C1

Heterogeneity in function of small artery smooth muscle BK_{Ca}

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Arteriolar myogenic vasoconstriction occurs when increased stretch or membrane tension leads to smooth muscle cell (SMC) depolarization and opening of voltage-gated Ca^{2+} channels. To prevent a positive feedback situation leading to excessive pressure-induced vasoconstriction, studies in cerebral artery smooth muscle have suggested that activation of the large conductance, Ca^{2+}-activated, K^+ channel (BK_{Ca}) provides an opposing hyperpolarizing influence to reduce Ca^{2+} channel activity. We have hypothesized that this mechanism may not, however, equally apply to all vascular beds. In particular, it is unlikely that BK_{Ca} plays a major role in arterioles of resting skeletal muscle where there is typically a high resistance to tissue blood flow. To establish the existence of such heterogeneity in vascular reactivity, studies were performed on cells dispersed from cremaster muscle arterioles and small cerebral arteries of rats. Whole cell K^+ currents were determined at pipette [Ca^{2+}] of 100 nM or 5 μM in the presence and absence of the specific BK_{Ca} inhibitor, iberiotoxin (IBTX; 0.1 μM). Similar total outward current densities were observed for the two cell preparations at the lower pipette Ca^{2+} levels. At 5 μM Ca^{2+}, however, cremaster VSM showed a significantly ($p < 0.05$) lower current density compared to cerebral VSM (34.5 $\pm$ 1.9 vs 45.5 $\pm$ 1.7 pA pF-1 at +70mV). Studies with IBTX suggested that differences in K^+ conductance between cremaster and cerebral cells at 5 μM intracellular [Ca^{2+}] were largely due to activity of BK_{Ca}. 17β-Estradiol (E2), reported to potentiate BKCa current via the channel's β-subunit, caused a significantly greater effect on whole cell K^+ currents in cerebral vessel SMCs compared to those of cremaster muscle. In contrast, the alpha subunit-selective BK_{Ca} opener, NS-1619, exerted a similar effect in cremaster and cerebral cells. Spontaneously transient outward currents (STOCs) were more apparent (in terms of frequency and amplitude) and occurred at more negative membrane potentials in cerebral SMCs, compared to cremaster SMCs. Also consistent with decreased STOC activity in the cremaster SMCs was an absence of detectable Ca^{2+} sparks (0 out of 76 cells) compared to that in cerebral SMCs (76 out of 105 cells). Measurements based upon mRNA and protein detection indicated a higher ratio of BK_{Ca} β:α-subunit expression in cerebral vessel SMCs compared to that from cremaster muscle. The data thus support vascular heterogeneity with respect to the activity of BK_{Ca}. Specifically, in cremaster arteriole SMCs, BK_{Ca} appears to show a relative decrease in β-subunit regulation of the channel compared to that in cerebral SMCs. We hypothesize that this reduced BKCa activity contributes to the increased vascular tone and resistance observed in cremaster arterioles versus comparable arteries from the cerebral circulation.

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

C2

Role of K^+ and Cl^- channels in the control of retinal blood flow *in vivo*

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The purpose of this study was to investigate the role of K^+ and Cl^- channels in the regulation of basal and endothelin-1 (Et-1)-evoked alterations in volumetric blood flow in the rat retinal circulation. Hooded Lister rats (male, 320-450g) were anaesthetised with an intraperitoneal injection of ketamine (130 mg kg^{-1}) and xylazine (17.5 mg kg^{-1}). Fifteen minutes prior to blood flow imaging, animals were intravitreally injected (10μl) with Hanks' solution, the vasoconstrictor peptide Et-1, or ion channel inhibitors in the absence or presence of Et-1. A group of non injected controls was also included. Volumetric blood flow was assessed in arterioles within the superior regions of the retina using acridine orange leukocyte fluorography and fluorescein angiography by means of a confocal scanning laser ophthalmoscope (HRA2, Heidelberg Engineering, Germany). Data are presented as means $\pm$ SEM. The n values reported refer to the number of animals used for each experiment (14-32 arterioles per group). Statistical analyses were carried out using a non-parametric Kruskall-Wallis test and Dunn's procedure for post hoc evaluation. In non-injected eyes, mean volumetric flow in single arterioles was 12.31 $\pm$ 0.23 nL s^{-1} (n=9) and this was unchanged in eyes injected with Hanks' solution (12.79 $\pm$ 0.25 nL s^{-1}, n=9, P>0.05). Injection of the Cl^- channel blocker, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS, 10 mM), increased volumetric flow (15.67$\pm$0.41 nL s^{-1}, n=9, P<0.001), while injection of penitrem A (1μM), an inhibitor of large-conductance Ca^{2+}-activated K^+ channels, or correolide (40μM), a Kv1 channel inhibitor, reduced flow rates (11.24$\pm$0.36 nL s^{-1}, n=11, P<0.01 and 11.31$\pm$0.28 nL s^{-1}; n=10, P<0.05, respectively). Intravitreal injection of Et-1 (104nM), induced severe vasoconstriction and a substantial reduction in flow (4.64$\pm$0.16 nL s^{-1}, n=8, p<0.001). This response was not affected by co-application of K^+ channel blockers (p>0.05 vs Et-1 alone), but was reversed in the presence of DIDS (14.71$\pm$0.272 nL s^{-1}, n=7, p<0.001 vs Et-1). These results suggest that K^+ and Cl^- channels are pivotal in controlling basal blood flow in the retina, while Cl^- channels may also play a key role in mediating the vasoregulatory actions of Et-1 in the retinal circulation.

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C3

Caveolin-1 and age-related changes in vascular contractility

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Caveolin-1 knock-out (KO) mice have a shorter life-span than wild-type (WT) mice (Park et al., 2003). Caveolae, and caveolin-1, can modulate vascular function while normal ageing has been associated with changes in structure and function of resistance arteries. We examined the effect of caveolin-1 ablation on vascular ageing.

Wild type (WT) and caveolin-1 knockout (KO) mice were killed by cervical dislocation at 3 and 12 months of age (3m, 12m). Third order mesenteric arteries were dissected, mounted on a pressure myograph (60mmHg, superfused with physiological salt solution (PSS), 95% air/5% CO_2, 37°C) and contractile responses to noradrenaline (NA) (10^{-9}-10^{-5}M) and KPSS (100mM) examined. Passive pressure-diameter/wall thickness relationships (5-140 mmHg) were determined in the absence of calcium and further calculations performed (Schofield et al., 2002) (n=30 3m WT, n=36 3m KO, n=23 12m WT, n=17 12m KO). Data is expressed as maximal responses (mean ± SEM) with statistical differences ($p<0.05$) examined by ANOVA and Bonferroni.

Caveolin-1 ablation significantly reduced NA constriction of vessels from 3 month mice (69 ± 2% KO (n=24); 81 ± 1% WT (n=30)). Ageing significantly reduced constriction in WT arteries (66 ± 2% 12m n=24), but had no effect on KO vessels (68 ± 2% 12m n=19). Constriction of arteries from 12 month WT and 3 and 12 month KO mice was comparable. Similar changes were observed with KPSS; while ageing in the WT mouse reduced constriction (72 ± 3% (3m, n=20) to 53 ± 4% (12m, n=19). Responses in KO mice at 3m were reduced *c.f.* WT (51 ± 3% n=29) but there were no further significant age-related changes (39 ± 4% n=19).

Ageing was associated with increased diameter and decreased wall: lumen ratio in arteries from WT and KO mice. However, the wall thickness and cross sectional area of KO mice were increased *c.f.* WT vessels indicating growth (growth index = $((CSA_1\text{-}CSA_2)/CSA_2)^*100$; 43% 3m; 21% 12m). Vessel distensibility was reduced by ageing in WT mice as shown by a leftward shift in stress-strain curves. Distensibility was also reduced in vessels from 3 month KO mice, however, these vessels were resistant to any further age-related changes.

Thus caveolin-1 ablation causes structural and functional changes in resistance arteries which are not altered by ageing. This suggests that caveolae play an important role in the normal vascular ageing process.

Park DS, Cohen AW, Frank PG, Razani B, Lee H, Williams TM, Chandra M, Shirani J, De Souza AP, Tang B, Jelicks LA, Factor SM, Weiss LM, Tanowitz HB, Lisanti MP (2003) Caveolin-1 null (-/-) mice show dramatic reductions in life span. Biochem., 42, 15124-31

Schofield, I., Malik, R., Izzard, A., Austin, C., Heagerty, A. (2002) Vascular structural and functional changes in type 2 diabetes mellitus. Circ., 106, 3037-43

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C4

Cellular localization of mitochondria contributes to the regulation of voltage-dependent potassium currents in the rat pulmonary, but not mesenteric arterial myocytes

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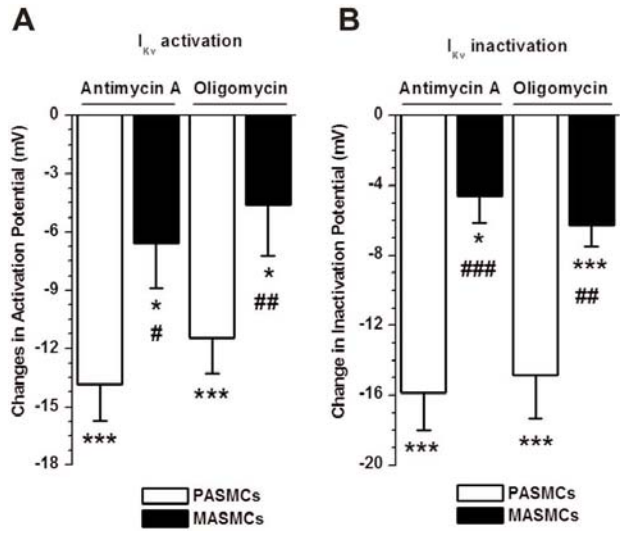
Mitochondria are one of the major oxygen sensors triggering hypoxic pulmonary vasoconstriction (HPV), a unique response of the pulmonary circulation to low oxygen unique. Various mitochondrial factors including reactive oxygen species, cytochrome c, ATP and magnesium, are also potent modulator of voltage-gated K^+ (Kv) channels in plasmalemmal membrane of pulmonary arterial smooth muscle cells (PASMCs). The main aim of this investigation was to determine whether differences between mitochondria localization in art PASMCs and systemic mesenteric arterial SMCs (MASMCs) could be a contributing factor to the divergent oxygen sensitivity in two different circulations. Myocytes were enzymatically isolated from arteries dissected from male Wistar rats (250-300g)(Smirnov *et al*., 2002; Firth *et al*., 2008); experiments were performed at room temperature. Cellular localization of mitochondria was compared using confocal imaging and immunofluorescent labelling (Gordienko *et al*., 2001; Gordienko & Zholos, 2004). It was found that mitochondria (labelled with MitoTracker Green) were located significantly closer to the plasmalemmal membrane (labelled with Di-8-ANEPPS) in PASMCs compared to MASMCs. On the other hand, sarcoplasmic reticulum (labelled with Brefeldin A BODIPY) was more closer to the plasmalemmal membrane in MASMCs than in PASMCs. Differences in the functional coupling between mitochondria and Kv channels were then evaluated with the patch-clamp technique and two specific mitochondrial inhibitors, antimycin A (a Complex III inhibitor) and oligomycin (an ATP synthase inhibitor)(Smirnov *et al*., 2002; Firth *et al*., 2008). These inhibitors were chosen as they have most pronounced effect on I_{Kv} amplitude and steady-state activation in rat PASMCs (Firth *et al*., 2008). Electrophysiological results showed that relative changes in the steady-state activation, inactivation of I_{Kv} (Fig. 1) and I_{Kv} block (not shown) induced by antimycin A and oligomycin were significantly greater in PASMCs than in MASCMs. These findings suggest a greater structural and functional coupling between mitochondria and Kv channels specifically exists in PASMCs, which could play an important role in HPV.



Mitochondrial inhibition has significantly greater effects on I_{Kv} in PASMCs than in MASMCs. **A** and **B**, Comparison of antimycin A and oligomycin induced changes in the half activation and half inactivation potentials, respectively. Open and filled bars represent the relative changes in the half activation and half inactivation potential in PASMCs (n=8-12) and MASMCs (n=9-11) for antimycin A and oligomycin, respectively. * P<0.05 and *** P <0.001. * compares the difference in the parameters between the control condition and in the presence of an inhibitor measured in the same cell using paired statistical test. # P<0.05, ## P<0.01, ### P<0.001. # compares the difference between the two cell types using non-paired statistical test.

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Smirnov SV *et al*. (2002). *J Physiol* **538**, 867-878

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C5

Expression of two pore domain potassium channels (K2P) in the placenta

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Vascular function of the placenta is closely linked with the well being of the fetus. Key to this is the effective transport of blood and gases which is possible due to the low resistance of the placenta. There is a poor understanding about the specific ion channels that are involved in regulating the vessel tone across the placenta. Two members of the K2P family TWIK-2 and TREK-1 are important for maintaining the resting membrane potential. This study aims to investigate the expression of these ion channels in arteries taken from the human chorion and villi. Hypothesis: TWIK-2 and TREK-1 expression is differentially modulated across the fetal and maternal interface of the placenta. Placentae were collected with written informed consent from patients undergoing elective Caesarean section at term (~37 weeks). Explants taken from the stem villous artery (SVA) and the third branch of the chorionic plate artery (CPA) were used to culture smooth muscle cells (SMC). After 20 days the cells were harvested and immunofluoresence was used to detect the expression of TWIK-2 and TREK-1. Briefly SMC were fixed with 3% PFA and pre incubated with primary antibody to detect expression of the K2P channels (Alomone, Jerusalem).Western blotting was performed on crude tissue lysate and separated by 12% SDS-PAGE to quantify expression of TWIK-2 and TREK-1 in CPA and SVA. The blots were stripped and re-probed with beta actin (AbCam, UK) to measure protein loading. Immunofluorescence for TWIK-2 and TREK-1 was detected in both CPA and SVA and staining for alpha actin confirmed the smooth muscle cell phenotype (n = 4 placentae). Staining was concentrated in the nuclei of SMC cultured from CPA, while in SVA, the staining was cytosolic and showed actin like stress fibres. Western blot analysis on isolated placental arteries showed that the TREK-1 dimer (100 kDa) band density is significantly higher in SVA (n =6 placentae; P= 0.04). No significant difference was seen with TWIK-2 (37 kDa) in the two different vessel types (n =6 placentae; P=0.47). We have successfully shown that TWIK-2 and TREK-1 are expressed in both the SVA and CPA. Our early findings suggest TREK-1 is up regulated in the SVA and the immunofluorescence staining shows the channel may be differentially regulated within the two vascular beds. These findings suggest that TWIK-2 and TREK-1 may have an important role in regulating vasodilatation of placental arteries and requires further investigation. This also raises the possibility that K2P channels are a potential target for treating placental disorders such as pre eclampsia where the vascular arteries are poorly perfused and do not respond well to vasodilators.

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C6

Spontaneous ATP release from nerves is the predominant determinant of spontaneous action potentials in the mouse urinary bladder

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Spontaneous purinergic neurotransmission was characterised in the mouse urinary bladder. Intracellular electrophysiological recording from smooth muscle cells of the detrusor muscle revealed spontaneous depolarizations, distinguishable from

spontaneous action potentials (sAPs) by their amplitude (<40 mV) and insensitivity to the L-type Ca^{2+} channel blocker nifedipine (1 μM) (mean ± S.E.M., 100 ± 29%; Student's two-tailed paired *t*-test, *P* = NS, *n* = 4). Spontaneous depolarizations were abolished by the $P2X_1$ receptor antagonist NF449 (10 μM) (frequency 8.5 ± 8.5% of controls, *P* < 0.01), insensitive to the muscarinic acetylcholine receptor antagonist atropine (1 μM) (103.4 ± 3.0%, *P* = NS), and became more frequent in latrotoxin (1 nM) (438 ± 95%, *P* < 0.05), suggesting that they are spontaneous excitatory junction potentials (sEJPs). Such sEJPs were correlated, in amplitude and timing, with focal Ca^{2+} transients in smooth muscle cells (measured using confocal microscopy), suggesting a common origin: ATP binding to $P2X_1$ receptors. sAPs were also abolished by NF449, insensitive to atropine (126 ± 39%, *P* = NS) and increased in frequency by LTX (930 ± 450%, *P* < 0.05) suggesting a neurogenic, purinergic origin, in common with sEJPs. By comparing the kinetics of sAPs and sEJPs, we demonstrated that sAPs occur when sufficient cation influx through $P2X_1$ receptors triggers L-type Ca^{2+} channels; the first peak of the differentiated rising phase of depolarizations - attributed to the influx of cations through the $P2X_1$ receptor - is of larger median amplitude for sAPs (2248 $mV.s^{-1}$) than sEJPs (439 $mV.s^{-1}$; Wilcoxon signed rank test, *P* < 0.0001). Surprisingly, sAPs in the mouse urinary bladder are triggered by stochastic ATP release from parasympathetic nerve terminals rather than being myogenic.

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C7

$P2X_1$ receptors are not involved in contractions and Ca^{2+} transients induced by PGE_2 in the mouse urinary bladder

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Prostaglandins (PG), particularly PGE_2, are implicated in the development of bladder overactivity. Although PGE_2 causes contraction of the mouse bladder, its mechanism of action has not yet been fully elucidated. This study used contraction studies and a confocal Ca^{2+}-imaging technique to further investigate the mechanism of action of PGE_2. Female CD-1 mice were killed by rising CO_2 and cervical dislocation; the bladder removed and strips were cut (from trigone to vertex) with urothelium left intact.

Exogenously applied PGE_2 (50 μM) caused an initial slow contraction of mouse bladder strips (peaking at around 2.23 ± 0.17 mN, number of animals, *n* = 7) and induced subsequent spontaneous contractions (with a mean frequency of 0.21 ± 0.01 Hz, and amplitude of 0.41 ± 0.05 mN, *n* = 7). Such activity *in vitro* is a characteristic of bladder overactivity. The PGE_2-induced overactivity was blocked by the L-type Ca^{2+} channel antagonist, nifedipine (1 μM) (*P* < 0.01, *n* = 6). Recent work from our lab has shown that spontaneous electrical activity at rest (in mouse urinary bladder) is neurogenic in origin; spontaneous action potentials result from stochastic ATP release from parasympathetic nerves (1), however the PGE_2-induced spontaneous contractions seen *in vitro* were unaffected by the selective $P2X_1$ antagonist NF449 (10 μM) (*P* = NS, *n* = 4).

A confocal Ca^{2+}-imaging technique was used to identify Ca^{2+} changes in bladder smooth muscle cells loaded with the Ca^{2+}-indicator, Oregon Green 488 BAPTA-1 AM (10 μM). Bath application of PGE_2 (50 μM) significantly increased the frequency of spontaneous whole cell Ca^{2+} flashes (from 0.04 ± 0.01 Hz to 0.18 ± 0.02 Hz, *P* < 0.01, *n* = 11), but had no effect on the amplitude of these flashes (137 ± 32%, as a percentage of control, *P* = NS, *n* = 5). The PGE_2-induced increase in the frequency of flashes was abolished by nifedipine (1 μM) (*P* < 0.01, *n* = 4), but were not significantly reduced in frequency by NF449 (10 μM) (83 ± 15%, *P* = NS, *n* = 4). In addition, the synchronicity of PGE_2 induced whole cell Ca^{2+} flashes observed may indicate that an increased level of coupling occurs in smooth muscle cells in response to the application of PGE_2.

These data suggest that PGE_2 directly or indirectly activates L-type Ca^{2+} channels (Ca_V1 family) to induce spontaneous contractions in bladder smooth muscle cells and that the purinergic $P2X_1$ receptor is not significantly involved in such a response.

(1) Young JS, Meng E, Cunnane TC, Brain KL. Spontaneous purinergic neurotransmission in the mouse urinary bladder. J Physiol. 2008; In press.

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C8

Nitric oxide regulation of capillary diameter via pericytes in rat cerebellum

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Regulation of cerebral blood flow by neural activity helps to supply the energy required by active neurons and is the basis for BOLD and PET functional imaging techniques. The modification of arteriolar smooth muscle tone by neurotransmitters and modulators has been extensively studied. Recent work has shown that neural regulation of cerebral blood flow can also occur at the level of the capillary, via smooth muscle-like cells called pericytes, which constrict in response to noradrenaline and dilate in response to glutamate (Peppiatt et al., 2006).

Nitric oxide has a well-established role in controlling basal vasodilatory tone in arteriolar smooth muscle, being produced by the endothelial isoform of nitric oxide synthase and diffusing into smooth muscle cells where it increases the intracellular cGMP concentration. In the brain and the periphery, nitric

oxide produced by neurons can also increase arteriolar dilatory tone, allowing increased blood flow to areas which have an increased energy requirement.
Here, we show that nitric oxide can also modulate pericyte vasodilatory tone in a manner depending on the oxygen concentration. Capillaries dilated by glutamate constricted in response to a non-specific nitric oxide synthase inhibitor at high (95%) but not low (20%) superfused oxygen concentrations. In addition, tonic nitric oxide levels provide basal dilatory tone to capillaries, as inhibition of nitric oxide synthase, in the absence of added glutamate, constricted capillaries at pericytes (this experiment was performed using 95% oxygen). This basal nitric oxide is derived from neuronal, not endothelial, nitric oxide synthase, as an inhibitor of the neuronal nitric oxide synthase isoform (1 μM 1400W) had the same effect on capillary tone as did the non-specific inhibitor (100 μM L-nitroarginine). These data extend to CNS capillaries the notion that nitric oxide can regulate vessel diameter, as is already established for CNS arterioles.

Peppiatt CM, Howarth C, Mobbs P, Attwell D (2006) Bidirectional control of CNS capillary diameter by pericytes. Nature 443:700-704.

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C10

Evidence of a mechanosensory role for CD31 in cardiovascular disease

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CD31 (PECAM-1) has been shown to form a mechanosensory complex with VE-cadherin and VEGFR which can modulate endothelial cell (EC) responses to shear stress through NF-κB activation.[1] Since CD31 is expressed on leukocytes, EC and platelets and has been shown to play a prominent role in the transmigration of leukocytes into sites of inflammation,[2] we hypothesized that it might contribute to the development of atherogenesis in a shear dependent manner. To test this hypothesis we generated Pecam1$^{-/-}$Apoe$^{-/-}$ mice to determine the role of CD31 in an established model of murine atherosclerosis. At 10 weeks of age, Pecam1$^{-/-}$Apoe$^{-/-}$ mice or control mice (age and sex-matched) were placed on a high fat diet (20% coco butter, 1.25% cholesterol) or retained on normal chow diet, for a further 13 weeks. Aortas were then excised and stained with oil red O (ORO) to determine plaque burden and serum lipid levels and leukocyte counts taken. No differences were found in weight gain, serum cholesterol or tri-glyceride levels between CD31 deficient and control groups on either a chow or high fat diet. White blood cell counts and the percentage of circulating neutrophils, monocytes and peripheral blood lymphocytes also remained similar between mouse groups. Mice kept on a chow diet showed no differences in percentage of plaque burden between CD31 deficient or control mice in the whole aorta. However, a detailed analysis of anatomically distinct areas of the aorta showed a large reduction in plaque burden in the inner curvature of the aortic arch, a well-defined area of disturbed non-laminar flow. Mice kept on high fat diet showed a larger plaque burden compared to those on chow diet, but no differences were seen in percentage plaque burden between CD31 deficient or control mice in the whole aorta. Complete analysis of the aortic regions revealed a reduction in plaque burden in areas of disturbed flow (aortic arch and inner curvature) and an elevation in plaque burden in areas of steady (laminar) flow (thoracic and abdominal aortas) in Apoe$^{-/-}$ mice lacking CD31 compared to the control groups.
Thus, under conditions of laminar flow, CD31 appears to act as a mechanotransducer of anti-atherogenic signals into EC, and therefore removal of CD31 leads to an increase in plaque burden. Conversely, in areas of complex flow, CD31 transduces pro-atherogenic signals, the loss of which moderates the disease process. This indicates that the mechanosensory role of CD31 is essential in the development of atheromas at areas of disturbed flow.

