Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C64

Effects of heat stress on left ventricular rotation and rotation rate in resting humans

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Introduction: Counter rotation in the left ventricular (LV) base and apex during systole and diastole plays an important role in the filling and emptying of the LV. Heat stress leads to increased cardiac work, evidenced by a rise in heart rate (HR) and cardiac output at rest. Whilst the influence of heat stress on LV dimensions has been assessed (Crandall *et al*. 2008) the effects on cardiac twisting (systolic rotation) and untwisting (diastolic rotation) remain unknown. In order to further elaborate the influence of passive heating on LV function, the present study sought to evaluate the hypothesis that passive heat stress increases LV rotation and rotation rate. Methods: Six active male subjects (21±2yr) completed the study, remaining fully hydrated throughout. Measurements were made at 4 different thermal conditions: 1) Control (Tcore ~37°C, Tskin ~32°C), 2) skin hyperthermia (Tc ~37°C, Tsk ~36°C), 3) skin and mild core hyperthermia (Tc ~38°C, Tsk ~37°C), and 4) high skin and core hyperthermia (Tc ~39°C, Tsk ~38°C). Echocardiographic images were acquired at each stage of heat stress. Two-dimensional images were analysed for LV basal and apical peak rotation (ROT_{bas}, ROT_{api}) and rotation rates ($ROTR_{bas}$, $ROTR_{api}$) and ejection fraction (EF). Mean arterial pressure (MAP) was measured online via a canula inserted in the radial artery. HR was assessed throughout the trial using a three lead ECG. A repeated measures ANOVA was used to detect effects over time and paired student t-test was applied post-hoc to ascertain differences between conditions. Alpha was set at 0.05, Bonferroni adjustment was made for repeated comparisons. Results: ROT_{bas} increased significantly between skin hyperthermia and high skin and core hyperthermia (-6.5±3.1 vs. -11.3±3.5°, $p<0.05$). ROT_{api} ($p>0.05$) was unaltered. Systolic and late diastolic $ROTR_{bas}$ increased significantly between control and high skin and core hyperthermia (-88±13 vs. -161±48 s^{-1} & 25±10 vs. 76±19 s^{-1}, $p<0.05$). Late diastolic $ROTR_{api}$ increased significantly between control and high skin and core hyperthermia (-6±12 vs. -47±31 s^{-1}, $p<0.05$). EF also increased between control and high core hyperthermia (61±4 vs. 76±7%, $p<0.05$). MAP remained constant throughout the experiment ($p>0.05$) and HR significantly increased between control and high core hyperthermia (62±14 vs. 119±14 beats*min^{-1}, $p<0.05$). Conclusion: Similar to previous studies we demonstrated an increase in systolic function with passive heating, as evidenced by an increase in EF. Our findings suggest that heat stress also increases LV systolic ROT_{bas}, systolic and late diastolic $ROTR_{bas}$ and late diastolic $ROTR_{api}$. The enhanced twisting and untwisting rate of the LV may facilitate the pronounced increase in EF observed with increased core temperature.

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C65

Distinct structure and pharmacological properties of the precapillary sphincters in ureteric microvasculature. *In situ* imaging study

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It has long been recognized that the control of intermittent blood flow in capillaries is controlled by the "precapillary sphincters". However, until now it has not been clear what type of cells performs this important physiological function. Suggestions are: smooth muscle cells (SMC) originating from precapillary arterioles (PA) (Rhodin 1967) encircling capillary orifices, precapillary pericytes expressing contractile proteins (Hirshi et al, 1996, Sims 1986) and even specialised contractile endothelial cells (Ragan et al, 1988). However, to the best of our knowledge no study has investigated the properties of these cells *in situ*. In the present study we used high-resolution X-Y-Z and fast temporal confocal imaging of *in situ* ureteric microvessels loaded with the Ca^{2+}-sensitive indicator Fluo-4 (Burdyga et al, 2003) in order to investigate morphology, Ca^{2+} signalling and mechanical activity of SMC and precapillary pericytes comprising rat ureteric precapillary sphincter (PS) (inner diameter, i.d.<10μm). The effects of central (phenylephrine, PhE) and local (endohelin-1, ET-1) factors as well as caffeine - a powerful vasoconstrictor on PA (i.d.=15-25μm) and PS (i.d.<10μm) have been investigated.

We have defined two types of PS: type 1 (length >40μm) in which endothelium was encircled by both SMC and precapillary pericytes; and type 2 (length <40μm) in which endothelium was encircled by a group of precapillary pericytes only. SMC made three full turns around the endothelium and occupied a length of 9.81±0.21μm (n=12). Precapillary pericytes formed an asymmetrical coat with thick massive body located on one side of the vessel giving several finger like processes wrapping around endothelium. Each precapillary pericyte occupied a length of 10.10±0.48μm (n=15).

