Birmingham, UK

Wilson et al., 1934 argued that modifying the placement of ECG electrodes on the limbs should have no effect on ECG wave amplitudes unless they are moved onto the torso. Despite this, limb electrodes are routinely placed on the torso during exercise stress testing or when it is inconvenient or impossible to position them on the extremities. Under these conditions, monitoring the ECG, and in particular the ST segment is of great importance when diagnosing suspected myocardial ischemia/infarction. There remains no agreement on exactly how such electrode modification affects the 12 lead ECG (Mason & Likar, 1966; Takuma et al., 1995).

We have therefore made 12 lead ECG measurements in 18 healthy subjects using both standard and modified electrode placements with the arm electrodes placed on the “anterior acromial region” and the leg electrodes on the “anterior superior iliac spine” (Takuma et al., 1995). Data was collected with a customised data collection system that averages ~ 120 heart beats per subject and performs automated offline analysis of the ECG waveform. Mean ± sem wave amplitudes were compared using a two tailed paired T-test with the Bonferroni correction for multiple comparisons.

As expected, mean R and T heights in the precordial leads were not significantly different using the modified electrode placement (all < 0.01mV). R height and T height changed significantly by at least 0.06 ± 0.01mV in all the limb leads (except for lead aVR). In a clinical setting, the amplitude changes in leads III and aVF could be considered as potentially misleading (i.e., ± 0.1mV). Modification produced a rightward shift of ~ 15∞ in
both QRS and T axes, but this would only be clinically important in patients with a borderline axis in the standard ECG. In no leads did clinically important ST depression or elevation occur (i.e., none had elevated or depressed J / ST(80) points by \( \geq 0.1 \text{mV} \)) and the largest change in ST height was only 0.03 \( \pm 0.03 \text{mV} \) (V3).

Moving the limb electrodes of a 12 lead ECG to the modified positions for exercise stress testing and emergency monitoring (when the limbs are inaccessible) does produce measurable and reproducible changes in ECG wave amplitudes in healthy subjects. It does not however, produce a misleading ST segment change. Modified electrode placement therefore appears acceptable for ST segment monitoring under these commonly used conditions.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC106**

Comparison of dynamic and isometric assessments of muscle angle-torque relationships in human knee extensors and flexors


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The assessment of length-tension relationship in human muscle is cumbersome, and is usually derived from isometric measurements of the angle-torque relationship. Slow dynamic contractions may offer an efficient and accurate alternative (Proske et al., 2004). The aim of this study was to compare these two methods for measurement of torque-angle relationship in human knee extensors and flexors.

Twenty-two male Gaelic football players (aged 21.9 \( \pm \) 1.4 yrs, height 184.5 \( \pm \) 6.0 cm, body mass 85.7 \( \pm \) 7.7 kg, all data mean \( \pm \) SD) participated in this study after giving written informed consent. Both dynamic concentric and isometric measurements of left and right legs were made in a single testing period one week after a familiarisation session. Volunteers were seated on an isokinetic dynamometer with knee range measurements of left and right legs were made in a single test-informed consent. Both dynamic concentric and isometric mean \( \pm \) SD, * = P<0.05 difference between points, ANOVA with 95% confidence intervals.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC107**

50 min. of aerobic exercise with a caloric expenditure of \( \sim \) 540 Kcal. modifies short-term leptin response to CHO intake

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Acute effect of aerobic exercise on serum leptin concentration has been subject of inconsistent results. A recent review concluded that, in order to modify serum leptin concentration a single session of aerobic exercise must either be at least longer that 60 min. or consume more than 800 Kcal (Bouassida et al 2008). The present study was designed to assess the effects of 50 min. of aerobic exercise with a caloric expenditure of \( \sim \) 540
Kcal, on short-term serum leptin levels in fasting subjects and following carbohydrate (CHO) intake. Ten healthy and physically-active males were randomly assigned to four testing conditions: a) FR: fasting-rest b) FE: fasting-exercise; c) BR: breakfast-rest; d) BE: breakfast-exercise. Blood samples were collected every 30 minutes during the 8-hour testing conditions for analysis of plasma leptin, insulin and blood glucose. Data were analyzed by two-way repeated measures ANOVA and simple effects follow up. During all testing conditions, significant reductions in the serum leptin levels were observed (p < 0.05). Our data show that: 1. Aerobic exercise did not induce direct alterations of serum leptin levels neither in fasting nor in fed subjects since no significant differences were detected when comparing FR vs. BE and FR vs. FE conditions. 2. Exercise diminished postprandial glucose and insulin concentrations: the BE condition elicited increases in glucose and insulin levels, but these were significantly lower than for the BR condition (p < 0.05). 3. A single meal (439 Kcal.) with a high CHO content induced an increase of serum leptin concentration: starting at 14:00 hours (five hours after CHO intake), serum leptin in the BR condition was significantly higher than in the FR condition (p < 0.05). 4. Aerobic exercise abolished the observed CHO effect on serum leptin levels since no significant differences were found when comparing FR and BE conditions. Thus, 50 min of aerobic exercise with a caloric expenditure of ~540 Kcal. is sufficient to modify leptin response to feeding.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC108**

**Low endothelial progenitor cell number in peripheral blood of atherosclerotic patients is not due to plasma-induced in vitro dysfunction**

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1Pharmacology, Universidad Complutense, MADRID, NC, Spain, 2Hematology, Hospital Clinico San Carlos, Madrid, Spain and 3Cardiac Surgery, Hospital Clinico San Carlos, Madrid, Spain

We aimed to study the number of endothelial progenitor cells (EPCs) in atherosclerotic patients who underwent bypass grafting, or valve replacement. Plasma from these patients was added to cultured EPCs from healthy blood donors in order to try to reproduce in vitro what found in vivo. EPC number was assessed by flow cytometry, as percentage of CD34+/CD144+/CD3−, CD34+/KDR+/CD3− and CD144+/CD105+/CD3− mononuclear cells. 44 bypass patients and 43 valvular patients were chosen. For EPC cultures, mononuclear cells were obtained from healthy blood donors and separated using ficoll. Cells were plated on fibronectin and incubated with MV2 medium. Apoptosis was assessed by DNA fragmentation. Protein expression was measured by Western blot. The study was conducted according to the Declaration of Helsinki (revised in 2000). The hospital ethic committee approved the protocol and we obtained informed consent from all subjects before sampling took place. Bypass patients had lower numbers of CD34+/CD144+ EPCs, compared to valvular (0.2239±0.032% vs 0.4370±0.08%, p=0.0187), and lower numbers of CD34+/CD144+/CD3− EPCs (0.3309±0.054% vs 0.7786±0.1922%, p=0.026). However, cultured EPCs from healthy donors exhibited a lower apoptotic rate after 24, 48 or 72h incubation with 1% plasma from bypass patients, compared to valvular (0.9998 vs 2.038, 0.9971 vs 1.951, 0.998 vs 1.599, respectively), being this effect prevented by TGFbeta blockers SIS3 and SB431542. Incubation with 1% plasma from bypass patients induced higher expression of CD105 and CD144, (measured by Western blot in at least two different experiments, using plasma from three different patients each one).

Atherosclerotic patients possess a lower pre-surgical number of EPCs, compared to valvular. EPC dysfunction in culture is not observed in EPCs from healthy donors incubated with plasma from atherosclerotic patients. The widely described in vitro EPC dysfunction in atherosclerotic patients may be due to intrinsic defects in these cells from the bone marrow.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

**PC109**

**Leg vascular conductance kinetics in older versus younger men during constant load calf plantar flexion exercise**

H.N. Reilly1, M. Egana1 and S. Green2

1Physiology, Trinity College, Dublin, Ireland and 2Physiology, University of Otago, Dunedin, New Zealand

Older men exhibit a preserved vascular conductance (VC) response to incremental leg extension exercise compared to active young men1. However, the age-related effects on the rate at which the leg vascular conductance response increases at the onset of constant load exercise (VC kinetic response) has not yet been examined. To examine the vascular conductance kinetics response during calf constant-load plantar-flexion exercise performed at different intensities in older and younger men Thirteen older (60-84 years) and thirteen younger (20-30 years) sedentary men were tested. Ethical approval was obtained from the Trinity College Dublin Faculty Research Ethics Committee. Subjects performed three constant load exercise bouts (6 min long) of intermittent calf plantar flexion exercise (6s duty cycle: 2 s contraction, 4 s relaxation) at an intensity of 30% maximum voluntary contraction (MVC) on a custom-built calf ergometer at a tilt of 67 degrees. This was then repeated at an intensity of 45% MVC. Calf BF was measured contraction by contraction using venous occlusion plethysmography. Kinetic analysis was performed by fitting a biexponential function to the mean (3 bouts) of the vascular conductance (BF/MAP) data. Student t-tests were used to detect differences between groups. Results are depicted as mean ± SD.
The time constant of the fast component was significantly shorter in the young (30%; 19.3±16; 45%; 37.5±13.20) compared to the older (30%; 82.2 ± 8.6; 45%; 54.3±48.7) group but the rest of the VC kinetic parameters, the mean response time and the end exercise amplitude at 360s were not different between both groups. This study showed that the rate at which vascular conducance responses increase during constant load static calf exercise is faster in younger men compared to older men.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC110

The effects of alternating hot and cold water immersion on recovery of muscle function after resistance training in humans


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Eccentric muscle contractions may result in temporary repairable damage to human muscle. This is associated with muscle soreness and a prolonged decline in muscle force production. Contrast Water Immersion (CWI) has been reported to accelerate recovery of muscle function after intense exercise (Vaile et al., 2007). CWI involves repeated alternate immersion in hot and cold water. The aim of this study was to evaluate the effects of CWI treatment on muscle force recovery after resistance training.

Thirty-five active human volunteers (mean±S.D.; height 173.9±7.4 centimetres, body mass 72±9.6 kilograms, age 22±2.6 years) participated in this study. The volunteers were randomly assigned to either a CWI group (n=18) or tepid water immersion control group (n=17). Each volunteer performed a familiarisation session, a pre-test session to record baseline measures, a 45 minute resistance training protocol, and four post-testing sessions at 24, 48, 72 and 96 hours after exercise. Measures were: knee extensor isometric Maximum Voluntary Contraction (MVC) force and electrically stimulated force at 20 and 50 Hertz measured at an internal knee angle of 120 degrees, concentric and eccentric torque measured during knee extension at 0.87 and 1.74rad.s⁻¹, and muscle soreness measured by questionnaire. The CWI group performed 4 cycles of a 1 minute immersion at 9±1°C alternated with 4 minutes of immersion at 39±1°C. The control group performed 20 minutes immersed in water at a temperature of 29±1°C. Subjects were seated in the tanks, immersed to the level of the anterior superior iliac spine. Treatments were administered immediately after resistance training and 24, 48 and 72 hours later, after post tests were completed. A repeated measures ANOVA was used to compare results of the two treatment groups over five time points and 95% confidence intervals were used to compare individual points. All measures changed over time (P<0.05) except for torque at 0.87rad.sec⁻¹. Soreness (arbitrary scale) increased to a maximum at 24 hours of 34.8±13.4 (CWI) and at 48 hours 37.7±14.9 (Control), and 20:50 Hertz ratio declined to a minimum of 0.65±0.09 (CWI) and 0.65±0.08 (Control) at 24 hours. There was a significant effect for differences between treatment groups only for the isometric MVC measure (P<0.05, see Figure 1).

Figure 1. Normalised isometric MVC force. Pre values (0) were 820.6±217.4 Newtons for CWI, and 751.1±217.4 Newtons for Control. * = P<0.05 (ANOVA with 95% confidence intervals).


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC111

The effect of caffeine ingestion on antigen-stimulated human T cell activation following prolonged cycling

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Caffeine is consumed by many athletes for its known ergogenic properties. However despite the high intention by athletes to use caffeine (Chester & Wojek, 2008), little research has concentrated on its effects on human immune cell function following high intensity exercise. A study by Bishop et al. (2005) demonstrated that caffeine ingestion 60 min before a 90 min cycle at 70% VO2peak increased the natural state of activation of CD4⁺ and CD8⁺ cells in vivo before and after exercise. However, this does not necessarily suggest enhanced effector function when faced with an antigenic challenge. Therefore, the aim of the present study was to investigate the effect of caffeine ingestion on human T cell (CD4⁺ and CD8⁺) function following
Interactions between pH-sensitive histidine residues in proton-gated, two-pore domain K⁺ channel TASK-3

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TASK-3 and TASK-2 are two-pore domain, background K⁺ channels gated by extracellular pH. TASK-3 dependence upon pH₀ is cooperative with Kd of 5.9 ± 0.05 and nᵣ of 2.1 ± 0.09 (n = 8; all errors quoted as SEM). The pH₀-sensor in TASK-3 is histidine 98 located at the extracellular entrance of the pore and TASK-3-H98N mutant is pH₀-independent (Rajan et al., 2000; Kim et al., 2000). The pH₀-gating of TASK-2 occurs with a pH₀₁/₂ of 8.0, is not cooperative and is mediated by neutralization of arginine 224, so that mutant TASK-2-R224A is pH₀-independent (Niemeyer et al., 2007). To find out whether both pH₀-sensors occurring in these dimeric channels are required in the gating process we have covalently linked the C-terminus of one channel with the N-terminus of the following to form concatenated structures containing either a normal set of pH₀-sensors (WT-WT), mixed structures containing one able and one neutralised pH₀-sensor (WT-Mut) or two pH₀-sensing disabled channels (Mut-Mut). Analysis was done by transient expression into HEK-293 cells and patch-clamp. Gating by pH₀ of TASK-3 WT-WT concatenated channels (n=8) or Mut-Mut (Mut = TASK-3-H98N, n=4) structures was similar to that of their non-concatenated equivalent channels. In contrast, the mixed WT-Mut and Mut-WT followed pH₀ in a non-cooperative manner and required more acidification for inhibition to occur. The respective pH₀₁/₂ values for TASK-3 WT-Mut and Mut-WT were 4.9 ± 0.04 (n=5) and 5.1 ± 0.12 (n=6). Assuming that N98 mimics a neutral form of H98, we have used the Kd of these mixed constructs (10⁻⁵ M) as Kd in a fit to the data of a simplified model for the gating of TASK-3 by protons in which there are two closed states with two or one charged H98 residues and an open state with both H98s neutralised. This analysis applied to the WT-WT data gave a value for Kd = 3.26*10⁻⁷ M consistent with the idea that neutralisation of a first H98 sensor of TASK-3 affects the pH₀₁/₂ of the second making it 30-times more susceptible H⁺ loss. WT-WT TASK-3 concatenated constructs behaved similarly to the non-concatenated channels with pH₀₁/₂ 8.2 ± 0.15 (n=4), whilst Mut-Mut (Mut = TASK-2-R224A) TASK-2 constructs lacked pH₀-dependence. The mixed WT-Mut and Mut-WT TASK-2 constructs had pH₀₁/₂ values (relative to WT-WT) of 7.8 ± 0.05, n=6 and 7.9 ± 0.10, n=6) very similar to that of WT and WT-WT channels. Our data show that both pH₀-sensors of TASK-3 and TASK-2 channels need to be neutralised for channel opening. There appears to be no interaction between sensors in TASK-2 channels accounting for the lack of cooperativity in the pH₀ effect. In TASK-3 however, the data indicate that neutralisation of one sensor profoundly affects the ability of the second sensor to become neutral thus accounting for cooperative, high-gain response to pH₀.

PC112

Interaction between pH₀-sensing histidine residues in proton-gated, two-pore domain K⁺ channel TASK-3

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TASK-3 and TASK-2 are two-pore domain, background K⁺ channels gated by extracellular pH. TASK-3 dependence upon pH₀ is cooperative with Kd of 5.9 ± 0.05 and nᵣ of 2.1 ± 0.09 (n = 8; all errors quoted as SEM). The pH₀-sensor in TASK-3 is histidine 98 located at the extracellular entrance of the pore and TASK-3-H98N mutant is pH₀-independent (Rajan et al., 2000; Kim et al., 2000). The pH₀-gating of TASK-2 occurs with a pH₀₁/₂ of 8.0, is not cooperative and is mediated by neutralization of arginine 224, so that mutant TASK-2-R224A is pH₀-independent (Niemeyer et al., 2007). To find out whether both pH₀-sensors occurring in these dimeric channels are required in the gating process we have covalently linked the C-terminus of one channel with the N-terminus of the following to form concatenated structures containing either a normal set of pH₀-sensors (WT-WT), mixed structures containing one able and one neutralised pH₀-sensor (WT-Mut) or two pH₀-sensing disabled channels (Mut-Mut). Analysis was done by transient expression into HEK-293 cells and patch-clamp. Gating by pH₀ of TASK-3 WT-WT concatenated channels (n=8) or Mut-Mut (Mut = TASK-3-H98N, n=4) structures was similar to that of their non-concatenated equivalent channels. In contrast, the mixed WT-Mut and Mut-WT followed pH₀ in a non-cooperative manner and required more acidification for inhibition to occur. The respective pH₀₁/₂ values for TASK-3 WT-Mut and Mut-WT were 4.9 ± 0.04 (n=5) and 5.1 ± 0.12 (n=6). Assuming that N98 mimics a neutral form of H98, we have used the Kd of these mixed constructs (10⁻⁵ M) as Kd in a fit to the data of a simplified model for the gating of TASK-3 by protons in which there are two closed states with two or one charged H98 residues and an open state with both H98s neutralised. This analysis applied to the WT-WT data gave a value for Kd = 3.26*10⁻⁷ M consistent with the idea that neutralisation of a first H98 sensor of TASK-3 affects the pH₀₁/₂ of the second making it 30-times more susceptible H⁺ loss. WT-WT TASK-3 concatenated constructs behaved similarly to the non-concatenated channels with pH₀₁/₂ 8.2 ± 0.15 (n=4), whilst Mut-Mut (Mut = TASK-2-R224A) TASK-2 constructs lacked pH₀-dependence. The mixed WT-Mut and Mut-WT TASK-2 constructs had pH₀₁/₂ values (relative to WT-WT) of 7.8 ± 0.05, n=6 and 7.9 ± 0.10, n=6) very similar to that of WT and WT-WT channels. Our data show that both pH₀-sensors of TASK-3 and TASK-2 channels need to be neutralised for channel opening. There appears to be no interaction between sensors in TASK-2 channels accounting for the lack of cooperativity in the pH₀ effect. In TASK-3 however, the data indicate that neutralisation of one sensor profoundly affects the ability of the second sensor to become neutral thus accounting for cooperative, high-gain response to pH₀.


Tools for optimising experiments and developing predictive models: an example for ion channel kinetics

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"The application of mathematics to natural phenomena is the aim of all science, because the expression of the laws of phenomena should always be mathematical" (Bernard, 1865). The need for mathematical tools has long been acknowledged by physiologists, in order to integrate data from past experiments with the testing of possible hypotheses about underlying mechanisms, and thus to guide future experimental design. A synergy between the wet-lab and modelling approaches enabled Hodgkin-Huxley, Noble and DiFrancesco to unravel the interaction of ion currents in many different cell types. The computational revolution led to a separation between "mathematical modellers" and "physiologists", reducing communication accordingly. A major criticism of current mathematical modelling in cardiac electrophysiology is that most of the results only replicate what is already known experimentally, even though (or because) the mathematical models have become evermore complex. We propose a set of tools to ensure validated and predictive models (applied here to ion-channel models and voltage clamp experiments). These tools investigate the relation between experimental data, its information content, and model structure. Currently, multiple voltage clamp step experiments are done to determine the kinetics of ion channels (activation, deactivation, inactivation and reactivation); most often these are undertaken in different cells, and the results have to be normalised for analysis. The full set of protocols produces redundant data and we show a much shorter protocol (length <15 s) that suffices to evaluate models' parameters.

We compare the information content of the optimized step protocol to action-potential and ramp clamp protocols. The optimization of experiments is a necessary step for providing predictive models, as there is a lack of knowledge of how much, and which kind of, experimental data suffices for a unique fit of a model's parameters. As a result many models are fitted using all the available data, which can be inconsistent and leaves no data for model validation. A model simply matching the experimental data does not imply a "good fit" if the model is overparameterised.

We analyse simulated experimental protocols and models to establish whether there is sufficient information to fit all of the models' parameters uniquely. This provides a separation between the 'training' data used to fit the model parameters and 'validation' data used to evaluate the predictive power of the model. This study of each protocol's parameter-information content allows us to design optimised patch-clamps for voltage gated ion channels. Fitting model parameters to such protocols enables us to evaluate and then ensure the model structures' ability to predict the current during any other clamp.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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Protein Kinase A phosphorylation of hK$_{2P}$3.1 and hK$_{2P}$9.1 carboxy-termini enables channel function

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Export of newly synthesised hK$_{2P}$3.1 (TASK1) and hK$_{2P}$9.1 (TASK3) channels from the endoplasmic reticulum (ER) and delivery to the cell surface is subject to quality control mechanisms. ER retrieval motifs that bind βCOP have been demonstrated on both the N- and C-termini of both channels (O'Kelly et al., 2002; O'Kelly & Goldstein, 2008; Zuzarte et al., 2009) while a C-terminal motif (MKRSSV), recruits 14-3-3 which overcomes βCOP binding and enables forward transport of the channels to the membrane (O'Kelly et al., 2002; Rajan et al., 2002; O'Kelly & Goldstein, 2008). 14-3-3 binding to this motif is phosphorylation dependent. Previous studies have shown that while there are two potential phosphorylation sites within this motif, 14-3-3 binding is dependent on phosphorylation of the terminal serine (S393 for hK$_{2P}$3.1 or S373 for hK$_{2P}$9.1; O'Kelly et al., 2002; Rajan et al., 2002). To date the kinase responsible for this phosphorylation is unidentified. This study presents evidence that phosphorylation of the terminal serine by protein kinase A (PKA) is required for K$_{2P}$3.1 and K$_{2P}$9.1 functional expression. In vitro kinase assays demonstrate that alanine substitution of S393 (hK$_{2P}$3.1) resulted in a significant reduction in phosphorylation by PKA. When expressed in Xenopus oocytes, mutant channels in which the consensus PKA site was ablated (S393A for hK$_{2P}$3.1 or S373A for K$_{2P}$9.1) showed a marked reduction in current ($4.0 \pm 0.37 \mu A$ and $0.59 \pm 0.16 \mu A$ at 60 mV for WT hK$_{2P}$3.1 and S373A respectively and $1.35 \pm 0.31 \mu A$ and $0.54 \pm 0.11 \mu A$ at 60 mV for WT K$_{2P}$9.1 and S373A respectively). Oocytes injected with the protein kinase A peptide inhibitor (PKI; 1 nM) 24 hours post WT hK$_{2P}$3.1 RNA injection and incubated for a further 16 hours failed to produce currents significantly different from that of water injected oocytes ($0.23 \pm 0.05 \mu A$ and $0.5 \pm 0.21 \mu A$ for WT hK$_{2P}$3.1 plus PKI injected and water injected cells respectively at 60 mV). Cells injected with RNA but not PKI gave acid sensitive currents similar to those seen previously. Similarly hK$_{2P}$9.1 injected oocytes treated with PKI also resulted in loss of WT currents ($0.3 \pm 0.07 \mu A$ and $1.36 \pm 0.26 \mu A$ for WT with and without PKI injection respectively at 60 mV). This data demonstrates that PKA phosphorylation of hK$_{2P}$3.1 or hK$_{2P}$9.1 on their terminal serine is required for functional expression of these channels.


Rajan S et al. (2002). J Physiol 545, 13-26
A Systems Biology Computational Comparison of Two Cardiac Pacemaker Cell Models
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Aim: Biophysical cardiac cell models consist of coupled ordinary differential equations with many regulatory biophysical parameters (> 100). Model responses are differentially regulated by such model specific parameters. This study quantitatively compares the underlying parametric regulation of model responses among two rabbit pacemaker models using a systems biology approach with a novel global sensitivity mutual information index (MI).

Methods: The rabbit pacemaker models developed by Kurata et al. [1] (Kurata) and Zhang et al. [2] (Zhang) were parameterically analysed in this study. Model responses in terms of action potential (AP) features were defined as maximum diastolic potential (MDP), overshoot potential (OS) and AP duration (APD₉₀) [3]. Independent parameters associated with channel conductances, gating steady states, time constants and intracellular ionic homeostasis in the models were identified with 194 in the Kurata case, and 176 in Zhang case. The global information-theoretic sensitivity MI index has been developed in a previous study [4]. It constitutes deterministic quantification of the correlation between modelling parameters and model responses. Through multiple evaluations of the model (> 10⁵) for randomly selected configurations of parameters, distributions of samples of model responses based on the perturbed parameter sets were obtained. With the large sampling, MI gave a quantitative statistical correlation between individual parameters and model responses. With such quantification, model parameters with respect to each model response were ranked from most relevant (MI = 1) to uninfluential (MI ~ 0). This enabled models comparison at parametric level.

Results: Standard AP profiles from the models are shown in Fig. 1A. Due to the differing parametric regulation of the models, the two AP profiles are different. This is reflected in the differential parametric responses as seen in Fig 1B. Relative MI shows that MDP is mostly regulated by a component of the I_{Ca,L} channel activation time kinetics (p_{K1}) in the Kurata case, whilst I_{Kr} channel conductance (g_{Kr}) in the Zhang case. Reversal potential of I_{Ca,L} channel (E_{Ca,L}) regulates the OS in both models. APD₉₀ is also regulated by differentially in the two models with a parameter of the I_{Ca,L} channel activation time kinetics (p_{K2}) in the Kurata case and a parameter of the I_{Ca,L} channel inactivation time kinetics (p_{Z1}) in the Zhang case.

Conclusions: This MI analysis shows that although there is a common set of parameters regulating model responses, there are subtle differences. Often different models rely upon distinct mechanisms to reproduce various desired responses and MI provides a global stastical measure for identification of the same. MI can further assist in parameter estimation during model development.

(A) Standard AP profiles from Kurata (solid line) and Zhang (dashed line) models. (B) Left column show relative MI data for Kurata and right column for Zhang. Top panels show data for MDP, middle for OS and bottom for APD₉₀. Parameters that maximally influence the respective model responses are shown. See text for details.

These results suggest that KV and KCNQ channels are impor-
table interactions between PIP2 and IP3.
These results show that endogenous PIP2 has a pronounced
In freshly dispersed single portal vein myocytes, single cation
channels were recorded at room temperature using the
inside-out patch configuration of the patch clamp technique
(Archer et al., 2008). Mean values are of n cells ± S.E.M. Statistical
analysis was carried out using unpaired and paired Student’s
t-test with the level of significance set at P<0.05.
To test the effects of PIP2 on OAG-evoked activity we compared
control cells with cells pre-treated with 20 μM wortmannin for
30 min to deplete PIP2 levels. Mean open probability of OAG-
(10 μM)-induced TRPC6-like activity was significantly increased
from 0.03 ± 0.01 (n=9) in control cells to 2.53 ± 0.37 (n=48) in
cells pre-treated with wortmannin at -50 mV. Application of
PIP2 inhibited OAG-evoked activity with an IC50 value of 0.74
μM at -50 mV. Inhibition of OAG-induced activity by 10 μM PIP2
was not affected by IP3 receptor blocker heparin (1 mg/ml), n=5). Nora-
drenaline-evoked channel activity was inhibited by anti-TRPC6
(88 ± 9 %, n=7) and anti-TRPC7 antibodies 85 ± 13 % (n=10) but
not by other anti-TRPC antibodies.
These results show that endogenous PIP2 has a pronounced
inhibitory action on TRPC6-like activity in portal vein myocytes
which can be removed by interactions with IP3. Moreover these
data suggest that these channels are likely to be composed of
TRPC6/TRPC7 heterotetramers and that TRPC7 proteins medi-
ate interactions between PIP2 and IP3.
miRNAs are involved in the down-regulation of Kir2.1 and IK1. We hypothesize that because a variation in expression of miRNAs has been shown in comparison with miR-1, which is a negative control for miR-212 down-regulated functional expression of pLuc2.1-3 repeated three times with different plasmid ratios).

We aim to demonstrate the relationship between miRNAs and Kir2.1 is a member of the gene subfamily coding for inward rectifier potassium channels. K_r2.1 passes inwardly rectifying K_+ current (I_Kr) which plays an important role in repolarising and stabilizing the membrane potential in cardiac myocytes; indeed down-regulation of inward rectifier current occurs in cardiomyopathy and is proarrhythmic (1).

Because the causes of _I_K1_ reduction are still unknown and because a variation in expression of miRNAs has been shown in cardiac hypertrophy and heart failure (2-4) we hypothesize that microRNAs are involved in the down-regulation of K_r2.1 and _I_K1_. We aim to demonstrate the relationship between miRNAs and down-regulation of K_r2.1 by identifying miRNAs that are up-regulated in cardiomyopathy (2-4) and predicted to target the K_r2.1 3'UTR (TargetScan; Miranda; PicTar), transfecting them into HEK293 cells and assaying for functional targeting using a luciferase reporter with putative target sites in the 3'UTR. miR-1 was chosen as control and to validate the method as it has been shown to target K_r2.1 (5).

Two luciferase reporter plasmids were constructed from pMIR-REPORT (Applied Biosystems); one with the K_r2.1 3' UTR miR-1 target site (pLuc2.1-1), and the other with predicted miR-132, miR-212 and miR-320 target sites (pLuc2.1-3). MiRNA expression plasmids were produced in the siRNA expression vector pSM30 (provided by Dr. G. Du, UTHSC, Houston) by inserting oligonucleotide cassettes of mature miRNA sequences.

HEK293 cells were transfected with a luciferase reporter plasmid, miRNA vector and a β-galactosidase plasmid as normalizer. After 48 hours the Dual-Light® assay (Applied Biosystems) was performed.

The method has been optimized with miR-1 and pLuc2.1-1. Luciferase/β-galactosidase was 40-44% lower in cells transfected with miR-1 vs pSM30 with no insert (p<0.01, n=3, t-test, repeated three times with different plasmid ratios).

miR-212 down-regulated functional expression of pLuc2.1-3 in comparison with miR-1, which is a negative control for pLuc2.1-3. Luciferase/β-galactosidase was 72% lower in cells transfected with miR-212 vs miR-1 (p<0.05, n=3, t-test).