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C11

A novel in vitro model for studying responses of endothelial cells under physiological flow conditions

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Haemodynamic forces occur naturally at branches of medium and large arteries and contribute to the development of atherosclerotic lesions. These forces modulate gene expression in the endothelium through complex mechanosensitive pathways. Low and oscillatory flow patterns promote a pro-atherogenic genotype, while high laminar flow activates athero-protective genes. To simulate pathophysiological, atherogenic blood flow patterns we have developed an *in vitro* perfusion system which allows exposure of endothelial cells grown in micro-slides to low or oscillatory flow patterns. Using this perfusion system we have examined the preconditioning effect of oscillatory flow on endothelial cells.
Preconditioning human umbilical vein endothelial cells (HUVECs) with oscillatory flow enhanced their responses to inflammatory stimulation. Thus, subsequent exposure of HUVECs for 18-22 hours with the inflammatory cytokine TNFα (5 ng/ml) resulted in increased detection of pro-inflammatory and chemoattractant factors such as IL-8 and MCP-1, when compared to non-preconditioned cells (Table 1). When com-

pared to static controls, we also demonstrated that preconditioning cells affected the distribution of the adhesion molecule ICAM-1, as determined by immunocytochemical staining. In an adhesion assay, preconditioning further affected the adherence of THP-1 monocytes to HUVECs.
In summary, this system allows the assessment of responses of the vascular endothelium to inflammatory factors in a pathophysiologically-relevant setting. Priming of HUVECs with oscillatory flow altered their protein expression and functional profiles and rendered them more sensitive to subsequent treatment with TNFα. This *in vitro* model is amenable to further studies examining the effects of cigarette smoke toxicants on the vascular endothelium.

	non-preconditioned		preconditioned	
Protein	Control	TNFα	Control	TNFα
IL-8	1.3±0.6	29.5±10.0	3.1±1.1	52.2±21.7
MCP-1	1.4±1.2	17.8±4.7	8.6±9.4	42.8±13.3

Table 1. Concentrations of inflammatory and chemoattractant proteins in media following exposure of HUVECs for 18-22 hours to 5 ng/ml TNFα. Data were obtained in cells cultured in static conditions (non-preconditioned) or preconditioned with oscillatory flow. Protein levels were determined by multiplex electrochemiluminescence detection using the MesoScale Discovery (MSD) platform. All concentrations are μg/ml. Data are means ± S.D. (n = 5-7 in each case).

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C12

Extracellular calcium-sensing receptor-mediated signalling and its role in human vascular smooth muscle cell proliferation and apoptosis

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Vascular smooth muscle cells (SMC) play a pivotal role in the pathogenesis of medial calcification (major cause of cardiovascular mortality in chronic kidney disease). We have recently demonstrated expression of the calcium-sensing receptor (CaSR) in human aortic SMC (HAoSMC) and human arteries and demonstrated a correlation between CaSR expression and medial calcification. The CaSR is involved in a number of diverse processes as hormone secretion, modulation of inflammation, proliferation, differentiation and apoptosis, however, functional significance of this receptor in vascular SMC is not fully understood. Here we examined CaSR-mediated intracellular signalling pathways and investigated the potential role of CaSR in regulating SMC proliferation and apoptosis.
HAoSMC were incubated with CaSR agonists (neomycin and gentamycin) and signalling inhibitors. ERK1,2 activation was assessed by Western blot. Inositol triphosphate (IP3) production was measured using Biotrak assay (Amersham). Cell proliferation was determined by BrdU incorporation and apoptosis assessed by flow cytometry of propidium iodide stained cells. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test.
Incubation of HAoSMC with 300μM neomycin resulted in 7.5-fold ($p<0.05$) increase in ERK1,2 phosphorylation. This induction was reduced ($p<0.01$) in the presence of 10μM PD98059 (MEK1 inhibitor), indicating that CaSR agonist-induced effects were mediated via classic MEK1/ERK1,2 pathway. ERK1,2 phosphorylation was almost completely abolished by 5μM U73122 (PLC inhibitor), indicating that PLC signalling was crucial for MEK1/ERK1,2 activation. No changes were observed with PI3K and PKC inhibitors. Confirming PLC activation, IP3 production was increased by neomycin/gentamycin ($p<0.05$) and reduced in the presence of U73122 ($p<0.05$). To confirm that ERK1,2 and PLC signalling were mediated via the CaSR, HAoSMC were transfected with CaSR siRNA. CaSR-knockdown resulted in attenuated ERK1,2 phosphorylation in response to neomycin (>50% of neomycin induction in control cells, $p<0.01$) while IP3 production was almost completely abolished. Treatment with neomycin increased HAoSMC proliferation >3-fold ($p<0.01$). This was reduced in CaSR-knockdown cells ($p<0.01$) and further inhibited by PD98059 and U73122 ($p<0.05$). Apoptosis was not affected by neomycin treatment or CaSR expression. However, inhibition of PLC signalling (incubation with U73122) produced a 3.5-fold increase in HAoSMC apoptosis ($p<0.05$), which was further increased by CaSR-knockdown (4.8-fold versus control siRNA, $p<0.05$).
In conclusion, these data suggest that CaSR stimulation leads to activation of the MEK1/ERK1,2 and PLC pathways and increases cell proliferation. CaSR-mediated PLC activation is crucial for SMC survival and protection against apoptosis.

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

C13

Proteomic analysis reveals distinct role of smooth muscle progenitors in extracellular matrix production

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Background. Recent studies on cardiovascular progenitors have led to a new appreciation of their secretome, as paracrine factors may support the regeneration of damaged tissues.
Methods and Results. Here we utilized a shot-gun proteomics strategy to define endothelial and smooth muscle progenitors (EPCs and SPCs) based on their distinct proteomic profiles in the secretome. Our data confirms previous results from

microarray experiments (Urbich C et al. 2005) that EPCs express high levels of cathepsins (Fig 1). In contrast, no cathepsins were detected in SPCs conditioned medium. SPCs also showed attenuated production of proteolytic enzymes and inflammatory cytokines, but secreted extracellular matrix molecules such as a variety of collagen chains and fibronectin. Compared to their mature smooth muscle counterparts, SPCs produced different isoforms of matrix proteins as evidenced by the truncation of angiogenic domains in collagen alpha-1 (I). Moreover, SPCs retained specific proteins from the bovine serum supplement, including insulin-like growth factor-binding protein 2, a known target gene of the hypoxia-inducible factor, pigment epithelium-derived factor, a potent inhibitor of angiogenesis, and proteoglycans regulating collagen assembly. As a functional consequence, SPCs showed reduced invasive capacity and their conditioned medium inhibited endothelial tube formation. Conclusion. The present study represents an important conceptual development in vascular biology suggesting that SPCs can regulate tissue remodeling and demonstrates the utility of contemporary proteomics to better characterize vascular progenitors intended for therapy in clinics.

Figure 1

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C14

Resistance artery structure and mechanics in hypertension and aging. Influence of integrin-extracellular matrix interactions

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Vascular structural and mechanical alterations in hypertension and aging contribute towards cardiovascular morbidity/mortality[1-3]. The aim of this study was to determine whether integrin-extracellular matrix (ECM) interactions contribute towards altered vascular structure and mechanics in hypertension and aging. The effect of disintegrin-treatment (kistrin, or echistatin) *ex vivo* on mesenteric artery structure and mechanics was assessed using wire-myography in spontaneously hypertensive rats (SHRs) and wistar-kyoto rats (WKYs). Integrin αV and α5 expression was also quantified using immunohistochemistry. Differences between data were tested using 2-tailed unpaired student's t-test, or one-way ANOVA followed by Bonferroni's correction for multiple comparisons. In arteries from 8 and 15 week-old SHRs versus WKYs, media/lumen ratios were greater (by 108 and 70% respectively; $P<0.01$) and media cross-sectional area (MCSA) and stiffness unaltered. Disintegrin-treatment of arteries did not alter structure or mechanics in SHRs versus control-treatment. With aging in WKYs, media thickness and MCSA were significantly increased (by 25 and 44% respectively in 15 week-old versus 8 week-old WKYs, $P<0.05$; by 43 and 46% respectively in >1 year-old versus 8 week-old WKYs, $P<0.01$) and stiffness unaltered. This was associated with unaltered integrin αV and α5 expression. In WKYs at all ages, disintegrin-treatment of arteries did not alter structure; however, stiffness was significantly reduced with echistatin-treatment versus control-treatment ($P<0.05$). These results suggest that integrin-ECM interactions are important determinants of passive stiffness in aging WKYs. Further insight into the role of integrin-ECM interactions on vascular structure and mechanics may have important implications for the reduction of cardiovascular morbidity/mortality.

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C15

The magnitude of improvement in flow-mediated dilatation following exercise training is similar in postmenopausal women with and without type 2 diabetes

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A decline in endothelial function as measured by flow mediated dilatation (FMD) is an early independent predictor of cardiovascular disease (CVD) and has been seen in diabetes (1)and following the menopause in women (2). Whether exercise training can improve endothelial function to the same magnitude in postmenopausal women with type 2 diabetes compared to those without diabetes is unclear and the purpose of the present study. 38 apparently healthy postmenopausal women (ND) and 15 postmenopausal women with type 2 diabetes (T2) volunteered for this study. All participants completed a maximal exercise test for the assessment of peak oxygen uptake (VO2peak), and were assessed for waist circumference and percentage body fat via skinfold analysis, FMD via reactive hyperaemia and ultrasound, blood pressure via shygmomanometry and HOMA via fasting blood sampling. Participants were then randomised into body mass matched exercise training or control groups. The exercise training group trained under supervision twice per week (+one home session) at 55, rising to 75% VO2peak for six months. The control participants continued life as normal. Following the six month intervention all baseline assessments were repeated and the impact of exercise training upon FMD compared between ND and T2 groups via two way mixed mode ANOVA (time x group). The control data were assessed separately and confirmed no change in any variable over 6 months in either the ND or T2 women. At baseline there were no significant differences in VO2peak (ND: 24.4 ±3.6, T2: 21.2 ±5.6 ml.kg.min-1), percentage body fat (ND: 37.9 ±5.2, T2: 40.6 ±5.6 %), blood pressure (ND MAP: 91 ±10, T2:100±12 mmHg) or FMD (ND: 4.2±2.9, T2: 4.1 ±2.9 %) between the ND and T2 women ($P > 0.05$). HOMA was greater in the T2 women (ND: 1.7 ±0.1, T2: 4.7 ±2.0 $P < 0.05$). Following training VO2peak improved more so in the ND than the T2 women (to 29.82±5.6 and 24.50±6.7 ml.kg.min-1, respectively (interaction $P = 0.06$), whilst waist circumference decreased by a greater magnitude in the T2 participants (ND: -0.5, T2: - 3 cm: interaction $P < 0.05$). Blood pressure and HOMA were not significantly affected by training ($P > 0.05$). FMD improved to 7.0 ±2.9 and 7.1 ± 2.0 % in the ND and T2 women respectively, but importantly there was no difference between groups in the magnitude of improvement (interaction $P > 0.05$). The impact of exercise training upon endothelial function was biologically significant even in a milieu of low oestrogen concentration plus insulin resistance.

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C16

Pressure-dependent myogenic tone in ischaemic vascular disease

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Advanced peripheral vascular disease, critical limb ischaemia (CLI) is associated with an inability to regulate blood volume within the capillary beds of the diseased part of the leg. Part of the sequelae accompanying CLI can be associated with dysfunction of resistance arterioles. Reactive oxygen species (ROS) were originally considered toxic metabolites. Increasing evidence suggests that they can play an important signalling role in essential physiological functions including pressure-dependent arteriolar myogenic tone (1). In addition to the well documented calcium/ calmodulin/ myosin light chain kinase dependency of myogenic contraction evidence suggests that G and F actin based structures contribute to myogenic tone (2). Here ROS and G/F actin mechanisms of arteriolar contractile responses in skeletal muscle arterioles isolated from the diseased part of the leg (DSM) vs. arterioles isolated form the healthy part of the leg (PSM, internal control) have been examined.

Methods: Resistance arterioles (lumen diameter ~80μM) were isolated from skeletal muscle biopsies taken from subjects with CLI. Pressure-dependent and pressure-independent mechanisms of vascular tone were studied (Danish MyoTech P110 pressure myograph).

Results: There was no difference in pressure-independent mechanisms of vascular tone (DSM vs. PSM). Pressure-dependent myogenic constriction was reduced (20.9 ± 2.3% vs. 4.2 ± 1.2% and 28.2 ± 3.1% vs. 6.8 ± 1.4%, PSM vs. DSM at 80mmHg and 120mmHg respectively; n=6 pairs). The antioxidant NAC, the inhibitor of NAD(P)H oxidase DPI and cytochalasin D, an inhibitor of actin polymerisation inhibited myogenic responses in PSM arterioles. NAC, DPI and cytochalasin D had no effect on pressure-dependent myogenic contraction measured in the DSM arterioles. NAC, DPI and cytochalasin D actions were selective to myogenic contraction as they had no effect on pressure-independent contraction.

Discussion: Based on these results we confirm that the myogenic response involves two discrete contractile components2. One pathway involves the conventional signalling pathway for vascular smooth muscle constriction. The other involves activation of NAD(P)H oxidase, elevation of ROS and actin polymerization. Since the NAD(P)H/ROS/actin polymerization-dependent response is absent in arterioles associated with CLI, we propose that deregulation of this second pathway negates the ability of these arterioles to resist pressure-dependent forced dilatation and generate a myogenic contraction and this dysfunction significantly contributes to the altered vascular function associated with CLI.

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PC2

UTP activates P2X receptor and regulate vascular tone in rat arterial smooth muscle

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It is well documented that uridine 5'-triphosphate (UTP) is released from several cells surrounding vascular smooth muscle cells including endothelial cells, platelets and sympathetic nerve terminals, and generally considered that UTP regulates vascular tone through selective activation of G-protein coupled P2Y receptors (Burnstock G, 2006). However, here we present evidence supporting that UTP mediates contraction of vascular smooth muscle through P2X receptor activation. Namely, we found that extracellularly applied UTP (~10μM) induced transient inward current in arterial (cerebral, mesenteric or aortic) myocytes, by use of whole-cell patch clamp configuration at a holding potential of -60mV. The current amplitude was enhanced dose-dependently, and current-voltage relationship of the current showed inward-rectification, which was similar to those evoked by activation of P2X channel. The current was also activated by high concentration of uridine 5'-diphosphate (UDP; 1mM), however, uridine 5'-monophosphate (UMP; 1mM) and uridine(1mM) were ineffective. Furthermore, the current induced by UTP (1mM) was inhibited by Gd^{3+} or La^{3+} (IC50=51.3μM or 21.8μM, respectively; n=3~5). Suramin and PPADS, both P2 receptor antagonists, also inhibited the current, and their IC50 were 0.43μM and 0.36μM, respectively (n=3~5). A previous study suggested that UTP activates TRPC3 channel through P2Y receptors in vascular smooth muscle cells (Reading SA et al., 2005). However, SKF96365 (30μM) and 2-APB (100μM), which are potent TRPC3 antagonists, failed to inhibit the UTP-induced current (n=3~5). Application of α,β-methylene ATP (10μM), a potent P2X receptor agonist, slightly potentiated the UTP (1mM) -evoked current (13.1±5.4% (n=8) from the basal current), but inversely, application of UTP (1mM) did not facilitate the α,β-methylene ATP (10μM) -evoked current. Similar effect was observed in the tension recordings in endothelium-denuded rat aorta ring preparations. These results indicate that UTP activates the same receptor which is sensitive to α,β-methylene ATP. Moreover, intracellular application of GDPβS or GTPγS (each 1mM), which is G-protein inhibitor or activator, respectively, did not modify the current at all. This result also indicates that the current was independent of G-protein coupled receptors.

Single channel analysis revealed that both UTP (1mM) and α,β-methylene ATP (10μM) activate channels with similar conductance (10.5pS) in the outside-out mode.

RT-PCR and Western blot analysis showed high expression of P2X1 subtype in cerebral and mesenteric arteries and aorta. However, TRPC3 transcript was not expressed in mesenteric artery and aorta. In cerebral artery, a faint band was observed for TRPC3.

Taken together, our results suggest that UTP regulates arterial tone through P2X receptor activation, but not through activation of P2Y receptor.

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PC4

Visualisation of vascular cannabinoid receptors and their potential interaction with α_1-adrenergic receptors

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The role of cannabinoid receptors (CB1 & CB2) within the cardiovascular system is unclear. The endogenous cannabinoid anandamide (AEA) mediates vasodilation *in vitro*[1] whilst *in vivo* a triphasic blood pressure response comprising pressor and depressor components has been reported[2]. A possible role for CB1 and CB2 receptors exists within vascular tissue. However, non-CB mediated responses in the vasculature have been observed and a role for the orphan receptor GPR55 has been postulated[3]. The aims of this study were to a) investigate the role of endogenous cannabinoids in mouse tail artery, a thermoregulatory vessel rich in α_1-adrenoceptors and b) examine, for the first time, the binding of a novel fluorescent ligand for CB receptors (Tocrifluor 1117).

Tail arteries were removed from 4 month old male C57 black mice. Vessel segments were either mounted in a wire myograph for functional studies or incubated in Tocrifluor 1117 (0.5μM) & QAPB (1μM, fluorescent α_1-adrenoceptor antagonist) for confocal analysis. Concentration response curves (CRC) to noradrenaline (NA) were performed in the presence and absence of the endocannabinoids AEA (1μM) and 2-arachidonylglycerol (2-AG, 1μM). Tocrifluor 1117 and QAPB were imaged under 488nm and 529nm excitation respectively. Tocrifluor 1117 binding was also examined in HEK293 cells stably expressing GPR55.

AEA 1μM caused a transient contraction in isolated tail artery segments (0.08g). The NA CRC was shifted to left in the presence of 1μM AEA (Log EC50 control –6.80 vs –7.82, $p<0.05$). The maximum contractile response was unchanged. In the presence of 2-AG (1μM) a small leftward (non -significant) shift of the NA CRC was observed. However, comparison of the effect of 2-AG alone (Log EC50 –7.36) and 2-AG plus

indomethacin (10μM, Log EC50 -6.33) revealed a significant difference (p>0.05). Tocrifluor 1117 binding was most evident on perivascular fat, adventital cells, nerves and smooth muscle. In several areas of media, colocalisation of Tocrifluor 1117 and QAPB was observed. In live HEK293 GPR55 cells, Tocrifluor 1117 generated a rise in Ca++ and promoted receptor clustering, visible as punctate fluorescence which developed over time.

Tocrifluor 1117 (fluorescent analogue of the CB1 antagonist AM251) is a potentially very powerful tool for identifying the cellular location of cannabinoid receptors, including GPR55 in living tissues. AM251 has been shown to activate GPR55[4]. The results of the study suggest that endogenous cannabinoids potentiate the actions of noradrenaline in tail artery possibly via co-localised cannabinoid (GPR55?) and α_1-adrenergic receptors in vascular smooth muscle. The importance of the CB/GPR55 receptors in perivascular fat and adventitia requires further study.

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PC5

Quantitative RT-PCR analysis of smooth muscle markers between mature and immature vascular myocytes

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Immature, non-contractile myocytes morphologically similar to the intestitial cells of Cajal, have been found resident in the vasculature[1]. It is thought that these cells are synthetic vascular myocytes and play a major role in vascular remodelling[2]. This work aimed to determine the quantitative differences in transcription of smooth muscle cell and interstitial cell marker genes between immature and mature vascular myocytes. Genes investigated were: smooth muscle (SM) α-actin, β-actin, myosin light chain kinase (MLCK), desmin, smooth muscle myosin heavy chain (SM-MHC) isoforms SM1 and SM2 (smooth muscle marker genes) and c-kit and vimentin (markers of interstitial cells).

Investigations were carried out on freshly isolated myocytes from mesenteric arteries of guinea-pigs which were killed in accordance with national guidelines for humane killing of experimental animals (Schedule 1). Single mature and immature myocytes were obtained by enzymatic digestion and collected using a wide-bore pipette mounted on a micromanipulator. Cells were prepared for qRT-PCR using the TaqMan PreAmp Cells-to-Ct Kit (Applied Biosystems). This involved cell lysis, DNA removal, reverse transcription of RNA into cDNA and gene-specific preamplification using custom assays followed by analysis in a qRT-PCR analyser. Custom TaqMan gene expression assays were designed for each gene using available guinea-pig sequences and sequences determined by homology. CT values were compared directly between cell phenotypes and fold differences (ratio of mRNA quantity in mature cells versus immature cells) for each target gene were tested for statistical significance using one sample Student's t-test (p=0.05).

The results are shown in Table 1.

C-kit mRNA was not detected in either mature or immature myocytes (n=13, N=4), but was present in the wall of the small intestine (n=3, N=1), confirming the validity of the assay.

These data confirm common cell lineage for immature and mature vascular myocytes and identify subtle differences in gene expression pattern between them, which hint at different physiological roles of these two phenotypes.

Table 1. Relative differences in marker mRNA quantity between mature and immature myocytes (the statistically significant ones are shown in bold).

Gene	*Replicates (n)*	*Animals (N)*	*Fold Difference (±SEM)*	*Signif*
SM α-actin	10	3	2.68±1.08	p=0
β-actin	21	4	1.66±1.27	p=0
Desmin	6	2	2.48±1.65	p=0
Vimentin	9	3	1.07±0.45	p=0
SM-MHC SM1	10	3	8.85±8.36	p=0
***SM-MHC SM2**	10	3	>>33.36	
MLCK	11	3	0.37±0.10	p=0

Table 1. Relative differences in marker mRNA quantity between mature and immature myocytes (the statistically significant ones are shown in bold).

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PC6

Impaired antioxidant stress protein expression in fetal endothelial cells in pre-eclampsia: effects of oxygen tension

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Pre-eclampsia (PE) is a pregnancy-specific disease resulting in systemic maternal endothelial dysfunction, associated with placental hypoperfusion and increased generation of reactive oxygen species (ROS) and pro-inflammatory mediators (Noris, 2005). We previously identified phenotypic alterations in human umbilical vein endothelial cells (HUVEC) isolated from PE pregnancies (Steinert et al., 2002; Afzal-Ahmed et al., 2007). In the present study, we examined whether PE affects activation of the redox sensitive transcription factor Nrf2 and whether changes in oxygen tension (21% versus 5%) affect Nrf2 mediated HO-1 protein expression in normal and PE HUVEC.

Normal and PE HUVEC were cultured in M199 + 20% serum. Nrf2 nuclear accumulation was assessed in cells stimulated for 1-4 h with 20μM 4-hydroxynonenal (HNE, lipid peroxidation product raised in PE). Cells were also adapted to 5% or 21% O2, treated for 4-24h with 10-40μM HNE and HO-1 expression determined by immunoblotting. HNE increased nuclear accumulation of Nrf2 which were not altered significantly in PE cells. HNE increased HO-1 expression in normal HUVEC (>2-fold), which was significantly attenuated in PE cells. HNE induced HO-1 expression was less pronounced in normal or PE HUVEC adapted to 5% O2. Our findings suggest that fetal endothelial cells from PE pregnancies may be more susceptible to oxidative damage as a result of impaired antioxidant enzyme expression and/or activity. As alterations in antioxidant gene expression in PE HUVEC persist in culture, it seems likely that the fetal vasculature is affected by increased ROS production in PE, leading potentially to fetal programming of cardiovascular disease in adulthood (Vatten et al., 2003; Tenhola et al., 2006).

