C105

Effect of flufenamic acid on VEGF-mediated increased hydraulic conductivity (L_p) in frog mesenteric microvessels

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VEGF is a potent vasodilator, angiogenic and vascular permeability factor. VEGF has been shown to increase permeability through activation of calcium influx into the endothelial cell cytoplasm. Furthermore, transfection of a non-endothelial cell line with both VEGF-R2 and the non-specific cation channel TRP6 resulted in a significant increase in calcium upon addition of VEGF (Foster *et al.* 2002). To determine whether TRP6 was involved in the mechanism of VEGF-mediated calcium influx, we have measured the effects of a differential modulator of the TRP channels, flufenamic acid (FFA) on the VEGF-mediated increase in permeability. Although FFA has actions on other targets as well, it inhibits TRP3 and enhances the activation of TRP6.

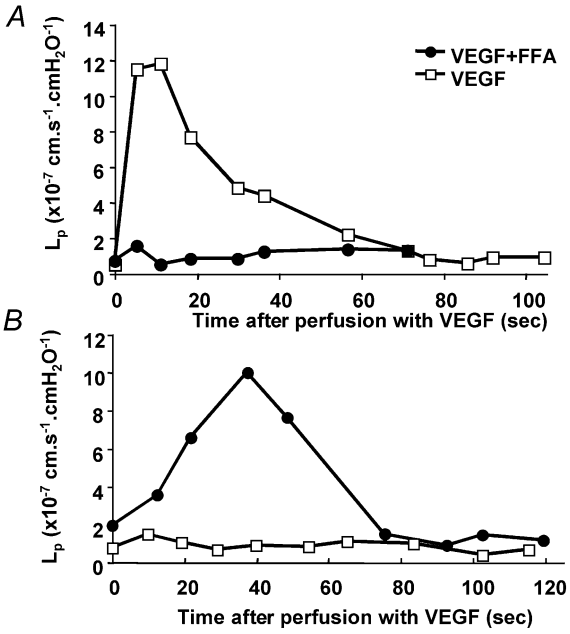

Figure 1.

Frogs (*Rana temporaria*) were anaesthetised by submersion in MS222 (1 mg ml^{-1}) and anaesthesia maintained by constant superfusion of the mesentery with frog Ringer solution containing MS222 (0.2 mg ml^{-1}). L_p (mean ± S.E.M. × 10^{-7} cm s^{-1} cmH$_2$O^{-1}) was measured during perfusion with 1 nM VEGF without or with 100 μM FFA in 20–30 μm diameter mesenteric microvessels, using a modification of the Landis Michel method (Michel *et al.* 1974). The vessel was then washed out for 20 min with 1% BSA and L_p measured during VEGF perfusion with or without FFA, respectively. At the end of the experiment frogs were killed by cranial destruction. Figure 1 shows the effects of FFA and VEGF on two vessels. In Fig. 1*A*, VEGF alone resulted in a significant increase in L_p from 0.5 to 11.8. In the presence of 100 μM FFA, VEGF only increased L_p from 0.7 to 1.5, i.e. a significant inhibition. In Fig. 1*B* VEGF increased L_p only slightly, from 0.8 to 1.7. However, after 10 min perfusion with FFA, VEGF increased L_p from 1.9 to 10.0. This pattern was typical and overall, in eleven vessels, five gave a response to VEGF that was inhibited by FFA, and six a response that was enhanced by FFA. The mean baseline L_p (during perfusion with BSA or FFA) was significantly lower in vessels in which FFA had an inhibitory effect (1.2 ± 0.8) rather than an enhancing effect (3.4 ± 0.9). When the vessel groups were analysed separately, in vessels in which VEGF caused a weak increase in L_p (2.3 ± 0.4-fold), FFA perfusion caused a significant enhancement of the response (to 3.6 ± 0.6-fold, $P < 0.01$, paired *t* test). This was greater than the response in the presence of FFA in the inhibitory vessels (1.2 ± 0.3-fold, $P < 0.02$, *t* test). In summary these data show that VEGF can either increase or decrease L_p during FFA perfusion, supporting the hypothesis that different TRP channels regulate VEGF-mediated permeability.