SMC of PA responded with Ca^{2+} oscillations and vasomotion when stimulated by either PhE (10μM) or ET-1 (10nM). Also in these myocytes, caffeine at low concentrations (0.5-1mM) enhanced or initiated Ca^{2+} sparks and at higher concentrations (2-5mM) produced Ca^{2+} waves and vasoconstriction. In marked contrast, SMC of PS were immune to caffeine (1-10mM) and PhE (10-100μM) but readily responded to ET-1 with Ca^{2+} oscillations and vasoconstriction. Precapillary pericytes were also

insensitive to either caffeine (10mM) or PhE (10-100μM) but readily responded to ET-1 (10nM) with a non-oscillatory Ca^{2+} transient which consisted of an initial spike followed by sustained component associated with strong vasoconstriction. The data suggest that precapillary pericytes are more likely to act as local sphincters controlling capillary blood flow responding to local factors.

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C66

Malfunction of the mitochondria causes reoxygenation-induced calcium overload in cardiac endothelial cells

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Endothelial cells (EC) exhibit a rise in cytosolic calcium (Cai) and a formation of intercellular gaps during ischemia. Both parameters aggravate during reoxygenation (Reox) and this is accompanied by endothelial barrier failure. As a consequence, edema appears in the affected organ, e.g. the heart, leading to malfunction. The aim of this study was to characterise the impact of mitochondria in mediating this Cai increase. Microvascular coronary EC from humanely killed rats were exposed to simulated ischemia (40min, pH 6.4) followed by Reox (40min, pH 7.4, 2.5mM glucose). Cai was monitored via fura 2, ATP loss was determined via the Mag-Fura-2 method, mitochondrial membrane potential (mMP) was analysed via JC-1 and gaps were identified planimetrically. The Fo/F1-ATPase inhibitor oligomycin (OGM, 10μM) and the mitochondria-specific scavenger Mito Q (1μM) were applied during Reox. The data presented describe the mean values ± s.e.m. in arbitrary units of fluorescent intensity. They were taken from at least 4 different experiments.

Under control conditions, the fura 2 ratio increased after 40 min of Reox significantly to 1.43±0.01 ($p<0.05$ vs. end-anoxia 1.25±0.02, n=140 cells). Gap formation increased to 240% ±20% ($p<0.05$ vs. end-anoxia 100%±1%; n=140 cells). During ischemia the JC-1 ratio decreased significantly from 1.02±0.08 to an end-anoxic value of 0.60±0.06 ($p<0.05$, n=80 cells) indicating a mMP depolarisation. Within the first 5 min of Reox the JC-1 ratio went back to normoxic level, but decreased again to 0.77±0.06 after 40 min of Reox ($p<0.05$ vs. normox., n=80 cells). During ischemia the Mag-Fura-2 ratio increased from 1.00 ± 0.00 to an end-anoxic value of 1.12 ± 0.01 pointing to an ATP loss ($p<0.05$ vs. normox., n=184 cells). With the onset of Reox the Mag-Fura-2 ratio decreased initially to almost basal level indicating an ATP recovery and then rose continuously to 1.16 ± 0.01 (n=184 cells) again pointing to an increasing ATP loss. Application of OGM, acting as an inhibitor of the Fo/F1-ATPase, reduced Cai after 40 min of Reox (1.36 ± 0.02 vs. w/o OGM, $p<0.05$; n=140 cells) and also reduced the ATP loss significantly (1.11 ± 0.01 vs. 1.16 ± 0.01 w/o OGM, $p<0.05$). The formation of gaps was also blocked by OMG (112% ±8% vs. 240% ±20% $p<0.05$). Application of Mito Q blocked the Cai rise (1.19 ± 0.08 vs. w/o Mito Q, $p<0.05$; n=120 cells) and reduced the ATP loss significantly (1.08 ± 0.02 w/o Mito Q, $p<0.05$; n=100 cells). In conclusion: During Reox Cai rises and a formation of gaps occur while the mitochondria depolarise and ATP is reduced. Increasing the cellular ATP content by inhibiting the Fo/F1-ATPase reduces both Cai rise and formation of gaps. The loss of ATP is possibly mediated by production of mitochondrial ROS since Mito Q improves both, ATP loss and Cai rise.