In summary, we have demonstrated miRNA targeting using the pSM30 vector and provided evidence for a miR-212 target in the 3'UTR of K_r2.1. These studies are intended to form the basis of future studies aimed at demonstrating down-regulation of _I_K1_ by specific miRNAs.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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Targeting of an inward rectifier K⁺ channel 3' untranslated region by microRNA up-regulated in heart disease

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MicroRNAs (miRNAs) are a class of noncoding small RNAs modulating gene expression. By annealing to the 3' terminal untranslated region (3'UTR) of the target mRNA, they lead to translational repression.

K_r2.1 is a member of the gene subfamily coding for inward rectifier potassium channels. K_r2.1 passes inwardly rectifying K_+ current (I_Kr) which plays an important role in repolarising and stabilizing the membrane potential in cardiac myocytes; indeed down-regulation of inward rectifier current occurs in cardiomyopathy and is proarrhythmic (1).

Because the causes of _I_K1_ reduction are still unknown and because a variation in expression of miRNAs has been shown in cardiac hypertrophy and heart failure (2-4) we hypothesize that microRNAs are involved in the down-regulation of K_r2.1 and _I_K1_. We aim to demonstrate the relationship between miRNAs and down-regulation of K_r2.1 by identifying miRNAs that are up-regulated in cardiomyopathy (2-4) and predicted to target the K_r2.1 3'UTR (TargetScan; Miranda; PicTar), transfecting them into HEK293 cells and assaying for functional targeting using a luciferase reporter with putative target sites in the 3'UTR. miR-1 was chosen as control and to validate the method as it has been shown to target K_r2.1 (5).

Two luciferase reporter plasmids were constructed from pMIR-REPORT (Applied Biosystems); one with the K_r2.1 3' UTR miR-1 target site (pLuc2.1-1), and the other with predicted miR-132, miR-212 and miR-320 target sites (pLuc2.1-3). MiRNA expression plasmids were produced in the siRNA expression vector pSM30 (provided by Dr. G. Du, UTHSC, Houston) by inserting oligonucleotide cassettes of mature miRNA sequences.

HEK293 cells were transfected with a luciferase reporter plasmid, miRNA vector and a β-galactosidase plasmid as normalizer. After 48 hours the Dual-Light® assay (Applied Biosystems) was performed.

The method has been optimized with miR-1 and pLuc2.1-1. Luciferase/β-galactosidase was 40-44% lower in cells transfected with miR-1 vs pSM30 with no insert (p<0.01, n=3, t-test, repeated three times with different plasmid ratios).

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In summary, we have demonstrated miRNA targeting using the pSM30 vector and provided evidence for a miR-212 target in the 3'UTR of K_r2.1. These studies are intended to form the basis of future studies aimed at demonstrating down-regulation of _I_K1_ by specific miRNAs.

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PC119

Cholesterol depletion does not prevent actin-based activation of non-voltage-gated sodium channels

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Cholesterol is a major lipid component of the plasma membranes, and it plays an important role in lipid organization, lateral heterogeneity and dynamics of plasma membrane. Lipid rafts, cholesterol-rich membrane domains, are assumed to play an essential role in the interactions between cell membrane and cortical cytoskeleton (Nebel et al., 2002). As we have shown earlier, the activity of non-voltage-gated sodium channels in K562 human leukaemia cells is critically dependent on F-actin organization (Negulyaev et al., 2000; Shumilina et al., 2003). Our present work was aimed to investigate possible role of lipid environment in the functions of sodium channels and their coupling with cortical cytoskeleton in K562 cells. The patch-clamp method was employed to examine the effect of methyl-β-cyclodextrin (MbCD), an agent that removes membrane cholesterol, on sodium currents. Single channel currents were recorded from cell-attached and inside-out patches essentially as described earlier (Morachevskaya et al., 2007). After incubation with MbCD (2.5 or 5 mM) an activation of sodium channels in response to cytochalasin B or D was observed in membrane fragments (inside-out mode) as well as in native cells (cell-attached mode). Unitary conductance of the channels in cholesterol-depleted cells (11.6±0.1 pS, n=8) was similar to those in control cells (11.9±0.7 pS, n=7). Inside-out experiments with the use of globular actin have indicated that filament assembly on cytoplasmic membrane side causes an inactivation of sodium channels. In sum, we conclude that cholesterol depletion did not affect essentially the biophysical characteristics, sodium channel activation and inactivation coupled with F-actin rearrangement. Our electrophysiological data imply that there is no association of non-voltage-gated sodium channels with cholesterol-rich membrane microdomains.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

The authors thank Dr. Youyou Zhao.

PC119

Cholesterol depletion does not prevent actin-based activation of non-voltage-gated sodium channels

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Modelling TRPM8 single channel gating
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Single channel patch clamp recording allows modelling of ion channel behaviour. Thus, a single kinetic model can formally describe the complex interactions during poly-modal activation of the channels. For TRPM8, activation depends on temperature, voltage and chemical signalling (Voets et al., 2007), and such modelling provides the most comprehensive approach to the study of the channel. Here we show the methods used to derive the kinetic signature of the TRPM8 channel from a set of single channel recordings.

Single channel (cell-attached patch mode) currents were recorded in HEK-293 cells stably expressing the TRPM8 channel. Data was recorded at room temperature and different voltages (from 40 to 140 mV). Currents were filtered (low pass Bessel) at 2 kHz and sampled at 10 kHz. Single channel transitions were identified by the half-amplitude threshold crossing criteria and time-course fitting (Colquhoun and Sigworth, 1995). Recordings were made from 7 different patches with at least 12000 openings in each recording (all values were expressed in means ± S.E.M.).

Three steps were used to derive a kinetic model from single channel recordings:
1. The data: Dwell time 1D histograms were constructed from transitions >0.16 ms using the square root of the number of events per bin with a constant bin width expressed on a logarithmic time axis (ms, 20bins/decade). These histograms were fitted with a sum of exponentials using pClamp 9. The appropriate number of exponentials was assessed both by visual inspection as well as a statistical estimation of the ‘goodness of fit’ (Maximum Likelihood and Least Squares Errors). These methods pointed to the need of 2 to 4 open states (mean open time ranged from 0.2 ± 0.02 to 8.95 ± 0.35 ms) and 4 to 6 closed states. The most likely transitions that are voltage regulated have also been identified.

2. Modelling: There are two Freeware software packages for Markov analysis of single channel data: HJCFIT (http://www.ucl.ac.uk/) and QuB (http://www.qub.buffalo.edu/). They were used to fit single channel sequences to different models and solve for the most likely rate constants. In order to discriminate between models, dwell time 2D distributions (Rothberg and Magleby, 1998) and dependency and dependency difference plots (Magleby and Song, 1992) were also used.

3. Testing the predictions: Lastly, the correspondence between the predictions derived from the model and the values initially used to describe the experimental recordings was tested. This study provided evidence that the response of TRPM8 channel to voltage variations can be described in terms of a model that includes channel transitions involving 2 to 4 open states and 4 to 6 closed states. The most likely transitions that are voltage regulated have also been identified.

Voets T et al. (2007). Handb Exp Pharmacol 179, 329-344
Rothberg BS and Magleby KL (1998). J Gen Physiol 111, 751-780

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Pharmacological activation of Peroxisome-Proliferator Activated Receptor b promotes rapid and calcineurin-dependent fiber remodelling and angiogenesis in mouse skeletal muscle

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Sedentary lifestyle is associated with the metabolic syndrome and promoting the oxidative capability of skeletal muscle has been proposed as a therapeutic approach to counteract metabolic disturbances. In cultured myotubes, overexpression of a nuclear receptor involved in the regulation of muscle development and metabolism (peroxisome proliferator-activated receptor β, or PPARβ), enhances fatty acid catabolism (1). Muscle-specific overexpression of PPARβ in mice promotes an increase in the number of oxidative myofibers and augments resistance against diet-induced obesity (2, 3). Recently, administration of PPARβ agonists has been reported to improve the metabolic phenotype of obese and insulin-resistant animals (4, 5).

This study presents in vivo effects of PPARβ activation on muscle morphology on wild-type mice, and describes the mechanism mediating these effects. Adult C57BL6 mice received daily subcutaneous injections of a PPARβ specific activator, or the calcineurin inhibitor cyclosporine A (CsA) or a combination of both. After different times (from 2 to 96 hours), tibialis anterior muscles were harvested immediately after death and used for histological and molecular analyses.

PPARβ activation leads to a muscle remodelling, characterized by increases of oxidative fiber and capillary numbers, and a decrease of fiber diameter, completed within 2 days of treatment. This muscle remodelling is accompanied by a quick and transient upregulation of myogenic and angiogenic markers. Both myogenic and angiogenic responses are dependent upon calcineurin activity as remodelling is completely blocked by CsA administration.

In conclusion, these data indicate that PPARβ activation is associated with a calcineurin-dependent effect on muscle morphology, that enhances the oxidative phenotype.

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the. These results have been performed in endothelial cells from venous origin, mainly in cultured human umbilical vein endothelial cells (HUVEC). Our aim was to compare the estradiol effects on changes induced by oxLDL on DDAH expression and ADMA production in cultured human umbilical artery endothelial cells (HUAEC) with results obtained in HUVEC. Primary HUAEC and HUVEC were exposed to 100 μg/mL of oxLDL, with or without 1 nM estradiol for 24 hours. ADMA was measured in culture supernatants by HPLC. DDAH-I and II mRNA expression and protein content was quantified by real time PCR and immunoblotting, respectively. ADMA production remained unaltered in HUAEC exposed to estradiol, whereas in HUVEC estradiol decreased ADMA production by 57% (p<0.001). In both cell types, oxLDL significantly increased ADMA production by ~50% (p<0.01). Finally, combined exposure to oxLDL plus estradiol completely abolished the increased production of ADMA induced by oxLDL (p<0.05 vs. oxLDL alone).

DDAH-I protein expression remained unchanged after all treatments. Exposure of HUVEC to oxLDL, reduced DDAH-II expression by 20% (p<0.01) at both the mRNA as well as the protein levels, which in turn increased ADMA levels. Estradiol alone increased DDAH-II mRNA and protein expression up to 170% (p<0.01). In cells exposed to estradiol in combination with oxLDL, DDAH-II protein levels were the same as those for estradiol alone.

Nevertheless, in HUAEC the effects were quite different. Estradiol alone did not modify DDAH-II expression, whereas oxLDL increased DDAH-II expression by 50% (p<0.05), probably as a consequence of increased ADMA levels. To check that possibility, DDAH activity was measured. DDAH activity was reduced by oxLDL by 50% (p<0.05). Estradiol alone did not modify DDAH activity, but restored the reduced activity caused by oxLDL (p<0.01 vs. oxLDL), thus resulting in decreased ADMA levels. Therefore, estradiol counteracts oxLDL-induced ADMA production in HUVEC, whereas in HUAEC, estradiol counteracts the raise of ADMA levels and the reduced DDAH activity induced by oxLDL, whereas the increased DDAH-II expression seems to be a consequence of increased ADMA levels.

Min. Ciencia e Innovación, ISCIII (FIS 06/0589, FIS 08/0634, RED HERACLES RD06/0009); Cons. Sanidad, GV (AP 09/2007, AP 121/08). PJ0 is a post-doc, and AS is a FPI fellow (BFPI 06/145), from Cons. Educación, Generalitat Valenciana, Spain.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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A growing mass of data strongly suggest that a number of vascular disorders induced by oxygen lack, including hypoxic pulmonary vasoconstriction (HPV), are associated with abnormalities of endothelial function but underlined mechanisms still remain unclear. The goal of this study was to shed light on the possible myoendothelial gap junctions contribution to the mechanisms responsible for HPV, and to clarify the phenomenon of paradoxical HPV inversion to relaxation under selective glycolysis inhibition. Membrane potentials were recorded from endothelial cells (EC) of guinea pig main pulmonary arteries (MPA) and aorta using whole-cell patch-clamp technique in current clamp mode. The vascular tone was measured on isolated rat aorta and MPA using contractile recording technique. All animals were anesthetized with sodium pentobarbital (50 mg/kg ip). Hypoxia (pO2 - 10 mmHg) caused rapid hyperpolarization in aorta endothelial cells from -44.7±4.1 to -37.4±2.1 mV (n=6). In contrast to this, EC in MPA were depolarized under hypoxic condition from -43.3±3.2 to -30.6±2.2 mV (n=5). Selective glycolysis inhibition with 10 μM iodoacetic acid (IAA) in combination with 10 mM sodium pyruvate led to inversion of these electrical responses in endothelial cells in both types of vessels, i.e. hypoxia hyperpolarized EC from MPA from -8.5±1.6 to -30.4±3.6 mV (n=6) and depolarized EC from aorta from -28.1±2.4 to -18.2±3.1 mV (n=6). In contractile recording experiments hypoxia elicited constriction in MPA with peak amplitude 45.5±5.3% (n=11) and aorta relaxation to 64.3±6.6% (n=8) of norepinephrine-induced pretone (300 nM). 10 μM IAA reversed HPV to dilatation with amplitude 67.6±7.1% (n=11) while relaxant hypoxia-induced responses in aorta were without changes and consisted 73.4±3.8% (n=8). Gap junction inhibitor, 18β-glycyrrhetinic acid (18β-GA, 30 μM), significantly decreased HPV amplitude to 7.6±5.8% (n=16) and led to inversion of hypoxia-induced aorta dilatation to constriction with amplitude 18.8±7.6% (n=10). After the treatment with 18β-GA selective glycolysis inhibition lost its paradoxical effect on HPV in MPA and hypoxia-induced dilatation in aorta (8.4±2.8% and 7.1±11.2%, respectively). It is likely that one yet unknown glycolysis-controlled mechanism in endothelial cells appears to be a common step for vascular contractility regulation under hypoxia, and that may underlie paradoxical responses of both pulmonary and systemic arterial smooth muscle to hypoxia. Our data suggest also that HPV may be due of depolarization of endothelial cells, which might be conducted from endothelium to smooth muscle cells via myoendothelial gap junctions.

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Estrogen receptor alpha mediates estradiol-stimulated endothelial prostacyclin production

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Clinical and experimental data support the consideration of endothelium as a target for estradiol and other estrogenic compounds. Estradiol acts in the endothelium to promote vasodilatation through release of several compounds, including synthesis of prostanoids, products of arachidonic acid metabolism. Two main prostanoids play an essential role in vascular physiology: thromboxane A2 (TXA2), which exhibits a proaggregant and vasoconstrictor profile, and prostacyclin (PGI2), a potent vasodilator. The different role of both types of estrogen receptors (ERα and ERβ) on the balance PGI2/TXA2 has not been studied. Our aim was to uncover whether estradiol enhances basal production of PGI2 or TXA2 in cultured human umbilical vein endothelial cells (HUVEC), to analyze the enzymatic mechanisms involved and to value the different role of both types of ER.

HUVEC were exposed to estradiol, selective ERα (PPT) or ERβ (DPN) agonists and antagonists (in specific: ICI182780; specific for ERβ: MPP) for 24 hours. PGI2 and TXA2 production was measured by ELISA. Production and expression of phospholipases, cyclooxygenases (COX-1 and COX-2), prostacyclin synthase and thromboxane synthase were analyzed by Western blot and quantitative RT-PCR.

Estradiol (1-100 nM) dose-dependent increased PGI2 production, up to 50 % (p < 0.001 vs. control), without affecting TXA2 production. COX-1 and prostacyclin synthase protein and gene expression were increased by 20 and 50 % respectively after exposure to estradiol (p < 0.001 vs. control), whereas COX-2, phospholipases and thromboxane synthase expression remained unaltered. All these effects were mediated through ERα, since were produced not only in the presence of estradiol but also in the presence of 10 nM PPT, and completely abolished in the presence of 1 μM MPP.

In conclusion, estradiol, acting through ERα, up-regulates COX-1 and prostacyclin synthase expression, thus directing prostanoid balance towards increased PGI2 production.

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Nitric oxide production and bioavailability in red blood cells from patients with Anorexia nervosa

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Anorexia nervosa (AN) is an eating disorder that leads to a marked loss of weight and important clinical complications. Nitric oxide (NO) is a gas involved in vascular homeostasis. Its synthesis occurs through the conversion of the semi-essential cationic amino acid L-arginine into L-citrulline and NO, by the action of the enzyme NO synthase (NOS). Arginase is an enzyme involved in urea cycle that competes with NOS by L-arginine. Bioavailability of NO depends not only on its production, but also on its degradation by reactive oxygen species (ROS).

The aim of this study was to investigate the NO production, urea cycle and oxidative stress markers as a possible indicative of bioavailability of NO in red blood cells from patients with AN. Eight female patients with AN from the Núcleo de Estudos e Saúde dos Adolescentes (NESA) / UERJ and eight age and sex-matched healthy volunteers participated in this study. The Pedro Ernesto Hospital Ethical Committee approved this study, and informed consent was obtained from each participant. Basal NOS activity was determined by the conversion of L-[3H]-arginine into L-[3H]-citrulline. Arginase activity in erythrocytes was determined by the conversion of L-[14C]-arginine to [14C]-urea. As an index of lipid peroxidation, the thiobarbituric acid-reactive substance formation (TBARS) was evaluated during an acid-heating reaction, as previously described by Draper et al.2. Superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation at 480 nm. Statistical significance was assessed using Student t-Test, with p <0.05.

The results showed a diminished NOS activity in patients with AN compared with controls (3.8 ± 0.4 vs. 8.2 ± 1.4 pmol/10⁶ cells/min; n=8). An increased activity of arginase was demonstrated in patients with AN in relation to controls (0.09 ± 0.02 vs. 0.12 ± 0.00 pmol urea/mg of protein/2h; n=8). An unchanged formation of TBARS (0.001 ± 0.000 vs. 0.003 ± 0.001 nMol/mg of protein; n=8) associated with elevated SOD activity (0.42 ± 0.07 vs. 0.12 ± 0.02 U/mg of protein; n=8) was found in AN patients in comparison to controls.

Our results demonstrated that in red blood cells from patients with AN the NO production is diminished and it can be in part attributed to an increased arginase activity. The activity of antioxidant enzyme SOD was elevated in AN while TBARS concentration was unaffected. The final correlation between oxidative stress and NO bioavailability needs to be further investigated.


Upregulation of Platelet L-Arginine-Nitric Oxide Pathway May Contribute to the Hypotensive Effect of Exercise Training

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Introduction
Previous studies realized by our group showed that a reduction in nitric oxide (NO) bioavailability is associated with a greater platelet activation in hypertension, which may contribute to the higher incidence of thrombotic events of this disease. Aerobic exercise cause important changes in vascular function, which contributes to blood pressure attenuation and hypertension outcomes. The aim of the present paper was to investigate the effects of aerobic training on L-arginine transport and NO synthase (NOS) activity, and also on lipid peroxidation in platelets of spontaneously hypertensive rats (SHR).

Methods
16 male SHR and 16 Wistar Kyoto rats (12 weeks old) were divided in two groups each (n=8): exercise (EX) and sedentary (SED). Exercise training was realized on a treadmill (5 d/wk; 60 min/d; velocity progressively increased up to 16 m/s) during 20 weeks. The animals were anesthetized with sodium thiopental (40 mg/kg) injected intraperitoneally and blood was collected from the abdominal aorta. Platelets were obtained by centrifugation and the following experiments were performed: L-arginine transport (by incubation with L-[3H]-arginine), NOS activity (by the conversion of L-[3H]-arginine in L-[3H]-citrulline, and lipid peroxidation (by the production of thiobarbituric acid reactive species, TBARS). SBP was measured weekly by tail cuff plethysmography. Data were compared with a one-way ANOVA, and significance level was set at 5%.

Results
SBP was significantly higher in SHR compared to WKY at the baseline (186 ± 8 vs. 132 ± 11 mm Hg). After 20 wk of training, SBP was significantly lower in SHREX (138 ± 8 mm Hg) than in SHREOX (214 ± 9 mm Hg) and SBP did not differ from WKY. There was a significant increase in platelet L-arginine transport (pmol L-arginine/10^9 cells/min) both in WKY (SED: 0.196 ± 0.054 vs. EX: 0.531 ± 0.052) and SHR (SED: 0.346 ± 0.076 vs. EX: 0.600 ± 0.049). NOS activity (pmol L-citrulline/10^8 cells) was significantly increased in SHREX (0.072 ± 0.007) compared to SHREOX (0.038 ± 0.007), but no changes were observed in WKY. Exercise did not affect and TBARS production in neither WKY nor SHR group.

Conclusion
These results confirm the importance of regular aerobic exercise as a non-pharmacological tool in hypertension management. An increase in NO bioavailability, through an upregulation of platelet L-arginine-nitric oxide pathway, may contribute to the hypotensive effect of aerobic exercise training in hypertensive animals.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Impaired activation of the Nrf2-Keap1 defence pathway in endothelial cells in gestational diabetes

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Gestational diabetes (GDM), a pregnancy associated disease affecting about 7% of pregnancies worldwide, is associated with an increased risk of type 2 diabetes and endothelial dysfunction in later life (Buchanan et al., 2005). Under physiological conditions, reactive oxygen species (ROS) serve as important signalling molecules (Autreaux et al., 2007), but an overproduction of ROS and/or impaired antioxidant gene expression will compromise cellular defences against oxidative stress (Gao & Mann, 2009). In the present study, we have examined the effects of advanced glycation end-products on expression of endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1), an antioxidant enzyme, regulated by the redox sensitive transcription factor NF-E2-related factor 2 (Nrf2) (Siow et al., 1999), in human umbilical vein endothelial cells (HUVEC) isolated from normal and GDM pregnancies.

HUVEC were cultured in M199 containing 20% FCS, and prior to experiments were deprived of serum (1% FCS) for 4 h and then challenged for 3-12h with AGE modified recombinant human serum albumin (AGE-HSA, 25-200μg/mL) or HSA. AGE-HSA (25 and 50μg/mL) increased eNOS expression 1.5-fold (arbitrary density: 25μg/mL: 0.37 ± 0.05 vs 0.61 ± 0.06, 50μg/mL: 0.36 ± 0.17 vs 0.62 ± 0.08, means ± S.E., n=3) in normal HUVEC (Fig. 1A). Notably, eNOS expression was similar in AGE-HSA or HSA-treated cells from GDM pregnancies. Although AGE-HSA also increased HO-1 expression after 6 h in normal HUVEC (Fig. 1B), HO-1 protein levels were not increased in endothelial cells from GDM pregnancies. As GDM is associated with impaired endothelial function, our findings provide insight into the molecular mechanisms underlying altered redox signalling in gestational diabetes.

Fig. 1. Effects of AGE-HSA on eNOS and HO-1 expression in fetal endothelial cells derived from normal pregnancies. Densitometric analysis of immunoblots three different HUVEC cultures. Values denote means ± S.E.M., ** p<0.01 (Student’s t-test)


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A calcium-dependent non-crossbridge stiffness in single intact skeletal muscle fibres from wild-type and transgenic MLC/mIGF1 mouse

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Previous studies on activated frog muscle fibres (1-3) showed the presence of a Ca2+-dependent non-crossbridge stiffness, called “static stiffness” (SS) which preceded tension generation following stimulation. This effect was attributed to a Ca2+-dependent stiffening of the titin filament. The experiments reported here were made to ascertain whether the SS was present also in mammalian muscle fibres from either wild-type (WT) or MLC/mIGF1 transgenic (TG) mice in which the local expression of the insulin like growth factor-1 (IGF1), under transcriptional control of Myosin Light Chain (MLC) promoter, induces muscle hypertrophy (4). Mice (3-6 month-old) were killed by rapid cervical dislocation, according to the EEC (Directive 86/609/EEC). Force and sarcomere length were measured with a fast force transducer (~50 kHz natural frequency) and a striation follower device. To reduce fibre damaging by the stretches, experiments were made on the tetanus rise at tension (P0) of about 0.5 the plateau tension. All the force values reported here are normalized for P0. The rupture force of the crossbridge ensemble, the critical force Pc and the sarcomere elongation at Pc (critical length, Lc) were measured. Before the release P∞ was 3.60 ± 0.15 (n=8) times P0. Critical length Lc was 12.02 ± 0.45 nm hs−1. In contrast with previous data on the tetanus rise (Bagni et al. 2005) during the quick force recovery P∞ was almost constant independently of the tension P∞ developed by the fibre at the time of the stretch. Lc increased immediately after the step release (0.2 ms delay) to 16.24 ± 0.75 nm hs−1 and remained elevated up to at the end of the phase 2 of the recovery (2 ms delay) when it started to decrease returning to the isometric value in about 20 ms. The ratio (P∞-P∞)/L∞, which represents the chord stiffness of the half-sarcomere, started to decrease progressively after the release and reached a maximum reduction of 27 ± 5 % at 2 ms delay. The return to the pre-release value occurred in about 20 ms. Data analysis suggested that: 1) crossbridge population remained almost constant during the quick force recovery; 2) the forced rupture of the actomyosin bond produced by the stretches is preceded by the reversal of the power stroke; 3) the average myosin lever arm movement during the power stroke can be measured by the changes of L∞; 4) after the execution of the

Reversal of the myosin power stroke induced by fast stretching of intact frog skeletal muscle fibres

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The mechanical properties of the actomyosin bond were investigated by applying to activated frog muscle fibres, fast stretches (~25 nm hs−1 amplitude and ~350 µs duration) which forcibly detached the crossbridge ensemble. Stretches were applied before and up to 20 ms after a conditioning step release (~4 nm hs−1 amplitude, 120 µs duration) during the time course of the quick force recovery. Experiments were made at 5±C. Frogs (Rana esculenta) were killed by decapitation followed by destruction of the spinal cord, according to the official regulation of the European Community Council (Directive 86/609/EEC). Force and sarcomere length were measured with a fast force transducer (~50 kHz natural frequency) and a striation follower device. To reduce fibre damaging by the stretches, experiments were made on the tetanus rise at tension (P∞) of about 0.5 the plateau tension. All the force values reported here are normalized for P∞. The rupture force of the crossbridge ensemble, the critical force P∞ and the sarcomere elongation at P∞ (critical length, L∞) were measured. Before the release P∞ was 3.60 ± 0.15 (n=8) times P0. Critical length L∞ was 12.02 ± 0.45 nm hs−1. In contrast with previous data on the tetanus rise (Bagni et al. 2005) during the quick force recovery P∞ was almost constant independently of the tension P∞ developed by the fibre at the time of the stretch. L∞ increased immediately after the step release (0.2 ms delay) to 16.24 ± 0.75 nm hs−1 and remained elevated up to at the end of the phase 2 of the recovery (2 ms delay) when it started to decrease returning to the isometric value in about 20 ms. The ratio (P∞-P∞)/L∞, which represents the chord stiffness of the half-sarcomere, started to decrease progressively after the release and reached a maximum reduction of 27 ± 5 % at 2 ms delay. The return to the pre-release value occurred in about 20 ms. Data analysis suggested that: 1) crossbridge population remained almost constant during the quick force recovery; 2) the forced rupture of the actomyosin bond produced by the stretches is preceded by the reversal of the power stroke; 3) the average myosin lever arm movement during the power stroke can be measured by the changes of L∞; 4) after the execution of the
power stroke, myosin heads are progressively detached and substituted by new heads attached with the pre-power stroke configuration. The complete substitution occurs in a relatively short time of 20 ms.


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The effects of peroxynitrite on rat sternohyoid muscle force

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Chronic intermittent hypoxia (CIH) is a central feature of the debilitating disorder—obstructive sleep apnoea. CIH has been shown to impair upper airway respiratory muscle function and perhaps due to oxidative and/or nitrosative stress. Peroxynitrite is both an oxidising and nitrating agent formed in vivo from the reaction of superoxide and nitric oxide. Peroxynitrite may play an important role in modulating skeletal muscle function. Studies have suggested that peroxynitrite is capable of producing diaphragmatic contractile dysfunction. We tested the hypothesis that peroxynitrite inhibits sternohyoid muscle contractile force in vitro. Earlier observations have suggested that muscle contraction accelerates the effect of exogenous oxidants due to the additive effect of oxidants produced from endogenous sources and therefore we also examined whether increased muscle contraction would accelerate and amplify the effect of peroxynitrite.

Adult male Wistar rats were anaesthetised with 5% isoflurane and killed by spinal transection. Longitudinal strips from the sternohyoid (upper airway dilator) muscle were mounted isometrically in water-jacketed tissue baths at 27∞C and either bubbled with a hyperoxic (95% O₂/5% CO₂) or anoxic (95% N₂/5% CO₂) gas mixture. Strips were set to optimal length and force-frequency relationship was assessed by stimulating the muscle at a frequency of 10, 50 and 100 Hz at 0, 30, 60 and 90 minutes. In a separate series of experiments, the force-frequency relationship was assessed every 10 minutes for two hours. Studies were conducted under paired conditions i.e. control vs. peroxynitrite (500mM)-treated muscle strips.

Peroxynitrite had no effect on muscle function under hyperoxic (7.6±1.1 vs. 6.5±1.8 N/cm², mean±SEM, control vs. peroxynitrite at 90 min, P>0.05, Student’s paired t test, n=9) or hypoxic conditions (6.6±0.9 vs. 6.8±0.8 N/cm², control vs. peroxynitrite at 90 min, P>0.05, n=9). Increased muscle activation i.e. tetanic contractions every 10 minutes did not unmask a peroxynitrite effect under hyperoxic (9.2±1.4 vs. 7.6±1.5 N/cm², control vs. peroxynitrite at 120 min, P>0.05, n=6) or hypoxic conditions (1.5±0.3 vs. 1.4±0.2 N/cm², control vs. peroxynitrite at 120 min, P>0.05, n=6). Preliminary studies using ebselen, a peroxynitrite scavenger, showed no improvement in hypoxia-induced muscle dysfunction. This suggests that endogenous peroxynitrite is not a major contributor to hypoxia-induced muscle dysfunction.

