Characterisation of Ca^{2+} sparks in human atrial cardiomyocytes

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In cardiomyocytes, excitation–contraction (EC) coupling is accompanied by a transient rise in the intracellular Ca^{2+} concentration, that is to a varying degree brought about by Ca^{2+} release from the sarcoplasmic retiuculum (SR), which is mediated by the opening of ryanodine receptors (RyR) in the SR membrane. The global Ca^{2+} transient is built-up by the synchronised recruitment of local Ca^{2+} release events, the so-called Ca^{2+} sparks, that are generated by gating of clusters of RyR (up to 100 channels). Activation of these Ca^{2+} sparks during EC coupling is a crucial step in the activation of myocyte contraction and depends on the intimate interaction between L-type Ca^{2+} channels in the plasma membrane on one hand and RyR in the membrane of the SR as the counterpart. Under various pathological conditions this interaction has been hypothesised to be partially disrupted. For example, in cardiomyocytes from spontaneously hypertensive rats, detailed analysis of Ca^{2+} sparks revealed that these events had a higher amplitude than in the control normotensive rats (Shorofsky *et al.* 1999).

We analysed Ca^{2+} sparks from atrial cardiomyocytes isolated from healthy human tissue obtained during by-pass operations in order to characterise these elementary Ca^{2+} events in greater detail. After enzymatic digestion of a small piece of human tissue, isolated cells were loaded either with fluo 3-AM or fluo 4-AM and the spatio-temporal properties of Ca^{2+} sparks were recorded with a Noran confocal microscope using linescan acquisition mode (278 lines per seconds). Sequences of linescan images were analysed using NIH Image and Igor software. In the majority of cells studied, Ca^{2+} sparks had a time to peak of 35.4 ± 2.1 ms and a mean amplitude of 132 ± 13 nM (all data presented as means ± S.E.M.). The duration, T_{50}, was measured at half of the Ca^{2+} spark amplitude and was equal to 37 ± 2.6 ms and their spatial spread (full width at half-maximum) was 4.2 ± 0.15 μm ($n = 44$ sparks in $n = 5$ cells). However, it was found that larger subcellular events also co-existed with 'normal' Ca^{2+} sparks. These events had a time to peak of 43.2 ± 4 ms and a mean amplitude of 118.6 ± 9 nM ($n = 19$ sparks in 5 cells). T_{50} was 33.7 ± 4.3 ms and their spatial spread increased to 9.75 ± 0.9 μm and was significantly different from the 'normal' Ca^{2+} sparks ($P \leqslant 0.05$, unpaired t test). We were surprised to find such a population of 'big sparks' in myocytes from 'healthy' (i.e. non-hypertrophied) human hearts, since large amplitude Ca^{2+} sparks have recently been reported to only occur under pathological conditions (Shorofsky *et al.* 1999).

Results such as those presented here will establish a baseline for the properties of Ca^{2+} sparks in human atrial tissue and will allow for the detailed comparison of their properties with the characteristics of Ca^{2+} sparks found in human hearts obtained from patients suffering various heart diseases.

Shorofsky, S.R. *et al.* (1999). *Circ. Res.* **84**, 424–434.

All procedures accord with current local guidelines.

Presynaptic $GABA_B$ receptors linked to kcnk3 regulate hypoxic chemotransmission in the rat carotid body

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The mammalian carotid body is a chemosensory organ that responds to changes in arterial hypoxia, acidosis and hypercapnia by increasing afferent chemosensory discharge in the carotid sinus nerve, which initiates corrective changes in ventilation. This is brought about by stimulus-induced neurotransmitter release from chemoreceptive glomus (type I) cells onto adjacent afferent petrosal nerve endings (Gonzalez *et al.* 1994). There is evidence that, at least in mouse, GABA is localized in glomus cells and thus may act as a neurotransmitter/modulator in chemosensory signalling (Oomori *et al.* 1994).

Carotid body glomus cells and petrosal neurons were isolated from 10- to 12-day-old Wistar rats following humane euthanasia, as previously described (Zhong *et al.* 1997). Using gene-specific primers, mRNA for both the BR1 and BR2 subtypes of the $GABA_B$ receptor was found in isolated glomus cells using RT-PCR. Immunohistochemical staining for these receptors using specific antibodies could also be found in these cells *in vitro* and *in situ*. In a functional co-culture model of glomus cell clusters and petrosal neurons, we examined the effects of the $GABA_B$ receptor blockers 5-aminovaleric acid (5-AVA) and hydroxysaclofen (OHS) on afferent chemoreceptor signalling due to hypoxia. In the presence of either 100 μM 5-AVA ($n = 14$) or 50 μM OHS ($n = 6$), the postsynaptic depolarization due to hypoxia (P_{O_2}, 5 mmHg) was significantly enhanced ($P < 0.05$ for either blocker, ANOVA). These effects were abolished by a selective inhibitor of PKA but not PKC. Hypoxia-induced depolarization of cultured glomus cell clusters could also be enhanced by these blockers. Furthermore, during patch-clamp recordings from glomus clusters, the selective $GABA_B$ receptor agonist baclofen enhanced whole-cell K^+ current, an effect significantly reduced in the presence of either 5 mM Ba^{2+} or 10 μM anandamide ($P < 0.05$ for either blocker, Mann-Whitney test).

These data suggest the release of GABA by glomus cells in response to hypoxia, with the neurotransmitter acting pre-synaptically to modulate the release of GABA and other neurotransmitters by PKA-regulated activation of KCNK3, an O_2-sensitive K^+ channel known to be expressed in these cells (Buckler *et al.* 2000).

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All procedures accord with current local guidelines.

Role of ionotropic glutamate receptors in the excitatory drive transmission to caudal medullary expiratory neurones of the rabbit

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Most of the expiratory neurones located in the caudal part of the ventral respiratory group (cVRG) are bulbospinal neurones (Bianchi *et al.* 1995). Although several lines of evidence indicate that the cVRG is not essential for respiratory rhythm generation, chemical activation of this structure via microinjections of the broad-spectrum excitatory amino acid (EAA) agonist, DL-homocysteic acid, suggests that cVRG expiratory neurones have ionotropic EAA receptors and may affect the pattern of breathing when strongly activated (Bongianni *et al.* 1994). This could be relevant to some physiological conditions, such as cough and other reflexes characterized by the activation of expiratory muscles. Cough probably involves the same neuronal network as subserves respiratory rhythm generation (Bongianni *et al.* 1998; Shannon *et al.* 2000). The aim of the present study was to investigate the functional role of ionotropic glutamate receptors in the excitatory drive transmission to cVRG expiratory neurones during eupnoeic breathing as well as during cough induced by mechanical stimulation of the tracheobronchial tree and other respiratory reflexes.