MitoQ was kindly provided by Dr. Murphy via Antipodean pharmaceuticals

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C67

Expression and role of PKC and Rho-kinase in control of agonist-induced calcium signalling in endothelial cells of rat tail artery

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Endothelial cells form a multifunctional signal transducing surface that regulates many fundamental processes in vascular system. A number of protein kinases have been implicated in controlling calcium signaling in a variety of cells. PKC_{α} and Rho-kinase have been implicated to play an important role in control of calcium entry through store operated channels in cultured endothelium [Mehta D *et al*, 2003; Ahmmed GU *et al*, 2004]. However expression and functional role of PKC and Rho-kinase in intact endothelium were not elucidated. In the present study we have used immunohistochemistry and confocal imaging to investigate role of PKC and Rho-kinase in intact endothelium of conduit artery. Rats were humanely killed under CO_2 anaesthesia; their tail artery removed from the ventral grove, cleaned of fat and loaded with Fluo-4 AM (Molecular Probes, 15um) with pluronic. Confocal imaging was done using Nipkow disc based confocal imaging system (Ultraview Perkin Elmer, UK). Minimum of 5 animals were used in each set of experiments. We have found that in endothelium of rat tail artery PKC_{α} was predominantly expressed. Direct activation of PKC in endothelial cells by PDBu 0.1μM caused activation of monophasic non-oscillatory calcium transient dependent exclusively on calcium entry which was inhibited by PKC inhibitor Ro-32-0432 (5μM), and store operated Ca^{2+} channel blocker SKF-96365 (50μM) but was resistant to Rho-kinase inhibitor H-1152 (0.2μM). Stimulation of intact endothelial cells by carbachol (0.1μM, 1μM, 10μM) produced translocation of PKC_{α} to

the plasma membrane and activation of a calcium transient which consisted of two components: initial fast - dependent on Ca^{2+} release and subsequent, sustained dependent on Ca^{2+} entry. Sustained component of CCh induced Ca^{2+} transient was 63.8±3.5% of the peak taken for 100% (n=10, 5 vessels). Inhibition of PKC by Ro-32-0432 (5μM) and Rho-kinase by H-1152 (0.2μM) reduced the sustained component of CCh induced Ca^{2+} transient to 15.9±4.3 and 34.9±4.6% (n=10, 5 vessels) of the peak, respectively. Thus the data obtained suggests that PKC and Rho-kinase are both involved in control of calcium entry activated by carbachol in the endothelial cells of large conduit arteries.

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C68

Involvement of TRPC channel in the homocysteine-induced endothelial dysfunction

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An elevated blood level of homocysteine (Hcy) is an independent risk factor for cardiovascular disease, but it is unclear how Hcy affects blood vessels. The canonical transient receptor potential channels (TRPC) are Ca2+-permeable cationic channels that we have shown to have a novel redox-related mechanism with relevance to inflammation (Xu et al., 2008a). In this study we examined the potential contribution of TRPC in homocysteine-induced endothelial dysfunction.

Human umbilical vein endothelial cells (HUVECs) were used according to the guideline of local ethic approval. Primary cultures of HUVECs were grown in endothelial growth medium (PromoCell, Germany), and the cell passage number less than 5 was used for experiments. TRPC-transfected HEK-293 cells were grown in DMEM-F12 (Invitrogen, UK) medium containing 10% fetal calf serum. Cells were maintained at 37°C under 95% air and 5% CO2. Cells were seeded onto 48-well or 96-well cell culture plates and cell proliferation assayed using WST-1 kit (Roche) (Xu et al., 2008b). Data are expressed as mean ± S.E.M. The unpaired student's t test or ANOVA was used where appropriate. All experiments were reproducible from at least three independent experiments.

Hcy at concentrations of 0.05-5 μM increased the proliferation of HUVECs, but high concentration (50 μM) decreased the proliferation, suggesting that the function of endothelial cells was regulated by the concentrations of Hcy corresponding to the modest increase in the circulating Hcy. To test the involvement of TRPC channels, we firstly examine the effect of Hcy on cell proliferation using TRPC-transfected HEK-293 cells. Using the culture medium without L-cysteine and L- methionine, Hcy (0.5-50 μM) significantly increased the proliferation of TRPC5-transfected cells in a dose dependent manner. The absorbance was increased by 2.52 ± 0.05 fold (n = 16, P<0.001) at 50 μM Hcy, while no effect was observed for the non-transfected cells. This stimulating effect on cell proliferation was related to channel activation. Secondly, we found the inhibition of TRPC4, TRPC5 and TRPC6 by the application of functional TRPC blocking antibodies resulted in the inhibition of proliferation in HUVECs. In addition, we found that the antioxidants (vitamin E and Se2+) displayed protective effects against Hcy-evoked cell proliferation.

These data indicate a new role of TRPC channels in the vascular endothelial cell pathophysiology, which should have an important significance for the understanding of Hcy-related disease processes.