In conclusion, peroxynitrite had no effect on sternohyoid muscle contractile force under hyperoxic or hypoxic conditions. A peroxynitrite-induced effect was not uncovered with increased muscle activation. This study suggests that upper airway mus-
DGE is a recently developed alternative to microarray gene expression profiling, currently the main platform used for global transcriptomic investigations. In contrast to microarray technology which is limited to the hybridisation of cDNA to probes printed on the array platform, DGE is not dependent on currently available sequence and thus provides a global, hypothesis free quantification of the transcriptome. The use of this technique will lead to a greater understanding of the molecular networks which control cellular function relating to muscle physiology in the horse.

Following the sequencing of the equine genome (EquCab2.0) in silico studies. Digital Gene Expression (DGE, Illumina) profiling was used to characterize the assembly of genes expressed in equine skeletal muscle and to identify the subset of genes which were differentially expressed following a ten month period of exercise conditioning. DGE is a recently developed alternative to microarray gene expression profiling, currently the main platform used for global transcriptomic investigations. In contrast to microarray technology which is limited to the hybridisation of cDNA to probes printed on the array platform, DGE is not dependent on currently available sequence and thus provides a global, hypothesis free quantitative analysis of the transcriptome. The use of this technique will lead to a greater understanding of the molecular networks that control cellular function relating to muscle physiology in the horse.

The study cohort comprised seven Thoroughbred racehorses from a single training yard with similar dietary and training management. The subjects undertook a regular exercise regime for ten months which consisted of 15-30 minute walk, followed by 1,000 m trot and 2,000 m canter once a day six times a week on an all-weather gallop as well as intermittent periods of higher intensity exercise (“work”) no more than once a week which consisted of 15-30 minute walk followed by 400 m trot, 500 m canter and 1,000 m gallop (sprint). Exercise physiological data (heart rate, velocity, plasma lactate concentrations etc.) were recorded throughout the conditioning period. Skeletal muscle biopsies were collected at rest from the gluteus medius following desensitisation of the site with 10 ml lidocaine (20mg/ml) in the same individuals at two time points: T1 - unconditioned, (9±0.5 months old) and T2 - conditioned (20±0.7 months old). RNA was isolated using the Trizol method, cDNA libraries were produced using the Illumina DGE sample preparation kit and directly sequenced using the Illumina Genome Analyser. This resulted in an average of 6.9 million tags per sample. Initial processing and alignment of tags to EquCab2.0 was carried out using the ELAND global alignment strategy (Illumina). Subsequent annotation of tags and statistical analyses were carried out using a custom written programme in the R-language (R Development Core Team, 2004). The online tool DAVID (Hosack et al. 2003) will be use for functional clustering and overrepresentation analyses of differentially expressed genes to identify gene pathways relevant to exercise. Results will be confirmed using real-time quantitative RT-PCR. This study will be the first to characterize global mRNA expression profiles in equine skeletal muscle following a period of exercise conditioning.

Digital gene expression profiling of the Thoroughbred horse skeletal muscle transcriptome following exercise conditioning

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The effect of vitamin E on skeletal muscle mass in intact and ovariectomized female rats

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The aim of this study was to determine the effects of vitamin E deficiency and supplementation on skeletal muscle mass in intact and ovariectomized female rats. One hundred and twenty 3-month-old female Wistar rats were used in this study. Half of the rats were ovariectomized (OVX) while the other half were left intact (sham operated). For ovariectomy, the rats were anaesthetized with Ketapex (100mg/ml) and Xylazil (20mg/ml) [combined in a mixture of 1:1 (v:v)] through intraperitoneal administration (0.25ml/100g body weight). The OVX rats were rested for two weeks for the wounds to recover. Intact and OVX rats were further divided into 6 groups and given different dietary treatments i.e. vitamin E deficient diets (VED, 75%VED, 50%VED, 25%VED), conventional rat chow diet (RC) and conventional rat chow diet with oral supplementation of 30mg/kg body weight of alpha-tocopherol vitamin E (RC+ATF). After 15 weeks, skeletal muscle mass, which is represented by lean soft tissue mass (fat-free mass), was measured using the DEXA (Dual Energy X-ray Absorbtionmetry) technique. We found that there was significant increase in skeletal muscle mass within all the individual groups of intact rats after 15 weeks of dietary manipulation [paired t-test: VED (P<0.05); 75%VED (P<0.001); 50%VED (P<0.001); 25%VED (P<0.01); RC (P<0.01); RC+ATF (P<0.001)]. However, when the after-treatment values of skeletal muscle mass of the various intact groups were compared, no significant difference was found. For OVX rats, there was also significant increase in skeletal muscle mass within all the individual groups after 15 weeks of dietary treatment [paired
resulting in an increased sensitivity to stress (1). Neonatal LPS (nLPS) exposure also causes long-term sensitization of the hypothalamo-pituitary-gonadal (HPG) axis to the inhibitory influences of stress in adulthood (2). Further, we have shown that nLPS results in delayed puberty and a concomitant decrease in kisspeptin (Kiss1) mRNA expression within the medial preoptic area (mPOA) in the female rat.

Whilst the mechanisms underlying the actions of nLPS on pubertal development remain unknown, it is possible that the stress neuroptide, corticotrophin-releasing factor (CRF), may play a role. The aim of the present study is to examine the role of CRF in both nLPS delayed puberty and normal pubertal development.

All surgical procedures were undertaken using ketamine (100 mg/kg i.p.) and Rompun (10 mg/kg i.p.) anaesthesia. Sprague-Dawley pups received either LPS (50 μg/kg, i.p.) or saline (0.05 ml) on postnatal day 3 and 5 (2), or had no neonatal treatments. At postnatal day 28, rats were implanted with intracerebroventricular (icv) cannulae connected to an osmotic mini-pump. The neonatal saline (nSAL) or nLPS treated rats received either a CRF receptor antagonist astressin (4nmol/day) or saline (0.2nmol/day or 0.4 nmol/day CRF (n=3-7 per group). Com-

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC138**

**Effect of growth hormone and insulin-like growth factor-I administration on atrogin-1 and MuRF-1 gene expression in control and arthritic rats**

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Adjuvant-induced arthritis is an animal model of chronic inflammation and rheumatoid arthritis. Chronic arthritis induces cachexia associated with an inhibition of the growth hormone (GH)-insulin-like growth factor-I (IGF-I) system together with an activation of the E3 ubiquitin ligase enzymes muscle atrophy F-box (atrogin-1) and muscle Ring finger 1 (MuRF-1) in the skeletal muscle. Muscle wasting is believed to accelerate morbidity and mortality in rheumatoid arthritis. The aim of this study was to analyze the effect of GH and IGF-I administration on E3 ubiquitin ligase system in the gastrocnemius muscle of arthritic rats. Arthritis was induced in adult male Wistar rats by an intradermal injection in the sole of the right paw of 1 mg of complete Freund’s adjuvant. Fifteen days after adjuvant’s injection, 300 μg/kg of GH or 200 μg/kg of IGF-I were administrated by subcutaneous injection 18 and 3 hours before being humanly killed. Arthritis induces anorexia, for that reason we included a pair-fed group to discard a possible effect of decreased food intake. Gene expression of IGF-I and GH receptor in liver and ubiquitin ligase enzymes atrogin-1 and MuRF-1 in the skeletal muscle were quantified using RT-PCR. Serum IGF-I was measured by radioimmunoassay (RIA). Arthritis decreased IGF-I and GH receptor gene expression in liver (P<0.05), as well as circulating IGF-I levels (P<0.05). In skeletal muscle, arthritis increased atrogin-1 and MuRF-1 gene expression (P<0.01). These effects are not the result of anorexia, since in pair-fed group the expression of those genes was not modified. Exogenous GH administration increased liver IGF-I mRNA and serum IGF-I levels (P<0.05). In control, but not in arthritic rats, GH and IGF-I administration decreased atrogin-1 (P<0.05) and MuRF-1 gene expression (P<0.05). However, neither GH nor IGF-I administration were able to prevent arthritis-induced increase in atrogin-1 and MuRF-1 gene expression in the gastrocnemius muscle. Although the GH-IGF-I system has an inhibitory effect on ubiquitin ligase enzymes, by decreasing atrogin-1 and MuRF-1 gene expression, in arthritis the GH or IGF administration was not able to reduce the expression of these enzymes in the skeletal muscle.

This work was supported by CYCYT (BFU2006-11899), a grant to M. Lopez-Menduiña (Ministerio de Educacion y Ciencia, BES-2007-16001) and a grant to E.Castillero (Gobierno Vasco,BFI06.31).

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**PC139**

**The role of corticotrophin-releasing factor in the timing of puberty in the female rat**

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Neonatal exposure to lipopolysaccharide (LPS) programs long-term changes in hypothalamo-pituitary-adrenocortical (HPA) axis activity, resulting in an increased sensitivity to stress (1). Neonatal LPS (nLPS) exposure also causes long-term sensitization of the hypothalamo-pituitary-gonadal (HPG) axis to the inhibitory influences of stress in adulthood (2). Further, we have shown that nLPS results in delayed puberty and a concomitant decrease in kisspeptin (Kiss1) mRNA expression within the medial preoptic area (mPOA) in the female rat.

Whilst the mechanisms underlying the actions of nLPS on pubertal development remain unknown, it is possible that the stress neuroptide, corticotrophin-releasing factor (CRF), may play a role. The aim of the present study is to examine the role of CRF in both nLPS delayed puberty and normal pubertal development.

All surgical procedures were undertaken using ketamine (100 mg/kg i.p.) and Rompun (10 mg/kg i.p.) anaesthesia. Sprague-Dawley pups received either LPS (50 μg/kg, i.p.) or saline (0.05 ml) on postnatal day 3 and 5 (2), or had no neonatal treatments. At postnatal day 28, rats were implanted with intracerebroventricular (icv) cannulae connected to an osmotic mini-pump. The neonatal saline (nSAL) or nLPS treated rats received either a CRF receptor antagonist astressin (4nmol/day) or artificial cerebrospinal fluid (aCSF) (n=4-7 per group). The neonatal none-treated rats were distributed between four experimental groups; no surgery control, aCSF (0.5μl/day), 0.2nmol/day or 0.4 nmol/day CRF (n=3-7 per group). Com-
Nitric oxide reduces angiotensin-II-induced vasopressin and oxytocin mRNA expression in hypothalamic paraventricular and supra-optic nuclei


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We have shown that inhibition of nitric oxide synthase (NOS) increases hormonal secretion of vasopressin (VP) and oxytocin (OT) and that nitric oxide (NO) donor reduces plasma concentrations of VP and OT in response to central stimulation of angiotensin-II (ANGII). In the present study we analyzed in hypothalamic paraventricular (PVN) and supra-optic (SON) nuclei the time course effect of central ANGII stimulation on mRNA expression of VP, OT and NOS by Real Time PCR. We also evaluated the influence of NO donor or inhibitor in these mRNA expressions. Wistar male rats (n=5-6), anaesthetized with 2.5% tribromoethanol (1ml/100g bw, ip) with a stainless cannula placed into the right lateral ventricle were injected with ANGII (50ng) and 30, 60, 90, 120 and 240 min later, the animals were decapitated and the PVN and SON nuclei microdissected. Another group of rats received, 20 min before ANGII, a central pretreatment with NO inhibitor (Nw-Nitro-L-arginine methyl ester, LNAME 250μg) or NO donor (S-Nitroso-N-acetylpenicillamine, SNAP 5μg) and PVN and SON were collected 60 min after ANGII stimulation. The results are reported as mean±SEM and the data were analyzed by t student test (signif. P<0.05). Values obtained from control animals were 1.0±0.2 arbitrary units (au). ANGII induced an increase in VP mRNA expression in both PVN and SON at 30, 60, 90 and 120 min after stimulation (peak at 60min: PVN 2.7±0.3, SON 2.2±0.1 au). An increase in OT mRNA expression was observed only at 30 and 60 min after ANGII (peak at 60min: PVN 2.2±0.3, SON 1.9±0.2 au). NOS expression after ANGII stimulation was increased at 30 and 60 min in the PVN and at all experimental periods in the SON (peak at 60min: PVN 1.6±0.1, SON 2.1±0.2 au). Therefore, the highest VP, OT and NOS mRNA expression induced by central ANGII occurred at 60 min after the stimulation. Animals submitted to injection of LNAME alone showed an increase in VP (PVN 2.4±0.2, SON 1.9±0.2 au) and OT (PVN 2.4±0.1, SON 2.1±0.3 au) mRNA expression, but a slightly reduction in NOS expression (PVN 0.8±0.1, SON 0.8±0.1 au). Pretreatment with LNAME did not change ANGII induced VP and OT expression, but it reduced NOS mRNA expression (PVN 1.1±0.1, SON 1.1±0.1 au). In ANGII stimulated rats, pretreatment with SNAP decreased the VP (PVN 1.7±0.1, SON 1.4±0.1 au) and OT (PVN 1.5±0.1, SON 1.5±0.1 au) mRNA expression, without effects on NOS expression. In conclusion, collectively with our previous results, the present data indicate that central angiotensinergic system regulates VP, OT and NOS expression and NO reduces VP and OT mRNA expression responses to ANGII, suggesting an inhibitory modulation of NO in the control of neurohypophyseal hormones synthesis and release.

Technical Assistance: Maria Valci A dos Santos, Marina Holanda and Milene M Lopes. Financial Support: FAPESP, CNPq and Capes.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Effect of growth hormone (GH) on the inflammatory process in pancreas obtained from old male senescence prone mice

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To investigate the effect of aging on various physiological parameters related to inflammation in the pancreas obtained from male, old and young senescence prone (SAMP8) mice and the influence of growth hormone (GH) treatment on the same parameters in the old group. Nineteen animals were used, divided into three groups: old (10 months), young (2 months), old GH-treated sc with 2mg/kg GH. All animals were treated for one month with GH or vehicle. The group of young animals was used as control. The expression of interleukin 4 (IL4), interleukin 10 (IL10), Interleukin 1β (IL1β), Interleukin 2 (IL2), Interleukin 6 (IL6), Tumor Necrosis Factor α (TNFα) and Monocyte chemoattractant protein 1 (MCP1) was determined by specific ELISA methods in homogenates of pancreas.
Anti-inflammatory cytokines like IL10 showed a significant decrease in older individuals whereas IL4 did not show differences with young mice. Aging significantly increased the levels of pro-inflammatory cytokines like IL1β, IL2, IL6, TNFα. But no differences in MCP1 were observed. Administration of GH to old male mice significantly increased the levels of IL4 and IL10. GH treatment reduced also significantly IL1β, IL2, IL6, TNFα and MCP1.

Aging leads to an increase of inflammatory processes in the pancreas. The administrations of sustitutive hormonal therapy with GH inhibited the production of pro-inflammatory cytokines and increased anti-inflammatory cytokine production.

This work was supported by grants: RETICEF RD 06/0013 (FIS) and SAF 2007, 66878-C02-01.

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PC142

The effect of synthetic analogues of the phyto-oestrogen 8-prenylnaringenin on tail skin temperature in a rat hot flush model

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Hot flushes are a distressing symptom of the menopausal syndrome affecting over 75% of women, many of whom seek medical treatment because of the severity of the symptoms. There has been growing interest in the use of phyto-oestrogens as alternative therapies for hot flushes due to recent reports highlighting adverse effects of oestrogen therapy. A potent oestrogenic compound in hops (Humulus lupulus) has been identified as 8-prenylnaringenin (8-PN) and hop extracts containing 8-PN have been used for treating menopausal symptoms, including hot flushes. Synthetic analogues of 8-PN, 8-neopentlnaringenin and 8-n-heptylnaringenin, have been demonstrated to be selective oestrogen receptor modulators in vitro. We have investigated the in vivo oestrogenic activity of these compounds using a rat model of the hot flush phenomenon in which the tail skin temperature (TST) is increased after oestrogen deficiency induced by ovariectomy.

Female Wistar rats were ovariectomized and implanted with chronic telemetry devices (TA10TA-F40 W/TP; Data Sciences International) under general anaesthesia induced by ketamine hydrochloride (100mg/kg) co-administered with medetomidine (0.5mg/kg) ip. On completion of surgery, anaesthesia was reversed by atipamezole (1mg/kg) sc. After a two week recovery period TST was monitored for 7 sec every 5 min throughout the experimental period. Following a three day monitoring period to establish baseline temperatures, 17β-oestradiol (E2), vehicle, 8-neopentlnaringenin or 8-n-heptylnaringenin were administered subcutaneously daily for five days. Administration of E2 (4μg/kg) significantly reduced the elevated TST on the fifth and final day of injection by 1.89 ± 0.14oC from the baseline TST (paired Students t-test, P<0.0002; n=6). In contrast, 8-n-heptylnaringenin (20mg/kg), a weak ERα agonist and strong ERβ antagonist, did not significantly reduce the increased TST when administered alone and failed to block the E2 induced decrease in TST when co-administered with E2. These results indicate that analogues of 8PN are effective in the rat model of hot flushes and deserve further investigation.

This work was supported by the BBSRC.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC143

Developing a Mathematical Model of Insulin release from Pancreatic β-cells: The Importance of Glutamate

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Pancreatic β-cells play a key role in the glucose homeostasis, secreting insulin in response to blood nutrient fluctuations. This is due to a complex relationship between metabolism and insulin secretion which comprises both triggering (ATP and Ca2+ dependent [1]) and amplifying (mitochondrial metabolite dependent [2]) pathways of insulin secretion. This study has attempted to develop a mathematical model of insulin release from pancreatic β-cells with particular regard to the role of glutamate. The model’s input is made up of three nutrient components: glucose, alanine [3] and glutamine [4] while the output of the model is represented by insulin secretion.

A systems biology approach was employed to integrate biological experimental work with mathematical tools to gain novel insights about the role played by glutamate in insulin secretion.

A simplified kinetic model of the glucose-stimulated insulin secretion in pancreatic β-cells which takes into account glycolysis, Krebs cycle, alanine uptake and glutamine-glutamate metabolism was built [5]. Experimental work was carried out on a functional clonal insulin-secreting cell line (BRIN-BD11) to validate the model. Cell viability and death assays were performed following incubation in various concentrations of nutrients. The utilization and production of the major components of the mathematical model include: glucose, alanine, glutamine, pyruvate, lactate, glutamate, ATP/ADP ratio and insulin. All the determinations described above were performed following BRIN-BD11 incubation in the presence of 10 mM of alanine and various concentrations of glucose (5, 16.7, 30 mM) and glutamine (0, 1, 2 mM). A quantitatively accurate description of the oscillating behaviour of pyruvate and others metabolites of the Krebs cycle and ATP was obtained. ATP, ADP, pyruvate and glu-
cose-6-phosphate concentrations were associated with coordinated oscillations.

Differences in the oscillating features were found when values obtained by experimental procedures were used since desensitization to glucose induced by 24hr incubation in high glucose (30mM) resulted in reduced oscillations of the metabolites and lower insulin secretion.

Cell viability was not altered by incubation in various nutrient combinations (maximum difference 12.1%).

Insulin secretion was biphasic in the presence of glucose and alanine (AUC value: 126 ± 12 and 117 ± 14 µg/mg protein during the 1st and 2nd phase respectively) but was not affected by acute (1–20 min) glutamine stimulation, indicating that only a small amount of glutamine-derived glutamate is converted into α-ketoglutarate in glucose stimulatory conditions. It is likely that changes in glutamate and α-ketoglutarate production represent a more important coupling pathway when low concentrations (below 5mM) of extracellular glucose are present.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC144**

**Impact of reactive oxygen species on renal function in anaesthetised rats**

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Reactive oxygen species (ROS) are a product of mitochondrial respiration undertaken by a number of enzymes such as xanthine oxidase and NADPH oxidase. Although the level of one ROS, superoxide anions, is limited by the enzyme superoxide dismutase (SOD), it can affect physiological processes. There is in vitro evidence (Gavin et al) that superoxide ions act directly to suppress epithelial transport along the nephron. The aim of this study was to examine in vivo the impact of either increasing the level of SOD, or following inhibition of its action on renal haemodynamic and excretory function.

Male Wistar rats (250-320g) were anaesthetised with 1ml i.p. chloralose/urethane (16.5/250 mg/ml) and cannulae were inserted into the right femoral artery, to measure mean arterial pressure (MAP), and vein to infused saline (9g/lNaCl) at 3ml/h. The left kidney was exposed via the flank, its ureter cannulated and an ultrasonic flowprobe fitted to the renal artery. A small cannula was inserted into the cortex for 4.5 ml to lie at the cortico-medullary border for the infusion of drugs (1.0 ml/l intrareally, i.r.). At the end of surgery, the i.v. saline was replaced with saline plus inulin (2g/l) for measurement of glomerular filtration rate (GFR) and the animals were allowed 1-2 h to recover. A 30 min basal urine collection was taken and thereafter an i.v. infusion of either tempol (a SOD mimetic) at 30 µmmol/kg/min, or diethyldithiocarbamate (DETc) at 2mg/kg/min was begun. After 30 min, a further 30 min urine collection was taken, the i.r. infusion was stopped and a 30 min recovery clearance was taken. Data, mean±S.E.M. were taken as significant when P<0.05 (ANOVA).

Infusion of tempol i.r. (n=5) had no effect on MAP, at 949± mmHg and was unchanged in the recovery period. Under these conditions there was a rise (P<0.05) in GFR, from 7.6±2.8 ml/min/kg, of 100% which was accompanied by increase in both urine flow, from 37.61±1.2 to 79.4±24.5 ml/min/kg, and absolute sodium excretion, from 2.6±0.9 to 8.22±3 µmol/kg/min (both P<0.05). Administration of DETc (n=6) was without effect on MAP but caused decreases in GFR, of some 40%, urine flow of some 30% and sodium excretion of 50% (all P<0.05).

These findings show that scavenging of superoxide anions within the kidney results in a marked increase in glomerular filtration and excretion of fluid and conversely blockade of SOD reduces filtration and fluid excretion. The changes in glomerular filtered load is most likely responsible for the changes in fluid excretion but this remains to be resolved.


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**PC145**

**Effect of cystine dimethylester lysosomal loading on the viability of LLC-PK₁ cells**

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**Background.** Cystinosis is an autosomal recessive disorder characterized by excessive accumulation of cystine in the lysosomes of the cell. The disease is caused by a defective cysteine transporter, cystinosin, in the lysosomal membrane which mediates cystine efflux from the lysosome to the cytosol (1). Cystinosis affects almost all cells and tissues but the kidney involvement remains the foremost clinical characteristic of the disorder which is manifested clinically by a generalized dysfunction of proximal tubular transport of glucose, amino acids, phosphate...
and essential ions resulting to Fanconi’s syndrome (2). Initial investigations on the pathogenesis of cystinosis were hampered by inability to achieve elevated intracellular concentrations of cystine. This hurdle was eventually solved when it was demonstrated that the methyl ester derivative of cystine leads to the accumulation of cystine in the lysosomes in vitro and in vivo (3,4).

**Aim.** In this study, we investigated the effect of cystine dimethyl ester (CDME) on the viability of LLC-PK₁ cells, an epithelial cell line originally derived from porcine kidneys and expresses many functions and characteristics of the proximal tubular cells.

**Methods.** A colorimetric assay based on the reduction of tetrazolium salt WST-1 into a colored formazan dye by succinate-tetrazolium reductase in viable cells was used as a measure of the net metabolic activity of cells following incubations at different concentrations of CDME (0.1 to 1 mM).

**Results.** Incubation of LLC-PK₁ cells with low CDME concentrations (0.1 and 0.2mM) for 60 and 120 minutes resulted in an increase in WST-1 absorbance associated with enhanced mitochondrial metabolism, which may be related to higher demand for ATP (139% of control at 0.1 mM CDME for 60 minutes to 372% of control at 0.2 mM CDME for 120 minutes, p<0.05). However, the viability of the cells subsequently dropped to less than 15% of control (p<0.05) upon incubation with higher concentration of CDME (1mM) for 30, 60 and 120 minutes, indicating the acute lethal effect of CDME at high concentration. It can be speculated that exposure of non-cystinotic cells to different concentrations of CDME (0.1 to 1 mM).

**Poster Communications**

**PC146**

**A urine concentrating defect in 11β-Hydroxysteroid Dehydrogenase Type 2 knockout mice**

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11β-Hydroxysteroid Dehydrogenase Type 2 (11βHSD2), which inactivates cortisol, is co-expressed with mineralocorticoid receptors (MR) in aldosterone target tissues where it protects the receptor from activation by glucocorticoids. Mutations in 11βHSD2 cause the syndrome of apparent mineralocorticoid excess in which hypertension is thought to be driven by volume expansion secondary to sodium retention[1]. 11βHSD2⁺/⁻ mice are also hypertensive but volume contracted[2]. This uncoupling of sodium and water reabsorption suggests a urine concentrating defect. We therefore assessed water balance and the effect on urine concentration of a water deprivation challenge. Male C57/Bl and 11βHSD2⁻/⁻ mice (n=5 per group) were individually housed in metabolic cages. After acclimatization, cumulative water balance was taken over 4 consecutive days, after which drinking water was removed for 24h. Following 1 week recovery, mice were injected with desmopressin (ddAVP; 1μg/kg; S.C.) and water removed for 24h. At the end of the experiment mice were culled by decapitation. Data are presented as mean±S.E.M. Statistical analysis was performed using ANOVA, Mann-Whitney or t-tests as appropriate.

Water turnover during the baseline period was higher in the 11βHSD2⁻/⁻ mice than in controls, with the knockouts being both polyuric (12.2±0.6 vs 2.1±0.1 ml/24hr P<0.001) and polydipsic (15.9±0.6 vs 5.2±0.2 ml/24hr P<0.001). Despite this cumulative water balance was not significantly different between the 2 groups. Urine osmolarity (Uosm) was significantly lower in the 11βHSD2⁻/⁻ than controls (446±17 vs 1658±69 mOsm P<0.001). Urine flow rate fell in both groups following water deprivation, but 11βHSD2⁻/⁻ mice continued to produce significantly more urine (1.7±0.1 vs 0.8±0.2 ml/24hr P<0.01). Water deprivation caused an increase in Uosm in both groups, however the increase was significantly blunted in the 11βHSD2⁻/⁻ mice (1892±109 vs 4888±509 P<0.001). In both groups of mice, ddAVP had no further effect on urine concentration following water deprivation. This suggested the maximal response to endogenous AVP had been achieved: notably the response was significantly lower in 11βHSD2⁻/⁻ mice (2246±243 vs 4318±202 mOsm P<0.001). Weight loss during water deprivation, used as an index of body water, was significantly greater in 11βHSD2⁻/⁻ than the controls (3.0±0.1 vs 1.8±0.2 g P<0.001).

In conclusion we have identified a urine concentrating defect in 11βHSD2⁻/⁻ mice, which may contribute to the contraction of plasma volume. Although 11βHSD2⁻/⁻ mice retain a residual concentrating capacity, the maximum value is blunted relative to controls. We cannot exclude a defect in AVP release but the failure of ddAVP to restore urine concentrating ability suggests a nephrogenic component to the diabetes insipidus.


I would like to acknowledge Gillian Brooker for her technical assistance.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Impact of Angiotensin 1-7 (Ang1-7) on renal haemodynamic and excretory function anaesthetised normotensive and hypertensive rats

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Increasingly, it is perceived that Ang 1-7 has significant physiological actions at the vascular and tubular levels of the kidney which are generally opposite to those of angiotensin II (AngII). Recent reports have shown Ang1-7 to cause a renal vasodilation and a diuresis and natriuresis and that these actions were enhanced in transgenic and renovascular models of hypertension (Burgelova et al, 2005) and concluded that the response to the peptide was related to the degree of activation of the renin-angiotensin system. This study tested the hypothesis that the renal haemodynamic and excretory responses to Ang 1-7 would be greater in the spontaneously hypertensive rat (SHR) than normotensive rats.

Male Wistar and SHR (300-350 g) were anaesthetised with 1 ml i.p. chloralose/urethane (16.5/250mg/ml) and cannulae placed in a femoral artery to measure blood pressure and in the renal vein for the infusion of saline (9g/l NaCl) at 3 ml/h. The left kidney was exposed, its ureter cannulated, an ultrasonic flow probe placed on the renal artery and a small cannula inserted 4.5 mm into the kidney for infusion of saline/vehicle or Ang1-7 at 1.0ml/h intra-renal(i.r.) at the cortico-medullary boarder. Following a 1-2 h recovery, four 20 min clearances were taken, 2 before and 2 during the intrarenal infusion of saline or Ang1-7 (3x10-7 M). Data, means ± S.E.M, were deemed significant when P<0.05 (Wilcoxon Signed Rank Test).

Infusion of saline i.r. in Wistar rats (n=5) had no effect on blood pressure (BP), renal blood flow (RBF), glomerular filtration rate (GFR), urine flow (UV), absolute (UNaV) or fractional sodium excretion (FENa). In the Wistar group in which Ang1-7 was infused i.r. (n=5), BP, at 92±5 mmHg, RBF, at 1.66±0.16 ml/min and GFR, at 2.71±0.21 ml/min/kg, were unchanged but UV, at 19.7±3.8 μl/min/kg, UNaV, at 0.29±0.11 μmol/min/kg, and FENa, at 0.69±0.27%, were increased (both P<0.05) by 2 to 2.5 fold. Infusion of Ang1-7 i.r. in the SHR (n=7) had no effect on either BP or RBF but increased UV, UNaV and FENa by between 2 and 3 fold (all P<0.05), respectively, which were responses very similar to those obtained in the Wistar rats. These findings demonstrated that infusion of Ang1-7 locally into the kidney at this dose, had minimal effects on renal haemodynamics but caused a marked increase in both water and sodium excretion. This would suggest that the peptide was having a direct action on tubular reabsorptive processes. Surprisingly, the magnitude of the excretory responses in the SHR were similar to those of the Wistar and did not support the proposed hypothesis. One possible reason may be due to the relatively normal levels of plasma renin activity reported to be present in the SHR (Bivol et al, 2007).