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PC7

From proliferative/migratory to contractile phenotype transition of vascular smooth muscle cells *in vitro* by heparin: role of the RhoGTPases

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In normal media of aorta, smooth muscle cells (SMC) display a contractile non-proliferative phenotype characterized by the expression of cytoskeletal marker proteins such as α-smooth muscle actin (α-SMA) and contractile activity in response to chemical and mechanical stimuli. During atherosclerosis or restenosis processes, SMC switch to a proliferative and migratory phenotype (synthetic phenotype) accompanied by a loss of α-SMA. In culture, SMC display a synthetic phenotype but are able to revert to a contractile behaviour upon addition of heparin. Heparin inhibits proliferation and migration of SMC and induces a contractile phenotype characterized by an overexpression of α-SMA. This in vitro study aims at investigating the role of the three homologs GTPases of the Rho family (RhoA, RhoB and RhoC) in the regulation by heparin of the expression of α-SMA and genes involved in the extracellular matrix remodeling such as matrix metalloproteinase 1 (MMP1).
Human aortic SMC were cultured in basal Smooth Muscle medium (bSMm) (Lonza) supplemented with 5% serum. The proliferative to contractile phenotype switch was induced by adding 0.04% heparin which resulted in a overexpression of α-SMA (x 2-4) and the formation of robust α-SMA positive stress fibers as seen by immunostaining. The involvement of key molecules in the cytoskeleton dynamics, the RhoGTPases RhoA, RhoB and RhoC was assessed in this process by using an inhibitor (Y27632) of their common effector, Rho kinase (ROCK) or by specifically silencing each RhoGTPase with siRNA as previously described (Deroanne et al. (2005); Ho et al. (2008)).
Addition of heparin to human aortic SMC strongly increased the basal expression of α-SMA only when serum was present in the medium. This increased expression does not depend on basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) or insuline. Y27632 significantly decreased the basal level of α-SMA without altering the enhancement factor induced by heparin (x 3,6). MMP1 mRNA level was concomitantly enhanced. Silencing RhoA had the same effect as Y27632 on α-SMA and MMP1 expression. RhoB silencing had no effect neither on the basal expression of α-SMA nor on its heparin-induced overexpression. Suppressing RhoC resulted in a significant enhancement of the basal α-SMA expression that was further increased by heparin. This transition towards a contractile phenotype was accompanied by a proportional reduction of MMP1 expression. As the silencing of RhoC induced a 2.5 fold increase in RhoA as previously described (Ho et al., (2008)), a double transfection with siRNA targeting RhoA and RhoC suppressed brought back α-SMA to its basal level. These results suggest that the basal level of α-SMA and MMP1 is under the control of RhoA, but not RhoB or RhoC.

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PC8

P2X-purinoceptor-mediated $[Ca^{2+}]_i$ mobilization in myocytes from rat preglomerular resistance arteries separated by means of iron oxide infusion

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Purinoceptors in the preglomerular renal resistance arteries regulate arterial diameter via P2X-purinoceptor (P2X-R) -mediated vasoconstriction and P2Y-purinoceptor -mediated vasodilatation [1] and, therefore, play an important role in the regu-

lation of renal blood flow and glomerular filtration rate. Selective P2X-R stimulation increases $[Ca^{2+}]_i$ in renal vascular smooth muscle cells (RVSMCs) freshly isolated from the preglomerular microvasculature [2] and triggers contraction of these myocytes. The aim of this work was to study at sub-cellular level the dynamics of $[Ca^{2+}]_i$ mobilisation and restoration following stimulation of P2X-R in RVSMCs. Experiments were performed on single RVSMCs freshly isolated from arcuate and interlobular arteries, and afferent arterioles, which were separated from rat kidney using an iron oxide sieving technique [3]. Changes of $[Ca^{2+}]_i$ in the cells loaded with the high affinity Ca2+ indicator Fluo-4 AM were visualised using fast (33-40 Hz) *x-y* confocal imaging. Data are presented as mean ± S.E.M. Gene expression was assessed with RT-PCR technique. At rest freshly isolated RVSMCs revealed spontaneous Ca^{2+} sparks and Ca^{2+} waves. Stimulation of P2X-Rs (ionotropic receptors [2]) with 10 μM α,β-methylene adenosine 5'-triphosphate (AMP-CPP) evoked a fast (mean time-to-peak 0.72±0.06 s; n=13) sub-plasmalemmal $[Ca^{2+}]_i$ upstroke (SPCU). This was two times faster than SPCU induced by stimulation of α_1-adrenoceptors (metabotropic receptors [2]). The SPCU was followed by a global rise of $[Ca^{2+}]_i$, which consisted of transient and sustained components [4], with the full duration at half-maximal amplitude of 4.08±0.30 s (n=19). The AMP-CPP-induced $[Ca^{2+}]_i$ mobilisation was closely followed (with a delay of 1.02±0.17 s; n=14) by a rise of mitochondrial $[Ca^{2+}]$ detected with Rhod-2. The relation of spatial patterns of AMP-CPP-induced $[Ca^{2+}]_i$ transients to the intracellular distribution of mitochondria and elements of sarcoplasmic reticulum (SR) suggests that: (1) spatial non-uniformities in SCPU are consistent with Ca^{2+} release from discrete sub-PM SR elements; (2) these SR elements are in close juxtaposition with mitochondria. RT-PCR analysis confirmed expression in single RVSMCs of genes encoding $P2X_1$-Rs, but not $P2X_3$-Rs or $P2X_5$-Rs. Our results demonstrated that activation of $P2X_1$-purinoceptors in RVSMCs evokes a significantly faster SPCU followed by a significantly shorter global $[Ca^{2+}]_i$ rise than in the case of α_1-adrenoceptor activation. It was also shown that these $[Ca^{2+}]_i$ transients are associated with mitochondrial Ca^{2+} uptake, thus suggesting a tight functional interaction between mitochondria and SR in RVSMCs. The mechanisms involved in genesis of P2X-R-induced SPCU are under current investigation.

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PC9

Intracellular calcium mobilisation and contraction induced by purinergic stimulation of vascular myocytes

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We tested an involvement of ryanodine receptors (RyRs), inositol 1,4,5-trisphosphate receptors ($IP3_3Rs$) and voltage-gated Ca^{2+} channels (VGCCs) in $[Ca^{2+}]_i$ mobilisation induced by stimulation of P_2X purinoceptors (P_2XRs) in smooth muscle cells (SMCs) from the guinea-pig small mesenteric arteries. Freshly isolated SMCs were loaded with the high affinity Ca^{2+} indicator fluo-3 and stimulated with 10 μM α,β-methyleneadenosine 5-triphosphate (α,β-meATP). Fast x-y confocal imaging revealed that activation of P_2XRs evoked an initial sub-plasmalemmal $[Ca^{2+}]_i$ upstroke (SPCU) at several restricted locations followed by propagating Ca^{2+} wave. The peak amplitude of α,β-meATP-induced SPCU was reduced: (1) by 37% (n=6) after inhibition of IP_3Rs (with 30 μM 2APB); (2) by 45% (n=6) after inhibition of RyRs (with 100 μM tetracaine) and (3) by 68% (n=6) after block of VGCCs (with 5 μM nicardipine). Although activation of P_2XRs does not engage metabotropic signalling pathway (involving activation of phospholipase C and intracellular mobilisation of IP_3), a contribution of IP_3Rs-mediated Ca^{2+} release to purinergic contractions was further demonstrated by isometric tension recording, in which smooth muscle rings from segments of second- or third-order mesenteric arteries were attached to isometric force transducer and stimulated with 10 μM α,β-meATP. Inhibition of either IP_3Rs or RyRs attenuated purinergic contraction. When VGCCs were inhibited with 5μM nicardipine, a relative contribution of RyRs-mediated Ca^{2+} release to purinergic contraction increased, thus suggesting that Ca^{2+} influx through VGCCs evokes predominantly IP_3Rs-mediated Ca^{2+} release. Immunostaining of RyRs and type 1 IP_3Rs in the isolated SMCs with identified SR and nucleus revealed that RyRs are predominantly located in the central/perinuclear SR elements, while sub-PM SR elements are enriched with IP_3Rs. These results suggest that not only Ca^{2+} entry through VGCCs but also RyRs- and IPP_3XRs-mediated Ca^{2+} release are involved in genesis of SPCU and contraction in response to P_2XR stimulation.

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PC10

A Mutation in the beta adducin subunit causes tissue-specific damage to myogenic tone

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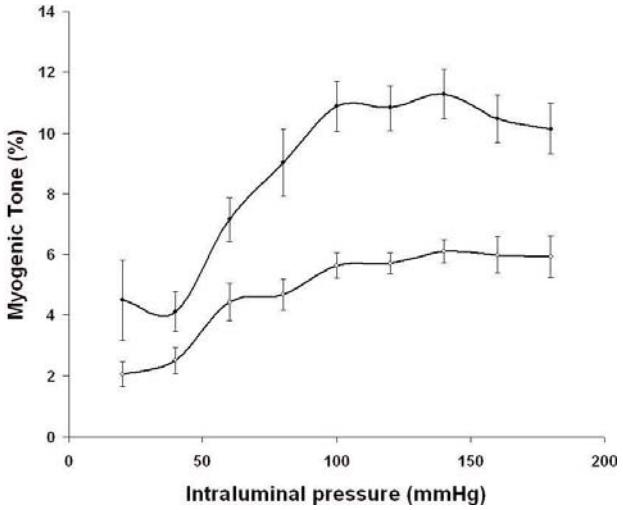
Introduction: Adducin is a heterodimeric protein composed of combinations of α, β and γ subunits. It is a prominent component of the cytoskeleton and contributes to a number of signal transduction pathways. Recently we have observed that rats with mutations in the β-subunit develop proteinuria. Conversely, rats with mutations in the α-subunit are protected from proteinuria despite a higher blood pressure. This study was designed to examine the effects of adducin subunit mutations on myogenic tone and to investigate how this may be related to the development of target organ damage.

Methods: Structure and function of isolated renal, middle cerebral and skeletal muscle arteries from congenic rats with mutations in α-subunits (NA rat) and in β-subunits (NB rat) were studied using pressure myography. Blood pressure was measured using a tail cuff method and urinary protein excretion was assayed. Experiments were performed before (6 weeks) and after (4 months) the development of renal disease.

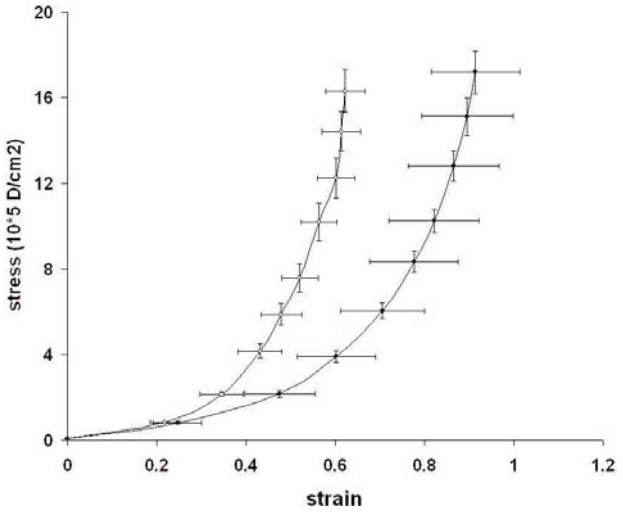
Results: NB rats developed lower systolic blood pressure, greater urinary protein loss and showed profound damage to myogenic tone in renal arteries at both ages studied. The reduction in tone was associated with an increase in arterial stiffness at 4 months (n=9, P<0.05). Myogenic tone in the middle cerebral arteries was also damaged at 4 months, but in skeletal muscle arteries was normal. In NA rats there was a reduction in myogenic tone in renal arteries from 6 weeks to 4 months of age and this was associated with a rise in urinary protein loss. Both the reduction in tone and increase in proteinuria were significantly less than that seen in the NB rat (Proteinuria in NB rat: 360 $\pm$25mg/day vs NA rat: 144$\pm$11mg/day, p<0.05, n=5). There were no differences in contraction to vasoconstrictors, responses of preconstricted arteries to acetylcholine or arterial structure between the strains.

Conclusion: While the α-adducin subunit mutation contributes to elevated blood pressure, the β-subunit mutation is more important for the generation of myogenic tone in renal and middle cerebral arteries. Whether the decline in tone in the NA arteries is due to the adducin α-subunit mutation or the rise in blood pressure is unclear. While other studies have shown that loss of myogenic tone in renal arteries is associated with proteinuria, this is the first study to demonstrate this pathology where the genetic defect is known.



Properties of renal arteries from NAand NB adducin rats

A. Myogenic tone is reduced in the renal arteries of 6 week old NB rats compared to NA (filled circles = NA rats n=9, open cicles=NB rats n=9, *=P<0.05)



B. Renal arteries from NB rats exhibited increased stiffness compared to NA rats at 4 months (n=9, P<0.05)

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PC11

Alpha-adrenoceptor activation and myogenic responsiveness in rat mesenteric arteries: possible role of TRPC channels

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The myogenic response is an important contributor to the vascular tone in resistance vessels. Though weak, it can be evoked in rat mesenteric arteries in the presence of noradrenalin (NA)[1]. This led to speculation that receptor and/or store-operated cation channels are involved. Canonical Transient Receptor Potential (TRPC) channels are linked to G-protein activation and are candidates for receptor operated Ca2+ permeable cation channels in vascular smooth muscle cells (VSMCs). TRPC1 and TRPC6 channels are expressed and functional in mesenteric artery VSMCs[2,3]. We hypothesized that upon alpha1-adrenoceptor activation, TRPC6 and/or TRPC1 channels are enabled, increasing sensitivity to heightened transmural pressure and leading to myogenic constriction.

Male Wistar rats were ethically euthanized. 2nd or 3rd order branches of the superior mesenteric artery were isolated and mounted in a pressure myograph at a physiological pH (7.35-7.45; 5% CO_2 / 25 mM HCO_3^-) and temperature. Vessel diameters were measured using Myoview software (Danish Myotechnology). At 60 mm Hg and in the presence of 2 nM neuropeptide Y, increasing concentrations of NA were added to the bath to achieve a stable vascular tone (8 - 12% under relaxed levels). A myogenic response was then evoked by increasing transmural pressure to 100 mm Hg. This was repeated in the presence of a TRPC channel inhibitor. Passive diameters were measured in a Ca^{2+} free solution for calculation of vascular tone (%).

The overall effect of pressure increase in the presence of NA was an increase in the amount of vascular tone from 12.3 ± 0.7 (60 mmHg) to 17.6 ± 0.8 % (100 mmHg, P<0.001; N=46). Gd3+ (2 μM), an inhibitor of non-selective cation channels, did not substantially affect 75 mM-KCl induced vasoconstriction (7.6 ± 9.0 % inhibition; N=9), but abolished the pressure-induced increase in vascular tone: 12.7 ± 2.4 to 18.5 ± 2.7 % (control) vs. 8.9 ± 3.1 to 7.8 ± 3.3% (Gd3+) (P<0.05 vs. ctrl. at 100mm Hg; N=6). The non-specific TRPC channel blocker SKF96365 (5 μM) did not substantially affect KCl induced vasoconstriction (1.9 ± 1.4 % inhibition; N=5), but inhibited the myogenic response: 13.5 ± 1.6 to 21.6 ± 2.2 % tone (control) vs. 12.9 ± 1.5 to 14.9 ± 3.0 % tone (SKF96365) (P<0.001 vs. ctrl. at 100mm Hg; N=7). P values were calculated with paired t tests.

Preliminary results using TRPC1 antibody (T1E3[4], 1:250) show no significant blocking effect on the myogenic response in arteries incubated overnight with antibody (9.6 ± 0.8 to 16.2 ± 1.8% tone; N=4) compared to arteries incubated with inactivated (boiled) antibody (8.7 ± 0.7 to 16.4 ± 1.6 % tone; N=4).

These results suggest that TRPC6 channels are involved in evoking myogenic responsiveness in rat mesenteric artery. Future experiments, possibly utilizing siRNA mediated TRPC6 knockdown, could elucidate this role of TRPC6 channels.

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PC12

Ca^{2+}-activated conductances modulate endothelin-induced Ca^{2+} signals in rat retinal arteriolar myocytes

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Electrophysiological recordings in retinal arterioles have demonstrated that application of endothelin promotes Ca^{2+}-sensitive, spontaneous transient inward and outward currents (Scholfield *et al*, 2007). The present study provides evidence that these currents act as important feedback mechanisms, modulating endothelin induced Ca^{2+}-signals. Retinal arterioles mechanically isolated from male Sprague-Dawley rats were loaded with fluorescent Ca^{2+}-indicator by incubation with fluo-4 AM (5 μM). Loaded vessels were placed in an organ bath on an inverted microscope and superfused with physiological solutions at 37ºC. High-speed confocal imaging (20 fps) was used to record cellular Ca^{2+}-signals from arteriolar myocytes. Normalized fluorescence (F/F_0) was calculated as a measure of [Ca^{2+}]. Ca^{2+}-oscillations were stimulated when endothelin (Et1, 10nM) was added. Oscillation frequency was increased from 0.123±0.018 s^{-1} (mean±SEM) under control conditions, to 0.340±0.021 s^{-1} in the presence of Et1 (N=89 cells in 4 vessels from 4 animals; P<0.001, non-parametric ANOVA, Dunn's multiple comparison post-hoc test). Oscillation amplitude ($\Delta F/F_0$) was also increased from an average of 0.28±0.02 to 0.57±0.03 for control and Et1 treatment periods, respectively (P<0.001). We have previously shown that 4,4'- diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) inhibits a Ca^{2+}-activated Cl^--conductance in these cells (McGahon *et al*, 2008). Addition of DIDS (1mM) in the continued presence of Et1 reduced the mean frequency of Ca^{2+}-oscillations back to control values (0.062±0.001 s^{-1}; P<0.001 vs Et1 alone; NS vs Control). DIDS also reduced the mean amplitude of the Ca^{2+}-oscillations to 0.40±0.04, but this was not statistically significantly different from the amplitude in the presence of ET1 alone. Iberiotoxin (100 nM), an inhibitor of large-conductance Ca^{2+}-activated K^+-channels, exaggerated the effects of Et1. Oscillation amplitude was increased from 0.24±0.01 in the presence of Et1 alone, to

0.33±0.02 when iberiotoxin was also added (N=51 cells in 4 vessels from 4 animals; P<0.001). Iberiotoxin, however, had no additional effect on oscillation frequency. It appears, therefore, that Ca^{2+}-activated Cl^- currents play an important role in determining the frequency of the Ca^{2+}-oscillations elicited by endothelin, while Ca^{2+}-activated K^+-currents limit the amplitude of these oscillations.

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The Wellcome Trust, JDRF

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PC13

The vasorelaxant effect of *Viscum album* leaf extract is mediated by calcium-dependent mechanisms

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Viscum album leaf extract (VA) has a folk reputation as an anti-hypertensive agent in Nigeria. Evidence suggests that it has a vasorelaxant effect that is endothelium dependent involving the release of nitric oxide (Ekpenyong et al, 1999). However, VA showed a relaxant effect in endothelium-denuded preparations (Ekpenyong et al, 1999) suggesting that it might also act on the vascular smooth muscle via calcium-dependent mechanisms. The present study was designed to investigate this possibility.

Fresh leaves of *Viscum album* were harvested and ground into powder. An aqueous extract was prepared and phytochemical analyses done. Sprague-Dawley rats (n=6) were anaesthetized using a 25% urethane and 1% chloralose mixture given intraperitoneally at a dose of 5ml/kg. Thoracic aortae were obtained and cut into 2 mm ring segments in Physiological salt solution (PSS; composition ($mmol.l^{-1}$): NaCl, 119; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 14.9; $CaCl_2$, 1.6; glucose, 11.5). Each aortic ring was mounted in a tissue bath containing PSS and continuously bubbled with a 95% O_2-5% CO_2 gas mixture at a temperature and pH of 37°C and 7.4 respectively (Adegunloye and Sofola, 1997). Relaxation response studies to VA (2-16 $mg.ml^{-1}$) were done following pre contraction with 10^{-7} $mol.l^{-1}$ noradrenalin or 60$mmol.l^{-1}$ KCl. The IC_{50} of VA in noradrenalin pre-contracted rings was 5.2 ± 0.79$mg.ml^{-1}$ while the IC_{25} in KCl pre-contracted rings was 7.1±0.56 $mg.ml^{-1}$. These concentrations of V. album were used in subsequent experiments. Concentration response curves (CRCs) to noradrenalin (10^{-9} to $10^{-5}mol.l^{-1}$), KCl (10-80$mmol.l^{-1}$) and $CaCl_2$ ((2.5×10^{-4} to 1.6×10^{-1} $mol.l^{-1}$) were constructed with and without VA. Phasic contraction to 10^{-5} $mol.l^{-1}$ noradrenalin was carried out in Ca-free EGTA (N′N′-ethylene glycol tetra acetic acid) PSS (Perry & Webb, 1991). The results are presented as mean ± SEM. The student's t test for paired data was used for statistical analysis. P<0.05 was taken as statistically significant. Phytochemical analyses showed the presence of flavonoids and tannins. The CRCs to noradrenalin or KCl were significantly (P<0.05) attenuated and shifted to the right in the presence of the VA. Also the CRC to $CaCl_2$ in the presence of noradrenalin or KCl was attenuated and shifted to the right by VA, while the phasic response to noradrenalin was diminished (P<0.05).

These results suggest that the vasorelaxant effect of VA may be mediated by a non-specific non-competitive inhibition of Ca^{2+} influx as well as inhibition of Ca^{2+} mobilization from intracellular stores. These calcium antagonistic effects may be due its flavonoid or tannin content.

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PC14

A novel mechanism for vasorelaxation by S-nitrosothiol-induced activation of PKG1a and type I PKA

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In previous studies we demonstrated a novel redox mechanism for directly activating PKG1a and PKAI by H2O2 mediated interprotein disulphide formation. These are examples of how oxidant signalling can directly integrate into phosphoregulation and alter vasotone. We hypothesised that nitric oxide (NO) species may also induce disulphide formation and hence kinase activation through formation of S-nitrosothiol intermediates. The nitric oxide donors SNAP and CysNO were assessed for their ability to generate protein S-nitrosylation in the isolated perfused rat heart using the biotin-switch method. Isolated rat heart perfusion at constant flow with the nitric oxide donor SNAP (100μM-1mM) did not alter protein S-nitrosylation, whereas CysNO (5-20μM) increased this protein modification in a dose-dependent manner. In addition to S-nitrosylation, CysNO also caused a dose-dependent increase in PKG1a and PKA-RI disulphide dimerisation, which was absent in SNAP perfused hearts. However, treatment of heart homogenate in vitro with SNAP induced an increase in both protein S-nitrosylation and PKG1a disulphide dimerisation, indicating that S-transnitrosylation is crucial for increased disulphide formation. When purified recombinant N-terminal PKG1a was exposed to CysNO, it induced intermolecular disul-

phide formation. This indicates that PKG1a disulphide occurs via formation of an S-nitrosothiol intermediate. It was demonstrated using studies measuring tension in isolated aorta that both NO donors are able to induce dose-dependent vessel relaxation. However, only SNAP mediated relaxation was blocked by the guanylate cyclase inhibitor ODQ, indicating that CysNO induces cGMP independent relaxation. Using PKG and PKA inhibitors in combination with ODQ is was shown that the cGMP independent relaxation generated by CysNO was dependent on PKG and PKA activation. This is consistent with CysNO directly activating PKG and PKA through the formation of a interprotein disulphide bond. These studies highlight the potential for beta-adrenergic like signalling, independently of cAMP elevation, by increased S-nitrosylation of PKA-RI.

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PC15

Endogenous superoxide dismutase (SOD) plays a greater role in hypoxia-induced muscle vasodilatation in rats exposed to 12% O_2 for 1-7 days than in normoxic rats

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The hindlimb vasodilatation evoked by acute systemic hypoxia is ~50% mediated by adenosine and 90% nitric oxide-dependent[1]. In normoxic (N) rats we showed that during acute hypoxia, xanthine oxidase (XO) metabolises adenosine to generate O_2^-, which is converted to H_2O_2 by endogenous SOD and contributes to the hindlimb vasodilatation[2]. Chronic hypoxia is likely to increase O_2^- release via XO and the mitochondria. Thus, we have now investigated in chronically hypoxic rats, housed in 12% O_2 for 1, 3 and 7 days (1, 3 & 7CH), the contribution of H_2O_2 generated by endogenous SOD in hindlimb.