Foster RM *et al.* (2002). *J Vasc Res* (in the Press).

Michel CC *et al.* (1974). *QJEP* **59**, 283–389.

This work was supported by the BHF (BB2000030 and PG98023).

All procedures accord with current UK legislation.

C106

Signal transduction in IL-1β-induced cyclo-oxygenase-2 expression in human myometrial smooth muscle cells

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Inflammatory cytokines such as interleukin-1β (IL-1β) and TNF-α are thought to be involved in the initiation of labour (Brockelhurst, 1999). Preterm labour accounts for one-third of premature births, and one of the leading causes is intra-uterine infection as evidenced by increased levels of IL-1β and TNF-α. IL-1β increases expression of the enzyme cyclo-oxygenase-2 (COX-2), which catalyses the limiting step in the production of prostaglandins PGE_2 and $PGF_{2\alpha}$, involved in muscle contraction in preterm and term labour. The mechanisms underlying IL-1β-induced increases in prostaglandins via COX-2 are not fully elucidated. IL-1β activates the three major mitogen-activated protein kinase (MAPK) pathways, and there is evidence implicating p38 MAPK and jun kinase (JNK) in cytokine-induced COX-2 expression (Guan *et al.* 1998). The COX-2 promoter contains several regulatory elements including NFκB sites, and it is possible that p38 MAPK may mediate COX-2 transcription via activation of NFκB possibly through involvement of protein kinase C (PKC) pathways.

Expression of COX-2 in human myometrial smooth muscle cells (HMSMCs), obtained from lower segment myometrial biopsies (the study was approved by the ethical committee of GKT School of Biomedical Sciences), was examined including inhibitors of MAP kinases (SB203580, 2 μM, for p38; U0126, 250 nM, for ERK 1/2; and SP600125, 20 μM, for JNK), PKC (GF109203X, 10 μM) and NFκB (MG-132, 42 μM; and PG490, 1 μM). Western blot analyses revealed that IL-1β increased COX-2 expression via signalling pathways involving p38 MAPK, PKC and NFκB. In parallel Northern blots we showed that inhibition of p38 MAPK and PKC reduced COX-2 mRNA levels in cells challenged with IL-1β for 3 h.

To examine NFκB translocation, we used electrophoretic mobility shift assays (EMSA) with antibodies for NFκB subunits

and inhibitors of MAPKs and PKC. Using two NFκB COX-2 promoter sites, we have shown that these inhibitors do not affect translocation of p65 and p50 subunits to the nucleus. MG132, a proteosome inhibitor known to prevent degradation of IκB, reduced NFκB translocation, whereas PG490, an inhibitor of nuclear NFκB transactivation was ineffective. These data suggest that p38 MAPK and/or PKC may affect NFκB-mediated COX-2 transcription through a mechanism other than translocation to the nucleus after Iκ kinase degradation, possibly through phosphorylation of NFκB dimers.

Brockelhurst *et al.* (1999). *Br Med J* **318**, 548–549.
Guan *et al.* (1998). *J Biol Chem* **273**, 28670–28676.

This work was supported by The Wellcome Trust.

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

C107

Rapid screening of ultrathin serial sections to detect endothelial gaps in the walls of microvessels in frog mesentery

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Recently, the reconstruction of microvascular walls has been used to determine the nature of endothelial openings or gaps induced by various permeability-raising stimuli (Michel & Neal, 1999). Attempts to assess the gap or pore density of the wall using randomly chosen electron micrographs of transverse sections of microvessel have proved successful when the openings were at a high density (Clough & Michel, 1988). The application of vascular endothelial growth factor (VEGF) to *Rana* mesenteric microvessels produces an increase in permeability and induces transcellular gaps (Michel & Neal, 1999). These gaps often occurred in clusters and were difficult to find with conventional electron microscopy of serial sections because the reconstructed area of the vessel wall was only a small percentage of the whole wall over which permeability was estimated.