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C69

Decreases in the multidrug transporter, P-glycoprotein, in the brain vasculature of subjects with Alzheimer's disease

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The blood-brain barrier is a poorly permeable interface between central nervous system (CNS) and peripheral circulation vital for homeostasis within the brain parenchyma. It consists of endothelial cells with specialised features including the presence of multidrug transporters of the ATP-Binding cassette (ABC) family, P-glycoprotein (Pgp), MRP4 and BCRP. These play important roles in limiting movement of substances into and enhancing their efflux from the brain. These special features of brain endothelial cells are maintained by signals from the surrounding brain. Such signals may be altered in CNS pathologies such as Alzheimer's disease (AD) which is characterised by accumulation of beta amyloid aggregates within the brain, particularly around cerebral vessels. Previous studies [1] have reported decreases in the glucose transporter, Glut-1, in the brain vasculature of AD patients. In addition, an inverse correlation has been found between amyloid deposition and Pgp expression [2] in non-demented subjects. The present study investigates the status of multidrug transporters in brain

microvasculature of subjects with AD and compares their levels of expression with those found in brain vasculature of normal human subjects.
Sections of frozen brain from hippocampal region, middle frontal gyrus and middle temporal gyrus were obtained from 7 subjects with AD and 7 age-matched normal subjects provided by the Neurological Foundation of New Zealand Human Brain Bank under ethical approval. These were subjected to dual fluorescence immunochemical staining using antibodies against Pgp, Glut-1, BCRP or MRP4 and von Willebrand factor. Expression of each protein was assessed using confocal microscopy, quantifying peak fluorescence values of cross sectional profiles across brain microvessels, 8-10 vessels being analysed per section. The conditions for use of each antibody that would permit detection of any expression differences were first established by comparing staining on cell lines that over- or under-express each of the transporters of interest. Results revealed that expression of P-gp was significantly reduced in brain microvessels in hippocampal regions of patients with AD compared to normal individuals ($p=0.005$). Decreases in Glut-1 were also evident on the microvessels in these same regions ($p<0.001$). Decreases seen in expression of MRP4 or BCRP did not reach significance.
The mechanisms responsible for the altered Pgp expression are not yet known but one possible pathway shown to influence Pgp transcription [3] and known to be altered in AD [4] is that involving Wnt/beta catenin. Involvement of this pathway in the alterations seen to blood-brain barrier characteristics in AD is now being explored.

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C70

Molecular mechanisms involved in high glucose-induced heme oxygenase-1 expression in endothelial cells

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Hyperglycaemia and diabetes are associated with enhanced oxidative stress, leading to the progression of diabetic vascular pathologies (Goldin et al., Circulation 2006;114: 597-605). The redox sensitive transcription factor Nrf2 mediates induction of protective genes, such as heme oxygenase-1 (HO-1), via activation of an antioxidant response element (ARE) (Mann et al., Cardiovasc. Res. 2007;75:261-274). HO-1 metabolises prooxidant heme to the antioxidants biliverdin and bilirubin (Siow et al. Redox Rep. 2007;12:11-15). The present study has examined the signalling pathways involved in high glucose enhanced superoxide generation, Nrf2 activation and HO-1 induction in bovine aortic endothelial cells (BAEC).
Confluent monolayers were treated for 0-24 h with DMEM containing 25mM D-glucose, in the absence or presence of the superoxide ($O_2^{\bullet-}$) scavengers SOD (200 Uml-1) and Tiron (10μM), inhibitors of NADPH oxidase (apocynin, 100 μM), flavoproteins (DPI, 10μM), eNOS (L-NAME, 50μM), or MAPK (SB203580, 10μM; SP600125, 20μM; U0126, 10μM). HO-1, nuclear Nrf2, and phosphorylated MAP kinases levels were determined by immunoblotting, Nrf2 translocation by immunofluorescence, and $O_2^{\bullet-}$ by L-012 chemiluminescence.
Treatment of BAEC with 25mM D-glucose (0-24h), but not D-mannitol, elicited concentration- and time-dependent increases in $O_2^{\bullet-}$, Nrf2 translocation and HO-1 expression. Increased HO-1 expression induced by high glucose was attenuated by Tiron and L-NAME, but not by extracellular SOD or NADPH oxidase inhibitors. Although high glucose induced rapid phosphorylation of p38MAPK and JNK, only inhibition of JNK abrogated high glucose-induced HO-1 expression, implicating the JNK signaling pathway in high glucose-induced activation of the Nrf2-ARE pathway and HO-1 expression.