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

ATP-mediated regulation of vasa recta diameter by pericytes in the renal medulla

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ATP-activated P2 receptors are expressed throughout the kidney in vascular smooth muscle, endothelial and tubular epithelial cells. Functional studies demonstrate that the renal vasculature is highly responsive to P2 receptor activation, and in vivo studies in rabbits have shown that the ATP analogues α, β-methylene ATP reduces both cortical and medullary blood flow [1]. In the renal medulla, blood flows via the vasa recta which are devoid of contractile smooth muscle. In vitro studies looking at isolated descending vasa recta have however shown that smooth muscle-like pericyte cells occur at regular intervals. Furthermore, it has been shown that these pericytes can contract isolated descending vasa recta in the presence of certain vasoactive substances [2]. In addition to studies on isolated capillaries, pericytes have been shown to control capillary diameter in situ, in whole retina and in the CNS [3]. In the retina pericytes have been shown to respond to ATP [3, 4]. Here we aim to (i) investigate whether extracellular ATP and UTP constrict in situ vasa recta pericytes and (ii) identify expression of P2 receptor subtypes in the medulla.

Kidney slices (200 μm) were obtained from Sprague Dawley rats (killed by cervical dislocation) and maintained in a physiological saline solution bubbled with 95% O2/ 5% CO2. Video imaging techniques were utilized to study whether ATP and UTP regulate vasa recta pericytes in live kidney slices; pericytes were identified by their ‘bump on a log’ morphology [3]. Immunohistochemistry techniques and confocal microscopy were employed to label and identify specific P2 receptor subtypes in fixed kidney slices.

Superfusion of ATP (10 μM) evoked significantly greater vasoconstriction (p<0.05, Student’s t test) of vasa recta at pericyte sites (24.37±4.94%, mean±s.e.m., n=5.) than at non-pericyte sites (2.15±2.11%, mean±s.e.m.). 100 μM UTP also evoked significantly greater vasoconstriction (p<0.05, Student’s t test) of vasa recta at pericyte sites (8.63±1.38%, mean±s.e.m., n=5) but did not significantly constrict non-pericyte sites (4.93±0.81%, mean±s.e.m.). Higher concentrations of UTP (1 mM) evoked a greater vasoconstriction of vasa recta at pericyte sites (15.48±1.84%, mean±s.e.m., n=5) but did not significantly constrict non-pericyte sites (5.17±0.84%, mean±s.e.m., p<0.05, Student’s t test). The P2 receptor antagonist suramin (100 μM) attenuated the ATP-evoked vasoconstriction by 56.12% (n=3). Immunohistochemistry experiments identified expression of P2X3, P2X4 and P2X7 receptor subtypes in the renal medulla.
Data presented here demonstrate that P2 receptors are expressed throughout the medulla in rat kidney slices and that both ATP and UTP can act specifically at pericytes to regulate vasa recta diameter. We propose that pericytes play a key role in regulating medullary blood flow at the capillary level in the kidney.


Funded by the Medical Research Council.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC149**

Pericyte-mediated regulation of vasa recta capillaries in situ

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Pericytes have been shown to control capillary diameter in situ, in whole retina and in the CNS (1). Pericytes occur at regular intervals on renal medullary vasa recta capillaries and are known to constrict isolated perfused descending vasa recta (2). Here we aim to (i) identify renal pericytes on vasa recta capillaries in live kidney slices, (ii) establish kidney slices as a viable model and (iii) investigate the role of pericytes in the regulation of vasa recta capillaries in situ.

Freshly isolated live kidney slices were labeled with calcein, propidium iodide and Hoechst and imaged using confocal imaging techniques to confirm viability of kidney slices (n=3). Pericytes and vasa recta capillaries were labeled with fluorescein-conjugated NG2 (neural-glial 2) and IB4 (isolectin B4), respectively, and pericyte location along vasa recta capillaries was confirmed in the medulla of kidney slices by confocal imaging techniques (n=10). Video imaging techniques were utilized to identify pericytes along vasa recta capillaries in live kidney slices and to investigate whether pericytes regulate the diameter of vasa recta capillaries in response to vasoactive agents in adult rat kidney slices. Kidney slices (200 μm) were obtained from adult Sprague Dawley rats (killed by cervical dislocation).

Here we show for the first time, pericyte-mediated constriction of vasa recta in situ, in live kidney slices. Superfusion of Angiotensin-II (Ang-II 100nM) resulted in an average constriction of vasa recta capillaries at pericyte sites of 29.26±4.67% (mean±s.e.m, n=14). This was significantly greater than at non-pericyte sites (p<0.001, Student’s t test) where capillary diameter changes were 7.56±1.98%, (mean±s.e.m, n=14).

Angiotensin-II (Ang-II 100nM) resulted in an average constriction of vasa recta capillaries at pericyte sites of 20.77±5.05%, (mean±s.e.m, n=7) than at non-pericyte sites of 7.63±2.04%, (mean±s.e.m, p<0.05, Student’s t test). The nitric oxide donor S-nitroso-N-acetyl-L-1- penicillamine (SNAP 100μM) evoked a significantly greater vasodilation of vasa recta at pericyte sites (14.05±3.52%, mean±s.e.m, n=12) than at non-pericyte sites (4.23±1.62%, mean±s.e.m, p<0.05, Student’s t test). Superimposing SNAP on Ang II significantly decreased (p<0.05, Student’s t test) the Ang II-evoked constriction (i.e. it dilated the vessel) by 84% (n=4) of the preconstricted diameter at the pericyte but had no effect elsewhere.

The techniques described here establish kidney slices as a viable model which can be used to investigate the role of in situ pericytes in the regulation of vasa recta. We have identified pericytes along vasa recta capillaries in the renal medulla and have presented data that shows vasa recta pericytes, like CNS pericytes, constrict vasa recta capillaries and may play a role in regulation of blood flow in the renal medulla.

Peppiatt CM et al., 2006 Nature 443(7112):700-704


Funded by the Medical Research Council.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC150**

Regulation of renal haemodynamics by reactive oxygen species in anaesthetised rats: a direct or indirect action via nitric oxide?

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The regulation of the blood flow through the cortex and medulla of the kidney is dependent on a range of factors one of which may be the level of oxidative stress that can modulate vascular tone. Previously (Ahmeda and Johns, 2004), we demonstrated that blockade of superoxide dismutase (SOD) within the kidney decreased perfusion of blood through both the cortex and medulla. This study aimed to investigate whether the vasoconstriction developed when superoxide anion production was increased was a consequence of a direct action on the blood vessels or an indirect one due to scavenging of nitric oxide (NO).

Groups (n=6) of male Wistar rats (250-300 g) fed a normal (0.3% Na+) or high salt (3% Na+) diet, were anaesthetised with 1 ml i.p. chloralose/urethane (16.5/250 mg/ml, respectively) and supplemented as required. The right femoral vein and artery were cannulated for infusion of saline (154 mM NaCl) at 3 ml/h, and measurement of arterial blood pressure (BP). The left kidney was exposed via a flank incision, placed in a holder and a small cannula inserted 4.5 mm into the kidney for intramedullary (i.m.) infusion of drugs at 0.6-1.0 ml/h. Two Laser-Doppler microprobes were inserted 1.5 and 4.0 mm into the
Intra-renal infusion of NDP-γMSH evokes a potent natriuresis and diuresis and is associated with collecting duct changes in ENaCα expression

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Gamma melanocyte stimulating hormone (γMSH) possesses cardiac pressor effects and promotes natriuresis by binding to melanocortin 3 receptor (MC3R) in the inner medulla (1). In this study we examined the in vivo and in vitro effects of a stable analogue NDP-γMSH (2) on mean arterial pressure (MAP), heart rate, fractional sodium excretion (FeNa), urinary flow rate, (UV) renal sympathetic nerve activity (RSNA) and expression of epithelial Na+ channel (ENaC), aquaporin-2 (AQP2) and MC3R.

Male Wistar rats were fed 0.3% (NNa) or 3% (HNa) sodium for 6 weeks from 3 weeks of age. They were anaesthetised with 1 ml (I.P.) of a chloralose–urethane (16.5 and 250 mg ml–1, respectively) and maintained with supplemental doses of 0.05 ml every 30 min. Indwelling cannulae were inserted for measurement of blood pressure, i.v. fluid infusion (0.15M saline) and collection of urine. Both kidneys were exposed, the left was cannulated for direct infusion of 1000nM NDP-γMSH (300fmol/min) into cortico-medullary border and the right for renal nerve isolation and measurement of RSNA with a bipolar stainless steel electrode. GFR was quantified using FITC–Inulin clearance. Kidneys were removed post infusion and homogenates were blotted for ENaCα, AQP2 and MC3R. Cultured inner medulla collecting duct cells, isolated from male Wistar kidneys (1) were treated with 100nM NDP-γMSH for 3 hours in the presence/absence of Cytochaldsin D & Brefeldin A. Total cell lyases and plasma membrane fractions prepared by differential centrifugation were blotted for ENaCα, AQP2 and MC3R.

Intraperitoneal and intravenous administration of a dose of 300nmol/kg/min of NDP-γMSH for 3 hours significantly increased FeNa (NNa, 8±2%; HNa, 14±2%; P<0.01) than NNa (10±4%; raised UV more in HNa, 45±12.7μl/min/kg (P<0.03) than NNa, 6.2±3.1± 2.9μl/min/kg; increased RSNA more in HNa, 46±10% (P<0.001) than NNa, 0.4±0.1%; raised GFR only in HNa, 3.20±0.17μl/min/kg (P<0.05), NNa, 1.86±0.15 μl/min/kg, MAP (NNa, 96±3mmHg; HNa 96±1mmHg) and heart rate (NNa, 369±16bpm; HNa, 415±15bpm) did not change during infusion. Results, presented as means±SEM, were compared using paired t-tests and post hoc bonferroni correction.

Data suggests that NDP-γMSH possesses same natriuretic/diuretic, but not cardiac pressor, effects as native γMSH. Natriuretic effects may be achieved through redistribution from apical membranes and a down regulation in expression of ENaC in collecting duct cells mediated by MC3R.

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PC151

Acute and chronic effects of nerve growth factor on cystometric parameters in female Wistar rats

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Nerve growth factor (NGF) elicits bladder hyperactivity following acute intravesical administration and is involved in experimentally induced cystitis (1), whilst 2 week intrathecal delivery of NGF (2) or administration of NGF into the bladder wall reduces bladder capacity and increases voiding frequency in

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
rats(3). We investigated the duration of action of acute intravesical NGF on voiding parameters in anaesthetised cystometry, and also the effects of chronic I.P. dosing of NGF in conscious rats.

Female Wistar rats (225-260g) were anaesthetised with isoflurane (2-5% in O2) and urethane (1.25g/kg I.V.). A jugular vein (anaesthetic infusion), carotid artery (BP measurement) and trachea were cannulated. The bladder was cannulated via the urethra and tied at the external meatus, creating a closed system for isovolumetric cystometry. Following baseline cystometrograms with saline (75μl/min) until reflex bladder contractions occurred (threshold volume), the bladder was filled to capacity with 20μg/ml NGF or vehicle (10% DMSO in saline) which was held in situ for 1 hour (h) before draining. Cystometrograms were then repeated at 1, 2, 3, and 4h post treatment. In a separate study rats were anaesthetised with isoflurane, and the bladder cannulated aseptically via the urethra. The bladder was filled with 1ml of either 20μg/ml NGF or vehicle which was held in situ for 1h before draining. The catheter was removed and animals allowed to recover for 24h before undergoing anaesthetised cystometry as described above. In conscious studies rats (225-260g) were dosed daily for 10 days with 0.1mg/kg NGF or saline 10ml/kg I.P. and voiding function assessed the following morning via metaboles over 3h. Mean ± S.E.M. from each study were compared by ANOVA for NGF vs. vehicle treated rats, P<0.05 was considered statistically significant.

Intravesical NGF (n=5) significantly decreased % baseline threshold volume vs. vehicle (n=4) at 2 (63.5±4.6 vs. 106.2±10.7), 3 (78.9±4.5 vs. 102.2±7.7) and 4h post treatment (75.7±7.5 vs. 108±7.4). Contraction frequency was significantly increased in the NGF treated group vs. vehicle at 1 (4±0.6 vs. 2±0.9), 2 (12±1.4 vs. 2±0.9), 3 (9±1.1 vs. 3±0.9) and 4h post-dose (10±2.1 vs. 2±0.9). At 24h post NGF there were no significant effects on either threshold volume or contraction frequency. In conscious rats an increase in voiding frequency and reduction in volume per void developed over time in the NGF treated group (n=4), reaching significance at day 5 for voiding frequency, and at day 6 for volume/void vs. vehicle (n=4). No significant difference between NGF and vehicle treatment was observed more than 24h post the last treatment day.

These results indicate that depending on the mode of administration, NGF can produce both an acute and more chronic bladder hyperactivity that persists for up to 24h post dose.


Yoshimura et al. The journal of Neuroscience 26(42): 10847-10855, 2006

Zvara & Vizzard. BMC Physiology 2007, 7:9

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Radiotelemetry allows measurement of both urodynamic and haemodynamic parameters in conscious unrestrained female beagle dogs

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Urodynamics have been traditionally recorded in anaesthetised or tethered animals. We have evaluated a model for simultaneous measurement of urodynamic and haemodynamic parameters in conscious, freely moving female dogs via radiotelemetry. All experiments were conducted in accordance with UK legislation and local ethical guidelines. Four female beagle dogs (9-15kg) were surgically anaesthetised with propofol (70-80mg) and maintained with isoflurane anaesthetic (2% in 1:2 O2/NO2 mix) for implanting telemetry transmitters (D70-PCT) to measure bladder pressure, blood pressure, ECG and temperature. An additional catheter was sutured into the bladder and attached to an access port located under the skin to allow filling and emptying of the bladder. Animals were given appropriate antibiotic and analgesic treatment prior to and following surgery. After recovery, animals were habituated to saline bladder filling via an infusion line connected to the bladder access port. Animals were singly housed in metabolism cages during studies to allow urine collection, the size of the cages allowed free movement. The optimum infusion rate for each animal was determined using a theoretical natural diuresis rate of 360ml/hr [Hamaide et al, 2003] as a starting point, and for each animal was identified based on consistency of bladder fill volumes and pressure. After each study animals were returned to their home pens. All 4 animals’ bladder fill data was consistent over subsequent fills in the same day and subsequent days. Table 1 shows maximum threshold and micturition pressure, bladder fill volume, MAP prior to first bladder fill, MAP prior to final fill and optimal infusion rate for each animal [mean ± SEM (n)].

Simultaneous measurement of haemodynamic parameters showed that cystometry did not cause stress to the animals. In conclusion this model allows simultaneous measurement of urological and cardiovascular parameters in the absence of anaesthetic or restraint providing a useful means to evaluate the effects of drugs on urological and cardiovascular function.

Table 1 Urological and haemodynamic parameters at the optimal infusion rate for each animal

<table>
<thead>
<tr>
<th>Animal</th>
<th>Urodynamic parameter</th>
<th>Haemodynamic parameter</th>
<th>MAP prior to first bladder fill</th>
<th>MAP prior to final bladder fill</th>
<th>Optimal infusion rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Threshold (H2O) (µl/ml)</td>
<td>Threshold (µl/ml)</td>
<td>118.4±6.7</td>
<td>118.4±6.7</td>
<td>300µl/h</td>
</tr>
<tr>
<td>2</td>
<td>Threshold (H2O) (µl/ml)</td>
<td>Threshold (µl/ml)</td>
<td>118.4±6.7</td>
<td>118.4±6.7</td>
<td>300µl/h</td>
</tr>
<tr>
<td>3</td>
<td>Threshold (H2O) (µl/ml)</td>
<td>Threshold (µl/ml)</td>
<td>118.4±6.7</td>
<td>118.4±6.7</td>
<td>300µl/h</td>
</tr>
<tr>
<td>4</td>
<td>Threshold (H2O) (µl/ml)</td>
<td>Threshold (µl/ml)</td>
<td>118.4±6.7</td>
<td>118.4±6.7</td>
<td>300µl/h</td>
</tr>
</tbody>
</table>

Vascular responses mediated by GPR30/GPER

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GPR30/GPER is a novel G protein-coupled estrogen receptor. Using a rabbit polyclonal antisera against the human GPR30 C-terminus, we identified GPR30 immunoreactivity in the cytoplasm of human aortic endothelial cells (HAEC). Administration of G-1, a selective GPR30 agonist or 17β-estradiol (E2) to HAEC produced a fast and sustained increase in intracellular Ca2+ concentrations [Ca2+]i. G-1 (0.01 μM, 0.1 μM and 1 μM) increased [Ca2+]i by 93 + 3.2, 176 + 4.2, and 558 + 6.9 nM, respectively. E-2 (15 μM) increased [Ca2+]i by 127 + 2.1 nM. Activation of GPR30 caused NO production in HAEC and NO-dependent aortic ring relaxation. G-1 (1 μM) or E2 (15 μM) increased ΔDAF (F/F0) fluorescence by 0.09 + 0.009 and 0.07 + 0.009, respectively, while acetylcholine (15 μM) increased ΔDAF by 0.31 + 0.01. Administration of G-1 (0.01 μM, 0.1 μM and 1 μM) produced HAEC hyperpolarization with an amplitude of 4 + 1.9 mV, 18 + 3.6 mV, and 28 + 3.8 mV, respectively. E2 (15 μM) hyperpolarized HAEC by 16 + 2.6 mV. Intravenous administration of G-1 (41.2 ng/kg to 41.2 μg/kg) to urethane-anesthetized Sprague Dawley rats produced a dose-dependent decrease (by 2.6 + 1.6 % to 14.7 + 1.1%) in mean arterial pressure. Taken together, our results indicate that GPR30 is involved in the vascular response to estrogen.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Thyroxine-induced heart hypertrophy: impact on cardiac function

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The impact of experimentally induced cardiac hypertrophy on cardiac haemodynamic parameters and inotropic responses is not fully understood. This study aimed to quantify the durational effect of thyroxine (T4)-induced cardiac hypertrophy on the haemodynamic and cardiac function parameters and to investigate how they responded when challenged with the β-adrenoceptor agonist, isoprenaline, before and after blockade of nitric oxide (NO) production with the NO synthase inhibitor NG-nitro-L-arginine (L-NAME).

Heart hypertrophy was induced by 7- and 14-day regimes of intra-peritoneal T4 treatment. Eight controls, nine T4-7 and nine T4-14 cardiac hypertrophied male Wistar rats (250-290g), respectively, were anaesthetized i.p. with 1 ml chloralose/urethane mixture (16.5/250 mg/ml, respectively) and prepared for in vivo measurement of haemodynamic and cardiac function parameters. Heart weights were higher in the group given T4 for both 7 days and 14 days than control group 14% and 19% (P<0.05), respectively. This was associated with an increase in heart rate, maximum dP/dt and contractility index by 16%, 34% and 14% (P<0.05) respectively in the T4-14 rats. The L-NAME increased the basal levels of blood pressure, heart rate, contractility index and maximum dP/dt by 39%, 23%, 32% and 27% (P<0.05) respectively in T4-14 rats. The L-NAME also augmented the isoprenaline’s inotropic effect in both T4-7 and T4-14 hypertrophied hearts but had no effect on basal myocyte contractility. The T4-14 heart hypertrophy rats showed reduced stress endurance capacity compared to both control and 7 days heart hypertrophy rats.

The haemodynamic and cardiac function parameters were significantly enhanced after 14 days thyroxine-induced cardiac hypertrophy indicating increased cardiac performance. Furthermore, the L-NAME caused significant augmentation of the isoprenaline inotropic effect, in addition to the enhanced effect on the basal cardiac performance in the 14 days hypertrophy hearts. A more severe degree of cardiac hypertrophy was associated with a reduction in stress endurance capacity.


Balligand JL, Kelly RA, Marsden PA, Smith TW, Michel T. Control of cardiac muscle cell function by an endogenous nitric oxide signalling system. Proc Natl Acad Sci USA 1993; 90: 347-351.

Supervisor: Professor Edward J. Johns; Department of Physiology, University College Cork, Republic of Ireland.
Cardiovascular short-term variability modulation by central V1a and V2 receptor blockade involve thermoregulation: transfer function study

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We have previously shown that central vasopressin receptors contribute to cardiovascular (CV) short-term variability in normotensive freely moving rats(1). In this study we investigate the effect of central vasopressin receptor blockade on blood pressure (BP) and heart rate (HR) short-term variability of conscious rats and its relationship to changes in body temperature (Tb) using transfer function. All procedures are in accordance with ECC Directive 86/609. Adult male Wistar outbred rats were anesthetized with xylazine/ketamine anesthesia (0.4ml 10% ketamine IP, 0.1ml 2% xylazine IP per animal) for insertion of TL11M2-PA-C50-PXT DSI implant for concomitant measurement of BP in aorta and Tb subcutaneously. After full recovery under the same anesthesia, rats were chronically instrumented with intracerebroventricular (icv) cannula (1). The role of central vasopressin receptors was assessed using selective non-peptide V1a, V1b and V2 antagonists injected icv, as described previously (1). At the end of experiments rats were euthanized with bolus overdose of thiopentone sodium. Arterial BP and Tb were digitalized at 1000Hz. Systolic BP (SBP), diastolic BP (DBP) and HR were derived from the arterial BP as maximum, minimum and inverse of interbeat interval, respectively. Time spectra and transfer function (gain, phase and coherence) were calculated on 15 overlapping 2048 point time series involving 410-s registration period. Spectra were analyzed in very-low-frequency (VLF: 0.0195 - 0.195Hz), low-frequency (LF: 0.195 - 0.8Hz) and high-frequency (HF: 0.8 - 3Hz) range. At neutral ambient temperature (22°C ± 2) 100ng of V1a antagonist increased coherence between Tb and SBP in LF spectral band (0.56 ± 0.03, p < 0.05). Moreover SBP and HR coherence in LF spectral band was increased by 100ng (0.76 ± 0.02, p < 0.05) and 500ng of V1a antagonist (0.8 ± 0.03, p < 0.05) 100ng of V2 antagonist increased coherence between SBP and HR (0.83 ± 0.04, p < 0.05), Tb and SBP (0.74 ± 0.1, p < 0.05) and Tb and HR (0.58 ± 0.03, p < 0.05). 100ng and 500ng of V2 antagonist increased coherence between Tb and SBP in VLF band (0.66 ± 0.15, p < 0.05; 0.59 ± 0.1, p < 0.05). Phase analysis in LF band under V2 receptor blockade indicate that changes in temperature precede those of SBP and HR. In rats exposed to 34°C ± 2 ambient temperature we noted increase of coherence between SBP and HR in LF spectral band under V2 receptor blockade (0.83 ± 0.02, p < 0.05). Results suggest that central V1a and V2 receptors contribute to CV short-term variability in LF and VLF spectral band involving baroreceptor reflex - dependent and - independent thermoregulation, respectively.

Poster Communications

PC156

Time and Frequency Domain Analysis of the Cardiovascular Response to Shaker Stress in Borderline Hypertensive Rats


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We investigated the effects of shaker stress on blood pressure (BP) and heart rate (HR) short-term variability and spontaneous baroreflex sensitivity (BRS) sensitivity in adult normotensive Wistar rats (WR) and borderline hypertensive rats with family history of hypertension (BHR). Experiments were performed in accordance with Directive 86/609/ECC: under xylazine/ketamine anesthesia (0.4 ml 10% ketamine IP, 0.1 ml 2% xylazine IP) TA11PA-C40 DSI implants were inserted in abdominal aorta and rats were treated with metamizol (200mg IP) for pain relief. At the end of experiments rats were euthanized with bolus overdose of thiopentone sodium. Only after full recovery (10 days) rats were included in experimentation. BP was recorded 20 minutes before, during acute exposure to shaking platform at 200 cycles/min/10 minutes, and 30 minutes after. This was followed by random exposure to 5 minutes long shaking period 18 times/day/3 days (1). BP was recorded during the last exposure to stress, as well as 30 minutes after. Arterial BP was digitalized at 1000Hz. Systolic BP (SBP), diastolic BP (DBP) and HR were derived from the arterial BP as maximum, minimum and inverse of interbeat interval, respectively. Evaluation of the spontaneous BRR was performed by using the method of sequences and calculation of baroreflex sensitivity-BRS and effectiveness index-BEI (2). Time spectra were calculated on 15 overlapping 2048 point time series involving 410-s registration period (3). Spectra were analyzed in very-low-frequency (VLF: 0.0195-0.195Hz), low-frequency (LF: 0.195-0.8Hz) and high-frequency (HF: 0.8-3Hz) band. Statistical significance was assessed with one-way ANOVA. Basal values of SBP, DBP and HR were comparable in both strains. Exposure of WR and BHR to acute shaker stress increased SBP and DBP (WR: 124.5 ± 4.5 mmHg, p < 0.05 and 95.3 ± 2.0 mmHg, p < 0.05; BHR: 149.3 ± 3.4 mmHg, p < 0.01 and 109.9 ± 3.9 mmHg, p < 0.05), and reduced VLF SBP and VLF DBP (WR: 2.8 ± 0.7 mmHg²/Hz, p < 0.05 and 1.5 ± 0.1 mmHg²/Hz, p < 0.05; BHR: 2.0 ± 0.3 mmHg²/Hz, p < 0.05 and 2.3 ± 0.4 mmHg²/Hz, p < 0.05). In HR spectra of WR, but not of BHR, LF HR (13.2 ± 0.4 bpm²/Hz, p < 0.05) and HF HR (5.6 ± 0.6 bpm²/Hz, p < 0.05) increased. Chronic stress increased HF SBP and HF DBP in both strains (WR: 0.5 ± 0.1 mmHg²/Hz, p < 0.05 and 0.4 ± 0.1 mmHg²/Hz, p < 0.05; BHR: 0.5 ± 0.1 mmHg²/Hz, p < 0.05 and 0.4 ± 0.1 mmHg²/Hz, p < 0.05), and enhanced LF HR (5.9 ± 1.3 bpm²/Hz, p < 0.05) only.
in WR. BRS and BEI did not change during acute and chronic exposure to stress in both strains. Spectral analysis results show that BHR exhibit reduced HR variability response to acute and chronic shaker stress in comparison to normotensive control, suggesting remodeling of cardiac regulation in absence of hypertension.


Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC160**

**The Effect of IL-6 on the Hypoxic Sensitivity of Vagal Nerve Paraganglia in the Rat**

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Paraganglia of the vagus (Xth cranial) nerve are minute clusters of glomus cells resembling the carotid body – an organ that senses changes in blood chemistry. They are chemosensitive to cyanide and reductions in the partial pressure of oxygen (PO₂) (O’Leary et al., 2004). We proposed that they may also be sensitive to circulating cytokines released from exercising muscles. Specifically, we wished to test our hypothesis that the myokine interleukin-6 (IL-6) can cause an acute change in superior laryngeal nerve (SLN) activity.

We exposed isolated superfused rat glomus cells - located at the bifurcation of the SLN – to hypoxia (~20mmHg) to measure chemosensitivity to changes in PO2. Next, we exposed the cells to IL-6 at concentrations of 0.1ng/ml, 0.3ng/ml and 1ng/ml during both normoxia (~100mmHg) and hypoxia while monitoring single axonal action potential frequency. This was followed by hypoxia in the absence of IL-6 to check that the preparation was still responsive. One single fibre per animal was tested and values are expressed as mean ± S.E.M.

The results confirm previous findings that these cells respond strongly to changes in PO₂, significantly increasing their discharge rate in response to hypoxia (from 0.71 ± 0.36 to 6.0 ± 2.4Hz). IL-6 did not affect the baseline discharge rate of the SLN units at any concentration; however, it did modulate the response to hypoxia. In 5/6 animals the smallest IL-6 concentration (0.1ng/ml) caused a small increase in peak frequency of the SLN (mean peak frequency 6.6 ± 2.6 Hz). Also, in 5/6 animals the largest IL-6 concentration (1ng/ml) caused a decrease in peak frequency of the SLN (4.7 ± 2.1 Hz). There was a statistically significant difference in peak discharge from the lowest to the highest concentration of IL-6 (Wilcoxon signed-rank test, p=0.03). This data shows that although the myokine IL-6 has no effect on basal discharge of rat vagal paraganglia during normoxia, it can modulate hypoxic responses. Therefore IL-6 may not play a role in modulating discharge during exercise when active muscles release it, but rather modulate discharge when paraganglia become inflamed, e.g. during chronic hypoxia (Liu et al., 2009).


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**PC161**

**Correlation of plasma B-type natriuretic peptide level with ejection fraction in primary hypertensive patients**

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Currently we are in the midst of a chronic disease epidemic of heart failure, one of the worst complications for primary hypertensive patients. Ejection fraction is an important measure for left ventricular function, as well as an indicator for the presence of heart failure (1&2). Earlier data suggests that B-type natriuretic peptide level partially reflects the ventricular pressure (3). The aim of this work is to investigate whether plasma concentration of BNP reflects the heart’s capability with the ejection fraction as the indicator. This study was approved by the ethical committee and consents from all the subjects were collected prior taking samples. It was conducted on 50 hypertensive patients referred for echocardiography to evaluate the ventricular function. Patients with diabetes mellitus, previous history of myocardial infarction were excluded from the study. The cardiologist making the assessment of left ventricular function was blinded to BNP levels. The BNP levels were assessed using the Triage Meter from Biosite Diagnostics. Results showed that BNP levels display a negative correlation with ejection fraction (Pearson correlation test) and it is clearly shown in the BNP versus ejection fraction scatter plot graph. The significant result (paired t test, p < 0.05) proves that both predictors are very important and relates to each other. The study which is the first to be conducted in Malaysia may be helpful in ruling out the diagnosis of heart failure or as the best screening method.
**Poster Communications**

**PC163**

**Expanding applications of automated planar patch clamp: A focus on internal exchange and heat activation**

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Planar patch clamp devices are finding their place in academic and industrial labs because of their ease of use and higher throughput as compared with conventional patch clamp. Planar borosilicate glass chips are used for performing whole cell, perforated patch, or cell attached electrophysiological recordings. High quality data is acquired with a high success rate for obtaining giga-seals (typically 60-80%). Given the design of the borosilicate glass chip, the internal recording solution is much more accessible to exchange. This means that experiments involving exchange of the internal solution can be performed with relative ease. Exemplar traces will be shown for block of KV1.3 channels by the internal perfusion of ions and small molecules. In addition, data will be presented showing planar lipid bilayers which were used to estimate the time required to fully exchange the internal solution.