All experimental procedures were conducted in accordance with Italian legislation. Microinjections (30–50 nl) of antagonists acting on either NMDA or non-NMDA receptors were made into the cVRG of spontaneously breathing rabbits ($n = 18$) under pentobarbitone anaesthesia (40 mg kg^{-1}, I.V., supplemented by 3–5 mg kg^{-1} every 30 min). Phrenic nerve and abdominal muscle activities were recorded. Blockade of both NMDA and non-NMDA receptors by kynurenic acid (KYN; 50 mM) suppressed spontaneous rhythmic activity of abdominal muscles and the inspiratory and expiratory components of the cough reflex. Microinjections of the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 mM) caused similar results. Microinjections of the NMDA receptor antagonist D(−)-2-amino-5-phosphonopentanoic acid (D-AP5; 10 mM) strongly reduced, but did not abolish spontaneous abdominal activity and the cough reflex. The activation of abdominal muscles induced either by tracheal occlusion at end-inspiration or by expiratory threshold loads (5 cmH_2O) was completely abolished by KYN and CNQX and strongly reduced by D-AP5.

The results indicate that the excitatory drive to cVRG expiratory neurones is mediated by ionotropic glutamate receptors during eupnoeic breathing, coughing, inflation reflex and expiratory threshold loading; non-NMDA receptors appear to play a major role. In addition, cVRG expiratory neurones appear to be of crucial importance in determining not only the expiratory, but also the inspiratory component of the cough motor pattern, thus supporting the view that these neurones are not merely upper motoneurones conveying the expiratory drive to the spinal cord (Bongianni *et al.* 1994).

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Reflex specific role of P2 receptors in the nucleus tractus solitarii

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β_2-Adrenergic receptor stimulation by clenbuterol *in vivo* induces apoptosis in the rat heart

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Studies *in vitro* have shown that signalling through the myocyte β_1-adrenergic receptor (AR) pathway induces, whereas β_2-AR signalling inhibits, apoptosis (Zaugg *et al.* 2000). Consensus is now growing for the use of β_2-AR selective agonists for inotropic support in heart failure. Here, we show that the net effect of β_2-AR stimulation *in vivo* may in fact induce apoptosis.

Male Wistar rats (*Rattus norvegicus*) (280 ± 18 g) were administered (S.C.) 5 mg of clenbuterol kg^{-1} (positive control and experimental groups) or the saline vehicle only (negative controls). The receptor pathways mediating the myocardial apoptosis were investigated by prior administration (S.C.) of selective β_1-AR (10 mg of bisoprolol kg^{-1}) or β_2-AR (10 mg ICI 118,551 kg^{-1}) antagonists 1 h before the clenbuterol. To investigate clenbuterol's role as a neuromodulator, the sympathetic nervous system was depleted of noradrenaline (NAdr) by administering (S.C.) 2 mg reserpine kg^{-1} 24 h prior to the clenbuterol. Four hours after clenbuterol or saline administration, rats were killed in accordance with the UK Animal (Scientific Procedures) Act, 1986, and the hearts rapidly excised and snap frozen. An antibody directed against caspase 3 (R & D Systems) was used to detect the incidence of apoptosis on 5 μm cryosections of the hearts. Apoptosis in the subendocardial region of each heart was quantified in six random fields using image analysis and the results (Fig. 1) are expressed as per cent area.

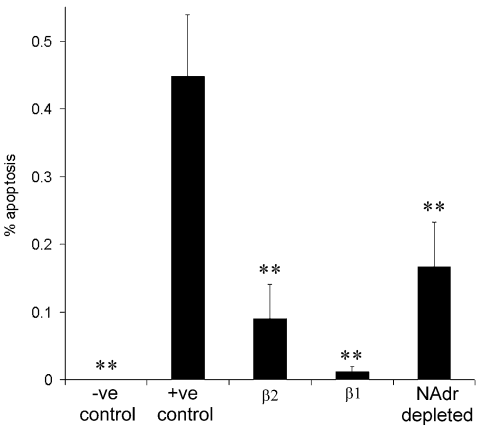

Figure 1. Apoptosis in the rat myocardium. −ve control: saline vehicle only; +ve control: clenbuterol only; β_1: bisoprolol + clenbuterol; β_2: ICI 118,551 + clenbuterol; NAdr depleted: reserpine + clenbuterol. All data are

means ± S.E.M. ($n = 8$). One-way ANOVA with Tukey *post-hoc* analysis was used to determine statistical significance from the +ve control group; $^{**}P < 0.01$.

No damage was found in the hearts from the negative control group. In contrast, in the hearts exposed to clenbuterol, a significant level of apoptosis was found. As expected, prior β_2-AR blockade significantly prevented the apoptosis induced by the β_2-AR selective agonist. Unexpectedly, however, β_1-AR blockade also significantly prevented the clenbuterol-induced apoptosis, as did the prior administration of reserpine. Through a process of elimination, we propose that the β_2-AR stimulation of the sympathetic varicosities by clenbuterol induced an enhanced release of NAdr, and it is this NAdr which, acting through the myocardial β_1-ARs, induced the apoptosis.

These data support previous *in vivo* and *in vitro* studies showing that apoptosis in the heart is mediated through the β_1-AR pathway. However, our study *in vivo* contradicts previous work *in vitro*, which has suggested that β_2-AR agonists may be used as inotropic support for heart failure patients. In conclusion, these data suggest that the net response to β_2-AR stimulation *in vivo* is to induce apoptosis.

Zaugg, M. *et al.* (2000). *Circulation* **102**, 344–350.

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Expression of heme oxygenase 1 and inducible nitric oxide synthase protein in human septic shock

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Carbon monoxide, produced by heme oxygenase (HO-1), and nitric oxide, produced by inducible nitric oxide synthase (iNOS), both cause vasodilatation by activating guanylate cyclase. Overproduction of nitric oxide by iNOS is commonly thought to be the principal cause of hypotension in sepsis. However, iNOS knockout mice, whose nitric oxide production was not increased when exposed to lipopolysaccharide, were no more resistant to sepsis than normal mice (Laubach *et al.* 1995).