Male Wistar 1, 3 and 7CH (n=9, 10 & 10) rats were anaesthetised with Alfaxan (12 mg.kg^{-1}.hr^{-1} i.v.) and routinely breathed 12% O_2. Arterial blood pressure (ABP) and femoral blood flow (FBF) were recorded and FVC was computed online (FBF/ABP) before and during a 5 min period of breathing 8% O_2 (acute hypoxia) before and during infusion of the cell permeant SOD inhibitor sodium diethyldithiocarbamate trihydrate (DETC; 5 mg.kg^{-1}.min^{-1} i.a.). Responses before & after DETC were compared with Student's paired t-test, & between groups by factorial ANOVA with Scheffe's post hoc test; *: P<0.05.

In 1, 3 and 7CH rats baseline P_aO_2 was lower than in N rats (47±1*, 45±2*, 46±2* vs 88±2 mmHg; mean±SEM), while baseline FVC was increased (0.011±0.001*, 0.010±0.001*, 0.010±0.001* vs 0.006±0.001 ml.min^{-1}.mmHg^{-1}), indicating a tonic hindlimb vasodilatation in 1-7CH rats. Breathing 8% O_2 caused a further decrease in P_aO_2 (33±1*, 30±1*, 31±1*), and increase in FVC (to 0.017±0.002*, 0.016±0.002*, 0.017±0.002*) in 1, 3 & 7CH rats to the same levels seen in N rats (28±2 mmHg; 0.016±0.001 ml.min^{-1}.mmHg^{-1}). During DETC infusion, baseline FVC was significantly reduced in 3CH, but not in 1 & 7CH rats. Further, acute hypoxia still caused a significant increase in FVC in 1, 3 & 7CH rats, but changes were smaller than before DETC (0.008±0.002 vs 0.003±0.001*, 0.007±0.002 vs 0.003±0.001*, 0.008±0.002 vs 0.003±0.001*). In N rats, the change in FVC tended to be reduced (0.009±0.001 vs 0.007±0.001 ml.min^{-1}.mmHg^{-1}).

Thus the conversion of O_2^- to H_2O_2 by endogenous SOD apparently contributes to tonic muscle vasodilatation in 3CH rats and plays a greater role in the muscle vasodilatation evoked by acute hypoxia in 1, 3 and 7CH than N rats. We propose that during early chronic hypoxia the generation of O_2^- via adenosine and XO or by mitochondria is increased, and/or SOD activity is increased so enhancing the vasodilator role of H_2O_2.

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PC16

Venular and arteriolar EDHF-mediated dilatation in cremaster muscle of the rat is sensitive to ROS scavenging

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Reactive oxygen species (ROS) are thought to play a role in EDHF, but endothelial sKCa and IKCa channels opening in response to increased endothelial [Ca2+]i, is also known to be a requirement. We have examined the vasodilatation response to carbachol in the isolated perfused cremaster muscle preparation.

The iliac artery of a freshly killed (Schedule 1) Wistar rat was cannulated orthogradely and branches that did not lead to the cremaster muscle were ligated. The cremaster microcirculation was flushed with St. Thomas' cardioplegic solution {in mmol.l-1: NaCl (110); KCl (7.9); MgCl2.6H20 (34); CaCl2 (1); and Hepes (11)} containing heparin (300 IU.ml-1). The skin over the scrotum was removed to reveal the cremasteric sack. The cremaster was cut to isolate it from the enclosed testes, care being taken not to damage any major vessel. The muscle was spread and pinned over a transparent Sylgard (Dow Corning) support and superfused with a Krebs-bicarbonate buffer solution maintained at 37□C at 1-2 ml.min-1. The perfusate was changed to a buffer contain-

ing albumin (10 mg.ml-1) with added FITC-albumin (5 mg.ml-1).
The cremaster muscle arterioles and venules (pre-phenylephrine diameter 47 ± 4.0 μm (MEAN ± SEM; n = 15 and 63.2 ± 6.7; n = 12, respectively) dose-dependently dilated to carbachol (artery 68 ± 0.6%, vein 84 ± 2.9%; p < 0.001 paired 't' test for both) which was significantly reduced by L-NAME and indomethacin (artery 48 ± 1.3, vein 70 ± 1.3%). The effect of these inhibitors in reducing the carbachol-induced dilatation was significantly greater in arterioles than in the paired venules (p < 0.05, paired 't' test), which may be accounted for by the differences in the smooth muscle layer thickness. The dilatation was virtually blocked by apamin and charybdotoxin (artery 6 ± 2.0%, vein 12 ± 1.5%, n = 4) and very much reduced by scavenging ROS with SOD and catalase (artery 13 ± 2.9%, vein 15 ± 3.9%, all n = 4).

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PC17

Human vascular endothelial cells show a time-dependent response to the stress of hypoglycaemia following their isolation and culture

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Inconsistent data regarding the ability of primary tissue to detect physiological falls in glucose concentration may be due, in part, to the time-dependent ability of a cell in culture to gain energy via oxidative phosphorylation, making it more sensitive to limitations in glucose supply. Thus, hypoglycaemia may induce a non-specific stress response in these cells that could be misinterpreted as low glucose sensing.
Human umbilical vascular endothelial cells (HUVEC) were enzymically and mechanically isolated from umbilical cords within 12 hours of delivery and distributed at high density onto 1% gelatin coated cover slips (Marin et al, 2001). Cells were cultured in Lonza Medium 199 containing foetal calf serum supplemented with penicillin, streptomycin and fungizone at 37□C in a 5% CO2 incubator, changing medium at every 3 days. Intracellular calcium concentration was measured by ratiometric (340/380nm) labelling with Fura 2 and the response to reducing the superfusate glucose concentration from 10mM to 0mM determined at 1, 2, 4 and 8 days after culture and compared to the response to 10uM ATP at the same time periods. Five cultures, each from 2 umbilical cords, were established and measurements made on 4 cover slips on each day of study. Time dependent differences in calcium response were analyzed by single factor ANOVA. Sheffe post hoc tests were performed as appropriate and significance was taken as P<0.05. Local ethical approval and informed consent were obtained.
A significant time dependent effect was observed in response to both falls in glucose and addition of ATP (P<0.001 and P<0.002 respectively) but the pattern of response differed (P<0.0001, two way ANOVA). The calcium response to zero glucose or ATP at day 1 was low (ratiometric mean±SEM: 0.005±0.003 and 0.004±0.004 respectively). At day 2 the response to zero glucose was significantly increased by a factor of 7.4 (0.037±0.008) but although the mean response to ATP was also increased by around the same amount, to 0.033±0.020, this did not reach significance. At day 4 the response to zero glucose was sustained (0.022±0.008) while the response to ATP (0.238±0.083) was substantially and significantly increased by almost 60-fold compared to day one. On the eighth day of culture, the response to glucose dropped to a level statistically similar to that of day 1 (0.004±0.002) while the ATP response was sustained at a level not significantly different to that on day 4 (0.161±0.035).
Our data shows that cultured HUVEC show a time-dependent response to the stress of hypoglycaemia following isolation and culture that differs in time course and magnitude from the response to ATP. What the mechanism involved is and whether it underlies the variability of the response to hypoglycaemia reported previously in the literature (Kumar, 2007) is not known.

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PC18

Does nicotinic acid adenine dinucleotide phosphate elicit Ca^{2+} release via two-pore channel subtype 2 in rat arterial smooth muscle cells?

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca^{2+} mobilising messenger in mammalian and non-mammalian cells. Studies on a variety of cell types suggest that NAADP evokes Ca^{2+} release from a lysosome-related store and via activation of a receptor distinct from either ryanodine receptors or inositol 1,4,5-trisphosphate receptors (IP_3R; 1,2). Consistent with this view, studies on pulmonary arterial smooth muscle cells (PASMC) have shown that NAADP elicits Ca^{2+} signals by mobilizing lysosome-related Ca^{2+} stores (3). Recent investigations have suggested that the two-pore channel subtype 2 (TPC2) acts as an NAADP receptor in HEK293 cells that stably over-express human TPC2 (hTPC2; 4). We therefore investigated the possible role of TPC2 in NAADP-mediated Ca^{2+} release in PASMC.

All experiments were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. PASMCs were isolated from male Wistar rats (150-300 g) that were humanely killed. Ca^{2+} imaging using Fura-2 fluorescence, and fluorescent imaging and analysis of subcellular labelling was carried out following previously described protocols (3).
RT-PCR analysis showed that TPC2 is expressed in PASMCs. Furthermore, in HEK293 cells that stably over-express hTPC2 and in acutely isolated rat PASMC deconvolution microscopy demonstrated that fluorescent labelling of TPC2 colocalised with LysoTracker Red labelled lysosomes (n = 3). HEK293 cells over-expressing hTPC2 exhibited a marked increase in the Fura-2 fluorescence ratio (F_{340}/F_{380}) from 0.75 ± 0.03 to 1.67 ± 0.15 (mean ± S.E.M., n = 44) in response to intracellular dialysis of NAADP (10 nM) from a patch pipette. Similarly, under control conditions, 10 nM NAADP elicited a global Ca^{2+} wave in PASMC increasing the F_{340}/F_{380} ratio from 0.64 ± 0.04 to 1.81 ± 0.13 (n = 22). Consistent with the notion that NAADP mobilises Ca^{2+} from a lysosome-related acidic store, the response to 10 nM NAADP was abolished by disruption of the lysosomal proton gradient using bafilomycin A1 (100 nM) in both PASMC (n = 7) and hTPC2 over-expressing HEK293 cells (n = 11). Furthermore, a specific NAADP antagonist, Ned-14 (100 μM) abolished NAADP-mediated Ca^{2+} release in both hTPC2 over-expressing HEK293 cells (n = 5) and PASMCs (n = 4). These data demonstrate that TPC2 exhibits analogous functional characteristics in both PASMCs and hTPC2 over-expressing HEK293 cells. These findings provide further support for the view that TPC2 is the molecular target of NAADP in both cell types.

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PC19

Ultrasound induced contraction of equine and bovine common carotid artery *in vitro*

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Ultrasound is known to produce a range of non-lethal responses in cells and tissues. Frequencies in the kilohertz ultrasound range have been shown to produce relaxation in large arteries (Fischell et al., 1991; Steffen et al., 1994). The present work explores the effects of insonation at MHz frequencies, representative of those used diagnostically and therapeutically, in an in vitro preparation of the carotid artery.
Fresh 1cm wide rings of equine and bovine common carotid artery obtained from the abattoir were mounted in a purpose-made myograph. They were immersed in a bath of Krebs-Ringer buffer at 37°C and were positioned at the focus of an ultrasound beam from a weakly focused 3.2 MHz source. Continuous wave insonation produced a contraction, the tension increase over the first 2 minutes was rapid; this was followed by a slower increase for the duration of the exposure up to 15 minutes. At a power of 145 mW contractile forces of 1.1 ± 0.09 mN (mean ± sd, n = 100) were measured, this was approximately 10% of the maximum force generated by noradrenaline (1mM). The magnitude of the response was weakly dependent on power in the range 72-145 mW and was not significantly different for pulsed and continuous wave stimulation where time averaged power was constant. The effect was unaffected by mechanical removal of the endothelium. The ultrasound beam generated insufficient acoustic pressure to produce a measurable effect and streaming at the vessel surface was very small compared to flow rates known to produce physiological effects. The temperature rise at the beam focus was approximately 0.3°C and we hypothesise that this contributes to the observed response, probably through changes in ion channel activity in smooth muscle cell membranes (Mustafa and Thulesius, 2005).

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PC20

Effects of endothelium activation on agonist-induced Ca^{2+} signalling in smooth muscle cells of mesenteric arteries and precapillary arterioles

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Endothelium derived relaxing factors (NO and/or EDHF) produce vasorelaxation: which could involve interaction between Ca signalling in endothelial cells and vascular smooth muscle cells (SMC). However, the mechanisms of reduction of $[Ca^{2+}]_i$ in SMC by NO and EDHF are still poorly understood. We have investigated possible roles of both NO-dependent and independent mechanisms in control of agonist induced Ca signalling in SMC of precapillary arterioles (PA) and small resistance arteries. A minimum of 17 vessels from 15 humanely killed rats were

studied using confocal imaging of Fluo-4 loaded ureteric microvessels and mesentery arteries (MA) *in situ*. In the MA phenylephrine (PhE) produced complex Ca signalling consisting of asynchronous Ca waves and synchronous Ca oscillations. Both carbachol (CCh 10μM) and NO donors: SNP (20μM) and SNAP (20μM) inhibited this complex Ca signalling in pre-stimulated MA. Nifedipine (10μM) selectively blocked synchronous Ca oscillations and vasomotion but had no effect on asynchronous Ca waves. Nifedipine- resistant asynchronous Ca waves were abolished by ryanodine (10μM) or 2-APB (50μM). Carbachol and NO donors reversibly blocked PhE - induced Ca oscillations in the presence of nifedipine. L-NAME (100μM) selectively abolished the inhibitory effects of CCh on PhE induced Ca transients in the absence and the presence of nifedipine but not those induced by NO donors suggesting that NO plays a key role in inhibition of PhE induced Ca signalling in SMC under normal physiological conditions. Ryanodine selectively blocked asynchronous Ca waves in MA but enabled SMC to generate fast propagating Ca spikes (flashes) followed by sustained rise of Ca. Both the Ca spikes and sustained rise in Ca were blocked by nifedipine. Carbachol but not NO donors reversibly inhibited both fast and slow components of PhE induced Ca transients in the presence of ryanodine suggesting a little role of NO in these effects.

In contrast to MA, in arterioles PhE induced exclusively Ca oscillations which were insensitive to ryanodine, nifedipine or removal of extracellular Ca but were inhibited by 2-APB. Neither CCh nor NO donors had any effects on PhE induced IP_3R mediated Ca oscillations in PA. Pretreatment of PA with TEA and BayK-8644 enabled them to respond to PhE with synchronous Ca spikes which were blocked by nifedipine but not 2-APB. These Ca spikes were reversibly blocked by CCh but not NO donors suggesting that NO-independent pathway is present in PA and can be physiologically active when the mechanisms of Ca entry via L-type Ca channels are activated. These data indicate marked differences in the mechanisms controlling Ca signalling evoked by agonists in SMC between macro- and microvessels and their modulation by endothelium derived relaxing factors.

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PC21

Acute simvastatin decreases contraction in mesenteric resistance arteries through a nitric oxide (NO)-mediated effect

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Introduction and objective: 3-Hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors, statins, provide beneficial acute effects independent of their lipid-lowering actions. One such effect is cardioprotection. However, the acute effects of statins on resistance arterial function are not completely understood. In the present study, we examined if acute simvastatin incubation modulated rat mesenteric resistance artery (MRA) function.

Methods: Male Wistar rats (3-4months) were killed by cervical dislocation. Third or fourth order MRA (2–4 mm in length) were dissected out and mounted on a wire myograph. Tissues were bathed in physiological salt solution (PSS) at 37ºC aerated with 20% O2/ 5% CO2, normalized and equilibrated for 30 minutes. Functional integrity was assessed by 1) contraction to 120 mM KPSS and 2) relaxation to acetylcholine (ACh, 10 μM) in U46619 (1 μM) pre-contracted rings. To examine the acute effects of simvastatin, MRA were then incubated with or without Simvastatin (0.1 or 1 μM) for 1 or 2hs and concentration-response curves to U46619 (10-10 - 10-6 M), ACh (10-9 – 10-5 M) and sodium nitropusside (SNP, 10-9 – 10-4 M) constructed. To evaluate the role of NO on the effects of simvastatin (1 μM, 2hs), experiments were repeated in MRA incubated with L-NNA (10 μM) (NO synthase inhibitor). Results were analysed using one- or two-way ANOVA using Bonferroni's post hoc test. Differences were considered statistically significant at $P<0.05$.

Results: Incubation with 0.1 μM simvastatin for 2hs had no significant effect on either maximal response (Control: 104±5 (N=12) vs. Simvastatin 0.1 μM: 112±7 % of KPSS (N=6) or the sensitivity to U46619 (Control: 6.78±0.09 vs. Simvastatin 0.1 μM; 6.80±0.15). However 1 μM simvastatin for 2hs significantly reduced the maximal response (52±13 % of KPSS-induced contraction (N=10); $P<0.05$) but did not change the sensitivity to U46619 (6.70±0.04). Simvastatin had no effect on responses to ACh or SNP at any concentration (ACh; Emax, Control: 89±3 vs. Simvastatin 0.1 μM: 92±3 vs. Simvastatin 1 μM: 79±5 % of relaxation and SNP; Emax, Control: 85±4 vs. Simvastatin 0.1 μM: 88±3 vs. Sinvastatin 1 μM: 83±4 % of relaxation). Similar effects were observed after 1 h incubation. L-NNA incubation reversed the effect of 1 μM simvastatin on the U46619-induced contraction of MRA (Emax, Control + L-NNA: 107±6 vs. Sinvastatin 1 μM + L-NNA: 100±12 % of KPSS-induced contraction).

Conclusion: Acute simvastatin treatment reduces the contraction of rat MRA to U46619 by a NO-dependent mechanism. This could explain the beneficial effect of acute statin on cardioprotection.

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PC22

PDGF regulation of REST mRNA in vascular smooth muscle

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The Repressor Element 1-Silencing Transcription factor (REST) is a negative regulator of genes implicated in variety of diseases including human neointimal hyperplasia (Ooi and Wood, 2007). We have previously reported its role in controlling pro-proliferative genes in vascular smooth muscle and downregulation of REST expression is important for smooth muscle proliferation (Cheong et al, 2005). However the mechanisms regulating REST levels remain unknown.

Human saphenous vein smooth muscle cells (VSMC) were grown from discarded human saphenous veins obtained at coronary artery bypass graft surgery with ethical consent. Data are presented as means ± sem and were statistically analysed by 1-way ANOVA. Statistical significance was defined as a value of $P<0.05$.

We observe that PDGF, a growth factor associated with atherosclerosis and restenosis, significantly inhibited the REST promoter-driven luciferase activity in VSMC by 43.2±11.1% (n=6). Using quantitative RT-PCR, we show that PDGF treatment resulted in a reduction of REST mRNA expression 34.1±7% (n=20-26). Activation of the PDGF receptor initiates MAPK/MEK, PKC, PI3K and calcium-calmodulin signalling (Hughes et al, 1996). Pretreatment with W-7 (a calmodulin antagonist) significantly prevented REST mRNA downregulation by PDGF, suggesting a role for the calcium-calmodulin pathway. Preincubation with KN-62 (a calcium-calmodulin dependent kinase II (CAMKII) inhibitor) attenuated the effect of PDGF on REST mRNA expression. Likewise, KN-93 (another CAMKII inhibitor) but not its control analog KN-92 was effective in preventing REST mRNA downregulation. Pretreatment of VSMC with siRNA against the δ-isoform of CAMKII also significantly prevented PDGF-induced REST mRNA downregulation. Neither PD98059 (a MEK-1 specific inhibitor), calphostin (a PKC specific inhibitor) nor wortmannin (a PI3K specific inhibitor) had an effect on PDGF-induced REST downregulation.

We here identify a PDGF-calmodulin dependent kinase pathway that regulates REST mRNA levels in vascular smooth muscle cells. This is the first pathway to be identified that impacts upon REST expression and marks an important step in understanding the molecular mechanisms controlling vascular smooth muscle proliferation.

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PC24

Apelin and coronary vascular resistance in post-ischaemic rat heart

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The highly basic peptide adipocytokine apelin-13 is the ligand for the G protein-coupled orphan receptor APJ (Tatemoto et al., 1998). The Apelin/APJ system is espressed in almost all tissues. On cardiovascular apparatus, apelin produces inotropic effects involving Na^+/H^+ and Na^+/Ca^{2+} exchangers (Szokody et al., 2002) and vasodilatatory effects involving nitric oxide (NO) (Tatemoto et al., 2001). Simpkin et al. (2007) observed that in isolated rat hearts the addition of apelin during reperfusion attenuates ischaemia-reperfusion (I/R) injury. Since global I/R induces myocardial contracture, we wanted to see whether Apelin-induced vasodilation and myocardial protection can counteract the effect of contracture on coronary vascular resistance (CVR).

Method. Thirty eight anaesthetized rats were killed by decapitation. The hearts were excised and perfused at constant flow with oxygenated Krebs–Henseleit buffer. Coronary perfusion and left ventricular pressure (LVP) were recorded. After stabilization, the hearts underwent 30 min of global ischaemia and 20 min of reperfusion. Apelin-13 was given at 0,5 mM concentration.

Five groups of hearts were performed. In Group I (n = 7; control) the hearts underwent I/R only. In Group II (n=7) they received apelin before I/R for 20min. The other groups received apelin during reperfusion for the same duration. While Group III (n=6) received only apelin, the NOS inhibitor L-NNA and the guanylate-cyclase (GC) inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one (ODQ) were added in Group IV (n=8) and V (n=6) respectively.

Results. Data are give as means ± SEM. In Group I a 26±11% increase of CVR occurred in reperfusion. Apelin before ischaemia (Group II) caused a 23±7% increase of CVR during stabilization and 80±18% at the end of reperfusion. When apelin was given during reperfusion (Group III) CVR was similar as in Group I (34±7%) and did not change if NO-synthase and guanylil-cyclase were blocked by L-NNA (Group IV, 45±20%) and ODQ (Group V, 42±16%) respectively. During reperfusion end-diastolic LVP was increased 20±9 folds in Group I, 14±4 in Group II and 22±10 in Group IV. It is likely that apelin before ischaemia induces a vasoconstriction by the activation by Na^+/H^+ and Na^+/Ca^{2+} exchangers not only in myocardium but also in coronary smooth muscle cells. During reperfusion myocardial contracture contributed to the further increase of CVR. Given after ischaemia apelin produced a vasodilatation that exceeded the effect of contracture. The absence of any effect of NO blockade suggests that apelin-induced vasodilatation may be mediated not only

by NO but also by some other factors (e.g. hyperpolarizing factor).

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PC25

Cardiac protein thiol modification by 15-deoxy-delta 12, 14 prostaglandin J2

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Cellular redox signalling is in part mediated by post translational modification of proteins by reactive oxygen species, nitrogen species or by products of their reactions. Enhanced lipid peroxidation has traditionally been causatively associated with many diseases; although important roles in redox signalling are now being recognised. Many lipid peroxidation products are reactive and are capable of reacting with and modifying protein thiols. Cyclopentenone prostaglandins (CyPGs) are model examples of reactive oxidised lipids, containing electrophilic carbon centres that allow covalent adduction with target proteins. To determine if lipid-protein adducts form in a cellular setting, a biotinylated derivative of 15-deoxy-delta 12, 14 prostaglandin J2 (15d-PGJ2) (an electrophilic lipid) was used to treat adult rat ventricular myocytes. There was a dose- and time-dependent increase in biotin-15d-PGJ2 labelling which was maximal at 50μM 15d-PGJ2 and after 120 minutes. 15d-PGJ2 treatment of isolated rat hearts decreased coronary perfusion pressure (vasodilation). Mesenteric vessels were treated with biotin-15d-PGJ2 in order to identify proteins that formed an adduct with 15d-PGJ2. These proteins were purified with avidin-agarose and identified by separation of tryptic digests by liquid chromatography with online analysis by mass spectrometry. Several proteins were identified that formed an adduct. However one particular protein that was modified, soluble epoxide hydrolase (sEH), we hypothesized might account for the vasodilation observed in isolated hearts. sEH catalyses the hydroysis of epoxides, such as epoxyeicosatrienoic acids (EETs), to diols (dihydroxyeicosatrienoic acids). We assayed sEH activity, and found 15d-PGJ2 inhibited its activity, both in vitro with recombinant sEH, as well in cardiac homogenates after hearts were exposed to this lipid. Furthermore, when an inhibitor of sEH, (AUDA), was perfused through isolated rat hearts, it mimicked the vasodilatory response of 15d-PJ2. Similarly, 14, 15-epoxyeicosatrienoic acid (14, 15-EET) also decreased coronary perfusion pressure. These results suggest that 15d-PGJ2 is able to form an adduct with sEH, which inactivates it. This results in cellular accumulation of 14, 15-EET, allowing them to exert their vasodilatory response.