Since many ion channels are sensitive to temperature and some compounds have been shown to exhibit different pharmacology at different temperatures, temperature can become an important parameter to control in electrophysiological experiments. By heating the bath solution and the solution entering the bath of a planar patch clamp system, the temperature of the external solution can be constantly maintained at a given temperature. Data will be presented showing hERG recordings at room temperature and at physiological temperature, including a full concentration response curve to quinidine at physiological temperature.

Not only can the amplitude and kinetics of currents be changed by temperature, indeed, some ion channels can actually be activated by elevated temperature. The TRPV1 channel, for example, can be activated by ligands such as capsaicin, but also has been reported to be activated by temperatures above ~42°C. Using a heated pipette, the temperature of the added solution can be increased and then rapidly applied to the cell. Very rapid changes in temperature can be achieved at the cell (within ms), from room temperature up to 60°C, whilst continuously recording. Exemplar traces will be presented showing heat activation of TRPV1 expressed in CHO cells using a range of temperatures. TRPV1 channels could prove to be important targets for the treatment of pain and, therefore, the temperature activation of TRPV1 could have important implications for drug design as well as investigations into physiological modes of action.

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**PC165**

**Phagocytic activity is increased in dissociated CD11b-positive cells prepared from brains of aged rats but this is not affected by treatment with amyloid-beta in vitro**

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Microglia are the resident macrophage-like cells of the CNS and are partly responsible for the clearance of amyloid-beta (Aβ) in the healthy brain. They display similar properties to peripheral macrophages and therefore they are phagocytic and capable of cytokine production. Alzheimer’s Disease (AD) is a common age-related neurodegenerative disease characterised by the formation of insoluble amyloid plaques that are associated with activated microglia, but it is unknown whether these microglia are involved in phagocytosis. Here we set out to assess whether phagocytic activity was altered in cells prepared from aged, compared with young, rats and to evaluate whether Aβ modulated phagocytic activity in these cells.

We investigated phagocytic activity in rat primary CD11b-positive cells using flow cytometry to assess uptake of quantum dots and found pre-treatment with Aβ 1-42 (2μM, 24h) exerted no significant effect on uptake. Phagocytosis of quantum dots (QD) was significantly decreased by pre-treatment of cells with H2O2 (*p<0.01; ANOVA; n=18) or NaF (***p<0.001; ANOVA; n=18). Phagocytosis was significantly increased in CD11b-pos-
itive cells prepared from aged, compared with young, rats (*p<0.05; Student’s t-test; n=26). We investigated microglial activation in brain tissue prepared from the same rats and found that there was a significant age-related increase in CD11b mRNA expression in both the hippocampus (*p<0.05; Student’s t-test; n=7) and the cortex (*p<0.05; Student’s t-test; n=7), while CD40 mRNA expression was significantly increased in hippocampus (***p<0.001; Student’s t-test; n=7). Expression of a lysosomal membrane protein, CD68, which is upregulated during phagocytic activity was also significantly increased in the hippocampus (**p<0.01; Student’s t-test; n=7) and the cortex of aged, compared with young, rats (*p<0.01; Student’s t-test; n=7).

We investigated the effect of Aβ1-42 treatment on phagocytic activity of CD11b-positive cells prepared from brain tissue of aged, compared with young, rats and found differential age-related effects. Treatment of cells prepared from young rats with Aβ1-42 significantly increased phagocytic activity (*p<0.05; Student’s t-test; n=27) but Aβ1-42 induced no further increase in the phagocytic activity of cells isolated from the brains of aged rats. The data suggest that the age-related increase in microglial activation is associated with an increase in phagocytic activity and that microglial cells prepared from aged rats cannot be further stimulated to increase their phagocytic activity by Aβ1-42.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC167
Temporal change in occipito-frontal propagation of repeated cortical spreading depressions in rats
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PC168
Vesicular glutamate transporter VGLUT2 is associated with synaptic projections from the lateral vestibular nucleus to the dorsal cochlear nucleus
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The dorsal cochlear nucleus (DCN) is the first relay of the central auditory system and is involved in the localization of sound in the vertical plane. The DCN is also the site of integration of other sensory inputs. Therefore knowledge of synaptic inputs to the DCN is of importance to understand possible interactions between the auditory and other sensory circuits in this structure. We recently developed a method that allows delivery of fluorescent dyes within precisely localised tissue areas (Barker et al., 2008). Using this system, in excised brain tissue from decapitated rats, we were able to deliver dextran amine rhodamine conjugates to the Lister Hooded rat DCN and trace retrograde projections coming from the lateral vestibular nucleus. Dextran amine conjugates were also delivered to the lateral vestibular nucleus and synaptic terminals could be localised within the DCN confirming anterograde projections from the lateral vestibular nucleus to the DCN. Vestibulocochlear projections were characterised within the sagittal and the coronal plane. The projections were mainly within the deep and fusiform cell layers of the DCN and fibre tracts could only be discriminated in the sagittal plane. Therefore to assess functional synaptic projections from the lateral vestibular nucleus to the DCN, whole cell recordings were performed in sagittal slices. Stimulating the lateral vestibular nucleus elicited glutamatergic synaptic currents in fusiform and granule cells that were blocked by glutamate receptor antagonists NBQX and D-AP5. Vesicular glutamate transporters (VGLUT’s) selectively package glutamate into synaptic vesicles. By combining the dextran amine neuronal tracing method with immunocytochemistry, we were able to show projections from the lateral vestibular nucleus predominantly colabeled with VGLUT2 in the DCN, whereas very few were labeled with VGLUT1. In conclusion, we have shown that the lateral vestibular nucleus sends functional glutamatergic projections into the DCN, mainly mediated by the VGLUT2 vesicular transporter. This suggests functional links between the auditory and the vestibular system and ultimately that balance and locomotion could interact with the sound localization in the vertical plane.


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PC169
Assessment of the intracellular activities of ROS and NO induced by passive stretching of isolated skeletal muscle fibres from aged wild type and dystrophic mice
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Skeletal muscle constantly produces reactive oxygen species (ROS) and nitric oxide (NO) which may play a role in signalling and regulatory pathways. Isolated muscles in vitro release NO to the extracellular space [1] and passive stretching of muscle increases the release of NO from rat skeletal muscle in vitro [2]. We have developed a model to study the generation of ROS and NO in real time in isolated single muscle fibres [3, 4].

The aim of this study was to evaluate the effect of passive stretching on the intracellular generation of ROS and NO in sin-
gle muscle fibres isolated from young and old wild type and young mdx mice. We used young (2-4 month-old) and old (26-28 month-old) C57BL/6 mice, and young mdx mice, a mouse model of Duchenne muscular dystrophy. Muscle fibres were isolated from the Flexor Digitorum Brevis and attached to a flexible silicone membrane which had been previously coated with a collagen Matrigel™ matrix. Fibres were loaded with different fluorophore probes: 2', 7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) which is a general detector of ROS, 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM-DA) which is a detector of NO, and dihydroethidium (DHE) which can detect superoxide. A passive stretching protocol was applied for eight minutes to fibres using the FX-4000™ Flexercell® system. Using fluorescence microscopy, the fluorescence emission from fibres at different time points was quantified by image analysis to monitor the intracellular ROS and NO. Experimental groups were consisted of 6-12 fibres. Statistical analysis: one-way ANOVA followed by post hoc LSD test for multiple comparisons, statisti- cal significance was set at P < 0.05. Results from positive control experiments indicated that the technique is able to detect changes in intracellular ROS and NO. The rate of increase in DAF-FM fluorescence (indicating NO) in fibres from young mice decreased significantly after the application of passive stretching. However, fibres from old mice showed a slight increase in DAF-FM fluorescence after the same stretching protocol, and fibres from young mdx showed no change with the protocol. Fibres from old and young mice displayed different patterns of ethidium fluorescence following DHE loading, with the activity of superoxide appearing to increase in fibres from young mice and decrease in fibres from old mice after the passive stretching protocol. We conclude that the application of passive stretching to isolated single muscle fibres produces minor changes in the intracellular activities of ROS and NO, and induces different patterns of intracellular fluorescence depending on the age of the mice and muscular dystrophy.


This work was funded by the Wellcome Trust, UK.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
The influence of cannabinoid CB2 receptor in adult rat mesenchymal stem cell viability

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Adult mesenchymal stem cells (MSCs) are a multipotent population of stem cells that can differentiate along the osteogenic, chondrogenic and adipogenic lineages. They offer a potentially exciting source of cells for engineering skeletal tissues for the treatment of osteochondral defects. Cannabinoids are lipophilic signalling molecules that interact with G-protein coupled cannabinoid (CB) receptors. Recently, the skeleton has been identified as a major endocannabinoid target through both the CB1 and CB2 cannabinoid receptors. The aim of this study was to examine the role of the CB2 receptor in the regulation of MSC viability. MSCs were isolated from the bone marrow of adult male Wistar rats and expanded in culture. MSCs were treated with the CB2 antagonist, AM630 (0.001-100μM) alone or in the presence of the CB2 agonist, JWH015 (10nM, 100nM and 1μM) for 24h and colorimetric TUNEL was performed to detect the percentage of apoptotic cells. Phospho-c-Jun N-terminal kinase (JNK) and c-Jun expression were assessed by western immunoblotting. Caspase-3 activity was measured using a colorimetric assay to assess cleavage of the caspase-3 substrate, Ac-DEVD-pNA.

AM630 (10 and 100μM) significantly increased the percentage of apoptosis; thus, in control conditions 9.08±0.38% (mean±S.E.M) of cells were apoptotic and this was significantly increased to 23.08±0.60% and 42.33±0.60% by AM630 at 10μM and 100μM, respectively (p<0.001, ANOVA, n=3, Newman-Keuls Multiple Comparison test) and this induction of apoptosis was prevented by JWH015 (100nM). AM630 (10μM, 24h) significantly increased phospho-JNK immunoreactivity from 1.58±0.20 (mean arbitrary units ± S.E.M) to 3.00±0.37 (p<0.05, n=5, Student’s t-test) and also significantly increased caspase-3 activity from 647.5±101.2 pmol pNA/min/mg to 2571±89.4 and this was significantly reduced to 1878±67.9 in cells exposed to AM630 in the presence of JWH015 (100nM; p<0.01, ANOVA, n=5, Newman-Keuls Multiple Comparison test). AM630 (10μM, 24h) induced a significant increase in phospho-c-Jun activity from 0.62±0.10 (mean arbitrary units ± S.E.M) to 1.20±0.08 and this was significantly reduced to 0.85±0.08 in cells exposed to AM630 in the presence of JWH015 (100nM) (p<0.05, p<0.01, ANOVA, n=5, Newman-Keuls Multiple Comparison test).

This study demonstrates that inhibition of the CB2 receptor leads to MSC apoptosis and suggests that activation of the CB2 receptor is necessary to maintain the survival of MSCs. This provides evidence of another cellular target for cannabinoid receptors which may be necessary for the maintenance of bone health.


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The role of the endocannabinoids system in the regulation of adult rat mesenchymal stem cell differentiation

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Adult mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate along the osteogenic, chondrogenic or adipogenic lineage. The exact physiological function of MSCs remains unclear at present, however, MSCs do play a role in tissue healing (2). Therefore, MSCs represent a source of adult stem cells which could have immense potential for use in regenerative medicine. The endocannabinoid system is comprised of the cannabinoid (CB) G-protein coupled receptors (CB1 and CB2), their endogenous ligands and degradative enzymes. The endocannabinoid system has recently been established as a major regulator of the musculoskeletal system (3). The aim of this study was to examine the role of the endocannabinoid system in the differentiation of MSCs along the osteogenic lineage.

MSCs were isolated from the bone marrow of adult Wistar rats and expanded in culture. MSCs were treated with osteogenic factors (OFs; 10mM β-glycerolphosphate, 10nM dexamethasone, 50µM ascorbic acid) to induce osteogenesis. In some experiments MSCs were treated with the CB1 receptor antagonists, AM251 or SR141617A (both 1μM). MSCs were treated for 2 weeks, RNA extracted and osteocalcin mRNA and CB1 mRNA were measured by qPCR. Osteocalcin immunoreactivity was assessed by immunocytochemistry. Hydroxyapatite deposits in the extracellular matrix were measured using a commercial kit following treatment of the cells with OFs, AM251 or SR141617A for 5 weeks. Osteocalcin mRNA was significantly increased from 0.3±0.1 (arbitrary units, mean±SEM) to 7.4±3.1 (p<0.01, unpaired t-test, n=3-6) by osteoinductive factors. Also, osteocalcin mRNA was significantly increased from 0.3±0.1 to 1.2±0.1 and 2.3±0.8 when MSCs were exposed to AM251 (1μM) and SR141617A (1μM), respectively (p<0.01 vs., control cells, unpaired t-test, n=6), indicative of osteogenic differentiation. Incubation with SR141617A in the presence of OFs further increased the expression of osteocalcin mRNA from 7.4±3.1 in cells treated with OFs alone to 15.7±5.1 (p<0.05, Newman-Keuls Multiple Comparison test, n=3-6). Hydroxyapatite deposits in the extracellular matrix were significantly increased from 1.9±0.1 (arbitrary units, mean±SEM) to 5.9±0.6 by OFs and further significantly increased to 7.3±0.3 when MSCs were exposed to AM251 in the presence of OFs (p<0.05, unpaired t-test, 8 replicates). CB1 mRNA expression was significantly increased from 1.6±0.8 (arbitrary units, mean±SEM) to 5.5±1.2 by OFs and further increased to 6.3±0.6 in MSCs treated with SR141617A (p<0.01, unpaired t-test, n=3-7).

These data show that the cannabinoid receptor system is upregulated during MSC osteogenesis and that osteogenesis is enhanced following pharmacological blockade of the CB1 receptor.

Role of cPLA₂ in Endocrine-sensitive and Endocrine-resistant breast cancer cell growth

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17β-estradiol (E₂) is a steroid hormone that regulates many biological activities - including cell proliferation and invasiveness - in breast cancer, contributing to tumorigenesis and progression. E₂ acts through its intracellular receptors (ERα and ERβ) to regulate gene transcription in the nucleus and activate rapid signaling pathways from the plasma membrane. Recent studies demonstrated a rapid, E₂-induced increase in intracellular Ca²⁺ concentration through activation of the calcium-dependent cytosolic phospholipase A2 (cPLA₂α) in the breast cancer-derived MCF-7 cell line (1). cPLA₂α catalyzes the hydrolysis of membrane glycerophospholipides to release arachidonic acid (AA), which is converted to biactive eicosanoid lipid mediators (including prostaglandins produced through cyclooxygenases) that can activate downstream proliferative signals (2). The rapid release of bioactive lipids may play a role in the transition of breast cancer from estrogen-sensitive to estrogen-resistant through the over-activation of the EGFR signaling pathway. The expression of cPLA₂α activation by western blotting and confocal microscopy. The E₂-induced rapid activation of cPLA₂α was dependent on EGFR/HER2 trans-activation, heterodimerisation and downstream signalling through ERK1/2 MAPK to phosphorylate cPLA₂α on Ser505. E₂ also promoted cPLA₂α trafficking to perinuclear membranes, and this effect was subsequent to, and dependent on, MAPK-induced phosphorylation on Ser505. EGFR and/or HER2 over-expressions correlates with a poor clinical outcome and resistance to endocrine therapy in breast cancer. The endocrine-resistant SKBR3 cell line over-expressed EGFR and HER2 and also showed elevated cPLA₂α expression (at both the mRNA and protein level) compared to MCF-7, confirming the correlation between the eicosanoid and the EGFR signalling pathways, as suggested by other reports in the literature (3). Pharmacological blockade of cPLA₂α with a specific inhibitor impacted on cell growth in both cell lines, by reducing E₂-induced proliferation and inducing apoptosis and necrosis, without affecting the invasiveness of surviving cells. cPLA₂α is likely to play a key role in regulating the already established growth-promoting effects of estrogen and COX-2 in breast cancer, balancing the cytotoxic effects of free arachidonic acid with the proliferative effects of prostaglandins. Furthermore, cPLA₂α could play a role in the transition of breast cancer from estrogen-dependence to estrogen-resistance through the over-activation of the EGFR signaling pathway.

Thomas W et al. (2006). Steroids 71(3).256-265

Heme oxygenase (HO)-1 as a potential biomarker in the pathogenesis of endometriosis

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The pathogenesis of endometriosis, an estrogen-dependent disease, remains elusive. Early work proposed the initiation of the disease is due to “retrograde menstruation” and this has been widely accepted. However, to date, the mechanism of endometriotic cell proliferation is still unexplained. Oxidative stress has been implicated as contributing to cell growth. The expression of heme oxygenase 1 (HO-1) is upregulated by oxidative stress and is capable of protecting cells against oxidant-mediated injury. HO-1 has been suggested as being involved in the mechanism of cell proliferation, and estradiol has also been implicated in proliferative events. In the present study, we determined the effects of oxidative stress and estradiol on endometriotic cell proliferation and on the expression of HO-1 using immortalized human endometriotic epithelial cells (12-2). Cells were treated with the oxidants H₂O₂ (10 μM) or menadione (25 μM), or the lipid peroxidation product acrolein (35 μM) for 48 hours and the proliferation of cells was determined by MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay. The results show that H₂O₂, menadione and acrolein significantly induced cell proliferation at 1 μM, 20 μM and 25 μM respectively, showing that H₂O₂ is the most potent compound. Cells were also pre-treated with estradiol at 10⁻⁸ M for 24 hours followed by the addition of compounds at similar concentrations for 24 hours. Interestingly, in the presence of estradiol at 10⁻⁸ M, the cells that were treated with lower concentrations of compounds showed a significant increase in proliferation.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC173

PC174


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but this was not the case in the presence of estradiol at 10^{-9} M. In contrast, at high concentrations, all compounds caused significant growth inhibition. The expression of HO-1 mRNA in treated cells was determined by Quantitative Real Time-Polymerase Chain Reaction (Q-PCR). A significant up-regulation of HO-1 mRNA was observed in all the treated cells and further increased in the presence of estradiol at 10^{-9} M. These results indicate that oxidative stress may contribute to the proliferation of endometriotic cells. In addition, there may be some interplay with estradiol to further increase cell proliferation. Expression results indicate that estradiol may be involved in the regulation of the HO-1. The up-regulation of HO-1 mRNA during proliferation indicates that this enzyme could be used as a reliable marker in the pathogenesis of endometriosis.

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PC175

Effects of altered KCl cotransporters expression on intracellular chloride levels and pH in cervical cancer cells

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KCl cotransporters (KCCs) play significant roles not only in ionic and osmotic homeostasis but also tumor biology. Overexpression of KCC1, KCC3 and KCC4, with KCC3 the most abundant isoform, has been shown to correlate with human cervical carcinogenesis (1). KCC1 and KCC4 are osmotically-sensitive and involved in volume regulation (2) while KCC3 contributes to cell proliferation (3). KCC3 overexpression enhances proliferation, migration and invasiveness of cervical cancer cells. Removal of the N-terminal 117 amino acids from KCC1 (ΔN117) produces a dominant-negative loss-of-function phenotype for KCC cotransport in human cervical cancer cells which exhibit inhibited cell proliferation, tumor growth and invasiveness (4). We have shown previously that expression levels of KCC3 correlate inversely with intracellular Cl\(^-\) levels ([Cl\(^-\)]\(_i\)) estimated by 36Cl\(^-\) equilibrium. There is no significant difference in cell volume between wild-type, KCC3-overexpressed, and ΔN117 mutant cervical cancer cells (4). To maintain electroneutrality, changes in [Cl\(^-\)]\(_i\) associated with altered KCC activity must result in alterations to the levels of other ions, most likely HCO\(_3^-\). In the present study, the properties of these KCC transfectants in regulating their intracellular pH (pHi) with changes in [Cl\(^-\)]\(_i\) have been investigated. 6-Methoxy-N-ethylquinolinium chloride (MEQ) was used to determine [Cl\(^-\)]\(_i\) in the three cell lines. [Cl\(^-\)]\(_i\) estimated by MEQ was 73.80±15.82 mM (n=5) in wild-type cervical cancer cells, 45.63±1.65 mM (n=3) in KCC3-overexpressed cervical cells and 104.01±6.66 mM (n=3) in ΔN117 mutant cells (Fig. 1A). The resting pHi was determined by cuvette fluorimetry using BCECF-AM (10 μM for 15 min at 37°C). As shown in figure 1B, there are no significant differences in resting pHi between KCC3-overexpressed cells and ΔN117 mutant cells in HEPES-buffered solution (HBS). However, ΔN117 mutant cells have a more acidic pHi than KCC3-overexpressed cells in bicarbonate-buffered solution (BBS). A number of Na-dependent and acid extruding transporters were assessed by RT-PCR. Only the expression of NHE3 correlated with [Cl\(^-\)]\(_i\) (Fig. 1C). The present results suggest that bicarbonate can replace Cl\(^-\) as the intracellular anion during changes of KCC transport activity.

Figure 1 (A) Intracellular chloride concentration measured by MEQ. (B) Intracellular pH measured by BCECF-AM. (C) Expression of membrane transporters measured by RT-PCR. 


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PC176

Genetic dissection of potassium chloride cotransporter (KCC) functions in epithelial development and carcinogenesis in Drosophila

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Electroneutral potassium chloride cotransporters (KCC) belong to the superfamily of electroneutral cation-chloride cotransporters and are encoded by four genes in vertebrates (KCC1 through KCC4) (1). The activity of KCC plays an important role in variety of physiological and pathological cellular functions,
including cell volume regulation, epithelial ion transport, and osmotic homeostasis. Our previous studies have highlighted important roles of KCC in tumor progression. Malignant transformation of cervical epithelial cells is associated with the differential expression of volume-sensitive KCC activities resulting from the up-regulation of mRNA transcripts for KCC1, KCC3 and KCC4 isoforms (2). Loss-of-function KCC mutant cervical cancer cells exhibit inhibited cell growth accompanied by decreased activity of the cell cycle proteins retinoblastoma and cdc2 kinase (3). In this study, we aim to investigate the in vivo functions of KCC in epithelial development and carcinogenesis using Drosophila melanogaster as the model. There is a single KCC homologue in Drosophila, but its functional properties remain largely unknown. Hypomorphic mutations in the Drosophila KCC homologue, CG5594, render flies susceptible to epileptic-like seizures associated with excitatory GABAergic signaling, consistent with the function of mammalian KCC2 (4). However, strong loss-of-function alleles are lethal, suggesting other critical developmental roles. Using the GAL4/UAS (Upstream Activation Sequence) expression, we have overexpressed CG5594 in multiple tissues (5). Crosses were performed at 25°C, and 1-2 day old adult progeny were anesthetized with CO2 for eye and wing analyses. Overexpression of CG5594 in differentiating cells of the eye using the GMR-GAL4 driver had no significant effect on the size of fly eye but produced a mild disarrangement of the ommatidial array (Fig. 1A). Additionally, CG5594 overexpression in the posterior compartment of the developing wing, under the control of engrailed-GAL4, resulted in drastic size reduction in the region of overexpression with associated reduction in cell size (Fig. 1B). We conclude that KCC can regulate cell growth and cell size of fly eyes and wings. Analyses of mutant clones in the fly eye and wing will provide an excellent assay system in which to investigate how modulation of KCC function influences epithelial development and how overexpression might lead to metastasis in an appropriate genetic background.


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PC177

Multiple purinergic receptors regulate anion secretion in the bovine oviduct epithelium

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Introduction: Fluid within the oviduct provides the appropriate environment essential for gamete transport, fertilization and early embryo development. However, little is known about the potential mechanisms underlying the formation of oviduct fluid. Our previous studies [1] have demonstrated a role for basolateral purinoceptor activation in promoting chloride secretion across the bovine oviduct epithelium. The present study explored the regulation of anion secretion by luminal purinoergic receptors.

Methods: Ion secretion was measured as changes in short-circuit current (I_{SC}(μA/cm²) across voltage-clamped polarized oviduct epithelial cell monolayers. Changes in intracellular calcium were measured by fluorescence microscopy using fura-2. mRNA expression was confirmed by reverse transcriptase PCR (RT-PCR). Results are expressed as mean ± SEM and statistical analyses were made by one way ANOVA and the Bonferroni/Dunn post hoc test.

Results and Discussion: ATP (100μM) application to the luminal surface of polarized bovine oviduct epithelial cells induced a rapid and transient increase in current of 28.82±1.7 μA/cm² followed by a sustained current of 1.91±0.1μA/cm² (n=18). Removal of extracellular chloride or bicarbonate ions or both reduced the ATP-induced I_{SC} response by 30%, 40% and 70% respectively (n=5). Furthermore, the ATP response was pre-
Evidence for a streptomycin-sensitive transport pathway in human sickle erythrocytes

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HbS erythrocytes from individuals with sickle cell disease (SCD) have altered membrane transport compared to HbA erythrocytes from healthy individuals (Brugnara, 2004). In particular, HbS cells show increased permeability to cations upon deoxygenation. This pathway, termed Psickle, contributes to erythrocyte shrinkage (Tosteson et al., 1952), accelerating sickling.

The identity of Psickle is elusive. We have demonstrated electrophysiologically that whole-cell conductance of HbS cells was higher than in HbA cells, and potentiated by deoxygenation. This pathway shares properties with Psickle (Browning et al., 2007). Deoxygenated HbS cells, unlike HbA cells, are permeable to certain organic osmolytes, including sucrose, and this pathway is inhibited by dipryrimadole and DIDS (Ellory et al., 2008). Here we show that streptomycin, an inhibitor of stretch-activated channels (Shen et al., 2003), inhibits both these pathways.

HbS and HbA cells were collected from volunteers with full ethical consent. Lysis experiments were performed at 37°C (Ellory et al., 2008). Briefly, streptomycin (0.1 to 10 mM) was added to erythrocytes (haematocrit 4%) suspended in buffered sucrose solution (300 mM) and the suspension maintained deoxygenated for 60 min. Lysis was determined by measuring haemoglobin release optically. Erythrocyte membrane currents were recorded at room temperature (Browning et al., 2007), using conventional whole-cell patch clamp techniques. I-V relationships were obtained by applying a series of 300 ms test potentials from -80 to +80 mV in 10 mV increments from a holding potential of 0 mV. Currents were analysed by averaging the current values of the final 50 ms evoked by each test potential.

In control experiments, about 25% of HbS cells lysed after 60 min in sucrose. About 60% of lysis was inhibited by streptomycin, IC50 0.13 ± 0.06 mM (n = 3) (Figure 1). Whole-cell current measurements were compared between HbA and HbS cells using Na+-containing bath and pipette solutions. A representative I-V curve is shown for HbS cells before and after the addition of 50 μM streptomycin (Figure 2). HbS cells demonstrated considerably larger whole-cell currents (p < 0.05) than HbA cells, although both had a reversal potential of approximately 0 mV. Application of streptomycin (50 μM) to the bath inhibited 74% of membrane current at +80 mV in HbS cells, compared to only 24% in HbA cells (not shown).

Results show that streptomycin can inhibit an electrophysiological conductance, thought to be Psickle, in addition to a selective permeability pathway in HbS cells. We hypothesise that this transport pathway may represent Psickle. Further studies can help elucidate the molecular identity of Psickle, which can have important therapeutic implications for SCD patients.

Figure 1. Representative data showing percentage of lysed deoxygenated HbS cells in 60 min with inhibition by streptomycin.

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Occlusion of small blood vessels is implicated and involves exposure of phosphatidylserine (PS) on the outer leaflet of the RBC membrane (Kuypers, 2008). This phospholipid is normally confined to the inner leaflet through the action of an aminophospholipid translocase. On exposure, it makes RBCs stickier. We have investigated the extent to which calcium entry through the deoxygenation-induced pathway, P_sickle (Lew and Bookchin, 2005), contributes to PS exposure. Blood samples were obtained with ethical approval from consenting volunteers homozygous for HbSS. RBCs were washed into low (LK) or high potassium (HK) saline, comprising (in mM) NaCl 140, KCl 4, glucose 5, HEPES 10 for LK saline, and NaCl 55, KCl 90, glucose 5 and HEPES 10 for HK saline, all pH 7.4 at 37°C. They were incubated at different extracellular [calcium]s for up to 18h. RBCs were subsequently treated with vanadate (1mM), harvested, and labelled with FITC-annexin (Becton Dickinson, BD). Percentage of RBCs with PS exposed on their external membrane was then measured by flow cytometry. Data are given as means±SEM for blood samples from n patients. After 18h, the percentage of RBCs expressing PS when incubated under oxygenated LK conditions was 5.3±1.4, 5.7±1.0, 5.7±1.1 and 6.2±0.9 (n = 4) at 0.5, 1.1, 2 and 5mM [calcium], respectively. At the same [calcium], when deoxygenated, values increased to 11.1±2.3, 11.1±1.4, 13.3±0.9 and 18.2±1.7. When deoxygenated at 5mM Ca^{2+} in LK saline, 10.3±4.4% (n = 3) RBCs exposed PS, falling to 5.4±1.7% in HK saline (compared with 5.3±0.8% in oxygenated conditions). The deoxygenation-induced increase in PS exposure was abolished by loading cells with the calcium chelator, MAPT-AM. In deoxygenated LK saline with 5mM calcium, the number of RBCs with PS exposure fell by about 15% when incubated with either dipyridamole (100μM) or clotrimazole (10μM).