We recently demonstrated increased HO-1 (Reade *et al.* 2002*a*) and decreased iNOS (Reade *et al.* 2002*b*) mRNA in mesenteric arterial smooth muscle (ASM) from patients with septic shock. We have now determined the protein expression of HO-1 and iNOS in these patients.

ASM was isolated from patients undergoing bowel resection for perforated viscus (who in the peri-operative period met the accepted criteria for septic shock), and from controls with bowel cancer. After mechanical removal of endothelium and adventitia, tissue was homogenised in protease inhibitor and frozen until sufficient samples had been accumulated. Western blotting was performed under reducing conditions, with membranes incubated in 1:2000 (iNOS) or 1:1000 (HO-1) primary antibody followed by 1:2000 peroxidase-labelled secondary antibody. Protein bands were quantified by computer analysis of the chemiluminescence detection film, then normalised to the protein concentration of the sample prior to dilution.

HO-1 protein expression was significantly increased in arterial smooth muscle from patients with septic shock (2.2 ± 1.7 units mg^{-1} control; 46 ± 15 units mg^{-1} septic) (means ± S.E.M.; $n = 10$ controls, 11 septics; $P = 0.015$, Student's unpaired t test) (Fig. 1). There was no increase in expression of iNOS; indeed iNOS protein was only detectable in ASM from two controls and three septics (Fig. 2).

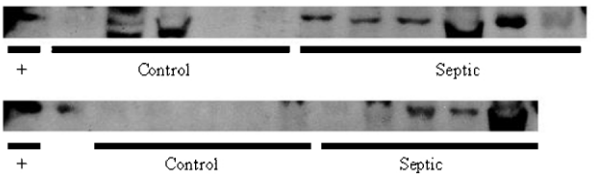

Figure 1. HO-1 in ASM from 10 control and 11 septic patients.

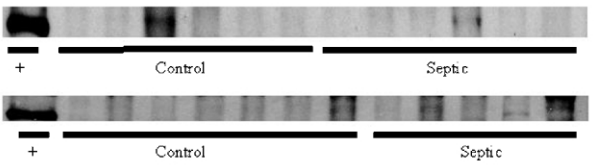

Figure 2. iNOS in ASM from 13 control and 11 septic patients.

We suggest that overexpression of heme oxygenase, rather than iNOS, might be responsible for the hypotension that characterises human septic shock.

This study was approved by the Central Oxfordshire Research Ethics Committee.

Laubach, V.E. *et al.* (1995). *Proc. Natl Acad. Sci. USA* **92**, 10688–10692.

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All procedures accord with current local guidelines.

In vivo gene transfer demonstrates that reduced endothelial nitric oxide synthase activity in the NTS lowers arterial pressure in the spontaneously hypertensive rat

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We demonstrated previously that endogenous endothelial nitric oxide synthase (eNOS) activity in the nucleus tractus solitarii (NTS) depresses baroreceptor reflex gain (BRG) in conscious normotensive rats (Waki *et al.* 2001). In this study, we investigated whether excessive eNOS activity in the NTS contributes chronically to the blunted baroreceptor reflex gain and sustained levels of arterial pressure found in the spontaneously hypertensive rat (SHR).

A recombinant adenoviral vector driving expression of a dominant negative mutant form of eNOS (so-called TeNOS; Lee *et al.* 1995) was microinjected bilaterally into the NTS (see Paton *et al.* 2001, for details) to disable endogenous eNOS activity in

the SHR and their progenitor, aged-matched controls – Wistar-Kyoto rats (WKY; 10–11 weeks old). We used radio-telemetry to acquire arterial pressure data and from this determined the spontaneous baroreceptor reflex gain (BRG) using both spectral analysis and the time-series technique (see Oosting *et al.* 1997) before, and 1–2 weeks following, viral transfection. Both transmitter implantation and transfections were performed under anaesthesia using ketamine (60 mg kg^{-1}) and medetomidine (250 μg kg^{-1}) given I.M. and reversed with a subcutaneous injection of atipamezole (1 mg kg^{-1}). Control rat groups consisted of NTS microinjection of an adenovirus that expressed enhanced green fluorescent protein (eGFP). Student's paired *t* test was used for statistical analysis and results were expressed as means ± S.E.M.

After TeNOS transfection in the SHR, BRG measured by both spectral analysis and time-series techniques significantly increased (time-series technique: from 1.00 ± 0.12 to 1.58 ± 0.26 ms $mmHg^{-1}$, $n = 4$, $P < 0.05$; spectral analysis: from 1.23 ± 0.18 to 3.03 ± 0.67 ms $mmHg^{-1}$, $n = 4$, $P < 0.05$) while mean arterial pressure (MAP) and HR significantly decreased (MAP: from 118 ± 4 to 108 ± 4 mmHg, $n = 4$, $P < 0.01$; HR: from 310 ± 8 to 259 ± 19, $n = 4$, $P < 0.05$). There was no significant alteration in any of the cardiovascular variables measured in the eGFP-transfected SHR ($n = 5$) but in the TeNOS-transfected WKY ($n = 5$), BRG increased, HR decreased and there was no change in MAP.

Our results indicate that endogenous eNOS activity in the NTS plays a major role in determining levels of arterial pressure, HR and BRG in pathological hypertension. Further, they demonstrate that restoring transmission of the baroreceptor reflex at the level of the NTS may help to normalize blood pressure.

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Nitroxyl anion induces redox-sensitive myocardial protective effects akin to ischaemic preconditioning in isolated rat hearts

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Nitric oxide (NO·) donors can mimic ischaemic preconditioning (IP) through the formation of reactive nitrogen species (RNOS). The involvement of nitroxyl (HNO/NO^-), the one-electron reduction product of NO·, in IP has been previously suggested (Pagliaro *et al.* 2001). Nitroxyl can be produced by L-arginine oxidation by NO synthase under certain conditions (Schmidt *et al.* 1996). At physiological pH, HNO/NO^- may be generated by Angeli's salt (AS, $Na_2N_2O_3$). We tested the hypothesis that AS provides IP-like effects that are significantly more potent than those observed with an NO· donor. Finally, we wanted to determine whether HNO/NO^--mediated myocardial protection might be attributed to a 'pro-oxidant' mechanism, inhibitable by switching myocardial redox conditions.