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PC26

Expression and role of RhoA/ROK pathway in control of agonist-induced Ca^{2+} signaling in endothelial cells of intact rat tail artery

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The endothelium is a confluent monolayer, lining the inner surface of blood vessels which acts as the main local regulator of vascular wall homeostasis. Binding of inflammatory mediators to G-protein coupled receptors increases endothelial cells permeability by increasing intracellular Ca^{2+} concentration. Several studies have shown that RhoA/Rho-kinase plays a key role in modulation of barrier properties of cultured endothelial cells and intact microvessels [W-S. Beata *et al*, 1998: C.M. Joes *et al*, 1999: R.H. Adamson *et al*, 2002]. Therefore we investigated role of Rho-A and its main effector, Rhokinase in control of calcium signaling in intact endothelial cells of rat tail artery using immunohistochemistry and confocal imaging. Rats were humanely killed under CO_2 anaesthesia; their tail 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 3 animals were used in each set of experiments. We have found that in endothelium of rat tail artery ROK-α but no ROK-β was expressed. Carbachol a muscurinic receptor agonist was used in our study to stimulate the intact endothelial cells. Application of carbachol (0.1uM, 1uM, 10uM) to intact endothelial cells produced 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 41 $\pm$ 0.7 of the peak taken for 100% (n= 372cells, 7vessels). The frequency of oscillation ranges from 0.05 to 0.3 Hz (n=372cells, 7vessels). Inhibition of Rho-A by C3-transferase (1μg/ml) or Rho-kinase by H-1152 (200 nM) reduced the initial fast component of CCh induced Ca^{2+} transient to 55$\pm$1.5 (n=134 cells, 3 vessels) and 52$\pm$2.1 (n=136 cells, 3 vessels) of the peak, respectively and either fully abolished or significantly decreased the amplitude and the duration of the Ca^{2+} oscillations while the amplitude of the sustained component expressed as a percentage of peak was 65$\pm$1.8% (n=134cells, 3vessels) and 29$\pm$1.6% (n=136cells, 3vessels), respectively. Taken together, the data obtained suggest that Rho-A/ROK pathway is involved in control of calcium signaling induced by CCh in intact endothelium of large conduit arteries.

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PC27

Cholesterol depletion alters coronary artery myocyte Ca^{2+} signalling in an agonist-specific manner

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Caveolae, flask-like invaginations of the cell membrane stabilised by caveolin proteins and rich in cholesterol, are abundant in vascular smooth muscle. Depletion of cell membrane cholesterol disrupts caveolae and may lead to alterations in cell signalling. Given the importance of the coronary arteries to health and the association of hypercholesterolemia with atherosclerosis, endothelial dysfunction and impaired vascular smooth muscle function, it is of interest and significance to determine the effect of cholesterol manipulation in this vessel. We have examined the effects of methylcyclodextrin (MCD), a cholesterol depleting agent, on agonist-induced *in situ* $[Ca^{2+}]_i$ signalling in rat coronary artery myocytes from an intact preparation, using confocal microscopy. N numbers varied from 14 to 30 cells, from a minimum of 3 animals. Incubation with MCD led to the significant and selective reduction of the response to 5-HT (10μM; 76.3$\pm$5%) and endothelin-1 (10nM; 86.6$\pm$4%), while the response to phenylephrine (100μM) remained unchanged. These data were confirmed using cholesterol oxidase as the cholesterol depleting agent. When cholesterol was replenished, using cholesterol-saturated MCD, Ca^{2+} signalling was restored. The response to high K^+ (60mM) and caffeine (10mM; under both normal and Ca^{2+}-free conditions) was unaffected by MCD. In additional experiments, enzymatically isolated myocytes were patch clamped. MCD induced a large increase in whole cell outward K^+ current and a significant decrease in cell capacitance.

Modulation of cell membrane cholesterol altered coronary artery myocyte Ca^{2+} signalling in an agonist-specific manner, indicating the importance of caveolae in the maintenance of myocyte signalling processes.

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PC28

Chronic hypoxia *in utero* (CHU) in the rat changes capillarity and oxidative function in skeletal muscle of the offspring

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We have previously shown that when pregnant Wistar dams are housed in a chamber containing 12% O2, the adult male offspring (CHU) show altered skeletal muscle vasodilator responses to acute hypoxia (breathing 8% O2). Relative to normal (N) rats, these dilator responses showed a reduced functional role for nitric oxide (NO) in rats exposed to CHU for the first half of pregnancy (early: ECHU) but not in those in those exposed to CHU for the second half (late: LCHU) of pregnancy (1). We also showed that this could not explained simply by a reduction in the expression of endothelial nitric oxide synthase (eNOS) protein.

In the adult rat, chronic hypoxia induces angiogenesis in skeletal muscle (2) and also modulates the profile of muscle fibre type (3). Thus, in this study, we have examined the oxidative capacity of the hindlimb muscle extensor digitorum longus (EDL) by measuring fibre type and capillarity in 9-10 week old ECHU (n=4) and LCHU (n=5) rats compared to normal (N) rats (n=4). Sections (10μm) were cut from snap frozen muscles. Results are expressed as mean$\pm$SEM and comparison to N rats was made by ANOVA. Capillarity was measured using rhodamine-conjugated lectin and fibre typing was performed using myosin ATPase staining. Western blot analysis of whole muscle homogenate from EDL confirmed our previous findings in other hindlimb muscles (1) that eNOS protein expression was not reduced in either ECHU or LCHU rats (ECHU: 9.6$\pm$19%; LCHU 27$\pm$19% relative to N). Capillary:fibre (C:F) ratio increased significantly in both ECHU ($p<0.01$) and LCHU ($p<0.001$) compared to N rats (N: 1.57$\pm$0.03, ECHU: 2.06$\pm$0.06, LCHU: 2.06$\pm$0.08). Type I (slow oxidative) fibre size and number were not affected by CHU. However, cross sectional area of type IIa (fast oxidative glycolytic) fibres was significantly increased in both CHU groups compared to N (ECHU 117$\pm$34%, $p<0.01$; LCHU 93$\pm$23%, $p<0.05$). The number of type IIb (fast twitch glycolytic) fibres was also decreased significantly in both CHU groups compared to N rats (ECHU: -19$\pm$6.1%, $p<0.05$; LCHU -32$\pm$3.1%, $p<0.001$). These changes in oxidative profile and capillarity suggest that exposure to chronic hypoxia in utero programmes the development and differentiation profile of skeletal muscle fibres in the EDL, possibly to optimize oxygen consumption and delivery. Since eNOS protein does not increase despite the increase in C:F it may be that eNOS expression within cells is decreased. Angiogenesis is a tightly regulated system, and this developmental programming may have long-term effects on muscle performance in adult life.

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PC29

Inhibition of endothelial cell migration by shear stress

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Angiogenesis is a multistep process involving endothelial cell (EC) migration and proliferation, tightly regulated by a complex system of promoting and inhibiting factors [1]. Stimulation of angiogenesis by increased shear stress *in vivo* is associated with an increase in production of vascular endothelial growth factor (VEGF) and nitric oxide (NO) in skeletal muscle [2,3]. The shear sensor that tranduces the increased shear into an angiogenic signal is poorly understood. Evidence suggests that CD31 expressed on ECs may act as part of a mechanosensory complex [4]. Thus, we aimed to investigate the hypothesis that shear stress and VEGF interact to modulate migration of ECs, and hence control angiogenesis. In addition, we investigated the roles of CD31 as a potential sensor and NO as potential effector of shear-induced changes in this model.

Using real time PCR, changes in expression of genes known to play a role in angiogenesis; VEGF, VEGF receptor (R) 1 & 2, endothelial NO synthase(eNOS), angiopoietin (ANG) 1 & 2, neuropilin (NP) 1 & 2, were investigated in human umbilical vein EC (HUVEC) cultured under stasis or shear stress (1.5 Pa) for 24h, ±VEGF in the final 4h. To assess migration, confluent HUVEC were wounded and then exposed to stasis or shear stress (0.3 Pa or 1.5 Pa) ±VEGF; wound recovery was measured at 0 and 16h. In similar experiments, the effect of NO or CD31 was investigated using the NOS inhibitor L-NAME or siRNA for CD31, respectively.

Gene expression analysis showed that shear alone increased eNOS, VEGFR2 and NP2 expression, but decreased expression of ANG 1 & 2 and NP1. These shear-induced changes were all maintained in the presence of VEGF. Shear decreased EC wound recovery (higher the shear = lower migration; $P<0.001$ 1.5 Pa vs. Static). Nevertheless, EC preferentially migrated in the direction of flow ($P<0.01$). VEGF enhanced wound recovery to the same extent under static and shear ($P<0.05$). Neither NOS inhibition nor reduced expression of CD31 modified the shear-induced inhibition in wound recovery.

Shear stress leads to differential regulation of a number of genes involved in the control of angiogenesis. Interestingly, shear appears to be regulating molecules that modify VEGF effects, rather than VEGF *per se*. VEGF appears to have little effect on any of these key angiogenic genes. Whereas *in vivo* results show that shear stimulates angiogenesis [2], our results show that shear decreases migration of ECs, suggesting that *in vivo* additional cellular responses are being modulated. CD31 does not appear to be the sensor involved in shear-induced inhibition of migration, and NO does not appear to be the effector of this response in this model.

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PC30

Intraplatelet L-arginine-nitric oxide and vascular reactivity in an animal model of postnatal stress

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Introduction: Animal models of postnatal stress can be considered depression models, and many of their abnormalities are similar to those revealed in human depression[1-3]. The objective of this study was to investigate the participation of vascular reactivity and the intraplatelet L-arginine-nitric oxide (NO) pathway in atherothrombosis and cardiovascular events common to depression.

Methods: All experiments were reviewed and approved by the Ethics Committee of Animal Experiments of the State University of Rio de Janeiro. Eight male Wistar rats underwent a unique maternal separation (UMS) for 8 minutes at a temperature of 22°C during their second day of life; and nine control rats were included in the study. The animals were anesthethized with pentobarbital (70 mg/kg, i.p.), blood was collected by aortic puncture and the mesenteric arterial bed was rapidly removed. The reactivity of the arterial mesenteric bed was measured as described by McGregor[4]. The basal activity of intraplatelet nitric oxide synthase (NOS) was measured by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline, and the influx of L-[^{3}H]- arginine was evaluated during an incubation period of 5 minutes at 37°C with L-[^{3}H]–arginine at 100 μM. Mann Whitney test was used to access the statistical significance ($p<0.05$). Data are presented as mean ± EP. Results: There was a difference in vasoconstriction (mmHg) caused by norepinephrine at concentrations of 1, 3 and 10 nmol in UMS rats (12 ± 2.5; 27.2 ± 3.4; 72 ± 9.6%, respectively) compared to controls rats (2.7 ± 2.6; 14.4 ± 3.9; 43.2 ± 7.5%, respectively). The vasodilator effect of acetylcholine was significantly increased in UMS rats compared to controls. In platelets, the basal activity of NOS in UMS rats (0.07 ± 0.01 pmol/10^8 cells) was reduced compared with controls (0.17 ± 0.03 pmol/10^8 cells.). There was also a decrease in total L-arginine transport from 0.42 ± 0.05 μmol/L/h to 0.24 ± 0.02 μmol/L/h in UMS rats.

Conclusion: Our findings demonstrate that postnatal stress presents with a dysfunction of mesenteric vascular reactivity associated with an inhibition of the L-arginine-NO pathway in platelets. These results are a possible additional mechanism to understand the pathophysiology of depression and cardiovascular complications.

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PC31

Facilitation of the myogenic response by pressure-induced changes in intracellular pH in small arteries

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The myogenic response plays an important role in blood flow autoregulation. In order to get more insight into the mechanisms of the myogenic response, the hypothesis was tested that changes in intracellular pH (pHi) are involved in the myogenic response. The study was performed on isobaric preparations of rat tail small arteries using videomicroscopic diameter determination and pH fluorimetry. In response to a pressure-increase pHi did not change throughout the early vessel dilation. During the subsequent vessel constriction pHi decreased with a similar time course as the constriction. For example, pHi was reduced by 0.12±0.01 units (n=7; $p<0.05$) upon a pressure increase from 10 to 80 mmHg. Subsequently, pHi recovered by 50.4±4.0% (n=7; $p<0.01$). In order to get insight into the mechanisms involved in the pHi alterations associated with the myogenic response, the effect of inhibition of proton transport mechanisms on the pressure-induced pHi changes was studied. Removal of extracellular bicarbonate did not reduce the early pHi peak or the later recovery phase. Application of EIPA and HOE 694, inhibitors of the Na/H-exchange, did not affect the early pHi peak, but reduced the amount of pHi recovery after a pressure increase from 10 to 80 mmHg by 48.7±3.2% (n=6; $p<0.05$). In addition, at 80 mmHg, but not at 10 mmHg, these inhibitors reduced the pHi recovery after an NH_4Cl-induced acid load. Based on the relationship between pHi and vessel diameter obtained by selectively altering pHi using NH_4Cl, it was determined that the pressure-induced pHi change accelerated the initial phase and increased the amplitude of the myogenic response. In summary, the myogenic response of rat small arteries is associated with changes in pHi, which facilitate the myogenic response. The early pHi peak seems to be coupled to the contractile process. The later pHi recovery is due, at least partly, to a pressure-induced activation of the Na/H-exchange.

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PC32

Stimulation of the AMP-activated protein kinase induces dilatation in hamster microvessels

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Recent studies suggest that the AMP-activated protein kinase (AMPK) inhibits BK_{Ca} channels in type I cells of rat carotid bodies (1). It has also been shown recently that AMPK could phosphorylate myosin light chain kinase in macrovascular smooth muscle (2), a mechanism resulting in decreased calcium sensitivity. While the earlier mechanism, when expressed in smooth muscle, would lead to vasoconstriction and inhibition of EDHF mediated dilation, the latter would lead to a vasodilation. We investigated whether a novel activator of the AMPK (3) A769662 (A76), affects microvascular tone and EDHF-dependent dilator pathways in hamster resistance arteries in which we have characterised EDHF mediated dilations on the molecular level (4).

Small resistance arteries (about 250 mm in diameter, n= 17 in total) were isolated from hamster gracilis muscle, cannulated and the smooth muscle layer loaded with the calcium indicator Fura2-AM as described previously (5). Vascular diameters were anlysed by videomicroscopy. All microvessels were pretreated with the COX inhibitor indomethacin (30μM) and the NOS inhibitor L-NAME (30μM).

When exposed to A76 (10^{-6} to 10^{-4} M), resistance arteries (preconstricted with 0.3 μM norepinephrine) showed a dose dependent vasodilatation (94.5 ± 4.0 % at 10-4M, mean ±SEM) associated with a decrease of $[Ca^{2+}]_i$ (94.5 ± 10.9% at 10^{-4}M) This vasodilation was not endothelium dependent. In arteries pre-constricted with 100mM potassium, A76 (10^{-4} M), induced neither relaxation nor decreased smooth muscle calcium. There was also no calcium independent dilation during an observation period of 15 min. Neither inhibition of IK_{Ca} / BK_{Ca} potassium channels with CTX (1μM) – which has been shown to inhibit EDHF mediated dilations previously - nor IBTX(10^{-7} M), a specific inhibitor of IK_{Ca} did alter maximal effects of A76 significantly whereas the K_{ATP} inhibitor glibenclamide (0,1mM) reduced dilation to 10.6 ±5.0 % as well as the decrease of $[Ca^{2+}]_i$ to 35.1 ± 0.5 %.

These results suggest that AMPK is a potent dilator of small resistance arteries in the hamster. The results obtained with glibenclamide suggest that K_{ATP} channels may be a target of AMPK in microvascular smooth muscle. In potassium depolarized vessels, no functional evidence has been found so far for a role of the AMPK in reducing calcium sensitivity as recently described in macrovessels.

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PC33

The role of muscarinic M_3 receptors in canine uterine artery vasorelaxation

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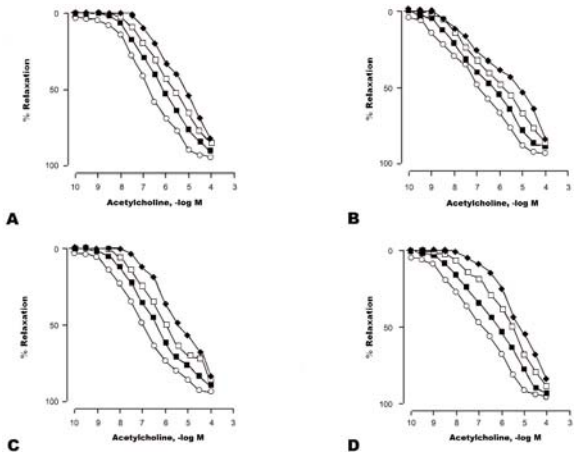
Vascular tone of canine uterine artery is mainly dependent on neurogenic impacts and functional integrity of its endothelium (Okamura et al., 1995; Pesic et al., 2003). Acetylcholine modulates blood flow causing vasodilatation by releasing nitric oxyde through the activation of muscarinic receptors located on the endothelial cells (Ignarro et al., 1987, Tare et al., 1990). To identify the receptor subtype functionally involved in acetylcholine-induced relaxation in canine uterine artery, arterial segments were taken from healthy non-pregnant mixed breed adult female dogs which underwent ovariohysterectomy as a method of sterilization. All surgical procedures followed the animal welfare regulations. Premedication with atropine (0.02-0.04 mg/kg), acepromazine (0.02-0.04 mg/kg) and ketamine (15 mg/kg) given intravenously preceded general anesthesia with halothane, nitrous oxide and oxygen combined. A range of muscarinic receptor antagonists such as atropine (non-selective), pirenzepine (M_1-selective), methoctramine (M_2-selective) and p-fluoro-hexahydro-sila-difenidol (p-FHHSiD) (M_1/M_3) were used and their pA_2 values were determined by Shild method. The relaxation of uterine arterial rings in response to acetylcholine in the presence or absence of selective muscarinic receptors antagonists was calculated using concentration response curves. Acetylcholine induced concentration-dependent and endothelium-dependent relaxation of arterial rings precontracted with phenylephrine (pEC_{50} = 6.90±0.02). The effects of competitive acetylcholine antagonists were concentration dependent and induced a significant rightward shift ($p<0.05$ for all antagonists tested, by Student t-test) of the concentration-response curves to acetylcholine (Fig.1). The Schild plot slopes for muscarinic receptor antagonists tested in this study yielded straight lines with mean slopes no different to unity indicating that the antagonism is competitive. Obtained pA_2 values are given in Table 1. The present study has shown that canine uterine artery contains functional cholinergic muscarinic receptors that mediate acetylcholine relaxation which is endothelium-dependent and concentration-dependent. On the basis of differential antagonist affinity we propose that this relaxation is mediated predominantly by stimulation of muscarinic M_3 receptors.

The pA_2 values, Schild plot slopes and coefficients of correlation (r^2) for muscarinic receptors antagonists on the canine uterine artery.

Antagonist	pA 2	slope	r^2	n
Atropine	9.91±0.06	0.89	0.998	12
Pirenzepine	6.60±0.04	1.11	0.999	14
Methoctramine	6.21±0.08	1.06	1	12
p-FHHSiD	8.05±0.1	1.02	0.996	12

Data are presented as mean ± SEM

n = number of animals



Antagonism of the relaxant effect of the acetylcholine by antagonists of muscarinic receptors. Acetylcholine concentration-response curves in the canine uterine artery with intact endothelium in the absence (white circle) and presence of $3x10^{-10}$ (dark cube), 10^{-9} (white cube) and $3x10^{-9}$ mol/L (diamond) atropine (A); $3x10^{-7}$ (dark cube), 10^{-6} (white cube) and $3x10^{-6}$ mol/L (diamond) pirenzepine (B); 10^{-6} (dark cube), $3x10^{-6}$ (white cube) and 10^{-5} mol/L (diamond) methoctramine (C); $3x10^{-8}$ (dark cube), 10^{-7} (white cube) and $3x10^{-7}$ mol/L (diamond) p-FHHSiD (D). Each point represents the mean of 4-13 experiments. Responses are expressed as a percentage of the phenylephrine (10^{-5} M) contraction.

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PC37

Single channel mechanism of the TRPM8 calcium channel voltage dependence

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TRPM8 channels are present in blood vessels where they are likely to be involved in the regulation of vascular tone (Yang et al., 2006; Inoue et al., 2006). Their activity depends on temperature, voltage and chemical signaling. However, single channel mechanisms of TRPM8 gating remain unknown. We examined the influence of voltage on TRPM8 gating using patch-clamp recording techniques (all values were expressed in means ± S.E.M.). In HEK293 cells stably expressing TRPM8, whole-cell currents during voltage steps (-100 to 150 mV, 10 mV increments) and ramps (-100 to 100 mV) showed a strong outward rectification. Rectification of several TRP channels has been suggested to arise from the voltage-dependence of gating (Nilius et al., 2005). Thus, single channel currents (cell-attached configuration) were measured at different voltages (room temperature, filtered at 2 kHz and sampled at 10 kHz) giving single channel conductance of 60.2±4.3 pS (n=6), and an increase in open probability (Po) from 0.03±0.01 at 40 mV (n=7) to 0.41±0.06 at 160 mV (n=7). To further study the mechanism underlying this voltage-dependent increase in Po, shut and open dwell times at 80 and 140 mV were compared. There was only a modest increase in the mean open time from 0.70±0.08 to 1.10±0.28 ms (n=4 patches; not significant, P=0.229, two-tailed *t*-test) whereas the mean shut time decreased from 15.55±2.15 to 4.04±1.08 ms (n=4; P=0.003) between 80 and 140 mV, respectively. Dwell-time histograms (transitions > 0.16 ms) were fitted with sums of exponentials (2 open and 4 shut in each case). They showed that membrane depolarization caused a reduction in the relative contributions of the long shut components. Thus, the two longest components (τ > 10 ms) accounted for 40% of all closures at 80 mV, but for only 14% at 140 mV. In contrast, the open time values were little affected by voltage. The 2-D dwell-time distributions (Rothberg and Magleby, 1998) and dependency plots (Magleby and Song, 1992) were consistent with voltage-dependent restructuring of channel gating. These results show that the main effect of membrane depolarization on TRPM8 gating is an increase in the frequency of channel opening. Furthermore, voltage-dependence of TRPM8 gating arises from the effects of membrane potential on the closed dwell times, while open times are essentially voltage-independent.

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PC38

Cell cycle-dependent expression of Kv3.4 channels and cell proliferation in human uterine artery smooth muscle cells

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The vascular smooth muscle cells (VSMCs) of the vessel wall are differentiated cells specialized in the contractile function required for the maintenance of vascular tone. However, VSMCs are not terminally differentiated and in response to local environmental factors can experience a phenotypic switch towards a synthetic phenotype, which leads to cell proliferation, migration and production of extracellular matrix components. Ion channels have been shown to participate in cell proliferation, as they can modulate the progression of the cells through the cell cycle. In this regard, it is known that in different cell types K+ channel expression varies with the cell cycle. Also, several reports indicate that the blockade of some K+ currents in VSMCs is antiproliferative. Recently, our group has demonstrated that the functional expression of Kv3.4 channels is related to proliferation of human uterine VSMCs. In this work, we sough to gain deeper insight on the relationship between the expression and function of Kv3.4 channels and the progression of cultured uterine VSMCs through the cell cycle. We have used selective cell cycle blockers to describe the expression pattern of Kv3.4 mRNA and protein and its functional contribution along the cell cycle in these cells. Besides, we have explored the effects of the blockade of Kv3.4 channels on the distribution of the cells in the different phases of the cell cycle. We found that Kv3.4 mRNA levels increased when cells enter in G1 phase, decline in G2/M and reach the lowest level in quiescent (G0) cells, but Kv3.4 protein levels and Kv3.4 currents remain elevated while cells are proliferating, being maximum during G2/M phase. On the other hand, Kv3.4 channels blockade leads to a decrease in the mRNA levels of D1, A2 and B1 cyclins, an increase in the proportion of cells in the G0/G1 phase, a decrease in the number of ki67-positive cells and a decrease in the proportion of cells incorporating BrdU. Altogether, our data indicates that the blockade of Kv3.4 channels induces a decrease in the number of cells entering the cell cycle, suggesting the existence of a close association between the functional expression of Kv3.4 channels and the proliferation rate of uterine VSMCs.