Goldin et al., Circulation 2006;114: 597-605

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Prof. Giovanni Mann, Dr. Richard Siow

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C71

A potential mechanism for impaired vasorelaxation in a dietary mismatched model of metabolic syndrome

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There is increasing evidence that poor early growth confers an increased risk of developing type 2 diabetes, hypertension, and other features of the metabolic syndrome in later life. It is hypothesized that this may result from a mismatch during early life exerting permanent effects on the structure and function of key metabolic organ systems, including the vasculature. The aim of this study was to explore the long-term impact of a dietary mismatch on endothelial function and specifically to investigate whether a decreased bioavailability of nitric oxide (NO) is a primary feature of endothelial dysfunction in this model.
Mice (C57BL/J) were exposed to either a high fat (HF, 45% kcal fat) or standard animal chow (C, 21% kcal fat) diet throughout pregnancy and lactation. The male offspring were then continued on the same diet as their dams or subjected to a nutritional mismatch and crossed over onto the alternative diet,

for 10 to 12 weeks post weaning, to give four dietary study groups (C/C, C/HF, HF/C and HF/HF) comprising 10 animals per group. Blood pressure (tail cuff plethysmography, SBP average 6 measures) and body weight were recorded at weekly intervals. Femoral artery vasorelaxation to ACh (1nM-10μM) was assessed using wire myography and basal NO production using 4,5-diaminofluoresceine diacetate (DAF-2 DA), an NO-sensitive fluorescent dye. Vascular segments were incubated for 45 min at 37°C with 5 μM DAF-2 DA in HEPES buffer (pH 7.4) and digital images collected using confocal microscopy (excitation at 490 nm; emission 535 nm). The images were analyzed using image software by measuring the mean OD of the fluorescence observed in the endothelium.

Offspring fed the HF diet post weaning (HF/HF and C/HF groups) gained more weight than the C/C and HF/C animals. Blood pressure was also significantly higher in the HF/HF and C/HF. Arteries from HF/HF and C/HF animals showed an impaired endothelium-dependent relaxation to ACh compared with C/C and HF/C animals ($P<0.05$). Basal NO production was greater in CC arteries compared with HF/HF, with staining most evident in the inter-junctional regions of the endothelium and in the underlying intima.

Our preliminary data demonstrate the impact of an in utero and postnatal HF diet on vascular function and suggest that the endothelial dysfunction observed is a result of impairment of NO production and/or bioavailability. It further suggests that both predisposition to disease, acquired in early life, and later lifestyle may contribute to the development of cardiovascular disease which are sustained into adulthood.

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C72

Endothelial dysfunction induced by maternal protein restriction is present at 5 weeks of age in male rat offspring

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Epidemiological studies demonstrate that low birth weight is associated with an increased risk of cardiovascular disease in adult life (Osmond *et al*., 1993). In the rat, the restriction of dietary protein during gestation leads to raised systolic blood pressure and endothelial dysfunction in the offspring (Langley & Jackson, 1994; Brawley *et al*., 2003). Yet whilst blood pressure has been shown to be elevated by 4 weeks of age, endothelial function has not been assessed before 80 days (Brawley *et al*., 2003). The aim of the present study was to determine if endothelial dysfunction was present with the onset of raised blood pressure.

Pregnant Wistar rats were fed a control (C; 18% casein) or protein restricted (PR; 9% casein) diet throughout pregnancy and returned to standard chow postpartum. Pups were weaned from their mothers at 21 days. At approximately 36 days blood pressure in male offspring was recorded using tail cuff plethysmography, before animals were sacrificed and thoracic aorta dissected and mounted in a wire myograph. Aorta segments were bathed in PSS heated to 37° C and continually gassed with 95% O_2 and 5% CO_2. Concentration response curves were conducted to phenylephrine (PE), the thromboxane mimetic U46619, acetylcholine (ACh), bradykinin (BK) and sodium nitroprusside (SNP). Responses to ACh were repeated in the presence of L-NAME (100 μM). Data is given as mean ± S.E.M. and differences were assessed by one-way ANOVA with Bonferroni *post hoc* correction. Significance was accepted at $p<0.05$.

Blood pressure at 5 weeks was similar between the groups (mmHg: C, 86.61 ± 4.15, n=9; PR, 88.50 ± 6.18, n=8; *p*=ns). Contractile responses to PE were similar between the groups, yet vasoconstriction to U46619 was significantly enhanced in the PR group compared to controls (pEC_{50}: C, 7.67 ± 0.05, n=7; PR, 8.01 ± 0.05, n=6, $p<0.01$). Endothelial-dependent vasodilatation to both ACh (pEC_{50}: C, 7.82 ± 0.10, n=7; PR, 7.52 ± 0.07, n=6; $p<0.05$) and BK (% max response: C, 29.2 ± 4.5, n=6; PR, 9.0 ± 3.4, n=5; $p<0.01$) was significantly impaired in the PR group compared to controls. Incubation with L-NAME completely abolished the ACh response in all groups and responses to the NO-donor SNP were similar in both groups (*p*=ns).