Wistar rats were anaesthetised with 1 g kg^{-1} urethane I.P. 10 min after heparin (2500 U, I.M.). Hearts were excised and retrogradely perfused with oxygenated Krebs-Henseleit solution at 37 °C. After 20 min stabilisation they were assigned to one of the six following treatment groups ($n = 12$ in each group): (1) hearts treated with intracoronary 1 μM AS; (2) with 0.5 μM diethylamine/NO (DEA/NO), an NO· donor; (3) with vehicle (100 nM NaOH), or (4) with buffer for 19 min prior to ischaemia/reperfusion (I/R) injury (30 min global ischaemia, 30 min reperfusion). (5) To test the influence of the redox state, AS was co-infused with *N*-acetyl-L-cysteine (NAC, 4 mM). In hearts of group (6), IP was induced by three cycles of 3 min of ischaemia followed by 10 min reperfusion prior to I/R. The extent of I/R injury was assessed by changes in left ventricular pressure (LVP), lactate dehydrogenase (LDH) release and quantification of infarct size. Mean ± S.E.M. data were analysed by Student's unpaired *t* test. $P < 0.05$ was considered significant.

In control hearts LDH release was 686 ± 14 U g^{-1} and infarct size was 39 ± 2 % of the left ventricle mass. AS and IP were more effective than DEA/NO in limiting cardiac injuries. In fact, in AS and IP groups LDH release and infarct size were reduced by 50–55 and 60–65 %, respectively. In the DEA/NO group LDH and infarct size were reduced by only 40 and 30 %, respectively. Post-ischaemic LVP recovery, which was 36 ± 6 % of pre-ischaemic levels in control hearts, was 55 ± 3, 53 ± 5 and 43 ± 4 % in AS, IP and DEA/NO groups, respectively. AS-induced protection was redox sensitive, as it was abolished by NAC co-infusion.

Data show that HNO/NO^-, generated by AS, affords myocardial protection akin to IP and greater than NO·, suggesting that RNOS like HNO/NO^- are not only necessary but also sufficient to trigger myocardial protection against I/R through a pro-'oxidative' and/or 'nitrosative' stress mechanism

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Schmidt, H.H.H.W. *et al.* (1996). *Proc. Natl Acad. Sci. USA* **93**, 14492–14497.

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Spontaneous fluctuations in cerebral blood flow during sleep and wakefulness in newborn lambs

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Arterial pressure (AP), intracranial pressure (ICP) and cerebral blood flow (CBF) undergo complex spontaneous fluctuations. In spite of the dampening effect of autoregulation, especially in the low-frequency (LF) range, CBF is affected by AP fluctuations. On the other hand, the relationship between CBF and ICP fluctuations in physiological conditions is still incompletely clarified. CBF also depends on changes in cerebral metabolism and chemical cerebrovascular regulation, such as those occurring across wake–sleep states (W, wakefulness; QS, quiet sleep; AS,

active sleep). The aim of the present work was to evaluate the relationship of LF spontaneous fluctuations in CBF *versus* those in AP and ICP across the wake–sleep cycle in newborn lambs.

Experiments were carried out on male Merino/Border-Leicester cross lambs. Lambs 2–5 days old ($n = 4$) were chronically implanted under general halothane and nitrous oxide anaesthesia with electrodes for electroencephalographic and electromyographic recordings and with catheters in the femoral artery and the subdural space for measuring AP and ICP, respectively. A transit-time ultrasound flow probe (Transonic Systems, Ithaca, NY) was positioned around the superior sagittal sinus for CBF recordings. 72-hour post-operative recovery was allowed. Artifact-free 120 s data sequences sampled at a rate of 100 Hz were selected during stable behavioural states and linearly detrended. Pearson's correlation coefficient was computed on ICP and CBF data after low-pass filtering at 0.3 Hz. AP and CBF variance was calculated in the LF range (0.016–0.3 Hz) by means of spectral analysis and Welch procedure on five overlapped Hanning-windowed 60 s subsets for each original sequence. After completion of the study, lambs were killed under general anaesthesia (150 mg kg^{-1} sodium pentobarbitone).

The mean correlation coefficient between ICP and CBF was positive and significantly different from 0 (one-sample *t* test, $P < 0.005$) in the different states and highest in AS (ANOVA and Tukey's *post-hoc* test, $P < 0.005$). In all states the coefficient of variation of CBF (CVCBF) was significantly higher than that of AP (CVAP) (paired *t* test, $P < 0.005$). CVCBF and CVAP were lowest in QS (Mann-Whitney test, $P < 0.05$).

Table 1.

	W	QS	AS
rPC	0.52 ± 0.04	0.51 ± 0.02	0.74 ± 0.03***
CVCBF %	4.67 ± 0.35***	3.26 ± 0.15	4.17 ± 0.36*
CVAP %	1.94 ± 0.09***	1.52 ± 0.04	2.33 ± 0.16***

rPC, correlation coefficient of CBF *vs.* ICP; CVCBF % and CVAP %, coefficient of variation of CBF and AP in LF range, respectively; means ± S.E.M. are reported. Significant differences *vs.* QS are indicated (*$P < 0.05$, ***$P < 0.005$).

The positive value of rPC indicates that ICP LF fluctuations are induced by those of CBF. Higher CVCBF *vs.* CVAP values indicate that CBF fluctuations cannot be explained by AP fluctuations alone. Lowest CVCBF and CVAP values in QS agree with the homeostatic features characterising this state.

All procedures accord with current National guidelines.