These results agree with previous findings that PS exposure increases upon deoxygenation. They also show that the effect is dependent on extracellular calcium and is prevented by its chelation intracellularly. PS exposure is largely prevented by removing the gradient for loss of potassium efflux by suspension in HK saline. There is modest inhibition by dipyridamole (a partial P_sickle inhibitor) and clotrimazole (which inhibits the Gardos channel). We conclude that PS exposure is induced by calcium entry following deoxygenation-induced P_sickle activation, and that intracellular calcium has its main effect via cell shrinkage due to solute loss following activation of the Gardos channel.


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**PC179**

**Ca^{2+} and phosphatidylserine exposure in red blood cells from patients with sickle cell disease**

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Patients with sickle cell disease (SCD) have HbS (rather than normal HbA) in their red blood cells (RBCs). How HbS results in the symptoms of SCD remains incompletely understood.
Voltage-dependent gating currents from the CLC-5 Cl⁻/H⁺ exchanger

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Much of our understanding of the CLC family has come from functional studies of the Torpedo CLC-0, a voltage-gated Cl⁻ channel, and from structural studies of prokaryotic CLC-ec1, a voltage-independent Cl⁻/H⁺ exchanger. Mammalian CLC-5, however, exhibits voltage-dependent activation and Cl⁻/H⁺ exchange. We carried out high resolution whole-cell patch clamp recordings of wild-type and mutant CLC-5 expressed in HEK293 cells, as described in Smith et al. (2009). Outward currents, corresponding to Cl⁻ into and H⁺ out from the cytosol, were activated by positive potentials (Fig. 1A). Upon repolarisation, brief (< 1ms) inward tail currents were observed, which according to the apparent reversal potential did not relate to the movement of permeating ions. As seen previously (Zdebik et al., 2008), neutralisation of an internal proton acceptor (E268A) resulted in ablated ionic flux across the membrane. With this mutant we observed non-linear capacity transient currents lasting approximately 1 ms (Fig. 1B), which had charge-movement kinetics ($V_{1/2} = 94.0 ± 0.8$ mV, $z_e = 1.3 ± 0.03$; mean ± s.e.m. of recordings from 5 cells) that correlated with the voltage-dependent activation of wild-type CLC-5 ($V_{1/2} = 108.6 ± 2.0$ mV, $z_e = 1.4 ± 0.05$, $n = 5$). The brief tail current that were observed with wild-type CLC-5 appeared to relate to gating rather than permeation and had similar charge-movement kinetics.

We found that the properties of the E268A transient currents were similar when extracellular Cl⁻ was replaced by Br⁻ ($z_e = 1.3 ± 0.10$, $n = 3$), an anion that also permeates wild-type CLC-5. In contrast, the voltage-dependence was reduced in the presence of the impermeant anions aspartate ($z_e = 0.9 ± 0.05$, $n = 3$) and methanesulphonate ($z_e = 0.9 ± 0.08$, $n = 3$). Furthermore, similar gating transients were recorded from cells expressing wild-type CLC-5 in the presence of the impermeant anions. The higher gating charge ($z_e$) observed with permeant anions suggest that these interact with or modify an intrinsic voltage-sensor with a $z_e$ of 0.9 and may provide the additional charge which moves through a fraction of the voltage field ($\delta = 0.4$). The impermeant anions are unlikely to contribute to the gating charge since it was similar with extracellular divalent SO₄²⁻ ions ($z_e = 0.9 ± 0.04$, $n = 3$) and persisted in near-complete removal of ions.

Our data suggest that the voltage-sensor in CLC-5 may be formed by an intrinsic protein domain in combination with permeating anions. As demonstrated by the lack of sustained Cl⁻/H⁺ current with the E268A mutant, “gating” of the pore also requires H⁺ likely to be delivered from the intracellular E268 proton acceptor.

Identification of tryptophan analogues as non-transported inhibitors of the proton-coupled amino acid transporter PAT2 (slc36a2)

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PAT2 (slc36a2) is a pH-dependent, Na⁺-independent transporter of small, dipolar amino and imino acids (1,2). PAT2 is defective in the renal reabsorptive disorder iminoglycinuria (3). Tryptophan analogues act as non-transported inhibitors of the amino acid transporters PAT1 (4) and ATB0⁺ (5). The purpose of this study was to identify the nature of the interaction of tryptophan analogues with PAT2. Rat PAT2 was expressed in X. laevis oocytes following standard procedures (2). Data are mean ± SEM (n) with statistical significance determined by ANOVA and Tukey’s multiple comparison post-test. Under optimal uptake conditions (pH 5.5, Na⁺-free), 5-hydroxy-L-tryptophan

Figure 1: Whole-cell currents recorded from HEK293 cells expressing wild-type (A) and E268A (B) CLC-5.


Supported by the Wellcome Trust.

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and $\alpha$-methyl-DL-tryptophan both significantly reduce (p<0.001) $[^3H]proline$ uptake (10μM, 5μCi.ml$^{-1}$, 40min, 22°C) in PAT2-expressing oocytes in a concentration-dependent manner with IC$_{50}$ values of 4.2 ± 0.8 (20) and 3.5 ± 0.6 (30) mM, respectively. Rheogenic H$^+$/proline symport via PAT2 was measured using two-electrode voltage-clamp (-60mV, pH 5.5, Na$^+$-free). In contrast, 5-hydroxy-L-tryptophan and $\alpha$-methyl-DL-tryptophan (both 20mM) failed to induce inward current in PAT2-expressing oocytes. However, both 5-hydroxy-L-tryptophan and $\alpha$-methyl-DL-tryptophan (20mM) significantly reduced (p<0.001) the proline- (0.2mM) induced current by 81.0 ± 2.3 (6) and 76.7 ± 3.6 (5) %, respectively. To confirm that both tryptophan analogues are non-transported inhibitors of PAT2, trans-stimulation of $[^3H]proline$ efflux (oocytes were preloaded with 5mM proline, 0.1μCi.ml$^{-1}$) was measured under optimal uptake conditions. The PAT2 substrate glycine (10mM) significantly trans-stimulated (p<0.001) $[^3H]proline$ efflux via PAT2 [266.5 ± 17.2 (10) pmol.oocyte$^{-1}$. (10min)$^{-1}$] compared to water-injected oocytes [13.6 ± 2.5 (10) pmol.oocyte$^{-1}$. (10min)$^{-1}$]. In contrast, both 5-hydroxy-L-tryptophan and $\alpha$-methyl-DL-tryptophan (both 10mM) failed (p>0.05 versus water) to trans-stimulate $[^3H]proline$ efflux via PAT2 [9.3 ± 1.0 (10) and 11.9 ± 1.4 (10) pmol.oocyte$^{-1}$. (10min)$^{-1}$], respectively. In conclusion, the combination of measurements demonstrate that 5-hydroxy-L-tryptophan and $\alpha$-methyl-DL-tryptophan are non-transported inhibitors of PAT2. These compounds should prove useful experimental tools to help elucidate the physiological role of PAT2.


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**PC183**

**Altered urothelial ATP signaling in human Overactive Bladder (OAB) patients**

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Overactive bladder (OAB) is characterized by frequency, urgency and urge-incontinence, in the absence of urinary infection. We have identified a previously unrecognized pyuria (≥10 white blood cell/ul urine) in a majority of patients diagnosed as having OAB; associated with more severe symptoms(1). It is established that extracellular ATP signalling, originating from stretch-evoked ATP release and necessarily involving activation of a variety of P2 receptors, is involved in bladder sensation(2). Furthermore, inflammation is associated with increased ATP release from epithelial cells(3). Taken together, we hypothesise that in pyuric OAB patients, there is increased ATP release and/or increased P2 receptor expression, caused by inflammation, which ultimately results in increased sensory nerve excitation and the exacerbation of OAB symptoms. Here we begin to investigate our hypothesis.

Bladder urothelium biopsies were obtained from i) asymptomatic patients, ii) pyuric OAB patients, and iii) non-pyuric OAB patients, using flexible cystoscopy. Basal and hypotonicity-evoked (i.e. stretch-evoked) ATP release from urothelium was quantified using a luciferin/luciferase assay, P2 receptor mRNA levels were investigated using quantitative real time-PCR, and P2 receptor expression was investigated in snap-frozen sliced tissue using immunohistochemistry.

Basal ATP release was 50-fold greater from the urothelium of pyuric OAB patients (P<0.01; n=10) than from non-pyuric OAB (n=9) or asymptomatic patients (n=9). In contrast, in all three patient groups, the concentration of ATP released following stretch was similar. Basal and stretch-evoked ATP release was significantly abolished (P<0.01; n=3) by addition of the P2 receptor antagonist suramin (1 mM). In asymptomatic patient urothelium, we detected significant levels of P2X$_{1,3,5,7}$ subunit and P2Y$_{1,2,6,11,14}$ receptor mRNA (n=6). Urothelium from pyuric OAB patients showed a significant increase in abundance of P2X$_5$ subunit and P2Y$_{1,2,6,11,14}$ receptor mRNA, and a significant decrease in abundance of P2X$_1$ subunit mRNA (P<0.01; n=3). Urothelium from non-pyuric OAB patients showed a significant increase in P2Y$_{11}$ mRNA (P<0.01, n=3). Immunohistochemistry confirmed our real time-PCR data (n=3).

In summary, this data demonstrates that in a subset of OAB patients (those with pyuria) there is increased basal ATP release from the urothelium, which is abolished by the P2 receptor antagonist suramin, and, altered P2 receptor expression. This data suggests that increased ATP release from the urothelium, involving activation of P2 receptors, may play a role in the heightened symptoms associated with pyuric OAB patients.


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Ursodeoxycholic acid exerts antisecretory actions on colonic epithelial cells

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Results: At high concentrations (500 μM) CDCA inhibited Na/K-ATPase pump activity in apically permeabilised monolayers and found that UDCA inhibited Na/K-ATPase pump activity to 16.2 ± 3.9% of that in control cells (n=4; p<0.001). In experiments designed to isolate basolateral secretory function, UDCA (50 μM – 1 mM) was devoid of prosecretory activity. However, pretreatment of T84 cells with UDCA (500 μM) significantly attenuated subsequent secretory responses to the Ca2+-dependent agonist, carbachol (CCh; 100 μM) and the cAMP-dependent agonist, forskolin (10 μM) to 11.9 ± 4.2% (n=9; p<0.001) and 43.0 ± 13.0% (n=6; p<0.05) of controls, respectively. The effects of UDCA were concentration-dependent with antisecretory actions being apparent at concentrations as low as 50 μM. However, UDCA (1 mM) did not alter transepithelial resistance implying it did not exert toxic effects. In further experiments we measured Na/K-ATPase pump activity in apically permeabilised monolayers and found that UDCA inhibited Na/K-ATPase pump activity to 16.2 ± 3.9% of that in control cells (n=4; p<0.001). In experiments designed to isolate basolateral K+ conductance, UDCA inhibited activity to 13.7 ± 2.4% of controls (n=5; p<0.001). Similar to UDCA, LCA was also without effect on basal Cl- secretion in T84 cells. However, pretreatment of cells with LCA (50–200 μM) significantly potentiated responses to CCh. LCA (100 μM) increased CCh-induced responses to 146.67 ± 8.7% of those in controls (n = 8; p<0.001). However, at concentrations > 500 μM, LCA exerted antisecretory actions similar to UDCA. Conclusion: Bacterial metabolism of CDCA alters its ability to regulate colonic epithelial secretion. While UDCA exerts mainly antisecretory effects, LCA enhances secretion at relatively low concentrations but is antisecretory at high concentrations. Our data also suggest that altering bile acid metabolism by pharmacological means or through antibiotic/probiotic manipulation of the enteric flora may prove useful in treating intestinal transport disorders associated with dysregulated epithelial transport.

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Epidermal growth factor enhances colonic epithelial secretory capacity through activation of phosphatidylinositol 3-kinase, mitogen-activated protein kinase and upregulation of multiple transport proteins

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Background: There is increasing evidence that growth factors, such as epidermal growth factor (EGF), are important regulators of epithelial transport function. Previous work from our laboratory has shown that EGF chronically enhances colonic epithelial secretory function through a mechanism involving increases in NKCC1 expression. Aim: Here we set out to further investigate molecular mechanisms underlying EGF potentiation of colonic epithelial secretory capacity. Methods: Monolayers of T84 cells, grown on permeable supports, were mounted in Ussing chambers and Cl- secretion measured as changes in short circuit current (Isc) across voltage-clamped monolayers of T84 colonic epithelial cells. Results were expressed as mean ± SEM for a series of n experiments. Statistical analyses were made by one way ANOVA using the Newman-Keuls multiple comparison test. P values <0.05 were considered to be significant.

Results: In addition to NKCC1, acute treatment with EGF (100 ng/ml for 15 min) induced increases in the mRNA levels of CTR (270 ± 20 % of controls; n = 3; p < 0.01), KCNN4 (150 ± 20 % of controls; n = 5; p < 0.05) and Na/K-ATPase β1 subunit (370 ± 100 % of controls; n = 4; p < 0.01) but did not alter expression of the α1 subunit of Na/K ATPase (130 ± 30 % of controls; n = 4). To determine signaling pathways involved in mediating the effects of EGF, we examined the effects of PI3-K (LY294002), ERK (PD98059) and p38 MAPK (SB203580) inhibitors on the ability of EGF to enhance ISc responses to either the Ca2+ or cAMP-dependent secretagogues, carbachol (CCh) and forskolin (FSK) (Table 1).

Conclusions: EGF chronically increases colonic epithelial secretory function through upregulation of key transport proteins that comprise the Cl- secretory pathway. PI3-K and ERK MAPK are important in mediating EGF potentiation of both Ca2+ and cAMP-dependent responses, while p38 MAPK is involved only in enhancing cAMP-dependent responses. The precise roles of these effector pathways in mediating EGF-induced increases in transport protein expression are currently under study. Our data suggest that agents targeting EGF-dependent signaling pathways may be useful in therapy of intestinal disorders associated with dysregulated epithelial transport.

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Table 1. ISc responses to CCh and FSK were measured 24 hrs after pretreatment with EGF (100 ng/ml for 15 min). Data are expressed as % control response to CCh (n = 6) or FSK (n = 5) alone (**p<0.01; ***p<0.001).

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Differential expression of UT-B urea transporters in the human gastrointestinal tract

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Facilitative UT-B urea transporters enable the passage of urea across cell membranes (1). Gastrointestinal urea transporters are thought to play a significant role in the symbiotic urea nitrogen salvaging process that occurs between mammalian hosts and their gut bacteria (2). This study investigated the expression of UT-B urea transporters along the human gastrointestinal tract, particularly in the colon.

Immunoblot analysis showed that UT-B proteins (purchased from AMS Biotechnology) were present throughout the human gastrointestinal tract and most strongly expressed in the caecum and colon. Within the colon, a 35 kDa signal was deglycosylated to a 30 kDa protein after one hour pre-incubation with PNGaseF enzyme. Glycosylated UT-B protein expression was greater in the proximal colon than in the distal colon. This differential pattern of colonic expression was also observed for several other proteins involved in nutrient transport mechanisms – such as NHE3 and NaK ATPase. Finally, at the cellular level UT-B transporters were located in the basolateral region of colonocytes situated in the upper portion of the colonic crypts.

These results illustrate a differential expression of glycosylated UT-B transporters along the human gastrointestinal tract, correlating to regions that contain the largest populations of intestinal bacteria. These data therefore suggest an important role for urea transporters in maintaining the symbiotic relationship between humans and their gut bacteria.


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Coleytramine: a Novel Non-nutrient Tool in Human Gastric Emptying Studies

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Two established physiological actions of the enteroendocrine peptide cholecystokinin (CCK) include inhibition of gastric emptying and mediation of satiation after a nutrient meal. There is evidence to suggest that the particulate resin colestyramine stimulates secretion of CCK in humans. It also directly stimulates the STC-1 enteroendocrine cell line. This preliminary study therefore examines the effect of colestyramine on gastric emptying and appetite in vivo in healthy humans in order to assess its potential use as a non-nutrient tool in gastric emptying studies. Nine healthy volunteers were given liquid test meals (500ml) containing 4g colestyramine, 12g colestyramine or water alone, on three occasions, in a randomised order. The effect of colestyramine on gastric emptying was determined non-invasively using the 13C-acetate breath test and the effect on appetite was assessed by visual analogue scales. Colestyramine significantly delayed gastric emptying (overall treatment effect, p < 0.001). The cumulative 13CO2:12CO2 expired over the 45-minute period after vehicle was 427.7 ± 28.1 mean/SEM, compared to 342 ± 26.7 after 4g, and 280.6 ± 14.3 after 12g colestyramine. Pairwise comparisons revealed that the significance of the emptying delay was greater in response to 12g (p<0.001) than 4g colestyramine (p = 0.048) compared to vehicle. The delay in gastric emptying in response to 4g was considerable variable between individuals, whereas the response to 12g was relatively consistent. Colestyramine also significantly reduced hunger (p=0.007), the amount of food participants felt able to eat (p=0.001) and bloating (p=0.01). It did not evoke nausea. After vehicle, it took subjects ~20 min to reach the average baseline hunger score, whereas the baseline score was not reached within the 45-min period with colestyramine. These data demonstrate that colestyramine significantly delays gastric emptying and reduces appetite in humans. Therefore, it has potential as a novel non-nutrient tool in gastric emptying studies in health and disease (eg diabetic gastroenteropathy). Colestyramine is particularly interesting in that it is not absorbed from the lumen and therefore cannot exert post-absorptive effects, which are problematic in interpreting the effects of absorbable nutrients. Furthermore, in light of its effects on gastric emptying and appetite, understanding the underlying mechanisms could lead to new therapeutic applications.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Influence of insulin on membrane-bound Ca2+ content in permeabilized rat hepatocytes

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Insulin induces change of Ca2+ content in hepatocytes (Benzeroual K. et al., 1997; Rodrigues M.A. et al., 2008). However, the role of IP3Rs and RyRs in insulin-induced change of Ca2+ content in hepatocytes is still unknown. Here we present results of our investigation of insulin effect on IP3Rs and RyRs in rat permeabilized hepatocytes.

Experiments were carried out on humanly killed 4-5 months old Wistar rats (n = 29, weight 0,18-0,2 kg). Isolated liver was perfused with insulin-content solution (external incubation solution contained (in mM): NaCl – 140,0; KCl – 4,7; CaCO3 – 1,3; MgCl2 – 1,0; HEPES – 10,0; glucose – 10,0; pH = 7,4 ; insu-
line - 0.04 u.) for 10 min at room temperature. Then hepatocytes were isolated by lidase digestion (16 u., 10 min, 37 ToC) and permeabilised by incubating with saponine (0.1 mg/ml, 10 min), which was added to internal incubation solution contained (in mM): NaCl – 20.0; KCl – 120.0; MgCl2 – 1.13; ATP (+Sigma, USA) – 2.0, CaCl2 – 1.3, HEPES – 10.0; pH = 7.0. Concentration of membrane-bound Ca2+ was measured using chlorotetracycline.

We showed that perfusion of liver with insulin-content solution causes statistically significant increase of membrane-bound Ca2+ concentration in permeabilized hepatocytes by 33.57 ± 7.93% (n = 29, P < 0.001) in comparison to membrane-bound Ca2+ concentration after liver perfusion with control solution (no insulin present). This liver perfusion with insulin also changed IP3 and ryanodine effect on membrane-bound Ca2+ in hepatocytes. We found that IP3 decreased the membrane-bound Ca2+ by 32.00 ± 4.08% (n = 9, P < 0.05) after perfusion liver by insulin-content solution, but caused an increase of membrane-bound Ca2+ content after perfusion with control solution (without insulin). Also ryanodine (5 and 500 nM) decreased concentration of the membrane-bound Ca2+ by 16.24 ± 5.21% (n = 7, P < 0.05) and 26.83 ± 9.45% (n = 4, P = 0.03), respectively. However, ryanodine (50 nM) did not elicit significant changes in the concentration of membrane-bound Ca2+ content in permeabilized hepatocytes after perfusion of liver with insulin-content solution. In control experiments ryanodine (5, 50 and 500 nM) caused statistically significant increase of membrane-bound Ca2+ content in permeabilized hepatocytes by 22.26 ± 7.54% (n = 7, P < 0.05), 22.95 ± 7.88% (n = 9, P < 0.05), 18.85 ± 7.50% (n = 6, P < 0.05), respectively.

Thus we conclude: 1) perfusion of liver by insulin-content solution prevents ryanodine’s (50 nM) action and changed on reverse action of ryanodine (5 and 500 nM) and IP3; 2) insulin changes concentration of membrane-bound Ca2+. We suppose that perfusion of liver by insulin-content solution increase content Ca2+ in intracellular store that is why action of ryanodine and IP3 is opposed to control.


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Altered calcium handling in monocrotaline-induced, right ventricular hypertrophy and failure in rats

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The altered contractility that occurs in cardiac hypertrophy and heart failure (HF) is often attributed to changes in Ca2+ handling properties (Hasenfuss and Pieske, 2002). Such changes have been less extensively studied in the right ventricle (RV), compared to the left ventricle (LV), thus the aim of this study was to investigate alterations in Ca2+ handling in RV hypertrophy and HF in pulmonary hypertensive rats. Male Wistar rats (200 g) were injected with monocrotaline at 30 mg/kg to induce RV hypertrophy (HYP) or 60 mg/kg to induce RV failure (FAIL) which was identified by clinical signs. Control animals were injected with an equivalent volume of saline (CON). Animals were humanely killed 3-4 weeks after injection and RV cardiomyocytes were enzymatically isolated. All experiments were conducted at 20-24°C. Cell shortening and Ca2+ transients were recorded in fura-4 AM loaded myocytes. Caffeine (20 mM) was used to assess the sarcoplasmic reticulum (SR) calcium load. Calcium sparks were measured by confocal microscopy using the fluorescent indicator fluo-4 AM. Statistical comparison between CON, HYP and FAIL myocytes was performed by 1-way ANOVA. All procedures accorded with current UK legislation.

Compared to CON hearts, there was a statistically significant increase in the ratio of heart weight: body weight by 50% in HYP and by 78% in FAIL heart (P < 0.05, n = 5-6 hearts in each group). Cell shortening was significantly decreased by 17% in HYP and by 29% in FAIL myocytes but Ca2+ transient amplitude significantly increased by 43% in HYP and 56% in FAIL myocytes, (P < 0.05 n = 17-20 myocytes in each group). SR load was also increased in HYP by 20% and FAIL by 61% myocytes (P < 0.05, n = 19-23 myocytes in each group). Consistent with an increase in SR load, the frequency, duration and width of calcium sparks significantly increased in
the development of hypertrophy and HF but spark amplitude progressively reduced (P<0.05, n=24-32 myocytes in each group). Ca2+-handling is altered in the RV hypertrophy and heart failure associated with monocrotaline-induced pulmonary hypertension. The increased SR load may explain the observed increase in Ca2+ transient amplitude and the increased number of Ca2+ sparks. However, these changes cannot be directly responsible for the reduced cell shortening, this is probably related to desensitization of the myofilaments to Ca2+ (Lamberts et al., 2007). Hasenfuss, G. et Pieske, B. (2002). J. Mol. Cell. Cardiol. 34, 951-969. Lamberts, R.R. et al. (2007). J. Physiol. 582, 695-709.

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**PC193**

**Delayed afterdepolarisations induced by dyadic calcium without sarcoplasmic reticulum calcium overflow. A simulation study**

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Delayed AfterDepolarisations (DAD) are depolarisations that occur after full repolarisation and have been related to sodium and/or calcium overload in the cell. They are triggered by “spontaneous release” of calcium from the sarcoplasmic reticulum (SR) leading to calcium oscillations and via the electrogenic sodium-calcium exchanger (NCX) to changes in membrane potential (1). Due to the variety of experimental findings the trigger of the release is still under debate: SR overload (2) vs. dyadic calcium (3).

We investigate the underlying mechanism of DAD induction using mathematical atrial and ventricular cell models of human, dog, rabbit, guinea-pig, rat and mouse (31 models). To compare inducibility of calcium oscillations, each model is reduced to its “calcium subsystem”; trans-membrane potential, intracellular sodium ([Na]i) and potassium concentrations are fixed at their end-diastolic values, all trans-membrane currents but the NCX are set to zero. Intracellular calcium handling is unchanged.

We find that in all models showing Calcium Oscillations in the SubSystem (CaOSS) the same parameter changes also produce DADs in the full model, which in the case of the Noble’98 model (4) lead to action potentials (Fig 1). We do not find parameter combinations that initiate DADs but do not show CaOSS. Thus the initiation of DADs in the full model is concurrent with the appearance of CaOSS.

Reduced models are tested for CaOSS by increasing [Na]i (increases [Ca]i via NCX) and the sarcoplasmic reticulum calcium ATPase (SERCA) current (reflecting increased sympathetic tone). All 19 reduced models that show CaOSS have i) Markov formulations for RyR, ii) the RyR modulated by either dyadic subspace (Cass) or cytoplasmic (Cacyt) calcium and iii) a SERCA formulation dependent on both Cacyt and CaSR (all but two). To investigate which of these model properties are essential for the production of CaOSS (and thus DADs) we perform in-silico experiments removing the dependence of RyR on Cass/Cacyt, or the dependence of SERCA on CaSR. The change in RyR abolishes CaOSS, whereas SERCA modulation changes just their frequency and amplitude. Modulation of RyR by CaSR is included in some models, but can be removed without preventing CaOSS – indicating that SR overload modulates DADs, but is not the key mechanism.

Our results confirm that the main trigger for CaOSS and DADs is an increased level of calcium within the cell, which is also the common feature of the experimental results of calcium-induced DADs. The “spontaneous release” of calcium from the SR occurs by the same mechanism as calcium induced calcium release, i.e., the modulation of the RyR opening by calcium in the dyadic subspace. Future work will investigate the correlation between Markov formulations of RyR and DAD appearance.


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Simulation of the effects of BAPTA and EGTA on inactivation of \(I_{\text{Ca}}\) in a model of the rat ventricular myocyte

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In cardiac ventricular myocytes, the fast Ca\(^{2+}\) buffer BAPTA has a greater effect than the slow Ca\(^{2+}\) buffer EGTA on inactivation of \(I_{\text{Ca}}\) (e.g. Brette at al. 2004, Sham, 1997). The aim of the present study was to refine an existing model of the rat ventricular myocyte (Pasek et al. 2006) to reproduce the differential effect of BAPTA and EGTA on inactivation of \(I_{\text{Ca}}\) in the rat myocyte and to investigate its possible cause. The principal modifications to the model were: (i) reformulation of the description of sarcoplasmic reticulum (SR) Ca\(^{2+}\)-release according to Shannon et al. (2004); (ii) reformulation of the description of voltage- and Ca\(^{2+}\)-dependent inactivation of \(I_{\text{Ca}}\) to simulate the experimental data of Brette et al. (2004); (iii) modification of the parameters of the SR Ca\(^{2+}\)-pump to give the relation between free Ca\(^{2+}\) in the cytosol and SR described by Shannon and Bers (1997); (iv) incorporation of equations controlling exchange of free EGTA and Ca\(^{2+}\)-EGTA and of free BAPTA and Ca\(^{2+}\)-BAPTA between the pipette, cytosol and dyadic space. In the model, both buffers could inhibit the cytosolic Ca\(^{2+}\)-transient, and thus contraction. However, only BAPTA inhibited efficiently the rise of Ca\(^{2+}\) in the dyadic space, thus causing significant inhibition of Ca\(^{2+}\)-dependent inactivation of \(I_{\text{Ca}}\). The principal reason for the different potencies of BAPTA and EGTA in reducing the rise of Ca\(^{2+}\) in the dyadic space was their different rates of Ca\(^{2+}\) binding (BAPTA: \(k_{\text{on}} = 500000 \text{mM}^{-1} \text{s}^{-1}\); EGTA: \(k_{\text{on}} = 5000 \text{mM}^{-1} \text{s}^{-1}\)). As a consequence, the concentration gradients of free BAPTA and Ca\(^{2+}\)-BAPTA, and of free EGTA and Ca\(^{2+}\)-EGTA, between the dyadic space and bulk cytosol were substantially different after the onset of depolarisation. In the case of BAPTA, the large gradients provided a large driving force, causing rapid transport of Ca\(^{2+}\)-BAPTA molecules out of the dyadic space, and movement of free BAPTA into the dyadic space, so that BAPTA appeared to act as a fast ‘shuttle’. The slower rate of Ca\(^{2+}\) binding to EGTA and development of substantially smaller free EGTA and Ca\(^{2+}\)-EGTA gradients between the dyadic space and cytosol reduced the ability of EGTA to affect Ca\(^{2+}\)-dependent inactivation of \(I_{\text{Ca}}\).

Brette F at al. (2004).


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Revisiting the differential effects of left sided and right sided sympathetic outflows on the whole heart: Studies in the isolated innervated rabbit heart

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Historical studies demonstrating differential effects between left and right sympathetic outflows to the heart have employed canine models (Ardell et al, 1988). The aim of this study was to re-examine these differences in the rabbit using a novel in vitro preparation. Adult male New Zealand rabbits (3.0±0.1kg; n=4) were used. The isolated heart preparation with intact autonomic nerves was obtained under propofol anaesthesia (1 mg/kg, i.v.) as previously described (Ng et al, 2001). Left ventricular pressure (LVP) was measured using a fluid filled balloon. The left and right paravertebral sympathetic chains were identified and isolated from adjacent tissues from the level of T4 up to T1/2 where fine custom-made silver bipolar electrodes were positioned and electrically isolated with dental cement. The threshold voltage that caused an increase in both heart rate (HR) and LVP was determined and left (LS) and right (RS) sympathetic chains were stimulated separately at twice threshold voltage at 2.3±1.1V (LS) and 1.2±0.4V (RS) respectively at 2Hz [Low], 5Hz [Med] and 10Hz [High] during 1) sinus rhythm and 2) during right ventricular pacing at 250bpm to examine chronotropic and inotropic effects. Data are mean±SEM, statistical analysis was performed using 2-factor ANOVA. Results: LS and RS increased both HR and LVP, to differing degrees (Fig1A). The peak HR achieved during RS was significantly greater than during LS (Table 1). Whilst there was a trend for LS to have a greater effect in increasing LVP than RS (see Table 1 and Fig1B), 2-factor ANOVA revealed a significant difference in the percentage change in HR and LVP between LS and RS (Fig1B). In conclusion, stimulating the right sympathetic outflow to the heart has a more prominent effect on the sino-atrial node but a weaker effect on the left ventricle, whereas the reverse is true when stimulating the left sympathetic outflow. This may reflect regional differences on sympathetic innervation to the heart.
The effect of caffeine on cardiomyocytes isolated from different age groups

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The aim of this study was to determine whether the effect of caffeine on calcium (Ca\(^{2+}\)) transients in isolated perfused rat cardiomyocytes is age-related. Caffeine is known to induce Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) (O’Neill SC, Eisner DA., 1990). Whether this effect of caffeine is the same in all age groups postnatally, is not presently known.