We addressed this problem by cutting ribbons of ultrathin serial sections (100 nm thick) of mesenteries of frogs that were humanely killed and mounting them on Formvar coated glass slides. After staining with Toluidine Blue, sections were rapidly scanned using a light microscope for the presence of endothelial gaps (endothelial breaks within the section). Areas of interest were delineated and the Formvar film with attached sections was floated off the glass and placed on a slot grid for observation in the electron microscope.

From resin-embedded vessels treated with pressure or calcium ionophore where gaps were known to be present, 503 serial sections showed that endothelial gaps were resolvable under oil immersion using a ×100 objective. From three resin-embedded vessels treated with VEGF 1, 206 sections were scanned for the presence of gap clusters. Four areas of gaps were delineated along the combined 120 μm length of the three vessels. The Formvar coating and attached sections separated easily from the glass allowing confirmation of the structures in the electron microscope.

This technique allows the rapid scanning of vast areas of vessel wall for endothelial gaps that occurred due to the action of permeability raising stimuli.

Clough G & Michel CC (1988). *J Physiol* **405**, 563–576.
Michel CC & Neal CR (1999). *Microcirculation* **6**, 45–54.

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All procedures accord with current UK legislation.

C108

A novel angiogenesis model in rat mesentery

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Vascular endothelial growth factor (VEGF) is the dominant growth factor in pathological and physiological angiogenesis. It promotes microvascular permeability, compliance and angiogenesis *in vivo* (Bates *et al.* 1999). To investigate the underlying physiological changes that occur during VEGF-mediated angiogenesis we have developed an angiogenesis model in a system amenable to functional characterisation. Adenovirus (AdV) expressing VEGF (Ad-VEGF) was produced by co-transformation of the vector pAdEasy-1 and pShuttle-CMV-$VEGF_{165}$ into BJ5183 bacteria and then transfection of the recombinant pAd-VEGF into HEK293 cells. AdV expressing β-gal (Ad-LacZ) and Ad-VEGF were purified by CsCl density gradient centrifugation, and dialysed against rat Ringer solution. Animal experiments conformed to Home Office guidelines. Male Wistar rats (300 g) were anaesthetised by halothane inhalation (5%), the gut exposed after laparotomy, and the mesentery superfused with Ringer solution at 37 °C. A connective tissue panel with few blood vessels and no overt angiogenesis was imaged using a digital camera and light microscope. 50 μl of Ad-VEGF or Ad-LacZ of ($1–3.3 \times 10^8$ $TCID_{50}$ ml^{-1}) and Monastral Blue (0.6%), diluted in rat Ringer solution, were injected into the fat pad adjacent to the panel. The mesentery was then replaced in the animal, the laparotomy sutured and the animal allowed to recover. Seven days later, the animal was anaesthetised, an incision made through the laparotomy scar, and the gut exposed as before. The same panel was identified from the Monastral Blue depot, and the tissue superfused with warm Ringer solution. The mesentery was imaged as before, and the rat killed by cervical dislocation. The degree of angiogenesis (angiogenesis index, AI) was calculated as the increase in area of vessels over 7 days (AI = 100 × (fractional vessel area on day 7 − fractional vessel area on day 1)/fractional vessel area on day 1), calculated using image analysis software (Openlab, Improvision). AdVEGF injection significantly increased AI (median ± interquartile range AI 434 ± 76%, $n = 6$), compared with the control group (140 ± 39%, $n = 7$, $P < 0.01$, Mann-Whitney U).

Immunofluorescence staining of the mesenteries with proliferating cells nuclear antigen (PCNA) showed dividing cells, confirming angiogenesis, rather than simply increased detection of vessels due to vasodilatation. These results demonstrated that AdV-mediated VEGF gene transfer was effective in inducing neovascularisation in a quantitative rat mesenteric model of angiogenesis. Since rat mesenteric microvessels have previously been characterised molecularly (e.g. using cDNA array), cellularly and physiologically, this model will allow similar characterisation during angiogenesis.