Intracellular signalling pathways mediating the angiotensin II-induced depression of the baroreceptor reflex in the nucleus of the solitary tract

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Circulation time in man from lung to periphery – pulse and non-pulse oximetry

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Measurement of circulation time (Ct) from lung to periphery has been investigated as a surrogate for cardiac output. The subject takes a breath of nitrogen and the circulation time is the time elapsing until a desaturation pulse is seen peripherally. Pulse oximetry, using the ear, shows shortening with exercise (12.1 ± 0.37 s, mean ± S.E.M., at rest; 9.1 ± 0.25 s at 100 W, $n = 20$), lengthening after β-blockade ($n = 6$) and lengthening in patients with echocardiographic left ventricular systolic dysfunction and clinical left heart failure (8 patients, 16.2 ± 1.1 s; 6 controls, 12.0 ± 0.5 s). The use of pulse oximetry to measure lung-ear delay discriminates heart failure from normal in many, but not all, subjects. In patients referred to a department of nuclear medicine for diagnosis of chest pain, ear and finger oximetry showed unacceptable variability when compared with delays between first pass nuclide arrival at lung and periphery. The nuclide delays for lung to carotid artery correlated significantly with the reciprocals of gated SPECT estimates of cardiac output (Q) (not significant for lung to finger).

In normal subjects a fast response oximeter (non-pulse, Waters oximeter) gave much shorter and more reproducible circulation time estimates than those obtained with pulse oximetry (Ct at rest, mean 5.5 ± 0.21 s (± S.E.M.) and in exercise at 50 W mean 4.1 ± 0.19 s; mean difference 1.4 ± 0.13 s; paired *t* test, $P = 0.002$). These include no significant instrument delay. There was a highly significant relationship between Ct using the fast (Waters) oximeter and the reciprocal of cardiac output (indirect Fick derived; $Ct = 0.28 \times 60/Q + 2.8$ s; Q in l min^{-1}; $P < 0.001$). Further investigation of fast response oximetry as an indirect indicator of cardiac output is proposed both for physiological measurement and as a potential clinical diagnostic tool.

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All procedures accorded with current local guidelines and the Declaration of Helsinki.

First evidence for transgenerational vascular programming in the rat protein restriction model

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Human epidemiological studies have shown a link between poor maternal nutrition and cardiovascular disease in later life (Roseboom *et al.* 2001). Previous studies have shown in rats that restriction of dietary protein in pregnancy causes maternal vascular dysfunction (Itoh *et al.* 2002; Koumentaki *et al.* 2002)

and results in offspring with raised blood pressure (Langley & Jackson, 1994) and vascular dysfunction (Brawley *et al.* 2002), which is also apparent in pregnant offspring (Torrens *et al.* 2002). However, no studies have investigated the effect of protein restriction during pregnancy on second generation (F2) offspring.

First generation female offspring of previously unmated Wistar rats, which had been fed a control (C; 18 % casein) or a protein-restricted (PR; 9 % casein) diet throughout pregnancy, were mated at around 100 days of age (approximately 200 g body weight). Male F2 offspring were humanely killed by CO_2 inhalation and cervical dislocation at *ca* 80 days of age. Small mesenteric arteries were mounted on a wire myograph, bathed in PSS at 37 °C and continually gassed with 95 % O_2 and 5 % CO_2. Following normalisation, concentration–response curves to phenylephrine (PE), acetylcholine (ACh) and sodium nitroprusside (SNP) were carried out. Data are expressed as means ± S.E.M., and significance tested by Student's unpaired *t* test.

Sensitivity to PE was increased in the PR group with no increase in the maximum ($-\log EC_{50}$: C, 5.87 ± 0.04, $n = 13$; PR, 6.06 ± 0.03, $n = 15$; $P < 0.001$; % maximal response: C, 119 ± 2, $n = 13$; PR, 122 ± 2, $n = 15$; $P = 0.27$). Vasodilatation to ACh was attenuated in the PR group (ACh; % maximal response: C, 90 ± 3, $n = 14$; PR, 73 ± 4, $n = 15$, $P < 0.01$). The maximal response to SNP was enhanced in the PR group (SNP; % maximal response: C, 77.3 ± 3.3, $n = 14$; PR, 87.5 ± 2.4, $n = 9$, $P < 0.05$).

In conclusion maternal protein restriction during pregnancy causes vascular dysfunction in the second generation even in the absence of additional nutritional challenges. These findings differ in some respects from those seen in the previous generation (Brawley *et al.* 2002), but offer a new insight into vascular programming.

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Electrocardiogram recordings in sea water

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A limited number of studies have been performed on the cardiac function of humans (Istepanian & Woodward, 1997) and cetaceans (Williams *et al.* 1993) in the marine environment. In most cases procedures and techniques for recording activities were invasive (King *et al.* 1953; Wardle & Kanwisher, 1974) or under non-physiological conditions (Hamlin *et al.* 1970).

Here we present an easy-to-use and easy-to-assemble, portable, non-harmful, waterproof recorder for monitoring electrocardiograms in sea water. The instrument was worn by the subject, freely carrying it during normal swimming activity. Three stainless-steel electrodes, each inserted into a plastic suction cup, were connected to a Holter recorder (QRS-LX16 from High Technology Devices srl) which pre-amplified, filtered and converted signals to digital, storing them in an 8-bit flash memory card. A polyethylene waterproof box, measuring 15 × 4 × 9 cm and weighing 660 g, was designed to protect the electric circuit from the marine environment. Data were transferred to a receiver system for analysis through dedicated software. A 500 Hz sampling frequency was used, obtaining up to 2 h of recording.

Heart activity monitoring in sea water was performed on five humans (*Homo sapiens sapiens*) and six bottlenose dolphins (*Tursiops truncatus*), awake and without anaesthesia. Experimental sessions took place on the surface and at a depth of 35 m in open calm sea with humans, and in 4–6 m deep tanks with dolphins. In humans, positive and negative electrodes were placed to the left and right of the hemi-thorax, respectively, below the pectoralis major muscle. The reference electrode was placed on the right iliac crest. In dolphins positive and negative electrodes were symmetrically positioned on the ventral thorax, to the left and right, respectively, of the middle of the sternum, caudally to pectoral fins. The reference electrode was placed on the belly region. The recorder was placed cephalically to the dorsal fin.

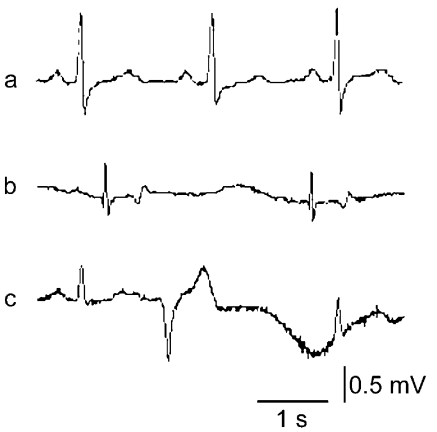

Figure 1. Samples of ECG recorded in sea water. a, human subject; b, bottlenose dolphin; c, ventricular premature beat recorded in a human subject.