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PC39

Expression and function of Kv7 channels in murine myometrium during early and late pregnancy

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Background: Voltage-gated potassium (Kv) channels are involved in the regulation of spontaneous myometrial contractions *in vitro* and are hypothesised to contribute to uterine quiescence in early pregnancy. The precise molecular identity of the channels has yet to be determined, but we have previously reported that Kv7 channels, encoded by KCNQ1-5 and/or β-accessory subunits encoded by KCNE genes (1-5), are present in non-pregnant mouse myometrium. In this study, we have examined the expression of KCNQ and KCNE genes in myometrium from early and late pregnant mice and compared expression to non-pregnant tissues. We have also investigated the functional impact of Kv7 channels in pregnancy using pharmacological agents.

Methods: Myometrial tissues were obtained from non-pregnant (at the time of oestrous), early pregnant (day 6-7) and late pregnant (day 17-18) C57/BL6 mice. Total RNA was extracted using Trizol and cDNA synthesised with Superscript III. qRT-PCR for KCNQ1-5 and KCNE1-5 was quantified using a standard curve and data expressed relative to the geometric mean of the two most stable housekeeping genes from a panel of 5. Myometrial strips were used for isometric tension recording and activity measured as mean integral tension (MIT) ±XE991/chromanol (Kv7 inhibitors) or retigabine/flupirtine (Kv7 activators).

Results: All of the KCNQ and KCNE isoforms studied were detected in mouse myometrium. There was a general suppression of all KCNQ isoforms, with the exception of KCNQ3, in early pregnancy ($n=6$, $p<0.001$) compared to myometrium from non-pregnant ($n=6$) and late pregnant ($n=6$) animals. This is in contrast to the high expression levels of KCNQ1 and KCNQ5 in myometrium from non-pregnant and late pregnant mice. KCNE isoforms were also gestationally regulated. KCNE1, KCNE3 and KCNE5 expression was decreased in late-pregnant compared to non-pregnant tissues ($p<0.05$)and KCNE2 and KCNE4 expression was increased in tissues from early and late pregnant mice compared to tissues from non-pregnant animals ($p < 0.001$). XE991 (10 μM) significantly increased spontaneous myometrial contractions in all tissues studied ($p < 0.05$) and retigabine/flupirtine (20 μM) attenuated myometrial contractility ($p<0.01$). Interestingly chromanol 293B (1-30 μM) had no impact on contractility.

Conclusions: The expression profile of KCNQ and KCNE isoforms in the myometrium is distinct to other smooth muscles. KCNQ isoforms are generally suppressed in early pregnancy but expression levels are restored to non-pregnant levels in late gestation. Kv7 channels modulators impact on non-pregnant and pregnant myometrial contractility suggesting a significant role for these channels in myometrial function.

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PC40

Inhibition of Ca^{2+} oscillations in rat retinal arteriolar smooth muscle by arachidonic acid

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The release of Ca^{2+} from the sarcoplasmic reticulum (SR) of smooth muscle in retinal arterioles is associated with muscle contraction and vascular constriction (Tumelty *et al*, 2007). There is evidence that arachidonic acid and its metabolites play a role in neurovascular coupling in the retinal microvasculature (Metea & Newman, 2006). We therefore decided to test the effects of arachidonic acid on Ca^{2+} signalling in rat retinal arteriolar smooth muscle.

Sprague-Dawley rats (300-500g) were killed with CO_2, enucleated, and retinal arterioles were mechanically dispersed and loaded with Fluo-4-AM (10μM). Ca^{2+} events were imaged using line-scan confocal laser microscopy and local and global changes in smooth muscle [Ca^{2+}] assessed using normalized fluorescence (F/F_0). Adjacent regions of the same vessel were imaged under control conditions and in the presence of arachidonic acid to limit photodamage. Data was collected from a minimum of 7 vessels isolated from at least 4 animals, and has been summarized as the mean±SEM.

Application of arachidonic acid (10μM) decreased the fraction of smooth muscle cells displaying spontaneous Ca^{2+}-oscillations, from 51±8% in control conditions to 21±6% after 5 minutes of arachidonic acid exposure ($P<0.05$, Wilcoxon signed-rank test). The average frequency of Ca^{2+}-oscillations in active cells was also decreased, from a control value of 0.057±0.005 $cell^{-1}\ s^{-1}$, to 0.038±0.006 $cell^{-1}\ s^{-1}$ after 5 min exposure to arachidonic acid ($P<0.05$, Mann Whitney U-test). Control protocols, in which the same vessel was imaged at 2 different sites at an interval of 5 minutes but with no application of arachidonic acid, showed no reduction in the number of active myocytes (40±10% and 39±9% of cells generated oscillations at 0 and +5mins, respectively; NS). The oscillation frequency in active cells was also unaffected, with an average value of 0.039±0.003 $cell^{-1}\ s^{-1}$ initially, and 0.044±0.005 $cell^{-1}\ s^{-1}$ after 5 minutes (NS). Arachidonic acid had no effect on the amplitude or full duration at half maximum (FDHM) of Ca^{2+}-oscillations (see Table 1). These data suggest that arachidonic acid inhibits the generation of global Ca^{2+} events in retinal arteriolar myocytes, which would be consistent with a vasodilatatory action in the retinal microcirculation.

Table 1: Characteristics of Ca^{2+}-oscillations

Recording conditions	*Amplitude ($\Delta F/F_0$)*	*FDHM (s)*
Control	2.16±0.10	1.918±0.143
+Arachidonic acid	2.37±0.22	1.720±0.221

Metea MR & Newman EA. (2006). *J Neurosci* **26**, 2862-2870.

Tumelty J et al. (2007). *Cell Calcium* **41**, 451-466.

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

PC42

Is the BKCa channelopathy the main reason for radiation-induced arterial hypertension?

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Exposure to radiation elicits arterial hypertension and endothelial dysfunction in mammals (Soloviev et al. 2002, 2003). The BKCa channel is one of the established radiation targets (Soloviev et al. 2005). The goal of this study was to clarify whether KCNMA1 gene silencing mimics the vascular effects of ionized irradiation in experimental animals since it is known that small interference RNA (siRNA) can be used to dissect the function of different splice variants of ion channel encoding genes. The siRNA to KCNMA1 gene (Metabion, Germany) was injected into the rats intravenously at a dose of 800 μmol/kg in accordance with standard Metabion protocol for siRNA annealing. On the 7th day after siRNA administration the thoracic aorta was taken from animals anesthetized with ketamine (37.5 mg/kg b.w., IP) and xylazine (5 ml/kg b.w. IP) to obtain isolated smooth muscle cells (SMC) and smooth muscle (SM) rings for patch-clamp technique, contractile recordings and real time PCR analysis (protein expression was confirmed by Western blot method). Whole-body irradiation (total dose 6 Gy) was performed with gamma rays delivered at a rate of 0.8 Gy/min from a cobalt source.

Outward potassium currents stimulated by a depolarized voltage step to +70 mV were 30±1, 11±1, and 13±1 pA/pF in control, irradiated (6 Gy) and KCNMA1 gene knockdown SMC, respectively (n=12, P<0.05). Paxilline (500 nM)-sensitive components were 25±2, 5±1, 9±1 pA/pF, respectively (n=12, P<0.05). The expression profile of BKCa mRNA transcripts in SM appeared to be significantly decreased in silencing SM similar to irradiated SM when the level of BKCa expression had decreased. The silencing of KCNMA1 gene led to a significant increase in arterial blood pressure in 30% of animals only, while radiation produced hypertension development in 90% of all observations. Standard acetylcholine test showed no abnormalities in endothelium-dependent relaxant responses in SM obtained from rats with silencing KCNMA1 gene in contrast to irradiated animals. At the same time KCMA1 gene silencing SM demonstrated an increased sensitivity to arterenol – mean values of pD2 (-log EC50) are 6.1±0.1 control, and 7.6±0.2 (n=10, P< 0.01) KCMA1 gene silencing SM.

Therefore, radiation alters the form and function of the BKCa channel and this type of ionic channel may contribute to related vascular abnormalities. Nevertheless, it is unlikely that BKCa can operate as a crucial factor for radiation-induced arterial hypertension. It is clear that the underlying mechanism producing the alterations of vascular function under irradiation is multifaceted.

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Soloviev A, Tishkin S, Parshikov A et al (2003) Br. J. Pharmacol. 138: 837-844.

Soloviev A, Tishkin S, Rekalov V et al. (2005) Proc Physiol Soc 568P, PC2.

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PC43

The effect of aldosterone on adipocyte-derived relaxation occurs via non-genomic and genomic effects on the vasculature

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Introduction: There is increasing evidence suggesting a link between aldosterone and components of the metabolic syndrome, particularly abdominal obesity and arterial hypertension. We have shown that the anticontractile property of perivascular adipose tissue is lost in patients with this syndrome; the aim of this study was to investigate whether aldosterone was able to influence this anticontractile response and whether this was due to genomic or non-genomic effects and if any influence could be reversed.

Methods: Small arterial segments (2mm, <200μm internal diameter) were dissected from healthy male wistar rats and studied using wire myography. The effects of aldosterone and its non-genomic and genomic antagonists, eplerenone and spironolactone respectively, were assessed under normoxic conditions following short and long incubations (10min and 3hours). Contractile responses to noradrenaline were calculated as a percentage of KCl contraction, and expressed as mean±SEM.

Results: Adipose tissue exhibited an anti-contractile effect on arteries (no adipocytes: 136±6% vs adipocytes 87±4%, n=10, P<0.0001) which was lost following 10min incubation with aldosterone (5nM) (adipocytes: 87±4%, n=10 vs. adipocytes + aldosterone: 124±6% n=9, P<0.001). Short incubation with eplerenone, but not spironolactone, restored the contractility to levels similar to arteries with adipocytes alone (88±3%, n=6). Three hour incubation of aldosterone caused a loss of the anticontractile effect similar to that observed with short incubation (134±5%, n=3) however, both eplerenone and spironolactone were only able to partially restore this effect (adipocytes + 3hr aldosterone: 134±5% vs. adipocytes + 3hr aldosterone + eplerenone: 122±2%, n=3, P=0.0358, adipocytes + 3hr aldosterone + spironolactone: 102±6%, n=3). Contractility of arteries without adipocytes was not significantly affected by any intervention.

Conclusion: Aldosterone ameliorates the anticontractile effect of adipose tissue via a predominantly non-genomic effect, because eplerenone can only partially restore contractility following longer incubations. Whether a combination of

eplerenone and spironolactone is able to fully restore the effects of aldosterone on contractility is yet to be established.

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PC44

The amplitude rather than mean strain determines the proliferative and apoptotic capacity of vascular smooth muscle cells *in vitro*

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Introduction: Restenosis represents the main drawback in the long term success of stenting procedures. Changes in vascular smooth muscle cell (SMC) proliferation and apoptosis are critical in the development of in-stent restenosis [1]. Cyclic strain is a key stimulus in the proliferative and apoptotic capacity of SMC [2]. The expansion of a comparatively stiff stent within a vessel alters the mechanical environment from low mean, high amplitude to a high mean, low amplitude cyclic strain. The aim of this work was to investigate the effect of mean strain and amplitude on the proliferation and apoptotic capacity of vascular SMC.

Methods: Bovine aortic SMC were subjected to cyclic strain at different mean strains and amplitudes using a Flexercell FX-4000T™ system with an applied sinusoidal waveform before cell proliferation and apoptosis were evaluated. Vybrant™ CFDA-SE dye and Alexa Fluor 488™ Annexin V and propidium iodide were used to determine cell proliferation and apoptosis, respectively, using FACS analysis. Cell counts were also performed using a hemocytometer.

Results: Bovine aortic SMC were strained at the same amplitude (2%) but at different mean strains (5% and 10%) for 48 h before levels of proliferation and apoptosis were measured. In parallel studies, cells were exposed to low mean strain (5%) and high amplitude (6%) versus high mean strain (11%) and low amplitude (2%). Changes in mean strain had no significant proliferative or apoptotic effect on SMC. In contrast, the proliferative and apoptotic capacity of SMC was highly dependent on the amplitude of the cyclic strain. A low strain amplitude (2% vs 6%) promoted cell proliferation (ANOVA, $p<0.05$) and inhibited apoptosis (ANOVA, $p<0.05$). We further validated the role of mean strain vs. amplitude using a novel bioreactor where SMCs were cultured inside a perfused stented Sylgard™ mock coronary artery in which physiological and pathological levels of strain were mimicked, see figure 1. Strains were set to be between 2% and 8% in the non-stented region. In the stented area strain was set to have amplitude between 1 and 1.5% and a mean strain increase of 25%. The level of proliferation was significantly increased in the stented region when compered to non-stented, see figure 2.

Conclusions: It is the amplitude of the strain that dictates SMC behaviour rather than the mean strain *in vitro*. These data provide evidence for the use of more compliant stent designs in order to reduce in-stent restenosis.

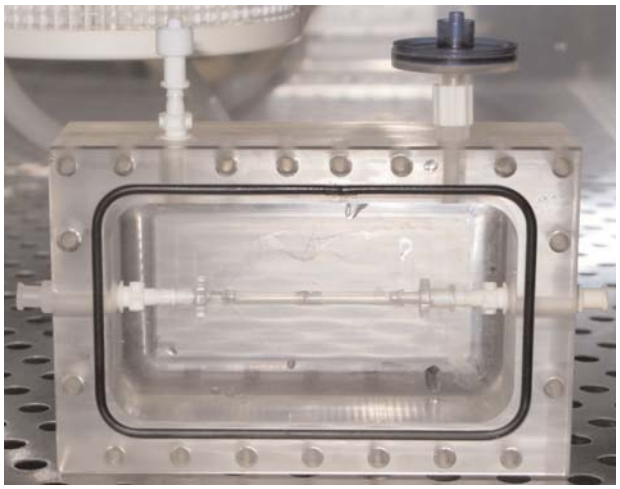

Figure 1: Bioreactor with a perfused stented Sylgard™ mock coronary artery.

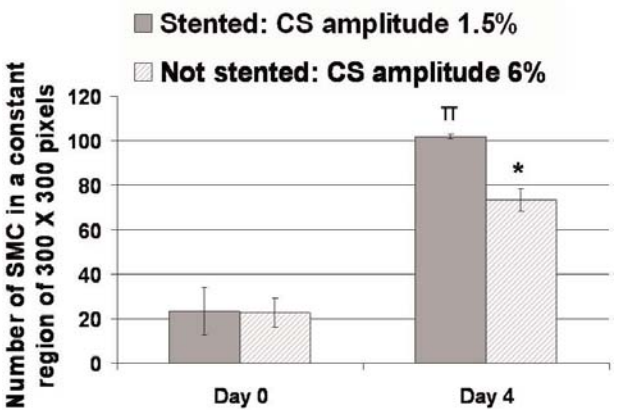

Figure 2 : SMC proliferation in a stented vs. non stented area inside a mock coronary artery before and after four days of cyclic strain (CS). (ANOVA, π $p<0.01$, * $p<0.01$).

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Medtronic AVE, Enterprise Ireland.

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

PC45

Evidence for IP_3R-mediated Ca^{2+} release following stimulation of P_2X purinoceptors in smooth muscle cells from the guinea-pig vas deferens

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A confocal Ca^{2+} imaging technique was used to detect sub-cellular $[Ca^{2+}]_i$ changes induced by activation of P_2X purinoceptors (P_2X-Rs) with 10 μM α,β-methyleneadenosine 5-triphosphate (α,β-meATP) in smooth muscle cells (SMCs) freshly isolated from the guinea-pig vas deferens. The spatio-temporal patterns of the α,β-meATP - induced $[Ca^{2+}]_i$ changes detected with fast x-y confocal imaging in fluo-3 loaded SMCs were related to spatial distribution of the sarcoplasmic reticulum (SR), visualised with Brefeldin A BODIPY, and type 1 IP_3Rs and RyRs, visualised by indirect immunofluorescence method. We have found that sub-plasmalemmal (sub-PM) SR elements possess both type 1 IP_3Rs and RyRs, while central/perinuclear SR elements are enriched with RyRs. α,β-meATP -induced $[Ca^{2+}]_i$ mobilisation consisted of: (1) an initial sub-PM $[Ca^{2+}]_i$ upstroke (SPCU) followed by (2) $[Ca^{2+}]_i$ wave which propagated through the entire cell volume. The peak amplitude of the α,β-meATP -induced SPCU was reduced: (1) by 38±3% (n=3) after inhibition of RyRs (with 100 μM tetracaine); (2) by 48±4% (n=4) after inhibition of IP_3Rs (with 30 μM 2-APB) and (3) by 71±5% (n=3) following block of voltage-gated Ca^{2+} channels (VGCCs) (with 5 μM nicardipine). The cumulative inhibition of IP_3Rs, RyRs and VGCCs reduced the SPCU by 65±5% (n=6). Depletion of intracellular Ca^{2+} stores by 10 min incubation with 10 μM cyclopiazonic acid (CPA) decreased SCPU by 55±7% (n=8). Subsequent inhibition of VGCCs, while keeping the stores depleted, decreased SPCU by 63±4% (n=8). Recording of isometric tension of smooth muscle strips from the guinea-pig vas deferens revealed that stimulation of P_2X-Rs with α,β-meATP induced biphasic contraction. Inhibition of IP_3Rs with 30 μM 2-APB decreased the transient component of the contractile response by 60±5% (n=4) and completely removed the tonic component of the contraction. These results suggest that IP_3R-mediated Ca^{2+} release is involved in genesis of SPCU and contraction in response to P_2X-R activation, which is not linked to Gq/11- phospholipase C - IP_3 signalling pathway.

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PC46

Cannabinoid receptor-mediated inhibition of sympathetic transmission in the mouse vas deferens

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Cannabinoids reduce neurotransmitter release from both central and peripheral nerve terminals, but the mechanisms by which this inhibition takes place in sympathetic nerve terminals, downstream of the receptors, is unclear. In this study, we investigated two aspects of cannabinoid signalling, using the vas deferens: (1) whether cannabinoids reduce the release of both cotransmitters from sympathetic nerves (noradrenaline and ATP); and (2) whether endogenous cannabinoids provide activity-dependent negative feedback of neurotransmission. Balb/c mice were killed by concussion and cervical dislocation; both vasa deferentia were removed. Longitudinal isometric contraction of each isolated mouse vas deferens was recorded in an organ bath, with trains of field stimuli (0.5 ms width; suprathreshold voltage; 10 pulses at 10 Hz) applied in pairs through ring electrodes at 120 s intervals. The noradrenergic component of contraction was measured in preparations pretreated with α,β-methylene ATP (1 μM); the purinergic component was measured in preparations pretreated with prazosin (100 nM). THC (100 nM) decreased the amplitude of the noradrenergic component of contraction, with a significant reduction at 30 min (36±9%, $P < 0.005$, $n = 6$), and maximal reduction after 70 min (52±9%, $P < 0.005$). In a cumulative concentration-response study (3 – 300 nM), THC had an EC_{50} of 9 nM (95% CI, 3 – 23 nM). The CB_1 receptor antagonist AM251 had no effect upon the noradrenergic component ($n = 6$). To test whether endocannabinoids could be released from the tissue following field stimulation, trains of either 10 or 200 pulses (at 10 Hz) were applied as conditioning stimuli; AM251 had no effect on subsequent test stimuli (10 pulses; delivered 15 s after the train; compared with a solvent control; $n = 6$). Intriguingly, there was no effect of THC (100 nM) on the purinergic component of contraction ($n = 7$ vasa deferentia). In conclusion, we report that THC inhibits noradrenergic neurotransmission with an EC_{50} of 9 nM, apparently without affecting the purinergic component; further work is required to confirm the latter observation. There appears to be neither an endogenous tone, nor release from the tissue upon electrical stimulation, of active endocannabinoids in the vas deferens.

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PC47

Contribution of inward rectifier K^+ currents to the excitability of vascular smooth muscle cells from BPN and BPH mice

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Inward rectifier (K_{IR}) and ATP-sensitive (K_{ATP}) K^+ channels play essential roles in vascular smooth muscle cells (VSMCs) as they contribute to maintain the resting membrane potential (E_M) and in this way regulate the contractile tone of resistance arteries. K_{ATP} channels underscore the functional bond between cellular metabolism and membrane excitability, and their blockade results in vasoconstriction and depolarization in various types of VSM. K_{IR} channels are known to be abundant in the VSMCs of resistance vessels, where their roles are far from being completely understood, although it has been established that they contribute to the resting E_M and resting tone of these cells and that their activation in response to moderate increases in extracellular K^+ induces vasodilation.

We designed this study to examine the expression and function of these channels in VSM and their regulation in essential hypertension, using VSMCs from mesenteric arteries obtained from a hypertensive inbred mice strain, BPH, and the corresponding normotensive strain, BPN. All animal protocols were approved by the Institutional Care and Use Committee of our Institution. Mice were killed by decapitation after isofluorane anesthesia. Real-time PCR using low-density TaqMan® arrays reveals mRNA expression of several K_{IR} and K_{ATP} genes in mesenteric VSMCs and also shows a reduced expression of some of these genes under hypertensive conditions. Functional characterization of K_{IR} and K_{ATP} channels was performed in freshly dispersed VSMCs with the whole-cell configuration of the patch-clamp technique, using $BaCl_2$ (100 μM) to block K_{IR} channels and pinacidil (10 μM) to activate K_{ATP} channels. Pinacidil application induced an increase of the current amplitude that was larger in BPN than in BPH VSMCs. We also found a decrease in the amplitude of the $BaCl_2$-sensitive current in VSMCs from BPH mice. The contribution of K_{IR} channels to the resting E_M was evaluated in perforated-patch experiments, where we observed a significant reduction of the $BaCl_2$-induced depolarization in hypertensive VSMCs.

Finally, the contribution of K_{IR} and K_{ATP} channels to determine vascular tone in mesenteric arteries of BPN and BPH mice was explored in pressurized arteries. Altogether, our findings indicate that the observed decrease in the functional expression of K_{IR} and K_{ATP} channels in BPH VSMCs from resistance arteries could participate in the genesis of the altered vascular tone during hypertension.

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PC49

Effects of elevated glucose and AGE-modified recombinant human serum albumin on antioxidant gene expression in human endothelial cells

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Hyperglycaemia and advanced glycation end-products (AGE) are associated with oxidative stress and endothelial dysfunction in diabetes mellitus (Mann et al., 2003). We previously hypothesised that the generation of reactive oxygen species (ROS) by NAD(P)H oxidase, mitochondria or uncoupled endothelial nitric oxide synthase may upregulate antioxidant gene expression in endothelial cells via activation of the redox sensitive transcription factor Nrf2, which binds to the antioxidant response element (ARE) in the promoter region of target genes (Mann et al., 2007). In the present study, we examined the effects of elevated glucose and AGE-modified recombinant human serum albumin (AGE-HSA) on antioxidant gene expression in human umbilical vein endothelial cells (HUVEC).

Endothelial cells were isolated by collagenase digestion and cultured in M199 containing 20% FCS. Confluent monolayers serum-deprived (1% FCS) for 4 h and then treated for 3-12 h with (i) elevated D-glucose (25 mM) and D-mannitol (20 mM + 5 mM glucose) as osmotic control) or (ii) AGE-HSA (100 μg/ml) and HSA (100 μg/ml) as a control. Elevated glucose increased heme oxygenase-1 (HO-1) expression after 6-12 h (Fig. 1), but had negligible effects on induction of either glutathione peroxidase-1 (GPx-1) or the phase II defence enzyme NAD(P)H:quinone oxidoreductase-1 (NQO1) (data not shown). Treatment of cells with AGE-HSA increased HO-1 expression after 3-6 h, which returned to basal levels after 12 h. AGE-HSA also increased expression of NQO1 but had negligible effects on GPx-1 expression. Our findings that hyperglycaemia and AGE-HSA induce adaptive antioxidant responses in fetal endothelial cells via the Nrf2/ARE pathway provide a basis for examining whether the phenotype of fetal endothelial cells is altered by in utero programming in gestational diabetes.