Cardiomyocytes were enzymatically isolated from 14, 21, 28 days old and adult rat hearts. Freshly isolated cardiomyocytes were loaded with the Ca\(^{2+}\) fluorescent dye Fura-2, then perfused with HEPES buffer (1mM Ca\(^{2+}\)) in a chamber under an inverted microscope at 34°C and stimulated at 0.2Hz. After equilibration cardiomyocytes were perfused with 20mM caffeine for 1 minute followed by washing with normal buffer. Ca\(^{2+}\) transients were monitored using photometry throughout. Data is expressed as mean ± standard error.

Exposure of adult cardiomyocytes to caffeine induced the characteristic marked increase in Ca\(^{2+}\) transient amplitude that was followed by complete abolition of the transients indicating emptying of the SR of Ca\(^{2+}\). This effect was also seen in cardiomyocytes from all other age groups. Upon reperfusion and washing out the caffeine, the SR gradually refills its Ca\(^{2+}\) stores and normal transients are restored. The rate of SR refilling was significantly slower in 14 day cardiomyocytes compared to those from adult (ANOVA, p = 0.027, n=10). The 14 day old cardiomyocytes on average took approximately 172 ± 12 seconds to refill the SR in comparison to adult cardiomyocytes which took 120 ± 10 seconds when exposed to the same concentration of caffeine for the same duration of time.

This work shows an age-related difference in rate of SR refilling following caffeine induced emptying. This could be due to differences in SR development. Additionally, refilling of the SR with Ca\(^{2+}\) in early postnatal development depends on extracellular Ca\(^{2+}\) coming via the Na\(^+\)/Ca\(^{2+}\) exchanger rather than the Ca\(^{2+}\) channel (Artman, M., 1992). The exchanger is likely to be slower in transporting Ca\(^{2+}\) across the sarcolemma which would explain the slower rate of SR refilling.


This work is supported by the BHF.

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Exercise training (four weeks induction at 40-60% 1RM then three sessions/week at 70% 1RM, three each of upper and lower body and two torso-based exercises). Before and after training, femoral artery blood flow was measured (Doppler ultra-sound) in three conditions: (i) postabsorptive state at rest, (ii) in both legs 120 min after oral feeding (Fortisip: 16% protein, 49% carbohydrate, 35% fat) providing energy at 4.25× BMR for 2.5 h supplied as a 3×bolus and 4 further aliquots every 30 min with one leg at rest, and (iii) the other leg recovering from a previous exercise bout of 6 × 8 repetitions of leg extensions (75% 1RM). All procedures were approved by University of Nottingham ethics committee. Leg strength (extension, curl and press) increased by 23.7±4.49, 39.6±2.86 and 32.3±4.13% respectively in the young, middle aged and old groups. Basal blood flow in the middle and old groups was identical (0.32±0.03 and 0.33±0.03 l.min⁻¹ respectively) with a strong trend for the flows to be less than in the young (0.51±0.1 l.min⁻¹). The young group showed no training effect in leg blood flow in response to feeding and exercise (66.2±12.3% pre-training vs. 74.6±14.0% post-training). In contrast, in both the middle aged and old groups, responses to feeding plus exercise (Figure 1) were significantly enhanced by training (middle 101±16% vs. 147±32% and old 59±15% vs. 115±47%). In middle aged subjects feeding alone did not increase leg blood flow in the untrained state, but induced responsiveness after training. The results show (i) that leg blood flow responses to exercise plus food are diminished in middle aged and old subjects and (ii) are restored to the pattern seen in young subjects by resistance training.

![Figure 1. Effect of resistance training on increases in femoral artery blood flow after feeding or exercise plus feeding in young, middle-aged and old subjects. Values are means ±SEM. ** = P<0.01 vs. basal pre-training; =P<0.01 vs. basal post-training; = P<0.01 vs. feeding plus exercise pre-training, all via ANOVA with Tukey’s post analysis.](image)

This work was supported by grants from UK BBSRC ((BB/XX510697/1 and BB/CS16779/1) European Union EXE-GENESIS program and The Dunhill Trust.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Molecular evidence for blood-brain barrier disruption in the absence of structural tissue damage during acute exposure to inspiratory hypoxia


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It has been suggested that inspiratory hypoxia may cause molecular opening of the blood brain barrier (BBB) in the absence of structural neuronal-parenchymal damage subsequent to a systemic accumulation of free radicals (Bailey et al., 2009). Therefore the current study aimed to establish changes in regional metabolism, specifically the trans-cerebral exchange of S100β and neuron-specific enolase (NSE) to distinguish potential “barrier disruption” from structural “brain tissue damage” caused by hypoxia.

Ten healthy males aged 27 (mean) ± 4 (SD) years were examined in normoxia and following 9h passive hypoxia (12.9%O2). Internal jugular venous and radial arterial bloods were collected simultaneously for the measurement of serum S100β and NSE by ELISA. Global cerebral blood flow (CBF) was measured using the Kety-Schmidt technique (Kety & Schmidt, 1945) and global cerebral plasma flow (CPF) determined as CBF x (1-haematocrit). Trans-cerebral net exchange was calculated as the arterio-jugular venous concentration difference x CPF. Data were analysed using a two-way repeated measures ANOVA and post-hoc Bonferroni-corrected paired samples t-tests.

Hypoxia was associated with a general increase in S100β (arterial inflow and venous output) whereas NSE decreased (Table 1). The net cerebral output of S100β and uptake of NSE observed during normoxia was shown to persist without any further changes being incurred during hypoxia. These findings provide the first regional molecular evidence to suggest that hypoxia causes subtle barrier disruption in the absence of neuronal-parenchymal brain injury.

Table 1 Brain-specific proteins

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample site</td>
<td>Arterial</td>
<td>Venous</td>
</tr>
<tr>
<td>S100β (µg/l)</td>
<td>36.55 ± 13.5</td>
<td>44.28 ± 12.98</td>
</tr>
<tr>
<td>NSE (µg/l)</td>
<td>3.88 ± 1.08</td>
<td>3.73 ± 0.64</td>
</tr>
</tbody>
</table>

Values are mean ± SD; NSE; α-vD, arterial minus venous concentration difference; main effect for condition indicates a pooled (arterial + venous) difference (P < 0.05) between normoxia vs. hypoxia; main effect for sample site indicates a pooled (normoxia + hypoxia) difference (P < 0.05) between arterial vs. venous.


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Influence of hyperoxia on the power-duration relationship during severe-intensity exercise in humans: a 31P-MRS study

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The purpose of this investigation was to explore the mechanistic bases of the hyperbolic relationship between power and the tolerable duration of severe-intensity exercise by assessing the kinetics of intramuscular phosphocreatine concentration ([PCr]) and pH during prediction trials performed both in normoxia and hyperoxia. It was hypothesised that the inspiration of hyperoxic gas would increase the asymptote of the power-duration relationship, i.e. the critical power (CP), without altering the curvature constant (W). It was also hypothesised that the tolerable duration of exercise, irrespective of the work-rate within the severe-intensity domain, would be associated with the attainment of the same, critically low [PCr] and pH values as determined using 31P magnetic resonance spectroscopy (31P-MRS).

Values are mean ± SD; NSE; α-vD, arterial minus venous concentration difference; main effect for condition indicates a pooled (arterial + venous) difference (P < 0.05) between normoxia vs. hypoxia; main effect for sample site indicates a pooled (normoxia + hypoxia) difference (P < 0.05) between arterial vs. venous.


Following ethical approval, seven male subjects (mean ± SD, age 30 ± 9 years) completed four constant work-rate, knee-extension exercise bouts to exhaustion (Tlim range: 3-10 min) both in normoxia (N) and hyperoxia (H; 70% O₂) inside the bore of 1.5 T superconducting magnet. The inspirants and work-rates were administered in a single-blind, randomised order. Individual power-time relationships were established using the hyperbolic model [time = W/([power-CP]) and the overall rate of decline in [PCr] was calculated as the mean response time (MRT). Paired samples t-tests were used to assess differences between conditions in CP and W'. End-exercise [PCr] and pH were compared across inspirants and work-rates using two-way repeated measures ANOVAs. Significance was accepted at P<0.05.

The CP was significantly higher in hyperoxia (N:16.1 ± 2.6 vs. H:18.0 ± 2.3 W; P<0.05), while the W' parameter was not significantly altered (N:1.92 ± 0.70 vs. H:1.48 ± 0.31 kJ; P>0.05). The [PCr] at the limit of tolerance (~8% of resting baseline) during each of the four trials was not significantly different either in normoxia or hyperoxia (F6,18 = 1.43, P>0.05). The end-exercise pH (~6.65) was likewise unaffected by work-rate and the inspired oxygen fraction (F6,18 = 1.43, P>0.05). The end-exercise [PCr] at the limit of tolerance (~8% of resting baseline) during each of the four trials was not significantly different either in normoxia or hyperoxia (F6,18 = 1.43, P>0.05). The end-exercise pH (~6.65) was likewise unaffected by work-rate and the inspired oxygen fraction (F6,18 = 1.43, P>0.05). The end-exercise [PCr] at the limit of tolerance (~8% of resting baseline) during each of the four trials was not significantly different either in normoxia or hyperoxia (F6,18 = 1.43, P>0.05). The end-exercise pH (~6.65) was likewise unaffected by work-rate and the inspired oxygen fraction (F6,18 = 1.43, P>0.05). The end-exercise [PCr] at the limit of tolerance (~8% of resting baseline) during each of the four trials was not significantly different either in normoxia or hyperoxia (F6,18 = 1.43, P>0.05). The end-exercise pH (~6.65) was likewise unaffected by work-rate and the inspired oxygen fraction (F6,18 = 1.43, P>0.05).

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Cardiac Cycle Timing Events are Reliably Measured Day-to-Day Using Digital Ballistocardiography

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Human myocardium has an amazing ability to contract reliably day-to-day as reflected by the timing events of the cardiac cycle. We measured the mechanical activity of the heart to record cardiac cycle timing events using digital ballistocardiography (dBG) in a group of healthy subjects (N=7; 3 females) with no known cardiovascular disease. We hypothesised that dBG would reliably record the timing events of the cardiac cycle from day-to-day. Subjects (mean ± SD, age= 31.7±10.5yrs) were assessed on three consecutive days (D1, D2, D3), at the same time of the day and under similar conditions in a quite laboratory setting. Each subject had the dBG-300 sensor attached to the skin using solid gel electrodes (single lead EKG). The dBG-300 sensor was placed over the sternum approximately 2cm above the xiphoid process. Thereafter, a 30-second ballistocardiogram was recorded and stored for later analysis. A total of 15 dBG waveforms (heart beats) were analysed for each subject per day and then averaged (Mean±SD). Results showed that average heart rate on D2 (58.4±11.7 bpm) was significantly (ANOVA) lower from D3 (60.2±7.7 bpm), but not D1 (59.1±8.2 bpm). When the dBG data was then corrected for heart rate there were no significant differences between any of the cardiac cycle timing intervals, including Q-wave (EKG) to: atrial systole (AS) (D1=40.6±12.6 msec; D2=43.5±13.9 msec; D3=44.9±11.5 msec); mitral valve close (MVC) (D1=42.6±9.8 msec; D2=41.3±11.5 msec; D3=42.2±8.1 msec); aortic valve open (AVO) (D1=75.5±8.0 msec; D2=75.4±9.6 msec; D3=72.0±9.8 msec); rapid ejection period (REP) (D1=145.1±10.9 msec; D2=141.5±10.0 msec; D3=138.7±5.7 msec); aortic valve close (AVC) (D1=329.7±30.3 msec; D2=333.3±28.9 msec; D3=331.0±27.2 msec); mitral valve open (MVO) (D1=433.7±31.3 msec; D2=435.1±29.5 msec; D3=436.2±16.0 msec); early diastole (ED) (D1=518.9±33.2 msec; D2=518.3±29.3 msec; D3=519.2±23.6 msec); and late diastole (LD) (D1=995.2±160.2 msec; D2=1025.6±236.2 msec; D3=996.1±139.6 msec). The percent difference for these variables day-to-day was 9.7% (AS), 3.1% (MVC), 4.6% (AVO), 1.9% (REP), 1.1% (AVC), 0.6% (MVO), 0.2% (ED), and 5.7% (LD). These data suggest that: 1) day-to-day cardiac cycle mechanics in human subjects is reliable; and 2) digital ballistocardiography can be used to reliably monitor differences in cardiac cycle timing intervals from day-to-day.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC203

Characterization of in vitro differentiated aged human skeletal muscle satellite cells by transcriptional profile

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Sarcopenia is an age-related non-pathological condition that includes a progressive loss of muscle mass, strength and function (Vandervoort et al., 2001). Several factors both intrinsic (metabolic pathway modifications, hormonal and cellular changes) and extrinsic (lifestyle and caloric intake) contribute to sarcopenia. The aging process is associated with a consistent decrease in the ability of muscle tissue to regenerate following injury or overuse due to the impairment of intervening satellite cells (Hawke and Garry, 2001). Satellite cells are muscle stem cells (Charge and Rudnicki, 2004). In response to stimuli such as myotrauma, satellite cells become activated, proliferate, and express myogenic markers and are termed myoblasts. Ultimately, these cells fuse to existing muscle fibers or fuse together to form new myofibers (myotubes) during regeneration of damaged skeletal muscle. Previously we
demonstrated that in old subjects the decreased muscle regenerative capability is not due to a reduced number of quiescent satellite cells but, more probably, due to an impairment of their differentiation program (Beccaﬁco et al., 2007; Fulle et al., 2005). Although this cells population was identiﬁed 40 years ago, little is known regarding molecular basis of activated elderly satellite cells in muscle repairing process.

The aim of the present study was to characterize the transcriptional proﬁle of myoblasts and myotubes obtained from elderly human skeletal muscle biopsies (after informed consent). We have isolated and cultured quiescent satellite cells on which we determined myogenic percentage using immunocytochemistry and the fusion index percentage of myotubes staining fast and slow myosin heavy chains. To obtain the goal, we performed microarray experiments on myoblasts and myotubes at early stages of differentiation (4, 24 and 72 hours) comparing the transcriptional proﬁle of older adults than young individuals.

The present study suggest that the failure of the differentiative program is due to the disregulation of genes involved in: i) oxidative damage accumulation in molecular substrates, probably due to impaired antioxidant activity and repair capability (upregulation of polymerase K and SHC1), ii) altered cytoskeleton turnover and extracellular matrix degradation, and iii) activation of atrophy mechanism via a speciﬁc FOXO-dependent program as well an impairment of the protein balance.


We thank Dr Luigi d’Amelio (Orthopaedic Division of Ospedale di Atri) for the surgical operations. This study was supported by a research grant from MIUR (Ministero Istruzione Università e Ricerca) and by University “G. d’Annunzio” of Chieti-Pescara local grants to Stefania Fulle.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Gut peptide hormones ghrelin and obestatin differentially effect structure and synaptic function of rat hippocampal neurones in vitro

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Ghrelin and obestatin, both products of the ghrelin gene which have been implicated in regulating appetite, have also been shown to have pro-cognitive effects in vivo. However, it is not clear if cognitive effects result from direct effects on relevant brain structures (e.g. hippocampus), or what, if any, interaction exists between both peptides. We examined the effect of both peptides, alone and in combination, on neuronal structure and synaptic function in vitro, by exposing primary hippocampal neuronal cultures to a range of doses of ghrelin (0.05, 0.5, 1.25, 5, 50 μM), obestatin or both in the medium for 48 hours prior to testing. Results of all experiments are shown in Table 1. Neuronal structure was analysed by calculating the area under curve (AUC) of the Sholl plots (Sholl 1953). No dose of ghrelin or obestatin had an effect on AUC, but a significant increase was seen at 5 μM dose when both peptides were co-applied. Additionally, while no ghrelin doses increased the length of the longest neurite, obestatin caused an increase at the 0.5 and 5 μM doses, and no increases were seen when both peptides were co-applied.

Synaptic release rate was calculated from the half life of FM 1-43 detecting with 65 mM KCl stimulation. Ghrelin at a dose of 5μM decreased synaptic release rate, as did obestatin at 0.05, 0.5 and 50 μM doses. No effect on synaptic release was seen when both peptides were co-administered. While both peptides were found to have effects on hippocampal neurones, no simple dose response relationship or interaction was found, suggesting an underlying complexity in the signalling of these peptides which has yet to be elucidated. In particular, the complex dose response to obestatin raises the possibility of more than one receptor for obestatin, which may have different affinities and which may function through interacting signalling pathways. Elucidating these pathways is likely to be key to understanding the pro-cognitive effects of both peptides.

Table 1.

<table>
<thead>
<tr>
<th>Sholl AUC</th>
<th>Contact</th>
<th>0.05μM</th>
<th>0.5μM</th>
<th>1.25μM</th>
<th>5μM</th>
<th>50μM</th>
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</thead>
<tbody>
<tr>
<td>Ghrelin (+) &amp; Obestatin (+)</td>
<td>123.4</td>
<td>121.3</td>
<td>122.5</td>
<td>123.2</td>
<td>123.5</td>
<td>124.2</td>
</tr>
<tr>
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<td>124.3</td>
<td>125.4</td>
<td>126.5</td>
<td>127.6</td>
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<td>124.7</td>
<td>125.8</td>
<td>126.9</td>
<td>127.1</td>
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</table>

<table>
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<th>Neuronal Length (μm)</th>
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<th>0.5μM</th>
<th>1.25μM</th>
<th>5μM</th>
<th>50μM</th>
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<tr>
<td>Ghrelin (+) &amp; Obestatin (+)</td>
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<td>801</td>
<td>823</td>
<td>845</td>
<td>867</td>
<td>889</td>
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<tr>
<td>Obestatin (+)</td>
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<td>812</td>
<td>834</td>
<td>856</td>
<td>878</td>
<td>891</td>
</tr>
<tr>
<td>Both (-)</td>
<td>791</td>
<td>813</td>
<td>835</td>
<td>857</td>
<td>879</td>
<td>892</td>
</tr>
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</table>

This work was supported by Science Foundation Ireland.
In vivo behaviours of squid motor units during denervation of neighbouring units

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The chromatophore system of the squid Loligo vulgaris, run by central motor neurones, generates images that can be recorded photographically (in the laboratory) at intervals while animals are being maintained in their holding tanks. The poster reports on image-analysis of two phenomena (supersensitivity, sprouting) visible in the output history of ‘residual’ chromatophore motor units in a partially denervated environment during the days following incomplete section of the pallial nerve on one side of the body [1,2].

Supersensitivity. The output amplitude of single units of multiply innervated dark chromatophores (spots) [3] is affected by their denervated neighbours through the intra-chromatophore coupling of a spot’s several muscle fibres (~25 on any one spot). The progress of this myogenic enhancement during the hours following section of the left pallial nerve (LPN) under light anaesthesia (<1% ethyl alcohol in seawater) is seen in Figure 1. Whenever the animal changes colour, the ‘residual’ unit on the left side of the mantle (yellow ring) behaves synchronously with similar ones on the right side (upper in the figure); expanded amplitude of individual spots is low at 18h post-operation (a), while neuromuscular endings of neighbouring units are still intact, and increases as muscle denervation supersensitivity (DSS) develops to a maximum at 40h (b, c). Interestingly, when the squid turns pale (d), DSS is suppressed through unknown mechanisms.

Sprouting. Figure 2 shows a second residual unit that had been spared by LPN section, a) at 5 days post-operation, b) at 16 days. Over the intervening 11 days, the motor field of this unit nearly doubled in area through the capture of neighbouring (denervated) spots of several size classes. Further enlargement of this unit did not occur in the remaining weeks before sacrifice.

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Optimized voxel-based morphometry of the rat brain reveals cortical and hippocampal volume decline with age


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Voxel-based morphometry (VBM) is commonly used for analysing differences in human brain volumes [1], but similar methodology has not been developed for use in the rat brain.
In this study, an optimized VBM protocol has been developed for the rat brain and using this, we have undertaken an analysis of age-related changes in brain volume in rats. High resolution structural MRI images were obtained from groups of young (N = 5) and aged (N = 7) rats using a Bruker 7 Tesla animal scanner (Bruker BioSpin, Ettlingen, Germany) and compared using FSL (FMRIB Software Library, 4.0) tools [2]. The animals were anaesthetised before entering the magnet with isoflurane (4% induction), delivered in oxygen. The isoflurane was then reduced to the minimum level to keep the animal asleep, with the depth of anaesthesia controlled by altering the percentage of isoflurane in response to changes in respiratory rate. Images were skull-stripped using the brain extraction tool (within MIPAV 4.0.2 software, [3]) and the grey matter partitions were registered to the same stereotaxic space using an in-house generic grey matter template image. The resulting images were averaged to create a study-specific template, to which the individual images were compared. Permutation-based non-parametric statistical analysis was undertaken. The data indicate that there was a significant reduction in volume in the hippocampus and cortex of aged, compared with young, rats. Specifically, volume reduction was observed in area CA1 of the hippocampus, as well as in the motor areas M1 and M2 of the cortex.

These findings indicate that this optimized VBM methodology can be used to analyse volumetric changes in the rodent brain.

Coronal image of the rat brain highlighting areas of significant tissue decline in the aged rat brain compared to young.


FMRIB Software Library tools freely available at http://www.fmrib.ox.ac.uk/fsl


This study funded by Science Foundation Ireland. We acknowledge the assistance of colleagues in the Trinity Centre for High Performance Computing.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Poster Communications

PC209

Effects of Interleukin-6 on Glucagon Secretion and viability of the pancreatic a-cell line (a TC1-9)

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There is a clear correlation between obesity and the development of Type 2 Diabetes Mellitus. The hallmarks of Type 2 Diabetes Mellitus (T2DM) are insulin resistance, insulin insufficiency and hyperglycaemia. Normal endocrine regulation of glucose homeostasis is lost in T2DM pancreatic due to β-cell and α-cell dysfunction and eventually pancreatic β-cell loss. Obesity (BMI>30kg/m²) is caused by excess adipose lipid storage, which is associated with a significant increase in various cytokines secreted by adipose tissue including IL-1β, TNF-α and IL-6. It is known that IL-6 is a pleiotropic cytokine that is secreted by many nucleated cells including adipocytes and is linked to insulin resistance and T2DM. The effects of pro-inflammatory cytokines on nutrient metabolism in the insulin secreting pancreatic β-cell have been studied in depth in Philip Newsholmes laboratory with results indicating a change in β-cell function from insulin production to cell defense. Despite this there has been relatively little research into the effects of IL-6 or other pro-inflammatory cytokines on pancreatic α-cells. Exposure to the pleiotropic cytokine IL-6 or indeed other pro-inflammatory cytokines may result in loss of regulatory control of glucagon secretion in obese and T2DM patients.

To determine the effects of IL-6 or β-cell secretory products on the functional integrity of pancreatic α-cell line, α TC1-9. α TC1-9 cells were incubated with various concentrations of glucose, glutamine and (sub lethal) combinations of IL-6, TNF-α, IL-1β, IFN-γ, for various incubation times (from 0.5 to 24 hours). The cell media was analysed for glucose and glutamine to determine consumption. Cell contents were analysed for ATP, glutamate and GSH + GSSG as indicators to changes in metabolic function. Viability was determined by the WST-1 assay. Glucagon levels in the culture media were also determined. α TC1-9 cells viability and function was determined at high glucose levels (up to 25mM) and high glutamine levels (up to 5mM). No effect on cell viability in high glucose or high glutamine was determined. IL-6 at sub lethal concentrations did not alter cell viability but altered parameters of cell metabolism and glucagon secretion. IL-6 alters parameters of alpha cell metabolism and function.

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Journal of Alzheimer’s Disease Volume 15:1 (September 2008)

Leonid Roytblat, Maxim Rachinsky, Allan Fisher, Lev Greenberg, Yoram Shapiro, Amos Doudevani and Simon Gelman

Raised Interleukin-6 Levels in Obese Patients

Obesity Research (2000) 8, 673–675; doi: 0.1038/oby.2000.86

187P
Joachim Spranger, Anja Kroke, Matthias Möhlig, Kurt Hoffmann, Manuela M. Bergmann, Michael Ristow, Heiner Boeing, and Andreas F.H. Pfeiffer

Inflammatory Cytokines and the Risk to Develop Type 2 Diabetes: Results of the Prospective Population-Based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study, Diabetes 52: 812-817

Aruna D. Pradhan; JoAnn E. Manson; Nader Rifai; Julie E. Buring; Paul M. Ridker

C-Reactive Protein, Interleukin 6, and Risk of Developing Type 2 Diabetes Mellitus, JAMA. 2001;286(3):327-334

Aoihe Kiely, Neville H McClenaghan, Peter R Flatt, Philip Newsholme, Pro-inflammatory cytokines increase glucose, alanine and triacylglycerol utilization but inhibit insulin secretion in a clonal pancreatic β-cell line

J Endocrinol 2007 195: 113-123

Abbreviations: IL, Interleukin; GSH, Glutathione; RIA, Radio Imunoassay

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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PC210

Aptosis and inflammation in cardiovascular aging: effect of chronic treatment with growth hormone in senescence-accelerated mice

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Aging induces deleterious effects in several organs, and is associated with complex changes in cardiovascular structure and function. This process might be due to damage caused by inflammatory mediators, free radicals and apoptosis (1-3). The purpose of this study was to investigate the effect of aging on different parameters related to apoptosis and NFkB (nuclear factor kappa B) expression in hearts from male senescence-accelerated mice (SAM-P8) and its controls male senescence-accelerated-resistant (SAM-R1). Also to study the influence of chronic administration of Growth Hormone (GH) on these parameters in old SAM-P8 mice. Forty male mice were used. Animals were divided into five experimental groups, two old groups of 10 month of age (SAM-P8/SAM-R1), one GH group of also 10 months age (SAM-P8), that was treated with the hormone (2 mg/kg/day/s.c.) and two young groups, of 2 months of age (SAM-P8/SAM-R1) that were used as young controls. After 30 days of treatment, animals were sacrificed by decapitation, and hearts were collected. The expression of BCL2-associated death promoter (BAD), BCL2-associated X protein (BAX), B-cell lymphoma 2 (BCL2) and NFkB, were determined by real-time reverse transcription polymerase chain reaction. Results were submitted to a two way ANOVA statistical evaluation using the Statgraphics program. The expression of NFkB (p<0.05) and BAX (p<0.01) were significantly increased in the old SAMP-8 control males, as compared to young mice. Age also diminished the levels of BCL2 (p<0.01), in this animals. In the case of SAM-R1 animals a significantly increased BAX as found (p<0.05), as compared to SAM-R1 young controls. The expressions of NFkB and BCL2 were similar in old and young SAM-R1 mice. The expression of BAD on SAM-P8 and SAM-R1 mice, showed no significant differences between young and old animals. Exogenous GH administration showed a significant decrease in levels of NFkB (p<0.01), BAX (p<0.01) and BAD (p<0.05). The expression of BCL2 was not significantly affected with the treatment in old SAM-P8. Our results suggest that the inflammatory process and some mediators of apoptosis could contribute to the observed alterations of the cardiovascular system associated with aging and that GH may play a potential protective effect during this process.


This work has been possible through a RETICEF RD 06/0013, grant from the Instituto Carlos III and another of SAF 2007 66878-C02-01. Katherine Forman was supported by Pre-Doctoral Fellowship, Chile

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PC211

Neonatal lipopolysaccharide exposure delays puberty and alters hypothalamic kisspeptin and kiss1r expression in the female rat

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Immunological challenge experienced in early life can have long-term programming effects on the hypothalamic-pituitary-adrenal (HPA) axis which permanently influence the stress response. Similarly, neonatal exposure to immunological stress enhances stress-induced suppression of the hypothalamic-pituitary gonadal (HPG) axis in adulthood, but may also affect earlier development, including the timing of puberty. To investigate the timing of the critical window for this programming of the HPG axis neonatal female rats were injected with lipopolysaccharide (LPS, 50μg/kg ip) or saline on postnatal days 3, 5, 7, 9, or 14 and 16 and monitored for vaginal opening and first vaginal estrus as markers of puberty. We also investigated the effects of neonatal programming on the development of the expression patterns of kisspeptin (Kiss1) and its receptor (Kiss1r) in hypothalamic sites known to contain kisspeptin—expressing neuronal populations critical to reproductive function: the medial preoptic area (mPOA) and the arcuate nucleus (ARC) in neonatally-stressed animals. We determined that the critical period for a significant delay in puberty due to neonatal LPS exposure is before 7 days of age in the female rat,
and demonstrated that Kiss1, but not Kiss1r mRNA, expression in the mPOA is down-regulated in pre-pubertal females. These data suggest that the mPOA population of kisspeptin neurones play a pivotal role in controlling the onset of puberty, and that their function can be affected by neonatal stress.

This work was supported by the Wellcome Trust and BBSRC (UK).