ECGs obtained showed a good signal-to-noise ratio which lasted for the whole recording period both in humans (Fig. 1a) and dolphins (Fig. 1b). The system reliably monitored anomalous cardiac activity detected in a human (Fig. 1c), as well as diving-induced bradycardia. Specifically, human heart rate decreased from 100 ± 3.6 beats min^{-1} (b.p.m., mean ± S.E.M.) on the surface to 88.6 ± 3.2 b.p.m. ($P < 0.05$, Student's paired *t* test) at 35 m, while bottlenose dolphin heart rate decreased from 79.5 ± 6.5 to 42.3 ± 4.2 b.p.m. ($P < 0.05$, Student's paired *t* test).

In conclusion, adoption of this instrument represents a reliable and powerful tool for future physiological studies. The innovative technical approach opens up new prospects in diving sports as well as in medical and veterinary diagnostic fields.

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Anteroventral third ventricle (AV3V) lesions impair cardiovascular responses to intravenous hypertonic saline (HT) infusion

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The present study sought to determine the effects of AV3V lesions on the cardiovascular responses to intravenous HT infusion. Male Wistar rats (300 g) anaesthetized with urethane (1.2 g kg^{-1}, I.V.), after induction with halothane (2 % in 100 O_2) had the femoral artery, femoral and jugular vein cannulated for mean arterial pressure (MAP) recording, urethane and HT infusion (3 M NaCl, 0.18 ml (100 g bw)$^{-1}$, in 60 s), respectively. Renal blood flow (RBF) was recorded by Doppler flowmetry (Transonic) and renal vascular conductance (RVC) was calculated as the ratio RBF (ml min^{-1})/MAP (mmHg) and expressed as percentage of baseline. At the end of experiments, under deep anaesthesia, rats were perfused transcardially with saline followed by 10 % formalin. The brains were removed, fixed in 10 % formalin, frozen and cut in 40 μm sections, stained with 2 % Neutral Red and analysed by light microscopy to confirm the lesions site. All data are expressed as means ± S.E.M. Two-way repeated measures analysis of variance (ANOVA) followed by Fisher's protected least-significant differences were used for comparisons. Differences were considered significant at $P < 0.05$. In sham-operated animals ($N = 8$), 10 min after HT infusion RBF and RVC increased to 137 ± 10 and 125 ± 7 %, respectively, and at 60 min to 141 ± 10 and 133 ± 10 %. We also observed increases in MAP (peak at 10 min: 11 ± 3 mmHg). Acute AV3V lesion (DC, 2 mA, 25 s, 30 min before infusion, $N = 6$) abolished MAP increases and the changes in RBF and RVC at 10 min (107 ± 7 and 103 ± 6 %, respectively) and 60 min (107 ± 7 and 106 ± 4 %) after HT infusion. These results demonstrate that the integrity of AV3V region is essential for renal vasodilatation that follows acute changes in the composition of the extracellular fluid compartment.

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All procedures accord with current local legislation.

Inhibition of mitochondrial F_0F_1 ATPsynthase by IF1 during coronary reactive hyperaemia in preconditioned goat heart

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In the goat ischaemic preconditioning (IP) reduces the total hyperaemic flow (THF) of a coronary reactive hyperaemia (CRH) obtained with a 15 s occlusion of the left circumflex coronary artery (LCCA) (Pagliaro *et al.* 2001). This study aimed to investigate whether the reduction of THF by IP is related to an impairment of ATPase activity by F_0F_1 ATP synthase by binding of its inhibitory protein IF1. The experiments were performed on six anaesthetised goats ventilated with a 2:1 gaseous mixture of nitrous oxide and oxygen. After sedation with diazepam (40 mg) anaesthesia was induced by I.V. injection of ketamine (15 mg kg^{-1}), and maintained by infusion of ketamine and hourly injection of 2.5 ml of fentanyl.

Aortic blood pressure and flow in the LCCA were recorded. CRH was induced before and after IP by a 5 min occlusion of the artery. Myocardial samples were taken in the absence and in the presence of preconditioning before, during and after each CRH. In particular, during CRH, samples were taken 15–20 s after the release of the occlusion, i.e. at the top of the hyperaemia, and after CRH was over, i.e. 4 and 6 min after the release. ATPase activity and IF1 content in ATP synthase were determined in all samples. Mean ± S.D. data were analysed by Student's t test for paired data. $P < 0.05$ was considered significant. Animals were humanely killed.

In the non-preconditioned heart, ATPase activity was 1.0 ± 0.13 U (mg total protein)$^{-1}$ before CRH, while it became 1.2 ± 0.15 at the top of CRH, and 0.76 ± 0.14 U mg^{-1} 4 min after the release of the 15 s occlusion of LCCA. After 6 min had elapsed from the release, ATPase activity was back to the control value (0.97 ± 0.1 U mg^{-1}).

After IP, CRH showed a significant ($P < 0.005$) 24 % decrease of THF from 164 ± 45 to 124 ± 27 ml. At the same time ATPase activity was reduced to 0.86 ± 0.16 U mg^{-1} before CRH. At the top of the hyperaemia it was 1.0 ± 0.06 U mg^{-1} and back to the control (0.88 ± 0.12 U mg^{-1}) 4 min after the release of the 15 s occlusion of LCCA. It is noticeable that the mean value of ATPase activity at the top of CRH after preconditioning was significantly lower than the corresponding value obtained before IP. Furthermore, the value of ATPase activity observed 4 min after the release of the 15 s occlusion of LCCA ATPase activity was not significantly different from the control while before preconditioning the corresponding value was significantly lower, indicating that modulation of F_0F_1ATPsynthase was due to the binding and release of F_1. Finally a negative linear correlation has been found between ATPase activity and IF_1 content (IF_1/F_1 ratio).

It may be concluded that, after IP, the reduction of THF could match the down-modulation of ATPase activity mediated by the binding of its specific inhibitory protein IF1.