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PC50

Pharmacological properties of pulmonary arteries from TASK-1 knockout mice

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Growing evidence suggests that the TWIK-related Acid Sensitive K^+ (TASK) channels have a functional role in the cardiovascular system. In pulmonary artery (PA), compelling evidence has suggested that TASK-1 is a major contributor to the background K^+ current that support the membrane potential (E_m) of the smooth muscle cells. However, due to poor selectivity of its modulators, the role of TASK-1 channels in the functional regulation of tone in pulmonary arteries remains elusive. The purpose of this study was to investigate the properties of PA isolated from mice in which the TASK-1 gene has been deleted, compared with their wild type controls (C57BL/6 mice).

Intra-PAs (200μm diameter) were isolated from C57BL/6 mice and mice in which the TASK-1 and TASK-3 genes had been deleted (TASK1/3 KO, Aller et al., 2005). TASK-3 is not expressed in PA (Gardener, Johnson et al., 2004). Vessels were mounted on a small vessel myograph for isometric tension measurement. The responses to various drugs were recorded and expressed relative to a reference contraction elicited by application of 50mM KCl at the beginning of each experiment. Statistical analysis was performed using Student's t-test, with 0.05 as a critical value.

Responses of PA to 50mM KCl were not significantly different between C57BL/6 mice (292 ± 54 mN, N=13 vessels) and TASK1/3 KO mice (237 ± 42 mN, N=11). The addition of 10mM KCl, the K^+ channel modulators 4-AP (1mM) and levcromakalim (10μM), or the L-type calcium channel blocker nifedipine (1μM), did not elicit any significant response in either the TASK1/3 KO (N=6) or C57BL/6 mice (N=6). The contractile response of PA to phenylephrine (PE) had a pEC_{50} of 6.6 ± 1.3 in C57BL/6 (N=4) and 6.8 ± 1.8 in TASK1/3 KO mice (N=4), with maximum responses (10μM) of 173 ± 37 % and 178 ± 15 %, respectively, of the response to 50mM KCl. For serotonin the pEC_{50} and maximum responses were, respectively, 6.9 ± 0.3 and 379 ± 94 % in C57BL/6 mice (N=4) and 7.1 ± 0.1 and 267 ± 31 %, respectively, for TASK1/3 KO (N=4). Statistical analysis of both pEC_{50} and the efficacy of PE and serotonin showed no difference between the TASK1/3 KO and their wild type control.

The PA isolated from TASK1/3 KO mice does not display any response to 10mM KCl, K^+ channel modulators or nifedipine, suggesting that the E_m is not depolarised and that artery is fully dilated as in the C57BL/6 mice. Responses to the agonists PE and serotonin were also unchanged. Further experiments are in progress to investigate other possible effect of TASK-1 deletion, in order to discriminate the contribution of the TASK channels to the overall background K^+ channels in PA smooth muscle cells. The results thus far do not, however, support a major physiological role for TASK-1 in mouse PA.

Aller MI et al.(2005). *J Neurosci* **25**, 11455-11467.

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PC51

Modulation of Kv2.1 assembly contributes to the changes in vascular smooth muscle cell excitability in a hypertensive mouse strain

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Essential hypertension involves a gradual and sustained increase in total peripheral resistance reflecting an increased vascular tone, which is associated with a depolarization of vascular smooth muscle cells (VSMCs) and relies on a change in the expression profile of voltage-dependent ion channels (mainly Cav and Kv channels) that promotes arterial contraction. Kv channels are principal contributors to determine the resting membrane potential of VSMCs. However, the changes in their expression and/or modulation during hypertension are poorly defined, mainly due to their large molecular diversity. Here we have undertaken the study of the molecular and functional characterization of Kv and KCa channels in VSMCs in essential hypertension by using VSMCs from mesenteric arteries obtained from a hypertensive inbred mouse strain, BPH, and the corresponding normotensive strain, BPN. Mice were killed by decapitation after isofluorane anesthesia. Real-time PCR using low-density taqman® arrays revealed a differential distribution of K^+ channel subunits mRNA between both strains, the most conspicuous change being the "de novo" expression of Kcng3 in hypertensive phenotype. To study the functional relevance of this change, we characterized the electrophysiological properties of freshly dissociated VSMCs of BPN and BPH mice with the patch-clamp technique. Both KCa current density, (500 nM paxilline-sensitive current) and Kv current density (paxilline-resistant current) were significantly smaller in BPH cells at all voltages. Application of selective Kv2 blockers as 50 nM stromatoxin or 20 nM guangxitoxin induced a reduction of the current amplitude that was significantly smaller in BPH cells, suggesting that the reduction in the functional expression of Kv2 currents in BPH cells contribute to the decreased Kv current in these cells. These findings could be explained by the "de novo" expression of Kv6.3 mRNA in BPH VSMCs, as Kv6.3 subunits associate with Kv2 channels to form heteromultimers with decreased current amplitude. In agreement with this, Kv6.3 protein could be detected by western-blot in BPH but not in BPN arteries. Moreover, intracellular application of anti-Kv6.3 antibody decreased Kv current amplitude in BPH VSMCs but had no effect in BPN ones, demonstrating a functional role of this subunit in hypertensive VSMCs. These data suggest that the expression of Kv6.3 channels in resistance vessels could contribute to the natural development of hypertension, and can help to understand the molecular basis of this complex and multifactorial disease.

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PC52

Inhibitory actions of mibefradil and flunarizine in human vas deferens

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It has been reported that Ca^{2+} currents in isolated human vas deferens smooth muscle cells exhibit components that are differentially sensitive to Ni^{2+} (T-type current) and verapamil (L-type current). Thus, Park et al., (2004) suggested that T-and L-type VOCs contribute to the tissue contractility. This study investigated the effects of T-type Ca^{2+}-antagonists, mibefradil and flunarizine compared to nifedipine, a prototype L-type Ca^{2+} antagonist. Given that mibefradil and flunarizine also block L-type VOCs, their potencies in this tissue as L-type Ca^{2+} antagonists were determined.

Human vas deferens specimens obtained after elective vasectomies (with consent of patients and College Ethical approval) were cut into strips of longitudinal muscle or rings of circular muscle. These were set up for tension recording and superfused with Krebs medium (36°C). Contractions were evoked by noradrenaline (NA) in the presence of Ca^{2+} antagonists and expressed as a percentage of drug-free controls (mean ± S.E.M). Statistical analysis was by one-way ANOVA followed by *a priori* t-test using within-groups variance from ANOVA. Differences between time-matched controls and drug groups were considered significant at $P < 0.05$.

NA (0.1-100 μM) evoked rhythmic and tonic contractions, which were inhibited by nifedipine (0.01-0.1 μM) or by mibefradil (1-10 μM) such that contractions to NA (100 μM) in longitudinal and circular muscles were reduced respectively by 88.4 ± 1.3%, (n=9) and 87.03 ± 2.7%, (n=8) in nifedipine (0.1 μM); by 89.1 ± 3.4%, (n=5) and 68.5 ± 3.8%, (n=7; $P< 0.05$) in mibefradil (10 μM) and modestly by 18.3 ± 6.3% (n=6) and 27.0 ± 10.8% (n=5) in flunarizine (10 μM). The drugs' potencies as antagonists of L-type VOCs were determined against contractions to high K^+ (120 mM, in the presence of FPL 64176, 1 μM). The contractions in longitudinal and circular muscle had different times to peak and decline but were inhibited comparably by nifedipine (IC_{50} longitudinal and circular muscle, ~2 nM) or by mibefradil (IC_{50} longitudinal muscle, 1.1 μM; circular muscle, 2.4 μM) and were insensitive to flunarizine (up to 30 μM). These results suggest that the effects of mibefradil and flunarizine, at concentrations found to be effective against noradrenaline-induced contractions of human vas deferens involve the blockade of L-type VOCs. The difference in the effect of mibefradil in longitudinal and circular muscle may involve factors that modulate the activation and/or sensitivity of L-type VOCs to the drug. Overall, the results indicate that noradrenaline-induced contractions of human vas deferens depend primarily on nifedipine-sensitive L-type VOCs than on mibefradil or flunarizine-sensitive T-type VOCs.

Park SY et al. (2004) J Urol 172, 628-633.

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

PC53

Human saphenous vein vascular smooth muscle cells mechanically damaged during vein harvesting as coronary artery bypass graft (CABG) co-express iNOS and ET-1

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The human saphenous vein (SV) suffers trauma during harvesting for CABG which reflects on the graft patency (Souza 1996, Souza et al. 2006, Dashwood and Loesch 2007). Commonly used harvesting procedures involve stripping the pedicle of connective tissue from the vein followed by the distension to overcome venospasm (Souza 1996). Such procedures cause structural damage of the vein vascular smooth muscle cells (VSMC) and rapid expression of immunoreactive iNOS (Loesch et al. 2006). Here, we used standard protocols of immunolabelling for confocal and electron microscopy (EM) and Western blot analysis to study the co-expression of iNOS and ET-1 in medial VSMC of SV harvested in patients undergoing CABG.

Following local ethics committee approval and patients' informed consent, segments of SV harvested for CABG (n=10) were collected during surgery. For EM, SV samples were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde prior to single and double immunolabelling with ExtrAvidin and immunogold methods using antibodies to iNOS (polyclonal SC-651, Santa Cruz Biotech) and ET-1 (monoclonal MCE-6901-01, Peninsula Labs). For the confocal microscopy frozen SV sections were labelled with iNOS and ET-1 antibodies in conjunction with Alexa Fluor® 568 and 488 (Molecular Probes), respectively.

Both confocal and EM revealed the co-expression of iNOS and ET-1 in VSMC of SV harvested for CABG (Fig. 1A-C and 2A-C). The immunoreactivity was predominantly localised in structurally damaged VSMC (Fig. 2). Western blot analysis showed increased iNOS and ET-1 levels in extracts from stripped and distended SV as compared to SV harvested without stripping and distension (Fig. 2D). Conventional harvesting of SV for CABG induces both iNOS and ET-1 in structurally distorted VSMC. The functional consequences of co-expressed iNOS and ET-1 in venous VSMC require further investigation.

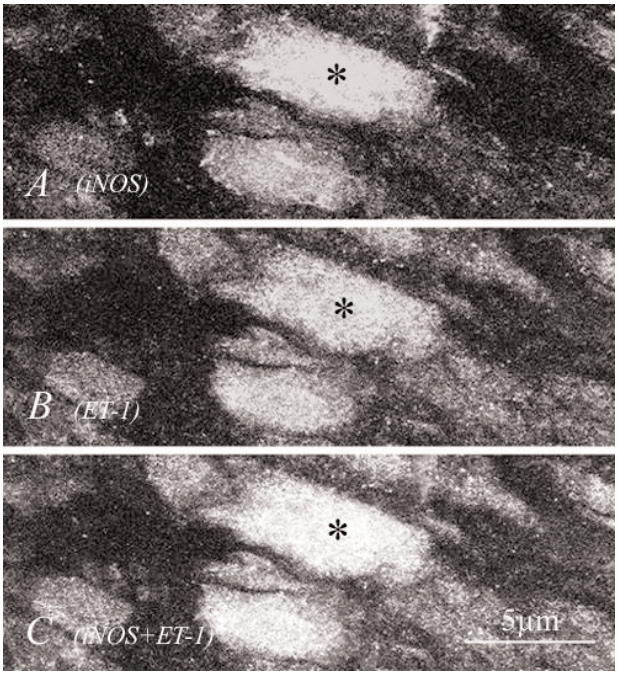

Figure 1

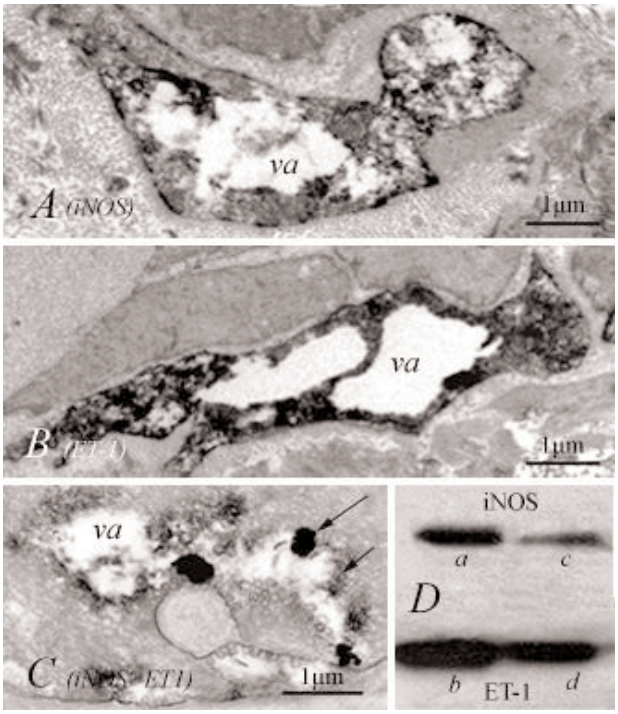

Figure 2

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Loesch A, Dashwood MR, Souza DSR (2006) Does the method of harvesting the saphenous vein for coronary artery bypass surgery affect venous smooth muscle cells? iNOS immunolabelling and ultrastructural findings Int J Surg 4:20-29.

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

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

PC54

Engineering microporosity in bacterial cellulose scaffolds

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The scaffold is an essential component in tissue engineering. By assessing a novel method, in which paraffin wax and starch particles with various sizes were placed in a growing culture of Acetobacter xylinum, three-dimensional nanofibril network of bacterial cellulose (BC) scaffolds with defined micropores were developed. The resulting BC had different morphologies and pore interconnectivity. Paraffin particles were incorporated throughout the scaffold, while starch particles were found only in the outermost area of the resulting scaffold. The porogens were successfully removed after culture with bacteria and no residues were detected with Electron Spectroscopy for Chemical Analysis (ESCA) or Fourier Transform Infra Red Spectroscopy (FT-IR).
The main purpose of use for the resulting scaffolds is tissue engineering of blood vessels, therefore SMC were selected as the cell type to culture in the scaffolds. The scaffolds were placed in Boyden chambers and seeded with smooth muscle cells (SMC). Platelet-derived growth factor was used as an attractant for cell ingrowth. Smooth muscle cell ingrowth and collagen production were investigated using histology and fluorescence procedures. Furthermore, organ bath techniques were used to evaluate the mechanical activity by stimulating the cells with relaxatory and contractile compound.
The SMC attached to and proliferated on and partly into the scaffolds. Smooth muscle cells were found throughout the BC scaffolds that were constructed using paraffin particles. Collagen production was detected microscopically after 2 weeks of culturing. There was no response to either relaxatory (papaverine) or contractile (adrenaline, noradrenaline, KCl or acetylcholine) stimulating compounds.

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

SA1

Dynamics of shear stress-induced remodelling

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Chronic changes in wall shear stress lead to vascular remodelling, characterized by increased vascular wall diameter and thickness, to restore wall shear stress values to baseline. Release of nitric oxide (NO) from endothelial cells exposed to excessive shear is a fundamental step in the remodelling process, and potentially triggers a cascade of events, including growth factor induction and matrix metalloproteinase (MMP) activation, that together contribute to restructuralisation of the vessel wall. MMPs, which are secreted as inactive zymogens (pro-MMPs), are rapidly cleaved and activated in in vivo models of chronic increased blood flow, and remain active until shear stress is normalized. Enhanced production of NO in high flow conditions, along with generation of reactive oxygen species through NADPH oxidase, combine to form peroxynitrite, which is important for MMP cleavage in the early phase of arterial remodelling. However, the later phase of this process implicates not only the activation of MMPs but also their ongoing synthesis. In this respect, we have uncovered a role for NF-kappaB as a key factor regulating the expression of MMP-9 and thus participating to the remodelling of vessels.

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

SA2

Stem cell differentiation into vascular cells induced by mechanical stress

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It was established that stem cells could repair lost endothelial cells and participate in the formation of neointimal lesions, because stem cells can differentiate into a variety of cells to replace dead cells or to repair damaged tissues. In this process, stem cells homing to the surface of injured vessels have to differentiate into vascular cells to exert their repairing functions (Xu Q. Circ Res. 2008;102:1011). Obviously, microenvironment where stem cells are attached play a crucial role in cell differentiation, although the spectrum of the initiators/stimulators responsible for such a differentiation remain to be clarified. It is well known that atherosclerotic lesions in the arteries are localized in some areas where blood flow is disturbed resulting in endothelial dysfunction/death in the presence of hyperlipidemia. Since recent findings suggest the potential role of stem cells in endothelial regeneration, it can be hypothesized that mechanical stress induced by blood flow can influence the differentiation process of stem cells (Xu Q. Nature Clin. Pract. Cardiovasc. Med. 2006;3:94). Support this hypothesis is recent findings that shear stress can induce differentiation of stem cells towards endothelial cell phenotype (Zeng et al. J Cell Biol. 2006; 174:1059), while stretch stress leads to differentiate into smooth muscle cells. It indicates that "good" blood flow (laminar shear stress) promotes endothelial differentiation from stem cells that tethering the surface of the vessel wall. How the stem cells sense and transduce the extracellular physical stimuli into intracellular biochemical signals is a crucial issue for understanding the mechanisms of stem cell differentiation. Collecting data derived from our and other laboratories showed that many kinds of molecules in the cells such as receptors, G proteins, cell cytoskeleton, kinases and transcriptional factors could serve as mechanoceptors directly or indirectly in response to mechanical stimulation implying that the activation of mechanoreceptors existing on the surface of stem cells is a crucial event. The sensed signals can be further sorted and/or modulated by processing of the molecules both on the cell surface and by the network of intracellular signalling pathways resulting in a sophisticated and dynamic set of cues that enable stem cell responses. The new findings indicate that signal pathway VEGF-Akt-HDAC-p53/p21 is crucial for stem cell differentiation into endothelial cells. The present presentation will summarize the data on shear stress-induced stem cell differentiation and the impact of such a differentiation on the pathogenesis of vascular diseases.

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

SA3

Stretch-dependent growth and differentiation in vascular smooth muscle

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Vascular adaptation to pressure and flow involves an intricate interplay of the endothelium, the smooth muscle layer and the extracellular matrix. Shear stress on the endothelium causes vasodilatation, which in turn increases tension in the vascular wall and causes stretch-induced growth (outward remodelling). Reduced endothelial function causes instead increased vascular tone, which leads to inward remodelling of the vascular wall and an increased wall-to-lumen ratio. Vascular smooth muscle cells exposed to physiological levels of stretch grow in a maintained contractile phenotype, in contrast to the

proliferative growth seen in vascular lesions and plaque formation. Organ culture of intact blood vessels is a useful technique to study molecular mechanisms of stretch-induced growth with maintained cell-cell and cell-matrix interactions. This technique has been applied to pressurized large and small arteries as well as to veins, revealing basically similar molecular mechanisms but with functional effects dependent on tissue organization and physiological role of the vessel. A convenient model is the rat or mouse portal vein, which has a dominantly longitudinal muscle layer that rapidly hypertrophies in vivo in response to increased portal pressure. Organ culture of portal veins under longitudinal stretch reproduces this growth response (1), and analysis of signal mechanisms shows that stretch causes synthesis of smooth muscle-specific contractile and cytoskeletal proteins, such as SM 22α, calponin, desmin and α-actin, by a sequence of events including biphasic (minutes and hours) phosphorylation of focal adhesion kinase (FAK), early (minutes) phosphorylation of the proliferation-related signal ERK1/2, and late (hours) Rho activation, cofilin phosphorylation and actin polymerisation (2,3). Synthesis of smooth muscle-specific proteins is regulated by the transcription factor serum response factor, in concert with cofactors such as myocardin and myocardin-related transcription factors, which are dependent on the state of actin polymerisation (reviewed in ref. 4). The actin filament stabilising agent jasplakinolide causes increased synthesis of smooth muscle proteins in the mouse portal vein, and also activates the ERK1/2 pathway, whereas effects of stretch on FAK phosphorylation or contractility are abolished (3). This suggests that actin filament dynamics are crucial for vascular remodelling responses. Cholesterol-rich membrane caveolae are important for integrating signal mechanisms in the plasma membrane, and studies in caveolin-1 deficient mice suggest that caveolae are needed for endothelium-dependent relaxation in response to flow, involving Akt phosphorylation and NO production. In contrast, stretch-induced responses in mouse portal vein do not involve Akt phosphorylation and are unaffected in caveolin-1 deficient mice (5). The signal mechanisms regulating contraction and growth responses seem to be integrated partly via the intracellular Ca^{2+} concentration, and evidence suggests that voltage-dependent Ca^{2+} influx via L-type membrane channels elicits smooth muscle differentiation via a Rho kinase dependent mechanism (6). In mouse portal vein, stretch-induced effects on cofilin phosphorylation, regulating actin polymerisation, are attenuated by L-type channel inhibition, correlating with decreased synthesis of smooth muscle marker proteins, while inhibition of Ca^{2+} influx via store-operated channels causes a global decrease in protein synthesis but does not inhibit stretch-induced cofilin phosphorylation or synthesis of smooth muscle-specific proteins. Thus Ca^{2+} exerts a dual influence on the regulation of protein synthesis in vascular smooth muscle. Since phenotype modulation of smooth muscle cells is associated with loss of voltage-dependent channels and gain of store-operated channels, the contractile and synthetic cellular phenotypes may be specifically susceptible to interventions targeting the different modes of Ca^{2+} entry.

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

SA4

Bio-mechanical activation and notch signaling - how vascular cells respond to stress !

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Over the last couple of years it has become increasingly clear that the Notch signaling pathway plays a pivotal role in the development and homeostasis of the cardiovascular system. The rapid increase in the number of publications that focus on the role of Notch signaling in regulating vascular cell function both in vitro and in vivo reflects the degree of interest in understanding the role of this pathway in vascular homeostasis. An emerging paradigm suggests that developmental gene regulatory networks are often recapitulated in the context of phenotypic modulation, vascular remodeling and repair in adult vascular disease. Notch receptor-ligand interactions, in conjunction with vascular endothelial growth factor (VEGF) and components of the Hedgehog (Hh) signaling pathway have all been implicated in vascular morphogenesis and modeling of the embryonic vasculature. The presentation will focus on the specific role of a Hh/VEGF/Ang axis in controlling vascular smooth muscle cell (SMC) growth (proliferation and apoptosis) through regulation of Notch signaling [1-5]. Using dynamic in vitro cultures of SMC under flow and pressure and ligated murine carotid arteries in vivo to mimic vascular injury, the components of these pathways in dictating the vascular SMC response to bio-mechanical injury will be addressed. Collectively, data will provide an insight into the coordinate regulation of Notch by sonic hedgehog (shh) and VEGF-A in adult SMC and thus may represent a future novel therapeutic target for intervention in vascular proliferative disorders.

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Am J Physiol Cell Physiol. 2005 289:C1188-96

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SA5

Mechanotransduction and the glycocalyx

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The surface of endothelial cells (ECs) is decorated with a wide variety of membrane-bound macromolecules that constitute the glycocalyx (GCX). As the most apical structure on the EC, the GCX senses the force (shear stress) of flowing blood and transmits it via the cytoskeleton throughout the cell to sites where transduction of force to biochemical response (mechanotransduction) may occur. In this presentation the structure of the GCX and many of the experiments that demonstrate its role in mechanotransduction and vascular remodeling will be reviewed. Experiments with enzymes that degrade specific glycosaminoglycan components have been used to show that the GCX mediates the shear-induced production of nitric oxide, a central process in cardiovascular control, while the same enzyme treatments do not affect shear-induced production of prostacyclin, another hallmark of EC mechanotransduction. These experiments reinforce the concept of distributed sites of mechanotransduction in EC. The characteristic remodeling of the EC cytoskeleton and intercellular junctions in response to shear stress are dependent on the GCX as well, and the experiments that support the role of the GCX in these processes will be reviewed as well.

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

SA6

Modulation of inflammatory responses of endothelial cells by changes in local shear stress

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Stable local haemodynamic microenvironments may determine the phenotype of endothelial cells (EC) in different regions of the circulation, but acute changes in flow might also modulate functional responses. We aim to understand how different levels or patterns of shear stress applied to endothelial cells regulate inflammatory responses, and in particular, leukocyte recruitment. For this purpose, we developed models in which human EC of various types (HUVEC from umbilical veins; HUAEC from umbilical arteries; HCAEC from coronary arteries) were cultured in glass capillaries coated with desired substrates. These constructs were conditioned by different levels of shear stress for different periods, or exposed to abrupt changes in shear. Conditioning could be combined with treatment with cytokines such as tumour necrosis factor-α (TNF) and interleukin-1β (IL-1), and adhesion and migration of flowing neutrophils analysed as an 'inflammatory' readout.