Bates DO *et al.* (1999). *Microcirculation* **6**, 83–96.

All procedures accord with current UK legislation.

C109

Endothelial cell facilitation of longitudinal spreading hyperpolarization and dilatation in rat isolated mesenteric artery

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In many small arteries, and in arterioles, hyperpolarization spreads bi-directionally along the longitudinal axis of the vessel. Spread appears to be endothelium dependent, and is suggested to underlie spreading vasodilatation (Emerson & Segal, 2000). In rat mesenteric arteries, myoendothelial gap junctions appear to contribute to the local spread of endothelium-dependent hyperpolarization (Edwards *et al.* 1999). However, homocellular gap junctions are also present in this artery (Sandow & Hill, 2000), raising the possibility for spreading hyperpolarization and vasodilatation.

Male Wistar rats (200–250 g) were killed humanely. Small mesenteric arteries were isolated, and then either pinned out for intracellular recording with sharp microelectrodes or cannulated and pressurized for diameter measurements. In both cases, tissue was superfused with Mops solution containing 100 μM L-NAME at 37 °C. Either ACh or levcromakalim were applied by pressure ejection from a micropipette. Values are means ± S.E.M. ACh evoked robust hyperpolarization at the local site of application, increasing the resting potential (−48.8 ± 4.7 mV, $n = 21$) by 14.3 ± 1.2 mV ($n = 8$). The hyperpolarization spread upstream and was detected 1 mm from the point of application (6.6 ± 0.8 mV, $n = 8$). The conducted (but not the local) hyperpolarization was blocked by cutting the vessel between the sites of stimulation and upstream recording. The spreading hyperpolarization was correlated with dilatation, where diameter increased to 74 ± 9 % of maximum at the local site and 69 ± 5 % 1 mm upstream of the point of ACh stimulation. However, in arteries where the endothelial cells had been selectively loaded with the Ca^{2+} indicator fluo-4, no change in $[Ca^{2+}]_i$ could be detected at the upstream site, contrasting with a 22 ± 3 % increase at the local site ($n = 3$). Direct smooth muscle stimulation with levcromakalim also evoked both local and upstream hyperpolarization (13.0 ± 1.6 mV, $n = 12$ and 4.3 ± 1.0 mV, $n = 9$, respectively) and dilatation (90 ± 4 %, $n = 4$, and 70 ± 12, $n = 4$ % maximum, respectively). However, in arteries where the endothelium had been destroyed, while local hyperpolarization was unaltered, the upstream responses were abolished.

These data show that spreading hyperpolarization and dilatation can occur in the mesenteric artery. Further, they indicate that the route for spread is through the endothelium and that the distant dilatation does not require any spreading change in endothelial cell $[Ca^{2+}]_i$.

Edwards G *et al.* (1999). *Br J Pharmacol* **128**, 1788–1794.

Emerson GG & Segal SS (2000). *Circ Res* **87**, 474–479.

Sandow SL & Hill CE (2000). *Circ Res* **86**, 341–346.

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All procedures accord with current UK legislation.

C110

The influence of the endothelium on the modulation of rat coronary artery diameter after elevation of extravascular pressure

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We have previously demonstrated that arterial diameter of isolated coronary arteries is governed by the relative, rather than the absolute, values of intravascular (IvP) and extravascular pressure (EvP). The elevation of EvP led to a reduction in arterial diameter. This was regained after IvP was increased to equal the transmural pressure (TmP) across the vessel walls prior to intervention (Azzawi & Austin, 2002). Here we examine the role of the endothelium in modulating vascular tone and in contributing to diameter regain achieved when IvP is increased.