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PC212

Eicosapentaenoic acid administration stimulates muscle regeneration and inhibits atrogenes gene expression in the gastrocnemius of arthritic rats

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Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid which is present in high concentration in fish oil. Adjuvant-induced arthritis is an animal model of cachexia that induces muscle wasting by increasing the ubiquitin-proteosome pathway. The aim of this work was to elucidate whether the effect of EPA on arthritis-induced muscle wasting is mediated by changes on muscle protein synthesis, regeneration and inflammation. For this purpose, arthritis was induced in male Wistar rats by an intradermal injection of Freund’s adjuvant. Three days after the injection, control and arthritis rats were divided into two experimental groups. One group was daily gavaged with EPA (1g/kg bw) and the other was gavaged with coconut oil (1g/kg bw). Rats were humanly killed after 12 days of treatment. EPA administration to arthritic rats ameliorated arthritis score and hindpaw swelling (P<0.01). Arthritis decreased skeletal muscle weight (P<0.01), whereas EPA administration reduced this effect (P<0.05). EPA prevented arthritis-induced increase of TNF-alpha gene expression both in liver and in gastrocnemius muscle (P<0.01). Arthritis increased the atrogene MAFbx and MuRF-1 mRNA in gastrocnemius, and this effect was prevented by EPA (P<0.01). Both EPA and arthritis increased differentiation marker myogenin mRNA and protein (P<0.01). Muscular proliferating cell nuclear antigen (PCNA) mRNA and protein were also increased in arthritic and EPA treated rats (P<0.01). These data suggest that EPA exerts an antiinflammatory effect on adjuvant-induced arthritis, and it attenuates muscle wasting by inhibiting proteolysis and stimulating muscle regeneration.

This work was supported by CYCYT (BFU, 2006-11899) a grant to Estibaliz Castillero (Gobierno Vasco, BFI06.31), and a grant to Maria Lopez-Menduiña (BES-2007-16001).

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PC213

The Effect of Clothing and Cultural Practice on Bone Mineral Density and bone turnover markers in Kuwaiti Pre-Menopausal Women

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Low bone mass is frequently encountered than expected in the Mediterranean and Gulf countries, which are sunny most of the time. As Kuwait is one of these sunny countries, we aimed to investigate the effect of outfitting style on bone mineral density (BMD) and on markers of bone turnover in middle-aged pre-menopausal Kuwaiti women.

Two groups of pre-menopausal single Kuwaiti females (20-35 yrs of age; 20 per study) were recruited. Written informed consent was obtained from each subject. A questionnaire was filled in by all subjects to obtain history and anthropometric data. Group I (GI) included females wearing black veil since puberty and not exposed to the sun. Group II (GII) included females wearing western clothes. Fasting blood samples were collected for measurement of baseline biochemistry, minerals, and markers of bone turnover (osteocalcin, N-telopeptide of type I collagen [sPINP] and C-telopeptide of type I collagen [sICTP]). BMD was measured by dual energy x-ray absorptiometry (DEXA) at the lumbar spine, femoral neck and total body. Statistical analysis was performed using SPSS 15. The two subjects were matched for age mean±SEM (GI versus GII) (22.5 ± 0.48 vs. 22.3 ± 0.49). The mean total body BMD of the two groups were similar (BMD1.16 ± 0.02 vs. 1.13 ± 0.015 g/cm2), T-score of total body (0.405 ±0.25 vs. 0.050±0.19). However, BMD of L2-L4 spine was significantly lower in veiled (GI) compared with non-veiled (GII) females (T-score ofL2-L4: -1.294 ± 0.33 vs. -0.06 ± 0.24, p<0.01). As for bone markers, sPINP and sICTP were significantly raised in veiled (GI) compared with non-veiled (GII) (p<0.01 and p<0.002, respectively). Bone mineral density is decreased in young pre-menopausal Kuwaiti females wearing veils compared with those without veils. They have evidence of raised bone turnover as shown by the elevation of markers of bone formation and resorption. It is therefore essential to assess vitamin D status in both groups to verify its link with the partial difference in sun exposure.

College of Graduate Studies Grant #YM04/08, Kuwait University

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.
Multiple Ca2+ stores are involved in phenylephrine stimulation of rat aortic smooth muscle

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Previous studies indicate that in pulmonary and coronary arterial smooth muscle cells beside IP3 and ryanodine sensitive Ca2+ pools, acid-filled Ca2+ store may play an important role in vasoconstriction. The aim of this study is to characterize the Ca2+ pools involved in phenylephrine (PHE) stimulation of rat aortic smooth muscle cells. Experiments were carried out using Ca2+-imaging technique on cultured rat aortic smooth muscle cells and force contraction of desendothelized rat aortic ring. Administration of PHE (1 microM) in Ca2+ free saline on cultured aortic smooth muscle cells induces a transitory elevation of [Ca2+]i by 561 + 3.6 nM (n = 38 cells). Following one hour pretreatment with bafilomycin (1 microM), a well known disrupter of acid-filled Ca2+ stores, the [Ca2+]i diminished up to 174 + 2.8 nM (n = 47 cells). The blockade of ryanodine receptors with ryanodine (10 microM; 15 min preincubation), or the IP3 receptors with 2-APB (2-aminoethyl diphenylborate - 100 microM; 15 min preincubation) also reduce the PHE-induced elevation of [Ca2+]i up to a level of 352 + 2.9 nM (n = 26) and 231 + 2.3 nM (n = 34), respectively. Meanwhile 1 h pretreatment with bafilomycin (1 microM) and 15 min with 2-APB (100 microM) totally abolished the elevation of cytoplasmatic Ca2+ induced by PHE (n = 52). Experiments carried out on desendothelizeed rat aorta rings reveal that 1 h treatment with bafilomycin (1 microM) inhibits the PHE-induced contraction with 58 + 3.2% (n = 6), while 45 min treatment with ryanodine (10 microM) or 2-APB (100 microM) reduce the contractions elicited by PHE with 31 + 2.8% (n = 6) and 47 + 3.6% (n = 6), respectively. Concomitant treatment with bafilomycin (1 microM; 1 h), and 2-APB (100 microM; 45 min) totally abolished PHE-induced contraction (n = 6). It may be thus concluded that PHE primary recruits Ca2+ from acid-filled and IP3-sensitive Ca2+ stores which, in turn, activate ryanodine receptors through calcium-induced calcium-release mechanism.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

Effect of ATP on interstitial cells of Cajal isolated from the rabbit corpus cavernosum

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Interstitial cells of Cajal (ICC) are thought to be pacemakers in the gastrointestinal tract, where they drive gastric and intestinal motility1. Similar cells have been identified in erectile tissue, although their role here is unknown2. In the present study
we have isolated ICC from the rabbit corpus cavernosum in order to study the effect of exogenous ATP on their membrane currents. Rabbıts were killed by lethal injection with pentobarbitone and their penises removed. The tunica albugínea was opened and the corpus cavernosum extracted and cut into 1 mm3 pieces which were stirred in an enzyme mixture, followed by washing in Hanks Ca2+ free saline to release single ICC and smooth muscle cells (SMC). The cells were then plated for study using the perforated patch clamp technique, using Cs+ rich pipette solution. When cells were held at –60 mV, ATP (20 μM) evoked large inward currents when the pipette and bath [Cl-] were symmetrical (135 mM). Under these conditions the ATP responses were reversibly reduced from 1105 ± 325 pA to 377 ± 97 pA by suramin (100 μM, a broad spectrum purinergic receptor antagonist (n=7, p <0.05). The selective P2Y receptor agonist, 2-methylthio ADP (1 μM), mimicked the effect of ATP. However, while the selective P2Y1 receptor antagonist MRS 2500 (100 nM) effectively reduced the responses to 2-methylthio ADP (from 460 ± 212 pA to 110 ± 82 pA, n=5, p < 0.05), it had a variable effect on the ATP responses, reducing them in only 2 out of 5 cells. This suggests that 2-methylthio ADP mediated its effects via P2Y1 receptors, but that ATP may act on more than one receptor subtype.

Also, niflumic acid (100 μM), a blocker of both Ca2+/activated Cl− currents and non-selective cation currents reduced the ATP-evoked currents from -536 ± 143 pA to -60 ± 26 pA (n=6, p <0.05). To test whether ATP-evoked currents were mediated by CI− or non-selective cation channels, the effects of ATP and caffeine (previously shown to evoke a Ca2+/activated Cl− current in these cells) were compared. When cells were held at -60 mV in symmetrical Cl− solutions, as above, both ATP and caffeine evoked inward currents. However, when ECl was adjusted to -40 mV, by reducing intracellular [Cl−], and cells then held at -10 to -20 mV, ATP still evoked an inward current, but the caffeine-evoked current shifted to the outward direction (n=6). When the extracellular [Na+] was reduced from 130 to 13 mM (by replacement with equimolar NMDG) the ATP current also shifted in the outward direction (n=3), whereas the caffeine evoked current was little affected. These data show that ATP can evoke inward currents in ICC from the corpus cavernosum that are likely to be mediated by non-selective cation channels.

Sanders KM, Ward SM. Interstitial cells of Cajal - a new perspective on The Physiological Society ethical requirements.

Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.


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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC218

Excitatory effects of ATP on rabbit urethra smooth muscle

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Myogenic tone in urethra smooth muscle is regulated by several neurotransmitters, including noradrenaline, acetylcholine and nitric oxide (Brading et al., 1999). ATP is also considered to be an important neurotransmitter in the urethra (Pinna et al., 1996), however its effects have not yet been fully characterised and therefore it’s physiological role remains unclear. The purpose of the present study was to characterise the effects of ATP on the contractile activity of rabbit urethral smooth muscle. New Zealand white rabbits were humanely killed and mechanical recordings were made from strips of proximal urethra (8 x 1 x 1 mm) using a multi channel Myobath system. Muscle strips were perfused with warmed Krebs solution bubbled with 95% O2-5% CO2 which contained atropine (1 μM) using a multi channel Myobath system. Muscle strips of proximal urethra (8 x 1 x 1 mm) were stimulated with ATP (10 μM) and NG-Nitro-L-arginine (NO-ARG, 100 μM). Strips were adjusted to a tension of 2-4 mN and allowed to equilibrate for 50 min before experimentation began.

Exogenous application of ATP (10 μM) induced robust contractions of the muscle strips. These responses typically consisted of an increase in tone, superimposed upon which were transient phasic contractions that varied in frequency and amplitude. The contractile effects were quantified by measuring the total area under the trace (mN.s). In 45 muscle strips ATP increased the tone from 43.6 ± 20.8 to 163 ± 40.1 mN.s (p<0.05). The ATP induced contractions were inhibited by the broad spectrum purinergic inhibitor suramin (100 μM) from 95 ± 18 to 37 ± 16 mN.s (n=17, p<0.05) but were not affected by the P2X receptor selective antagonist NF279 (1 μM, 9 ± 31 mN.s under control conditions versus 101 ± 21 mN.s in its presence, p>0.05). In contrast, the ATP effects were greatly attenuated by the P2Y1 receptor antagonist MRS2500 (100 nM) and were mimicked by the P2Y receptor agonist, 2-methylthio ADP (2-MeSADP). For example, in 18 muscle strips the mean ATP evoked contraction was reduced from 118 ± 23 to 65 ± 15 mN.s by MRS2500 (p<0.05) whereas 2-MeSADP (1 μM), induced a contraction measuring 372 ± 108 mN.s compared to 153 ± 44 mN.s produced by ATP (10 μM) in paired experiments (p<0.05, n = 14).

These results suggest that ATP could act as an excitatory neurotransmitter and that these effects are likely to be mediated via P2Y receptors.


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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC219

Modulation of trabecular meshwork cell function by cross-linked actin networks

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Worldwide glaucoma is a leading cause of irreversible blindness and its incidence is increasing as the population ages as it is an age-related disease. Its onset is insidious as it rarely causes symptoms and many patients are not aware of any visual field loss. Primary Open Angle Glaucoma (POAG) is the main type of Glaucoma, next being secondary angle closure glaucoma and is an economically significant disease. Chronic raised intraocular pressure leads to damage at the optic nerve head with consequent visual field loss and little is known about the precise underlying pathologic mechanism(s). Cross-Linked Actin Networks (CLANs) are triangular polygonal arrangements comprised of actin; these form geodesic dome arrangements. We have recently demonstrated that bovine TM cells exposed to dexamethasone upregulate CLAN incidence far more robustly compared to human cells, this gave us a model to probe functional affects of CLANs on TM cells. The aim of this study was to determine the functional effects of CLANs in the bovine TM and also to determine to heat shock protein response. Primary TM cells were cultured from bovine eyes (obtained from an abattoir) and we employed a collagen contraction model to examine the effect of CLANs on cell contraction. Moreover we examined the effects of CLANs on cell viability and apoptosis using standard techniques. In addition we determined the expression of Heat shock proteins 70, heat shock cognate 70 as well as the enzyme manganese superoxide dismutase (MnSOD) using Western Blotting and the inflammatory cytokine Interleukin-6 (IL-6) by E.L.I.S.A. Results showed that CLANs inhibited matrix contraction in the 2D model but not 3D model of contraction (P<0.01). There was no significant increase in apoptosis in association with CLAN incidence. There was a significant increase in expression of inducible (ten fold change) Hsp70 that was time-dependant and also hsc70 (P<0.001). MnSOD expression was also increased in response to CLANs as
was the expression of inflammatory cytokine IL-6 as measured using ELISA (P<0.01). These data demonstrate a functional impairment of TM cells in response to CLANs and that it is not related to cellular death. Moreover, there is significantly increased expression of Hsps, antioxidant enzyme and a proinflammatory cytokine, that may impinge on cellular function.

CLAN in Trabecular meshwork cell imaged by confocal microscopy. The usual arrangement of F-actin is that of stress fibres of varying length.


Fight for Sight. Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

PC221

Ultrastructural characterization of interstitial cells of Cajal from the guinea-pig bladder

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ICC were first discovered by Ramon y Cajal in the intestine in 1911 using methylene blue and Golgi staining techniques (Cajal, 1911) and are now established as pacemakers and mediators of neurotransmission (Sanders, 1996). It was previously believed that ICC were present in only the gastrointestinal tract but there is now convincing evidence that the urinary tract, including the urethra, urinary bladder, renal pelvis and ureters also contain populations of cells with many characteristics of ICC, though these studies are at a comparatively early stage (Brading and McCloskey, 2005). The aim of the present study was to: identify interstitial cells of Cajal (ICC) in the wall of the guinea-pig bladder using transmission electron microscopy and antibodies to the Kit receptor conjugated to nanogold particles; to characterize the ultrastructural properties of bladder ICC and to examine their relationships with nerves and smooth muscle cells.

Bladders were removed from guinea-pigs (n=4), killed by cervical dislocation in accordance with Schedule 1, UK Home Office regulations. Bladder tissues were prepared for transmission electron microscopy using standard protocols.

Kit-positive ICC were identified in sections from the lamina propria and detrusor, consistent with previous findings with confocal microscopy (Davidson and McCloskey, 2005). In the lamina propria, ICC formed an interconnecting network of branched cells and were also found in close proximity to small blood vessels where their branches made contacts with the vessel wall. In the detrusor, ICC were found on the edge of smooth muscle bundles and the contacts between SMC and ICC were typically 50-100nm. ICC were frequently associated with intramural nerves, again typically within 100nm.

Ultrastructurally, ICC contained mitochondria, rough and smooth endoplasmic reticulum, thin and intermediate filaments, caveolae, Golgi apparatus, free ribosomes and cytoplasmic vesicles. They were distinct from fibroblasts by the presence of a basal lamina and differed from smooth muscle cells in the absence of thick filaments and dense bodies.

The ultrastructural characteristics of ICC in the guinea-pig bladder were characterized with transmission electron microscopy and were consistent with those of gut ICC. ICC were positively identified with c-Kit antibody labeling in the lamina propria and detrusor regions and were closely associated with each other, nerves and smooth muscle cells, consistent with previous published work (Davidson and McCloskey, 2005) at the light microscope level.


Sanders KM (1996) Gastroenterology 111, 492–515


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PC222

Investigation of the ultrastructural properties of interstitial cells of Cajal from the guinea-pig urethra

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Interstitial cells of Cajal (ICC) have been identified in the wall of the rabbit urethra with c-Kit antibodies (Lyons et al, 2007) and share morphological characteristics with ICC from the gastrointestinal tract. These cells exhibit spontaneous electrical and Ca2+-activity (Sergeant et al, 2000) and may act to control contractility and tone of the urethral smooth muscle. ICC are ultrastructurally distinct from smooth muscle cells and fibroblasts and the aim of the present study was to examine the ultrastructural properties of urethral ICC with transmission electron microscopy.

Bladders and urethras were removed from guinea-pigs (n=4), killed by cervical dislocation in accordance with Schedule 1, UK Home Office regulations. The proximal urethra was prepared for transmission electron microscopy using standard protocols. We have previously shown that c-Kit-positive ICC are present within the muscularis of the rabbit urethra (Lyons et al, 2007). In the present study, the muscularis region of the urethra was studied with electron microscopy. Cells with the typical properties of ICC were identified in the circular and longitudinal...
PC223

**Intercellular communication and spontaneous activity in the guinea-pig bladder**

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Spontaneous activity is a phenomenon common to many smooth muscle tissues including the gastrointestinal tract, some blood vessels and urinary tract tissues including the bladder. Spontaneous contractility of bladder smooth muscle are non-voiding contractions which are thought to underlie the tone of the bladder wall during filling (Turner and Brading, 1997). While this activity is considered to be myogenic, its origin and modulation is incompletely understood. The aim of the present study was to assess the contribution of the mucosal layer to spontaneous contractility and to examine the effect of pharmacological agents which target gap junctions.

Bladders were removed from guinea-pigs, killed by cervical dislocation in accordance with Schedule 1, UK Home Office regulations. In vitro tension recordings were made from bladder strips mounted in organ baths which typically developed spontaneous activity during the equilibration period. Spontaneous activity was unaffected by tetrodotoxin (0.1μM), indicating its myogenic origin (n=19 strips from 8 animals, p>0.05). There was no significant difference in the amplitude or frequency of spontaneous contractions between strips which were intact (7.2±0.8mN, 3.5±2.3 per minute) and those which had the mucosal layer removed (8.1±0.9mN, 2.8±1.7 per minute, p>0.05, n=24 strips from 12 animals, unpaired t-test). Gap 27, a Cx43 gap junction blocker transiently inhibited spontaneous activity in both intact and denuded strips (n=5 from 3 animals). The gap junction uncoupler, heptanol (300μM) completely inhibited activity in intact and denuded strips within 6.13±0.45min and 2.87±0.78 min respectively (p<0.05, n=6 strips from 2 animals, unpaired t-test). Full recovery was obtained on washout. The addition of a gap junction opener, AAP10 did not affect the amplitude or frequency of spontaneous activity in intact or denuded strips (p>0.05, n=3) indicating that gap junctions are fully open under control conditions. Moreover, in intact and denuded strips, the time taken by heptanol to inhibit activity was not significantly affected by pre-treatment with AAP10 (n=5, p>0.05 in each case).

The findings of the present study have shown that myogenic spontaneous activity of the bladder requires functional communication via gap junctions and that this activity does not depend on the presence of the mucosal layer.


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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC224**

**c-Kit-positive Interstitial Cells of Cajal in the Human Bladder**

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The cellular mechanisms underlying pathological bladder conditions including urge urinary incontinence are poorly understood. An increasing body of evidence suggests that interstitial cells of Cajal (ICC) may have significant roles in normal bladder activity (Brading & McCloskey, 2005) and also in conditions leading to overactivity (Kubota et al, 2008). Bladder ICC have morphological similarities to ICC of the gastrointestinal tract and have been identified with the established marker, anti-c-Kit, in animal tissues (Davidson & McCloskey, 2005). The purpose of the present study was to identify ICC in human bladder samples and examine their relationships with nerves and smooth muscle.

Bladder biopsies were obtained with informed consent and full ethical approval from patients undergoing urological investigation. Whole-mount, flat sheet tissue preparations were labeled with antibodies using standard immunohistochemical protocols and imaged with a confocal microscope. Single cells were dissociated from biopsy samples using a cocktail of enzymes and viewed with bright-field microscopy. Tissues labeled with anti-c-Kit had several sub-populations of immunopositive cells (n=18 samples). c-Kit-positive cells in the lamina propria were branched, stellate-shaped with a central nucleus and formed frequent connections with each other.
When the biopsy samples contained underlying detrusor smooth muscle bundles, c-Kit-positive cells with lateral branches from an elongated cell body were located on the boundary of the bundles. In some biopsies, discrete, rounded, non-branched c-Kit-positive cells were present; these were deemed to be mast cells. Double-labeling with anti-c-Kit and anti-vAChT to label cholinergic nerves demonstrated close relationships between ICC and cholinergic nerves (n=4 samples) in both the lamina propria and the underlying detrusor. Vimentin-positive cells were abundant throughout the lamina propria and formed a network of interconnecting cells with different morphologies including stellate and elongated bipolar cells (n=4 samples). Vimentin filaments are present in fibroblasts, ICC and other mesenchymal cells and in human bladder are therefore likely to represent a mixed population of cell types. Enzymatic dispersal of biopsy samples yielded a heterogeneous population of cells, the majority of which were spindle-shaped smooth muscle cells and a smaller number of branched cells which resembled ICC, previously investigated in guinea-pig tissues (Johnston et al., 2008).

The human bladder contains populations of c-Kit-positive ICC in the lamina propria and the detrusor. These ICC are structurally associated with cholinergic nerves and are similarly distributed to the ICC of the guinea-pig bladder (Davidson and McCloskey, 2005).

Kubota Y et al. (2008). Neurourol Urodyn. 27, 330-40
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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC225**

**TRPM8 activation causes depolarisations accompanied by high-frequency spontaneous hyperpolarisations through a Ca^{2+} signalling complex with BK_{Ca} channels in rat tail artery myocytes**

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The menthol receptor, Transient Receptor Potential Melastatin member 8 (TRPM8), is the principal sensor of cold in primary sensory neurones (Bautista et al., 2007). However, recently it has been described as a prominent vascular TRPM protein (Inoue et al., 2006; Yang et al., 2006). Previously we have shown that TRPM8 mRNA is expressed in a number of rat arteries including aorta, ventral tail, mesenteric and femoral artery (Melanaphy et al., 2008). In aortic, pulmonary and tail arterial smooth muscle TRPM8 activation increases cytosolic [Ca^{2+}] and triggers myocyte contraction (Yang et al., 2006; Borisova et al., 2008). However, the localisation of the TRPM8 protein in vascular myocytes and the electrophysiological characteristics of its activation remain unknown.

Ventral tail artery was obtained from humanely-dispatched Sprague-Dawley rats (12 weeks). Single vascular myocytes were then isolated by enzymatic digestion for immunocytochemistry and patch clamp analysis. Staining of isolated rat tail artery myocytes with TRPM8 rabbit antibody revealed TRPM8 immunoreactivity predominantly localised on the cells perimeter. In voltage-clamped tail artery myocytes, held at -20 to -40 mV, 300 μM menthol accelerated STOCs discharge from 2.8±0.4 s^-1 to 6.3±0.4 s^-1 (mean ± S.E.M.; paired t-test, P<0.01; N=4 (N = no. of cells)) and often resulted in a transient increase in whole cell current. In perforated patch clamp configuration, 300 μM menthol depolarised tail artery myocytes from a mean membrane potential of -56.8±3 mV by 8.3±1 mV (paired t-test, P<0.001; N=7). Most interestingly, menthol simultaneously increased the frequency of spontaneous hyperpolarisations from a mean of 0.3±0.1 s^-1 to 1.4±0.4 s^-1 (paired t-test, P<0.05; N=6). These hyperpolarisations also increased in amplitude from a mean of -1.18±3 mV to -22.8±3 mV (unpaired t-test, P<0.05; N=1, n=7-10 (n = no. of observations)). There were no other changes observed in the characteristics of the hyperpolarisations, e.g. time-to-peak and decay time constant. Taken together these findings suggest that TRPM8 activation causes acceleration of elementary Ca^{2+} store release events (sparks), resulting in increased BK_{Ca} channel activity.

We conclude that the calcium permeable cation channel, TRPM8, is located on the periphery of tail artery vascular myocytes, and TRPM8 activation increases [Ca^{2+}], resulting in increased BK_{Ca} channel activity. TRPM8 activation produces two distinct patterns of electrical activity, (i) sustained membrane depolarisation due to cation influx, and (ii) brief spontaneous hyperpolarisations of increased size and frequency due to TRPM8/Ca^{2+} sparks/BK_{Ca} channel coupling.


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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC226**

**Ca^{2+} signalling mediated by purinergic receptors in rat aortic smooth muscle cells**

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Vascular smooth muscle cells form most of the walls of blood vessels and their contractile ability determines blood flow and blood pressure. During vascular injury (eg. atherosclerosis),
they can also proliferate and exhibit the synthetic phenotype. Elevation of intracellular Ca\(^{2+}\) initiate contraction, while during vascular injury they may promote cell growth. CAMP has opposing effects on smooth muscle cell activity and interactions between Ca\(^{2+}\) and cAMP are clearly important. Here, we have characterised Ca\(^{2+}\) signals evoked by the purinergic receptor agonist, ATP in cultured rat aortic smooth muscle cells (RASMCs). We have investigated whether increases in cytosolic Ca\(^{2+}\) concentration affect adenylyl cyclase (AC) activity. For Ca\(^{2+}\) measurements, cells were loaded with the Ca\(^{2+}\) indicator fluo-4 and fluorescent measurements were made in a 96-well population based flexstation assay. All results are expressed as means ± S.E. ATP mobilized Ca\(^{2+}\) in a concentration-dependent manner (EC\(_{50}\) = 3.5 ± 1.1 μM, n = 3) and the peak response was unchanged in the absence of extracellular Ca\(^{2+}\). Involvement of P2Y purinergic receptors was implicated because the response to ATP was completely abolished by the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX). cAMP measurements were made using radioimmuno assay and all AC stimulations were made in the presence of the phosphodiesterase inhibitor, U73122 (IC\(_{50}\) = 1.7 ± 0.27μM, n = 3) and there was no response to the P2X receptor agonist, α,β-methyl ATP. cAMP measurements were made using radioimmuno assay and all AC stimulations were made in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX). ATP (100μM) inhibited forskolin-stimulated AC by 24 ± 1.8% (n = 4, p = 0.001, student’s t-test). However, this inhibition persisted in the absence of extracellular Ca\(^{2+}\) (31 ± 1.02%, n = 3, p = 0.0022) and after intracellular Ca\(^{2+}\) stores were emptied using thapsigargin (1μM) (29 ± 2.04%, n = 3, p = 0.005). This effect was abrogated after treatment of cells with pertussis toxin (n = 3). To summarise, ATP mobilized intracellular Ca\(^{2+}\) via P2Y receptors and inhibited forskolin-stimulated AC activity in RASMCs. The inhibition of AC activity was independent of increases in cytosolic [Ca\(^{2+}\)] and is likely to be mediated by Gi G-protein.

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Where applicable, the authors confirm that the experiments described here conform with The Physiological Society ethical requirements.

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**PC227**

**ATP signalling in equine digital vessels**

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Extracellular ATP plays a role in the maintenance of vascular tone. ATP released from perivascular nerves as a cotransmitter with noradrenaline (NA), and from endothelial cells in response to changes in blood flow and hypoxia. The role and distribution of ATP-activated P2 receptors (P2R) is characterised for many blood vessels but not in equine digital vessels. Haemodynamic disturbances leading to ischaemic and reperfusion injury of the equine digit are thought to be involved in the debilitating condition laminitis. This study aims to characterise ATP signalling in equine digital vessels, before investigating ATP signalling in laminitic digital vessels, where abnormal ATP signalling may play a role. Equine digital arteries (EDA) and veins (EDV) were collected from horses killed in an abattoir. Limbs were collected within 10 min of slaughter and iced Krebs solution was infused into the digital circulation to remove blood. Sections of isolated EDA and EDV were used in organ-bath experiments, whereby electric field stimulation (EFS) in the absence and presence of antagonists to P2R and α\(_1\)-adrenoceptors was examined. Concentration-response curves were constructed to NA and ATP on low and raised tone. Immunofluorescent localization of P2R subtypes were performed. Data are mean±s.e.m. On low tone preparations EFS (28 V, 0.3 ms, 4-32 Hz) induced vasoconstriction in EDA and EDV; in both cases this was abolished by tetrodotoxin (1 μM; n=6). In EDA, vasoconstriction was partially inhibited (by 28±9%) by the P2R antagonist suramin (10 μM; n=6), but not by the P2R antagonist PPADS (10 μM; n=6), and predominantly inhibited (by 78±12%) by phenolamine (10 μM; n=6). In EDV, vasoconstriction was partially inhibited by suramin (10 μM; n=6) and PPADS (10 μM; n=6) to a similar degree (by 43±18% and 39±7%, respectively), and to a lesser extent (by 18±6%) by phenolamine (10 μM; n=6). Exogenous NA and ATP mimicked EFS in EDA; immunostaining for P2X\(_1\), P2X\(_2\) and P2X\(_7\) receptor subunits was seen in vascular smooth muscle (SM) and P2X\(_1\), P2X\(_2\) and P2X\(_3\) subunits in endothelium. Denuding the EDA of endothelium did not alter EFS- or ATP-evoked vasoconstriction. Exogenous NA and ATP mimicked EFS in EDV; immunostaining for P2X\(_1\), P2X\(_2\) and P2X\(_7\) receptor subunits was seen in vascular SM. ATP failed to induce vasodilation on raised tone preparations in either EDA or EDV. ATP and NA are co-transmitters in sympathetic nerves supplying the equine digital vasculature, NA being the dominant partner in arteries and ATP being the dominant partner in veins. ATP did not produce either endothelium-dependent or -independent vasodilatation; P2X\(_1\), P2X\(_2\) and/or P2X\(_{1/2}\), or, P2X\(_7\) receptors are likely to mediate vasoconstriction on EDA and EDV, respectively. We will now use this information to investigate whether ATP signalling is altered in the digital vasculature of horses with laminitis.

Burnstock G. Dual control of vascular tone and remodelling by ATP released from nerves and endothelial cells. Pharm. Reports 60:12-20, 2008

Alice Fordham and Silvia Janska contributed equally.

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