Initial studies showed that conditioning of HUVEC for 24h at increasing shear stress acted to powerfully suppress responses to TNF, but not IL-1, judged by neutrophil recruitment (Sheikh et al., 2003; 2005). However, in subsequent studies, responses to both cytokines were suppressed by shear conditioning for HUAEC and HCAEC. Studies in which culture medium constituents, such as basic fibroblast growth factor, were swapped, indicated that this difference between the endothelial cells arose from culture conditions rather than from an in vivo imprinted phenotype. The fact that the original 'static' cultures of each cell type showed similar abilities to support adhesion and migration of neutrophils also indicated that the phenotypes of EC were plastic and could be re-set by conditioning in vitro. Taking this further, we analysed expression of selected genes in HUVEC immediately after digestion from veins, after standard culture in vitro and then after shear conditioning. Changes were induced by initial culture, which were reversed in part at least by the return to a shear environment. Thus it seems that endothelial phenotype is highly pliable, with enviromental factors, such as shear stress and growth factors, modifying responses in an interlinked but reversible manner.

We thus investigated whether the less responsive state induced in vitro by shear stress would change when flow was ceased. This might be relevant to ischaemic conditions in vivo (for instance linked to thrombo-embolism, surgical interventions or organ transplantation), where an inflammatory response typically follows reperfusion. We found that response of EC to TNF only increased slowly over 24-48h after cessation of flow, and that if a very low level of shear stress was retained, then the response remained suppressed (Matharu et al.,2008). In all of the above, functional

changes could be linked to changes in expression of receptors such as E-selectin and the shear-sensitive transcription factor KLF-2, and in activation of NFkB. However, anomalies in the correlations between the different responses indicated that other modulatory events occurred outside of these well-described mediators.
Nevertheless, most of the changes noted were over hours and linked to modulation of gene expression. In studies of flow reduction, however, we also noted an early pro-adhesive response. In the period around 60-120min after cessation of flow, neutrophils adhered to otherwise unstimulated HUVEC, when included in medium used to ' reperfuse ' it (Matharu et al., 2008). Others have shown that an early oxidative response follows flow cessation (Manevich et al., 2001). Here, the transient neutrophil adhesion observed was attributable to oxidant-induced upregulation of expression of P-selection on the EC.
Taken together, these studies suggest that local conditioning of endothelial cells contributes to vessel- and organ-specificity in inflammatory responses, and predisposition of certain sites to development of inflammation. At the same time, acute responses of EC to disruption of flow may contribute to outcome of ischaemia and reperfusion.

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

SA8

Mechanotransduction of shear stress and regulation of microvascular resistance

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Regulation of wall shear stress (WSS) in microvessels is an important local mechanism regulating microvascular resistance, thus tissue blood flow and at the same time aiming to optimize circulatory energy consumption. The pressure drop across a microvascular unit indicates the loss of circulatory energy that is primarily due to WSS. In arterioles, an increase in WSS results in a substantial, endothelium dependent dilation, whereas in venules, it elicits only a limited increase in diameter. In arterioles, only dilator mediators, such as nitric oxide (NO), prostaglandins (PGI2/PGE2) endothelium derived hyperpolarizing factor(s) EDHFs, are released, whereas in venules, in addition to these mediators, constrictor prostaglandins are also released. WSS is the function of wall shear rate (WSR) and blood viscosity (hematocrit and plasma viscosity), which parameters can be substantially different in arterioles and venules. Thus it is likely that regulation of WSS is achieved by different mechanisms in arterioles and venules. In the arterial side WSS is determined primarily by high WSR (high velocity/narrow diameter), whereas in the venular side WSS is determined primary by the hematocrit related viscosity. Accordingly, it seems that at the arteriolar side WSS is regulated primarily by substantial increases in diameter, which can be achieved, since arterioles have a substantial basal tone and because WSR is high, thus diameter changes have less impact on apparent viscosity. In the venular side however, WSR is low, thus changes in diameter can substantially affect hematocrit-induced apparent viscosity of blood. Thus in the microcirculation there is a complex interrelationship between rheological parameters, structural and functional properties of microvascular network. The nature of endothelial mediation of WSS seems to be gender specific, and it can change with age and in diseased conditions. The primary sensors of changes in WSS are likely to be the glycocalyx, as part of endothelial surface layer and platelet endothelial cell adhesion molecule (PECAM), whereas the cytoskeleton and integrins are the next serially coupled molecules of mechanotransduction. There are still several controversial issues however, such as the role of increase in [Ca2+]i and reactive oxygen species in the release of various endothelial mediators and the nature and mediation of WSS-induced responses in cerebral microvessels. The fascinating role of endothelium in the mechanotransduction of shear stress into vasomotor response is still an open field for further discoveries.

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

SA9

Haemodynamic forces as *in vivo* angiogenic stimuli

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Chronic vasodilator treatment intensifies levels of shear stress in capillary beds, stimulating a specific form of angiogenesis termed longitudinal splitting, while sustained muscle overload induces the more familiar sprouting form of capillary growth (Egginton, 2001). Similar findings are observed in both rats and mice. These differently directed mechanical forces (luminal and abluminal, respectively) expand the capillary bed to a similar extent over a similar time-course, but by different growth processes characterised by unique features in structure, gene expression and protein complement (Williams et al. 2006).
Elevated capillary shear stress is transduced into an angiogenic response irrespective of the vasodilator mechanism employed, with the essential involvement of endothelial nitric oxide synthase. Surprisingly, the rate of capillary growth thus recruited

is largely mirrored by the rate of capillary regression on withdrawal of vasodilator treatment, involving reciprocal changes in VEGF and eNOS, but low levels of endothelial mitosis or apoptosis. *In vitro* responses of endothelial cells (EC) to elevated shear stress leads to differential regulation of a number of genes involved in the control of angiogenesis. Interestingly, shear appears to regulate molecules that modify VEGF effects, rather than VEGF *per se*. EC motility is increased by VEGF, although shear appears to inhibit EC migration when confluent layers are wounded in parallel to flow axis.

Capillary growth following overload is critically dependent on matrix metalloprotease activity and, as with shear-dependent growth, the presence of elevated VEGF levels. Angiogenesis does not appear to be dependent on a threshold stimulus, as seen by the differential in capillarity and EC proliferation in response to graded muscle overload. However, the response is mediated by a threshold, rather than a graded response in VEGF or Flk-1, suggesting the degree of angiogenesis is likely controlled by interactions among pro-angiogenic stimuli. There was little evidence for synergistic potentiation when applied in combination with either high flow (pharmacological dilatation) or low flow (surgical ischaemia), suggesting that feedback control limits the extent of angiogenesis in skeletal muscle.

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

SA10

Wall shear stress distribution in the arterial system. Reconsiderations based upon *in vivo* measurements

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Wall shear stress (WSS), the drag of the flowing blood exerted on endothelial cells, is an important determinant of endothelial cell function and gene expression. WSS can be estimated from wall shear rate (WSR) and local blood viscosity, WSR being defined as the radial derivative of blood flow velocity at the wall. In large arteries in man, WSR is derived from velocity profiles, non-invasively recorded by means of ultrasound or Magnetic Resonance Imaging (MRI). In our studies we use ultrasound, because of its better spatial and especially temporal resolution than of MRI. The velocity profiles are recorded with a two-dimensional ultrasound imaging device combined with a dedicated acquisition and processing system as developed in our institute. WSS is estimated from the derived WSR and whole blood viscosity, because the thin plasma layer can be ignored relative to the size of the ultrasound sample volume (sample length 300 μm; 50% overlapping). In arterioles WSS is measured directly or estimated from WSR and plasma viscosity, WSR being derived from velocity profiles recorded with labeled blood platelets or nanometer particles as velocity tracers. Originally, the displacement and the radial position of the velocity tracers were determined by hand, a time consuming procedure. Recently a computerized two-dimensional particle tracking technique has been developed to determine radial position and displacement of the particles, automatically providing velocity profiles. In arterioles plasma viscosity can be used to calculate WSS, because the velocity tracers come as close to the wall as 0.2-0.5 μm.

The *in vivo* measurements have shown that the theoretical assumptions regarding WSS in the arterial system and its calculation are far from valid. In both arteries and arterioles, velocity profiles are flattened rather than fully developed parabolas. This implies that WSR has to be derived from recorded velocity profiles. Assuming a parabolic velocity profile will on the average underestimate derived WSR by a factor of 2-3. In humans mean WSS varies along the arterial tree and is higher in the common carotid artery (1.1-1.3 Pa; 1 Pa=10 dyn cm^{-2}) than in the brachial artery (0.4- 0.5 Pa) and the common (0.3-0.4) and superficial (0.5 Pa) femoral arteries. Only in the common carotid artery mean WSS is close to the theoretically predicted value of 1.5 Pa. The lower mean WSS in conduit arteries can be explained by the high peripheral resistance in these arteries, reducing mean volume flow and inducing reflections. Dilation of the femoral artery vascular bed results in mean WSS values in this artery not significantly different from those in the common carotid artery. This observation indicates that at rest mean WSS is largely determined locally. Although small, the difference in mean WSS between the common and the superficial femoral artery is significant, the former artery seeing reflections from both the deep and the superficial artery, while the latter one only sees reflections from its own vascular bed. Also in the carotid artery bifurcation differences in mean WSS have to be appreciated. It is of interest to note that in both the femoral and the carotid artery bifurcation the differences in mean WSS are associated with local differences in intima-media thickness (IMT): the lower mean WSS is, the larger IMT will be. Also in animals mean WSS is not constant along the arterial tree. In arterioles mean WSS varies between 2 and 10 Pa and is dependent on the site of measurement in the arteriolar network. Across species mean WSS in a particular artery decreases linearly with increasing body mass on a log-log scale, in the infra-renal aorta from on the average 8.8 Pa in mice to 7.0 Pa in rats and 0.5 Pa in humans (flow velocities being similar). A similar pattern can be found in the carotid artery, varying on the average from 7.0 Pa in mice to 4.7 Pa in rats and 1.2 Pa in man.

The observation that mean WSS is far from constant along the arterial tree indicates that Murray's cube law on flow-diameter relations cannot be applied to the whole arterial system. At the present state of the art it can be concluded that the exponent of the power law varies from 2 in large branches of the aortic arch to 2.55 in coronary arteries and 3 in arterioles. The *in vivo* findings also imply that in *in vitro* investigations no average calculated shear stress value can be taken to study gene expression by endothelial cells derived from different vascular areas or from the same artery in different species. The cells have to be studied under the shear stress conditions they are exposed to in real life.

Sensing and transduction of shear stress is likely to be in part mediated by the endothelial glycocalyx, because pretreatment of endothelial cells with hyaluronidase, leading to substantial reduction of glycocalyx dimensions, attenuates shear stress induced release of nitric oxide and shape changes of these cells. Therefore, modulation of shear stress sensing and transduc-

tion by altered glycocalyx properties, for example, in atherogenesis, should be considered.

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SA11

Endothelial phenotype plasticity in unstable flow regions of the cardiovascular system: differential microRNA expression

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Arterial endothelial phenotype heterogeneity significantly influences athero-suceptibility and athero-protection in vivo. Differential transcript profiling of endothelium in susceptible arterial regions of normal adult swine displays a balance of pro-pathological and protective transcript profiles when compared with adjacent regions that rarely, if ever, develop atherosclerosis. The endothelial phenotype in vivo and in vitro is highly sensitive to the local blood flow characteristics via mechanotransduction and transport mechanisms. Athero-susceptible locations map to regions of hemodynamic (and biomechanical) spatio-temporal complexity where transient vortices within flow separation zones promote flow reversal, oscillatory shear stresses, low flow velocities and low mean shear stresses, steep spatial shear stress gradients, and occasional turbulence (chaotic flow). Differential phenotypes are detectable in endothelium in vivo at the mRNA, protein, post-translational, and functional levels. We now demonstrate that differential microRNA expression that targets specific gene and protein expression is part of the regulation of endothelial phenotype.

Regulation of mRNA stability and translation occurs by highly conserved small non-coding microRNAs (miRNAs). Microarrays identified 3 miRNA families (let-7, miR10, miR26) as upregulated in endothelium from an atheroprotected region of thoracic aorta relative to a nearby atherosusceptible region (aortic arch). By qRT-PCR, expression levels of miR10a and 10b were 4.9 and 20.7-fold higher respectively at protected (n=8) vs susceptible (n=10) regions; in contrast miR7d and miR26b were elevated <2-fold. The copy number of miR10a was greater than that of miR10b and its preferential expression in endothelium in situ was detected by immunofluorescence. 854 putative targets of miR10a/b were organized into interactive pathways using IngenuityTM. Sequences of 138 of the most interactive genes were entered into the Sfold program that assesses target secondary structure as an important predictor of miRNA-target hybridization sensitivity. Among miR10a/b targets showing high total hybridization energy were Flt-1 (VEGFR1), Hox-D10 and VEGFA. Endothelial expressions of these genes were suppressed in protected vs susceptible regions in a reciprocal relationship with miR10a/b. Cultured endothelial cells overexpressing miR10 suppressed Flt-1 gene expression. The data show miRs to be flow responsive and suggest miR10a/b to be important regulators of endothelial gene expression in atheroprotection/susceptibility.

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SA12

Effects of shear flow on selectin expression in endothelial cells co-cultured with smooth muscle cells

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ABSTRACT

In vitro co-culture of endothelial cells (ECs) with smooth muscle cells (SMCs) induced rapid and sustained increases in EC expression of E-selectin. By using inhibitors, dominant-negative mutants, and siRNA, we found that activations of JNK and p38 are critical for the co-culture-induced E-selectin expression. Gel shifting and chromatin immunoprecipitation assays showed that SMC-co-culture increased the NF-κB-promoter binding activity in ECs. Inhibition of NF-κB activation blocked the co-culture-induced E-selectin promoter activity. Protein arrays and neutralizing antibodies showed that IL-1β and IL-6 produced by EC/SMC co-cultures contribute to the co-culture-induction of EC signaling and E-selectin expression. Pre-shearing of ECs inhibited the co-culture-induced EC signaling and E-selectin expression. These findings serve to elucidate the molecular mechanisms underlying the SMC-induction of EC E-selectin expression and the shear stress-protection against this SMC-induction.

INTRODUCTION

The aim was to elucidate the mechanisms that regulate the SMC-induced E-selectin expression in ECs and its inhibition by shear stress. This article reviews several publications in our labs [1-5].

MATERIALS AND METHODS

Cell culture. ECs were isolated from fresh human umbilical cords. SMCs were obtained from Clonetics (Palo Alto, CA).

Preshearing of ECs. ECs were seeded onto the outer side of the membrane (10-μm-thick, 0.4-μm pores, pre-coated with fibronectin) of a transwell. After incubation for 24 h, the membrane with ECs was incorporated into a flow chamber on the underside of the transwell for shear stress applications at a high (HSS, 12 dyn/cm2) or low level (LSS, 0.5 dyn/cm2) for 4 or 24 h.

Co-culture of ECs and SMCs. After EC preshearing, the inner side of the membrane was seeded with SMCs under static condition, thus forming an EC/SMC co-culture system. Controls had no cells or ECs instead of SMCs on the inner side. To study the effect of distance of EC/SMC separation, ECs seeded on the

membrane were separated by 1 mm from the SMCs plated on an outer chamber (EC/M/SMC).

RESULTS AND DISCUSSION

Pre-exposure of ECs to HSS, but not LSS, for 24 h inhibits SMC-induced E-selectin expression in ECs. EC/SMC co-culture induced an increase in E-selectin mRNA expression in ECs within 1 h. Separation of ECs from SMCs by 1 mm retarded the E-selectin expression. Pre-shearing of ECs at HSS for 24 h inhibited the co-culture-induced E-selectin expression; this was not seen with LSS. Thus, SMCs induced EC expression of E-selectin via a paracrine effect that can be inhibited by HSS.

SMC-induced EC expression of E-selectin and its inhibition by shear stress are mediated by the JNK and p38 pathways. The phosphorylation of ERK, JNK, p38, and Akt in ECs showed transient increases after co-culture with SMCs. The co-culture-induced E-selectin expression was inhibited by inhibitors for only JNK and p38. Pre-shearing at HSS, but not LSS, for 24 h inhibited the co-culture-induced JNK and p38 phosphorylation. JNK- or p38-specific siRNA caused significant inhibition of the co-culture-induced E-selectin expression. The increase in E-selectin-Luc promoter activity in ECs by SMC-co-culture was prevented by pre-shearing at HSS, but not LSS. Thus, the SMC-induction of EC expression of E-selectin is mediated by JNK and p38 and blocked by HSS.

SMC-induced EC expression of E-selectin and its inhibition by shear stress are dependent on NF-κB. Inhibition of NF-κB abolished the co-culture-induced E-selectin promoter activity. Co-culture with SMCs increased the NF-κB-DNA binding activity in EC nucleus, which was inhibited by HSS (but not LSS). Thus, the co-culture induced E-selectin expression is mediated by NF-κB, and this effect is inhibited by HSS.

IL-1β and IL-6 produced by EC/SMC are the major factors contributing to the SMC-induced signaling and E-selectin expression in ECs. Using a human cytokine array system, we identified IL-1β and IL-6 as the proteins released from EC/SMC at significantly higher levels than EC/EC (>4-fold). Neutralizing antibodies against IL-6 and/or IL-1β inhibited the co-culture-induced increases in E-selectin mRNA, JNK and p38 phosphorylation, and NF-κB-DNA binding activity.

IRAK and gp130 are involved in regulatory effects of SMC-co-culture and shear stress on EC E-selectin expression. The SMC-induced E-selectin expression in ECs was suppressed by siRNAs against gp130 (IL-6 receptor) and IRAK (complex with the IL-1β receptor upon its stimulation). The co-culture-induced phosphorylations of gp130 and IRAK were inhibited by pre-shearing at HSS (but not LSS) for 24 h.

These results indicate that the SMC-induction of E-selectin in ECs involves the paracrine action of IL6 and IL-1β on their receptors to activate the JNK, p38 and NF-κB, and that this effect can be inhibited by high shear stress.

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SA13

Shear stress, inflammation and atherosclerosis

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Atherosclerosis is the disease with the highest mortality in the western world. Despite its large socio-economical impact, the underlying mechanisms are only partially known. It has been accepted for decades that atherosclerosis is a lipid driven disease, despite the fact that risk factors related to lipid metabolism only partially explain atherogenesis. Furthermore, new therapies specially focussed upon lipid metabolism only partially reduce plaque size. Recently two concepts – inflammation and blood flow/shear stress – have undergone a renaissance and gained a lot of interest as complementary explanations for plaque formation and these concepts will be the topic of the present manuscript.

The role of inflammation became apparent from a series of mouse studies where systematically parts of the immune system were knocked down, before the induction of atherosclerosis. These studies identified inflammation as an independent mechanism attributing to plaque formation, and based upon these results and further studies atherosclerosis is considered a lipid driven inflammatory disease. The effect of blood flow in atherosclerosis is based upon the observation that plaques are not evenly distributed over the arterial system. These predilection sites are at or near side branches, i.e. where blood flow is non-uniform, or at the lesser curvature of bends, i.e. where blood velocity is relatively low. The effect of blood flow on the vessel wall is through shear stress which alters the physiology of endothelial cells. Shear stress (τ N/m2 or Pascal (Pa)) arises from the friction between two virtual layers in a fluid, and is induced by the difference in movement of the two layers (dv/dr s-1; in case of a cylindrical tube) and the "roughness" (or viscosity Pa.s) between these layers ($\tau = dv/dr * \eta$). Shear stress also arises at the interplay between blood and the endothelial layer, where it induces a shearing deformation of the endothelial cells. This shearing deformation affects the phenotype of the endothelial cells and thereby the inflammatory component and plaque progression/composition.

This paper describes the interaction between shear stress and inflammation. We will first describe recent findings on the sensing mechanism of shear stress by the endothelium. Subse-

quently, pro-inflammatory pathways modulated by shear stress in endothelial cells, followed by the effect of shear stress on plaque progression and plaque composition. At the end we will discuss new findings related to longitudinal plaque heterogeneity.

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SA14

Engineering vascular grafts

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Coronary artery and peripheral vascular disease are associated with significant morbidity and mortality. In these patients, surgical intervention including small diameter bypass grafting with autologous veins or arteries is a common treatment. However, many patients lack suitable autologous vessels, either because these vessels are diseased themselves or because of previous surgery, and in these cases, synthetic grafts are often used. Unfortunately, many of these grafts fail because of the low number of endothelial cells and the proportion of the endothelialised surface remaining after exposure to flow, which results in acute thrombosis and subsequent occlusion of the vessel.
At the University of Manchester, we are developing small calibre vascular grafts for coronary or peripheral bypass and vascular access grafts for haemodialysis. These grafts are based on electrostatically spun polyurethane and polycaprolactone with controlled porosity and biodegradability and are coated with specific vascular matrix molecules to regulate cell adhesion, migration, and growth factor bioavailability. This talk will focus on the approaches we are using to improve both the initial adhesion of endothelial cells to the graft surface and the retention of these cells to this surface following restoration of flow. Our studies have revealed significant new insights into the biology of endothelial cell attachment to surfaces coated with specific vascular matrix molecules, with important implications for the design of the next generation of vascular grafts.

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SA15

Waveform analysis and microcirculatory function

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Risk factors for cardiovascular disease mediate their effects by altering the structure and function of wall and endothelial components of arterial blood vessels. Pathological change in the microcirculation plays a pivotal role in promoting end-organ dysfunction that not only predisposes to further organ damage but also increases the risk for future macrovascular events. The microcirculation is recognised as the site where the earliest manifestations of cardiovascular disease occur that may play a pivotal role in driving the atherosclerotic process in conduit vessels(1).
Ultrasound and the Doppler effect have been long used to measure blood velocity and its temporal and spatial variation within the vascular tree in order to diagnose and monitor vascular disease. Changes in morphology of the linear flow velocity spectral envelope is not representative of any single vessel but is determined by changes in the properties and total cross-sectional area of downstream vascular networks(2). Quantitative analysis of Doppler-time velocity waveforms that reflect measures of flow pulsatility (eg resistive index, pulsatility index) can mirror changes in downstream vascular resistance and may predict future adverse clinical outcomes(3). In a series of studies in different patient groups we have shown these derived indices often provide misleading information in relation to the haemodynamic actions of drug interventions and are not sensitive in detecting early microvascular dysfunction in different vascular beds in humans(4,5). Novel algorithms that enable quantitative analysis of the Doppler velocity spectral envelope over the duration of the cardiac cycle provides more sensitive information in relation to the haemodynamic action of drugs and identification of early microvascular abnormalities in humans. Data will be presented showing the superiority of this approach in identifying early microvascular abnormalities in waveforms obtained from different arterial territories in different disease states associated with increased cardiovascular risk.
Techniques capable of detecting microvascular damage and monitoring response to therapeutic interventions, especially in vulnerable target organs of interest, may improve risk stratification and could represent a valuable surrogate for future cardiovascular outcome.

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PL1

Regulation of gene expression by hypoxia

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Decreased oxygen availability (hypoxia) is a hallmark feature of the microenvironment in a number of conditions including arthritis and inflammatory bowel disease (IBD). Recent advances in our understanding of oxygen-dependent cell signaling have uncovered several mechanisms by which hypoxia impacts upon the development of inflammation through the coordinated expression of adaptive, inflammatory and apoptotic genes. Two central transcription factors involved in the regulation of this response are Hypoxia Inducible Factor (HIF) and Nuclear Factor-κB (NF-κB) which display different degrees of sensitivity to activation during hypoxia. Furthermore, HIF and NF-κB demonstrate an intimate interdependence at several mechanistic levels. Recent studies indicate that these pathways may represent important new therapeutic targets in diseases characterized by hypoxic inflammation.

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