The data demonstrates that protein restriction during gestation leads to vascular dysfunction in isolated thoracic aorta segments, which is present from 5 weeks of age and is independent of any increase in blood pressure.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

C73

Characterisation of epidermal primary afferents in the mouse

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The epidermis is richly innervated with sensory afferents most of which are of the "non-peptidergic" group (Zylka *et al.,* 2005; Belle *et al.,* 2007) and in the present study some of the characteristics of these afferents have been determined.

Studies were conducted on C57/Bl6 mice (SA36 strain) carrying the *thy1.2-egfp* gene expressed in non-peptide epidermal afferents (Belle *et al.,* 2007). For anatomy, animals were terminally anaesthetised with sodium pentobarbitone i.p. (80mg/kg) prior to whole body perfusion fixation with 4% paraformaldehyde and for electrophysiology they were anaesthetised with halothane and killed by decapitation. Dorsal root ganglion

further depolarised the membrane potential by 6mV (n=5, p<0.05) and 4mV (n=5, p<0.05), respectively, however, was unable to restore the abolished electrical activity. Phenylephrine restored the electrical activity blocked by niflumic acid, with most measured parameters comparable to those in nifedipine alone.
Conclusion: Altogether, these results suggest that the increased slow wave frequency observed by the addition of phenylephrine is likely due to its effects on PICs as phenylephrine increased the frequency of both pacemaker potentials and slow waves. The effects of phenylephrine were dependent upon Ca^{2+} cycling by internal Ca^{2+} stores within the endoplasmic reticulum and mitochondria, but not by the activation of Cl^- channels.

Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC86A

erg current in mouse gonadotropes

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PC87

Reversible disruption of AE2 expression at the rat blood-brain barrier following transient focal astrocyte loss

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The endothelial cells of the blood-brain barrier secrete HCO_3^- ions into the brain. On the present evidence it is likely that the normal secretion occurs via Na^+-driven HCO_3^- transport into the endothelial cells together with efflux via Cl^-/HCO_3^- exchange [1]. The leading candidate for the efflux transporter is AE2 as mRNA for AE2 is prominently expressed in the primary cultured endothelial cells and AE2 protein is associated with the microvessels in brain slices and is seen in Western blots from the cultured cells [2]. It is well documented that astrocytes are involved in induction and maintenance of various blood-brain barrier features but there are few reports on their influence on ion transporters.
We here report an investigation on the effects of astrocytes on endothelial cells employing a method of selective ablation of astrocytes in the brain [3]. This method uses systemic injection of 3-chloropropanediol which produces focal loss of GFAP positive astrocytes in certain brain regions including the inferior colliculus. In this region there was virtually complete loss of GFAP expressing astrocytes from 3 to 6 days after injection. There were marked changes in expression of occludin and claudin 5 from PECAM-1 expressing microvessels over a similar time course and these were reversed soon after repopulation of the region with astrocytes. P-glycoprotein expression also markedly decreased and recovered with a similar time course [4]. The present study follows changes in expression of AE2 protein in microvessels using the same methods.
AE2 and the endothelial cell marker PECAM-1 were visualized on microvessels in frozen rat brain sections using fluorescence microscopy. This method included antigen unmasking with 1% SDS [5] as reported previously [2]. PECAM-1 remained clearly expressed throughout the next 28 days, but the distribution of AE2 first became discontinuous and then largely disappeared over 3 days. Recovery was underway at 14 days and appeared to return to normal levels by 28 days coincident with the reappearance of GFAP immunoreactive astrocytes. These in vivo results suggest that with AE2, as with P-glycoprotein, there may be a substantial decrease in expression in brain endothelial cells once these cells are removed from the influence of astrocytes and grown in culture. Hence the previously reporteded transport rates of HCO_3^- in cultured cells may have underestimated the transport rates occurring in vivo.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC88

Nitric oxide and hydrogen peroxide are involved in hypoxia-reoxygenation induced upregulation of P-glycoprotein in rat microvascular brain endothelial cells

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The microvascular brain endothelial cells that form the blood-brain barrier (BBB) are exposed to oxidative stress during hypoxia-reoxygenation (H/R) such as occurs in ischemic stroke. H/R triggers signaling cascades that may affect various features of the BBB including upregulation of the multi-drug transporter protein, P-glycoprotein (Pgp) [1, 2]. This could have important implications for drug delivery to the brain following ischemic stroke. In the present study, the immortalised rat brain endothelial cell line, GPNT [3], was used as an *in vitro* system to examine the effects of H/R on Pgp expression and to investigate the role of reactive oxygen species (ROS) and nitric oxide (NO) in the signaling cascades involved in these effects.