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Muscle afferent inputs to cardiovascular control during isometric exercise vary with muscle group in patients with chronic heart failure

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The influence of skeletal muscle afferents on the pressor response (PR) to isometric exercise in patients with chronic heart failure (CHF) has been reported as greater (Piepoli *et al.* 1996), no different (Sterns *et al.* 1991) or smaller (Carrington *et al.* 2001) when compared with age-matched control subjects. This discrepancy might, in part, be explained by the muscle group chosen for study since muscle fibre type (Carrington *et al.* 1999) and training status (Fisher & White, 1999) are known to influence the PR in healthy subjects. With ethics committee approval, we examined cardiovascular responses during voluntary isometric plantarflexion (CALF) and voluntary isometric handgrip (HAND) and during subsequent post-exercise circulatory occlusion (PECO). Continuous blood pressure (Finapres, Ohmeda) and heart rate (ECG) responses were recorded in six stable CHF patients, mean (S.D.) age 65.5 (8.3) years (New York Heart Association class II–III) during an 8 min protocol (Fisher & White, 1999). This comprised 2 min rest, 2 min CALF or HAND ischaemic isometric EXERCISE (EX, at 30 % maximum voluntary strength), 2 min PECO and 2 min recovery.

The change in blood pressure at the end of CALF and HAND exercise was not significantly different but HAND PECO produced a significantly greater blood pressure response than CALF PECO (Table 1).

Table 1. Mean ± S.E.M. changes from rest in systolic (SBP), diastolic (DBP) and heart rate (HR)

Parameter	CALF		HAND	
	EX	PECO	EX	PECO
SBP (mmHg)	32 ± 6	7 ± 2	23 ± 4	14 ± 3*
DBP (mmHg)	14 ± 2	3 ± 1	14 ± 3	8 ± 1*
HR (beats min^{-1})	6 ± 2	0 ± 2	8 ± 3	1 ± 2

*Significant difference between muscle groups, $P < 0.05$, Wilcoxon signed ranks test.

Since the influence of central command and muscle mechanoreflex is absent during PECO, muscle chemoreflex activity must be greater in HAND than CALF. This may be explained by a higher proportion of fast twitch fibres in the forearm than in the calf muscles (though our contractile data indicate a relatively fast calf muscle in these subjects). Alternatively, it may be that the weight-bearing, locomotor role of the calf muscles constitutes a conditioning stimulus in CHF patients, which leads to desensitisation of the muscle chemoreceptors and therefore, a smaller PR (Carrington *et al.* 1999). We conclude that it would be wrong to make general statements about muscle chemoreflex inputs to cardiovascular control in CHF patients based upon measurements made on only one muscle group and without reference to muscle fibre type and training status.

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Calcium channel antagonist inhibits ischaemia-like changes in the ECG evoked by stimulation in the periaqueductal grey matter – implications for non-cardiac chest pain

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Although chest pain is symptomatic of myocardial ischaemia, up to 50 % of patients who present with pains in the chest have angiographically normal coronary arteries and are diagnosed with 'non-cardiac' chest pain (Mayou, 1998). Psychiatric examination of this patient group has revealed a high incidence of anxiety states such as panic disorder (e.g. Fleet *et al.* 1996). Panic attacks are characterised by extreme fear and intense autonomic disturbances that are often accompanied by chest pain (DSMIV). In animals, stimulation in the dorsal part of the periaqueductal grey matter (dPAG) evokes panic-like behaviour with intense autonomic activation (Lovick, 2000) and changes in the ST segment of the ECG (Lovick & Drew, 1998). We have now explored the possibility that a coronary constriction may underlie the changes in the ECG waveform that occur during stimulation in the dPAG.

Male Wistar rats (300–390 g body weight) were anaesthetised with 20 % urethane (0.7 ml 100 g^{-1}, I.P.) and instrumented to record blood pressure, heart rate, ECG (approximately V4 position), femoral arterial blood flow and tracheal air flow. Rectal temperature was maintained at 37 °C. Experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986. Stimulation in the dPAG (50–120 μA, 1 ms pulses, 80 Hz for 10 s) produced a pressor response (50.0 ± 2.7 mmHg, mean ± S.E.M.), tachycardia (47.4 ± 4.4 beats min^{-1}), an increase in femoral arterial conductance (58.2 ± 6.5 %) and hyperpnoea (60.3 ± 7.5 breaths min^{-1}). In all rats ($n = 15$), the duration of the interval between the peak of the S and T waves (ST_{int}) increased significantly from 18.2 ± 0.6 to 22.0 ± 0.6 ms ($P < 0.005$, paired t test) and the ST segment of the ECG waveform (ST_{seg}) was depressed by 0.052 ± 0.01 mV. Administration of the slow calcium channel antagonist amlodipine (1, 2 and 3 mg kg^{-1} I.V., $n = 5$ for each dose) produced a dose-related attenuation of the PAG-evoked cardiovascular changes and the PAG-evoked increase in ST_{int} and ST_{seg} of the ECG waveform. At the highest dose used (3 mg kg^{-1}), the dPAG-evoked increase in ST_{int} (from 18.55 ± 1.36 to 23.13 ± 1.4 ms) was attenuated significantly by 86.4 % (from 19.52 ± 1.39 to 20.14 ± 1.28 ms) ($P < 0.05$, $n = 5$) and the PAG-evoked depression of ST_{seg} was reduced by 80 % from 0.082 ± 0.023 to 0.0163 ± 0.0018 mV ($P < 0.01$). The effect was maximal 120 min after drug administration and recovery had not occurred after 3 h when the experiment was terminated by an overdose of anaesthetic.

We suggest that during activation of the dPAG, localised coronary spasm may give rise to regional ischaemic changes in the heart that are reflected by changes in the waveform of the ECG. Such a regional ischaemia could contribute to episodes of chest pain in patients with 'non-cardiac' chest pain and panic disorder.

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Haemodynamic responses at the onset of voluntary and stimulated isometric exercise in man

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We have previously reported an increase in vascular conductance of the contralateral lower limb at the onset of voluntary and stimulated isometric calf exercise (Fisher & White, 2001), but the mechanisms underpinning this change remain unclear. In dynamic exercise a similar phenomenon has been attributed to a muscle pump-induced increase in venous return and consequent sympathetic withdrawal (Joyner *et al.* 2001). However, this mechanism cannot explain our previous observation as (1) local circulatory arrest preceded exercise and (2) in itself this did not alter vascular conductance. The present study sought to investigate whether there are central circulatory changes at the onset of voluntary and electrically evoked isometric calf exercise that could explain our observations.