Wistar rats were humanely killed by stunning followed by cervical dislocation and septal coronary arteries were dissected out. Each artery was mounted on a modified pressure myograph where they were constantly superfused with physiological salt solution (pH 7.4, 37 °C, 95 % air and 5 % CO_2). A lid was secured over the myograph chamber which allowed EvP to be altered (via a 95 % air and 5 % CO_2 source). The internal diameter of vessels was determined using a video dimension analyser. Data are given as means ± S.E.M.

At an IvP of 60 mmHg, and thus a net outward TmP of 60 mmHg, coronary arteries (mean internal diameter of 256 ± 10 μm, $n = 8$) developed myogenic tone (140 ± 10 μm). Elevation of EvP to 40 mmHg, which effectively reduced the net outward TmP to 20 mmHg, reduced the diameter of all vessels by 12 ± 2 %. Subsequent elevation of IvP to 100 mmHg, at a maintained EvP of 40 mmHg, to return net outward TmP to 60 mmHg increased arterial diameter to 105 ± 6 % of that observed at the original TmP of 60 mmHg (IvP of 60 mmHg and EvP of 0 mmHg). Endothelial denudation was then achieved by introduction of 4–5 air bubbles through the lumen. This abolished the endothelial-dependent dilator response to acetylecholine. Myogenic tone was increased in all vessels (mean increase in tone = 33 ± 12 μm). Elevation of EvP to 40 mmHg, reduced the diameter of all vessels by 24 ± 3 % (Student's paired t test, $P < 0.01$). Subsequent elevation of IvP to 100 mmHg, increased arterial diameter to 89 ± 2 % ($P < 0.01$).

The effect of the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 0.1 mmol l^{-1}) was then examined (mean internal diameter = 243 ± 11 μm, $n = 10$, tone = 120 ± 14 μm). Elevation of EvP to 40 mmHg reduced the diameter of all vessels by 11 ± 1 and 19 ± 4 % before and after inhibition, respectively ($P < 0.05$). Subsequent elevation of IvP to 100 mmHg, increased arterial diameter to 102 ± 1 and 89 ± 4 % before and after inhibition, respectively ($P < 0.05$). These results demonstrate that the endothelium may be an important modulator of arterial responsiveness to IvP-EvP intervention.

Azzawi M & Austin C (2002). *J Physiol* **543.P**, 73*P*.

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All procedures accord with current UK legislation.

C111

Inhibition of endothelium-dependent hyperpolarization in the rat isolated mesenteric artery with the thromboxane-mimetic, U46619

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Smooth muscle depolarization and contraction with U46619 in mesenteric arteries is associated with a depression in the subsequent non-nitric oxide, endothelium-dependent hyperpolarization and relaxation (Plane & Garland, 1996). This is in marked contrast to stimulation with α-adenoreceptor agonists, when reproducible nitric oxide-independent hyperpolarization and relaxation is obtained (Garland & McPherson, 1992). The present study addresses the mechanism which may underlie this effect.

Male Wistar rats (200–250 g) were humanely killed by cervical dislocation and exsanguination. Small mesenteric resistance arteries were isolated and mounted in a Mulvany-Halpern myograph for simultaneous measurement of smooth muscle membrane potential and tension charge (Garland & McPherson, 1992). All experiments were done in the presence of L-NAME (100 μM). ACh (30 nM–3 μM) evoked endothelium-dependent and reproducible smooth muscle cell hyperpolarization, increasing the resting potential from -53 ± 1 to -75 ± 2 mV (mean ± S.E.M., $n = 5$). This hyperpolarization was abolished in the presence of 50 nM apamin. Repeated exposure to ACh was associated with a small but significant depression in the maximum amplitude of hyperpolarization, so that following a third exposure to ACh the potential only increased to -69 ± 3 mV ($n = 5$; $P < 0.05$, ANOVA). Stimulation with 1 nM–0.1 μM U46619, followed by washout and subsequent exposure to ACh, was associated with a progressive decline in the ACh-evoked hyperpolarization. After three exposures to U46619, ACh failed to evoke a hyperpolarization (resting potential -52 ± 1 mV; with 3 μM ACh present, -55 ± 1 mV, $n = 8$). If ACh was applied during ongoing stimulation with U44619, it evoked membrane repolarization with an overshooting hyperpolarization to -62 ± 3 mV. These increases in potential were associated with 86 ± 8 % relaxation ($n = 8$). By the third exposure, ACh only reversed the depolarization to U46619 to close to the original resting potential (-50 ± 2 mV, $n = 8$), although relaxation was not altered. Depression in the ACh hyperpolarization was not observed if the arteries were exposed for similar periods to phenylephrine rather than U46619.