GPNT cells were cultured until 70% confluent and harvested into 0.1% SDS either 24h after application of 200μM hydrogen peroxide (H_2O_2) or 24h after reoxygenation, following 6h hypoxia (H/R). In each case, reduced serum medium (RSM) was used. Hypoxia was achieved by placing cells in deoxygenated RSM and maintaining them at 1% O_2 in N_2 with 6% CO_2. Pgp protein levels were assessed via Western blot analyses. Cells were also analysed for production of ROS or NO within 1h after application of H_2O_2 or after H/R. This was done via flow cytometry using the ROS-sensitive dye, 2',7'-dichlorofluorescin diacetate (DCF-DA) (loaded at 10μM for 10min) or the NO-sensitive dye 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) (loaded at 5μM for up to 1h). On exposure to 200μM H_2O_2, intracellular ROS levels increased, showing DCF fluorescence of 339 ± 67% of the untreated control after 20 min (n=4, $p<0.05$, paired t test). The upregulation of Pgp protein seen after exposure to H_2O_2 was abolished following pre-treatment with 6000U/ml PEG-catalase (n=3). The NO donor, SNP (1-2mM) also increased Pgp protein levels (n=3). After H/R, there was a small increase in intracellular ROS levels, reaching a maximum 10min after reoxygenation. Intracellular NO levels were increased 1h after reoxygenation, being 243 ± 75% of untreated controls (n=4, $p<0.05$, paired t test). The increases in Pgp protein levels seen after H/R were partially inhibited by either PEG-catalase or by the nitric oxide synthase inhibitor, L-NAME (1-2mM) (n=3).

These results suggest that generation of both H_2O_2 and NO are involved in the increased expression of Pgp in rat brain endothelial cells following H/R and add to our understanding of the mechanisms involved in the changes to the BBB that accompany stroke. After stroke, BBB integrity becomes compromised. Thus upregulation of Pgp may provide compensatory means to protect the brain though it may also lead to decreased drug access [4].

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PC89

The effects of leptin on aortic rings with and without endothelium isolated from streptozotocin-induced diabetic and control rats

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Aims: The aim of this study was to examine the effects of leptin on aortic rings with and without endothelium isolated from streptozotocin (STZ)-diabetic and control rats and in the presence of an inhibitor of nitric oxide synthase.

Methods: STZ (50 mg/kg, i.p.)-induced diabetic (n=12) and age-matched control (n=12) Sprague-Dawley male rats were anaesthetized with i.p. injections of 60 mg/kg ketamine and were sacrificed with decapitation. Thoracic aortic rings with and without endothelium were mounted in isolated tissue baths. Concentration-response curves to leptin (10-13 – 10-9 M) were constructed under basal tone and after precontracted with 10-6 M phenylephrine in the presence or absence of Nω-nitro-L-arginine methyl ester (L-NAME, 10-5 M). The study protocol was approved by The Animal Experimentation Ethics Committee of Selcuk University.

Results: Leptin caused a concentration-dependent relaxation in the precontracted endothelium intact aortic rings from diabetic and control rats. Responses to leptin in diabetic aorta were significantly increased compared to those of control. The maximum response (Emax) of control aortic rings to leptin was 38.3 ± 4.6%, while that of diabetic rings was 56.5 ± 5.2% ($P<0.05$). EC50 values for leptin were similar for aortic rings from diabetic (7.5 ± 1.6 x 10-12) and control rats (5.5 ± 1.2 x 10-12, $P> 0.05$). L-NAME pretreatment caused complete inhibition in the relaxant responses to leptin of the control aortic rings while it induced a reduction in these responses of the diabetic rings (Emax:21.8 ± 4.3%, $p<0.05$; EC50: 7.0 ± 1.4 x10-12). Leptin-induced relaxations were abolished when the endothelium was denuded. Leptin had no effect on basal tone of endothelium intact and denuded aortic rings from control rats. In diabetic rings, leptin (10-13-10-9 M) elicited dose dependent contraction (Emax: 22.3 ± 4.2%, $p<0.05$, n=8). Removal of endothelium significantly increased contractile effect of leptin on basal tone in diabetic rings (Emax: 55.2 ± 5.7%, $P<0.05$).

Conclusion: Reduced nitric oxide-dependent relaxant response to leptin may have been compensated by another endothelial mechanism(s) in diabetic rat aorta. On the other hand, leptin causes contractile effect on the basal tone in aorta smooth muscle isolated from STZ-induced diabetic rats.