With local ethics committee approval, 13 healthy subjects (11 males), mean age (± S.E.M.) 24.1 ± 1.4 years, undertook 2 min of seated isometric plantar flexion at 30 % MVC performed either voluntarily (VOL) or electrically evoked (STIM). Circulatory occlusion commenced just prior to exercise and was sustained for 2 min after exercise. Heart rate (HR) (Cardiorater) and blood pressure (MAP) (Finapress) were recorded on a beat-to-beat basis. Indices of central haemodynamics were measured using impedance cardiography (Minnesota Impedance Cardiograph, Model 304B) with a tetrapolar band electrode configuration (Instruments for Medicine, Inc.). Stroke volume (SV) was estimated using the Kubicek formula (Kubicek *et al.* 1974) and cardiac output (CO) taken as the product of SV and HR. All exercise values are presented as 15 s ensemble average changes from rest. Statistical analysis was performed using repeated measures ANOVA ($P < 0.05$) and *post-hoc* paired *t* tests.

Table 1 shows resting HR, MAP, SV and CO values and the average changes produced by VOL and STIM over the first 15 s of exercise. SV and CO showed no significant change from resting values during either exercise protocol. During VOL but not STIM, MAP increased from resting levels and was significantly higher than STIM at this time. HR increased significantly from resting in VOL and less markedly in STIM though statistically there was no significant difference between these responses.

Table 1. Mean resting values (± S.E.M.) and changes from rest in cardiovascular parameters during first 15 s of voluntary and stimulated exercise

	Rest		15 s Exercise	
	VOL	STIM	VOL	STIM
MAP (mmHg)	92.5 ± 3.34	95.3 ± 3.30	4.6 ± 1.76*†	0.1 ± 2.40
HR (beats min^{-1})	75.2 ± 5.0	76.4 ± 5.1	5.2 ± 2.53*	3.12 ± 2.20
SV (ml)	92.8 ± 7.19	92.2 ± 7.87	−0.4 ± 2.20	−0.9 ± 2.30
CO (l min^{-1})	6.7 ± 0.40	6.7 ± 0.40	0.4 ± 0.28	0.3 ± 0.18

MAP, mean arterial blood pressure; HR, heart rate; SV, stroke volume; CO, cardiac output; VOL, voluntary exercise; STIM, stimulated exercise. *Significant difference from rest ($P < 0.05$), †significant difference VOL *vs.* STIM ($P < 0.05$).

These data show that central haemodynamic changes are minimal at the onset of VOL and STIM. Therefore it is clear that subjects are unlikely to have inadvertently caused an increase in venous return e.g. by abdominal fixation. Thus the initiation of sympathetic withdrawal (Joyner *et al.* 2001) is unlikely to explain the transient increase in vascular conductance observed in the contralateral lower limb during both voluntary and stimulated isometric exercise (Fisher & White, 2001). This provides further support for a local vasodilator effect in this limb that is mediated by central pathways in response to both voluntary and stimulated exercise.

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The effect of respiration rate on heart rate variability and baroreflex sensitivity

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This study investigated the effect of different breathing rates on cardiac autonomic tone and blood pressure control. The influence of nose and mouth-only breathing was also assessed. After obtaining ethical approval and informed consent, sixteen healthy subjects (8 males, 8 females, aged 33.9 ± 2.5 years, mean ± S.E.M.) breathed spontaneously for 5 min and then randomly at 6, 9 and 12 cycles per min (cpm) whilst having continuous measures of heart rate (MP100, BIOPAC Systems Inc., USA) and blood pressure (Portapres, TNO-Netherlands) performed. Cardiac autonomic tone was assessed by heart rate variability (HRV) in the time domain and by power spectral analysis (PSA) in the frequency domain. Blood pressure control was evaluated by baroreflex sensitivity (BRS) derived from a transfer function analysis of the PSA data. ANOVA and Bonferroni-adjusted *t* tests were performed. Data are given as means ± S.E.M. Time domain measures showed that compared to spontaneous breathing, standard deviation of normal RR intervals (SDNN) (ms) 81.5 ± 9.3 *vs.* 52.2 ± 5.2 (a primary index of HRV), root mean square of successive differences (RMSSD)

(ms) 51.2 ± 6.5 *vs.* 36.3 ± 4.7 and percentage of normal RR intervals greater than 50 ms from the previous beat (pNN50) (%) 17.0 ± 3.8 *vs.* 9.8 ± 3.0 (both measures of parasympathetic activity) were all significantly increased at 6 cpm ($P < 0.01$), and SDNN (ms) 63.4 ± 7.3 *vs.* 52.2 ± 5.2 and pNN50 (%) 14.0 ± 4.1 *vs.* 9.8 ± 3.0 at 9 cpm ($P < 0.05$). Total power (ms^2) (a frequency domain measure of HRV) was increased at 6 cpm 7813.0 ± 1959.0 *vs.* 3045.0 ± 591.0, as was BRS ($ms\ mmHg^{-1}$) 10.6 ± 1.1 *vs.* 8.7 ± 1.0 ($P < 0.05$). When compared with baseline, nose-only breathing demonstrated a decrease in LF/HF ratio (a measure of sympathovagal balance) 1.0 ± 0.3 *vs.* 3.1 ± 1.0 ($P < 0.01$), normalised low frequency power (nLF, an index of sympathetic activity) 42.0 ± 5.0 *vs.* 62.0 ± 4.6 ($P < 0.05$) and an increase in normalised high frequency power (nHF, a measure of parasympathetic activity) 58.0 ± 5.0 *vs.* 38.0 ± 4.6 ($P < 0.05$). Compared with mouth breathing, nose-only breathing decreased nLF 42.0 ± 5.0 *vs.* 50.0 ± 5.0 and increased nHF 58.0 ± 5.0 *vs.* 50.0 ± 5.0 ($P < 0.05$). These data suggest that reduced breathing frequency (particularly 6 cpm) is associated with increased parasympathetic activity, cardiac autonomic tone and blood pressure control, and that nose-only breathing is associated with altered sympathovagal balance, predominantly reflecting increased cardiac vagal activity.

All procedures accord with current UK guidelines and the Declaration of Helsinki.