These data indicate a progressive inhibition in the ability of ACh to stimulate endothelium-dependent hyperpolarization as a consequence of repeated smooth muscle stimulation with U46619. The fact that the hyperpolarization to ACh could also be blocked with apamin, raises the possibility that U46619 may inhibit SK_{ca} channels.

Garland CJ & McPherson GA (1992). *Br J Pharmacol* **105**, 429–435.

Plane F & Garland CJ (1996). *Br J Pharmacol* **119**, 191–193.

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All procedures accord with current UK legislation.

C126

Altered atypical β-adrenoceptor-mediated vasodilatation in the aorta from the spontaneously hypertensive rat

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The present study was undertaken to investigate the functional expression of atypical β-adrenoceptors (β-AR) in aorta from Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) at 12 weeks old.

Rats were anaesthetised with pentobarbital (30 mg kg^{-1} I.P.) and thoracic aortic rings were removed, isolated and suspended on stainless-steel wires in 5 ml organ baths containing Krebs solution (Mallem *et al.* 2002). Rings were constricted with phenylephrine (α_1-AR agonist) and cumulative concentration–relaxation curves to atypical β-AR agonists were constructed. Results are expressed as the percentage of relaxation from the steady-state contraction level induced by phenylephrine (mean ± S.E.M. of n experiments).

In intact WKY aorta, CGP 12177 (CGP) or cyanopindolol (partial β_3-AR and atypical β-AR agonists with β_1/β_2-AR antagonistic properties) produced concentration-dependent relaxation ($pD_2 = 6.17 \pm 0.05$; $E_{max} = 95.9 \pm 1$ %; $n = 5$, for cyanopindolol). In order to determine the role of the endothelium and nitric oxide (NO) in atypical β-AR-mediated relaxation, responses to CGP or to cyanopindolol were evaluated in endothelium-denuded aorta or after pretreatment with a NO synthase inhibitor (L-NMMA, 100 μM). Both CGP and cyanopindolol-induced relaxation were not modified by endothelium removal or after L-NMMA treatment. The CGP-induced endothelium-independent relaxation was not altered in the presence of 10 μM nadolol (β_1/β_2-AR antagonist) or 1 μM SR 59230 (β_3-AR antagonist) but was significantly reduced in the presence of CGP 20712A or bupranolol (atypical β-AR antagonists in higher concentrations) ($P < 0.05$ by ANOVA). The involvement of the cyclic AMP-dependent pathway in response to atypical β-AR agonists was evaluated in denuded aortic rings of WKY aorta. Endothelium-independent relaxation to CGP was inhibited by SQ 22536 or MDL 12330A, non-selective adenylyl cyclase inhibitors ($P < 0.05$ by ANOVA). In SHR aortic rings, CGP or cyanopindolol produced concentration-dependent relaxation similar to that obtained in WKY aorta ($pD_2 = 6.09 \pm 0.05$; $E_{max} = 96 \pm 1$ %; $n = 5$, for cyanopindolol). In contrast, the concentration–relaxation curve to CGP or cyanopindolol was significantly inhibited in endothelium-denuded rings or after L-NMMA treatment, suggesting that the atypical β-AR response was altered in SHR rats ($P < 0.05$ by ANOVA). Pertussis toxin treatment of SHR (10 μg kg^{-1}, I.P.) partly restored the endothelium-independent relaxation to CGP.