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PC90

The influence of shear stress on physiological angiogenesis and regression

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Chronic vasodilator treatment intensifies levels of shear stress in capillary beds, stimulating a specific form of angiogenesis, termed longitudinal splitting (Egginton, 2001). The use of the α1-adrenoreceptor antagonist, prazosin is well-established for investigation of this process in skeletal muscle (Baum, 2004). Various studies have explored changes in protein expression during shear stress-induced angiogenesis. However, little attention has focussed on the physiological response to cessation of vasodilator treatment and subsequent regression.

Male mice, of the C57BL/10 strain, received prazosin dissolved in tap water (50mg/L) for a period of 4 weeks. In addition to control animals, time points were considered during prazosin treatment (14, 28 days) and the regression phase (3, 7, 14, 28, 42 days; n=4). At sampling, the tibialis anterior muscles were removed, following stunning and cervical dislocation.

Assessment of capillary-to-fibre was used to confirm the microvascular response. Angiogenesis was demonstrated by a 15% increase in capillarity after 4 weeks of vasodilator treatment. Upon cessation of treatment, an equivalent decrease represented vessel regression. Changes in protein expression were explored using immunoblotting, with membranes being protein-stained to ensure equal loading of samples. ANOVA was used to assess statistical significance.

Interestingly, VEGF levels were seen to decline in response to increases in shear stress, with statistical significance seen ($P<0.01$), supporting the theory of hyperperfusion (Baum, 2004). Despite this decrease, angiogenesis occurred, reinforcing the suggested role of nitric oxide as an angiogenic factor (Williams, 2006). As expected, increases in eNOS levels were seen in response to prazosin treatment. A mirroring decline from peak eNOS levels was seen during the regression phase, reaching significance after 6 weeks ($P<0.05$). The expression of the main angiogenic VEGF receptor, Flk-1, increased with shear stress, perhaps compensating for reduced levels of its ligand.

Interestingly, Ang-2 levels increased bimodally reflecting its pleiotropic effects, with a 23% increase seen after 2 weeks of prazosin treatment. Following a brief decrease, a further 74% increase occurred during vessel regression. The effects of this cytokine were clearly dependent on associated levels of VEGF. We conclude that this form of angiogenesis involves both a rapid phase on induction and regression following withdrawal of stimulus.

All procedures follow current UK legislation

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PC91

Effects of diabetes and insulin resistance in pregnant rats on *ex vivo* vascular reaction to magnesium

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PC92

Graded, movement-coupled hyaluronan secretion into joints and potential signal pathways *in vivo*

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Hyaluronan (HA) governs interstitial hydraulic permeability. In synovial fluid it also contributes to joint lubrication and intra-articular (i.a.) fluid conservation via filter-cake formation. Since i.a. HA injections and exercise reportedly improve moderate osteoarthritis, we investigated the effect of graded joint movement on HA secretion and potential signal pathways *in vivo*.

Endogenous HA was washed out from pairs of cannulated knee joint cavities in anaesthetised rabbits (pentobarbitone 30 mg kg^{-1}, urethane 500 mg kg^{-1} i.v.). The joints were subjected to intermittent, passive cycling at a fixed frequency (0.5Hz) for different durations (0, 1, 3, 9min in every 15min, duration 0-60%) or 20% duration at different frequencies (0, 0.17, 0.5, 1.5Hz) to determine stimulus-response curves. Newly secreted HA was harvested by washout after 5h and analysed by HPLC. Putative signalling pathways were assessed using i.a. pharmacological agents and contralateral control vehicle in moved or static joints.

Movement at 0.5Hz and 20% duration almost doubled the HA secretion rate qHA ($p<0.0001$, paired t test, n=35). The coupling was a graded one, with curvilinear stimulus-response curves to both frequency and duration of movement ($p=0.0001$, ANOVA). Since stretch stimulates HA secretion in cell culture via a Ca^{2+} - protein kinase C (PKC) – MEK-ERK pathway (1), we next probed the pathway's functionality in vivo. The Ca^{2+} ionophore ionomycin more than doubled qHA in static joints ($p=0.02$, paired t test, n=5), as did PKC activation by phorbol ester (PMA) ($p= 0.001$). Moreover the PKC inhibitor bisindolylmaleimide I (BIM) substantially inhibited PMA-stimulated secretion in static joints ($p<0.02$, n=10,16, t test), as did the MEK-ERK inhibitors U0126 and PD98059 ($p\leq0.001$, n=5 respectively, paired t test). Despite these positive results in static joints, BIM, U0126 and PD98059 each failed to inhibit movement-stimulated HA secretion significantly ($p=0.96,0.23, 0.32$ respectively, n=5,8,11, paired t test). By contrast, the phospholipase C (PLC) inhibitor U73122 almost totally abolished the stimulation of