In conclusion, these results show that (1) atypical β-AR are functionally expressed in vascular smooth muscle of rat aorta, (2) they mediate a NO-independent vasorelaxation partly through a cAMP-dependent pathway, and (3) atypical β-AR relaxation is altered in hypertension and G_i protein pathway could be involved in this phenomenon.

Mallem MY *et al.* (2002). *Arch Mal Coeur Vaiss* **95**, 647–650.

All procedures accord with current National and local guidelines.

C127

Visceral adiposity, obesity, weight loss and endothelial function in humans

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Obesity is an independent risk factor for the development of coronary artery disease and is associated with endothelial dysfunction (ED), a critical early event in atherogenesis. We aimed to explore the relationship between measures of fat distribution and flow-mediated vasodilatation (FMD), a surrogate marker for endothelial function, and the influence of weight loss on this.

We assessed endothelial function in 73 subjects (37 ± 10 years old, mean ± S.D., 60 % female) over a range of body mass indexes (BMI, 18–73 kg m^{-2}). No subject had diabetes or a history of cardiovascular disease. Height, weight and waist and hip circumferences were measured. Brachial artery FMD was assessed after an overnight fast and analysed using an automated wall-tracking system. Blood for lipid profile and glucose was taken. Correlations were assessed with non-parametric tests.

FMD showed a weak negative correlation with BMI ($r = -0.31$, $P < 0.01$). When dichotomised by the upper limit of normal for BMI (30 kg m^{-2}), FMD was worse in the obese group (6.8 ± 3.1 *vs.* 9.3 ± 3.4 %, $P < 0.001$, Student's unpaired t test). Waist-hip ratio (WHR), which gives an indication of visceral adiposity, was closely negatively correlated with FMD ($r = -0.60$, $P < 0.0001$). Weight ($r = -0.43$, $P < 0.02$) and waist circumference ($r = -0.53$, $P < 0.01$) showed modest correlations with FMD. When dichotomised by upper limit of normal for WHR (0.95 for men, 0.8 for women) FMD was worse in the higher WHR group (6.7 ± 2.9 *vs.* 9.4 ± 3.5 %, $P < 0.0005$). Higher WHR was associated with raised LDL cholesterol ($P < 0.005$), triglyceride ($P < 0.005$) and systolic blood pressure ($P < 0.0005$), but not fasting glucose.

Of this cohort, eight underwent laparoscopic gastric banding or gastric bypass in order to achieve weight loss. FMD was reassessed after a minimum of 6 months or 10 % weight loss, whichever occurred earlier. Two patients gained a small amount of weight post-operatively whilst the remaining six lost between 6 and 38 kg. Percentage change in body weight was very closely correlated with absolute change in FMD ($r = 0.90$, $P < 0.002$); of the other anthropometric measures, change in waist circumference best predicted improvement in endothelial function ($r = 0.79$, $P < 0.02$).

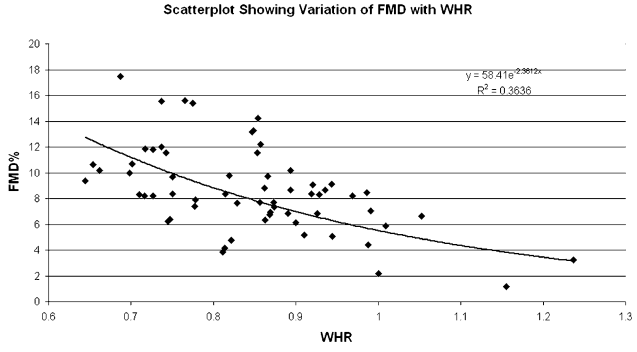

Figure 1.

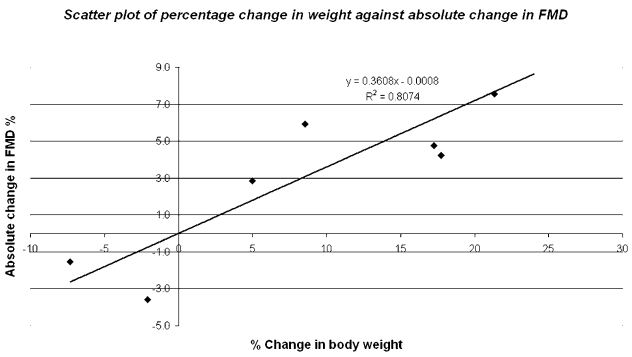

Figure 2.

These data demonstrate a close association between FMD and WHR. WHR may be a better marker than BMI when assessing patients' cardiovascular risk profile. Weight loss is associated with an improvement in FMD, probably through a reduction in visceral adiposity, and may significantly improve endothelial function in subjects who, despite weight reduction, remain morbidly obese.

Celermajer DS *et al.* (1992). *Lancet* **340**, 1111–1115.
Hashimoto M *et al.* (1998). *Int J Obes* **22**, 477–484.
Hubert HB *et al.* (1983). *Circulation* **67**, 968–977.

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

C128

Role of oxidant stress in angiotensin II-induced contractions of human resistance arteries in healthy older subjects

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Angiotensin II (AII) increases vascular superoxide production (Griendling *et al.* 1994; Berry *et al.* 2000). We investigated the role of superoxide in the reactivity of human resistance arteries to AII. Resistance arteries were isolated from subcutaneous gluteal biopsies obtained under local anaesthesia (lignocaine 2 %) from healthy volunteers ($n = 8$, age 70 ± 2 years, mean ± S.E.M.; 31 arteries). The study was approved by the local Hospital Research Ethics Committee and written informed consent obtained from all participants. Arteries were mounted on a Mulvany-Halpern small vessel myograph and pretreated with potassium chloride (124 mM). Arteries were then treated with the antioxidant vitamin C (1 mM, pH buffered), the superoxide dismutase mimetic TEMPOL (1 mM), the xanthine oxidase inhibitor allopurinol (1 mM) or vehicle, for 30 min before a single concentration–response curve to AII (0.1–100 nM) was obtained in each tissue, in the continued presence of vitamin C, TEMPOL or vehicle. Solutions were bubbled with 95% O_2–5% CO_2 throughout all studies. Results (means ± S.E.M.) are shown as % response *vs.* contraction to potassium chloride. In the presence of vitamin C the potency of AII and the maximum response were reduced (AII *vs.* vitamin C: pEC_{50} 8.5 ± 0.2 *vs.* 8.2 ± 0.1, maximum response 69 ± 15 *vs.* 34 ± 10 %; $n = 5$; ANOVA $F = 7.73$; $P = 0.03$). There was little difference in AII

responses following treatment with TEMPOL (AII *vs.* AII + TEMPOL: pEC_{50} 8.6 ± 0.2 *vs.* 8.5 ± 0.1; maximum response 87 ± 13 *vs.* 80 ± 8 %; $n = 7$; $F = 0.14$; $P = 0.67$). However, pretreatment of tissues with allopurinol caused a major decrease in response to AII (AII *vs.* AII + allopurinol: pEC_{50} 8.6 ± 0.1 *vs.* 8.3 ± 0.1; maximum response 86 ± 11 *vs.* 22 ± 3 %; $n = 6$; $F = 35.97$; $P = < 0.0001$). Decreased potency of Ang II after treatment with vitamin C is consistent with involvement of pro-oxidant pathways in the contractile response of human resistance arteries to Ang II. The lack of effect of TEMPOL suggests that superoxide is not the major reactive oxygen species responsible. The results with allopurinol suggest a superoxide-independent role for xanthine oxidase in Ang II-mediated contraction in healthy older subjects.

Berry C *et al.* (2000). *Circulation* **101**, 2206–2212.
Griendling KK *et al.* (1994). *Circ Res* **74**, 1141–1148.

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

C129

Lymphatic area and lymphatic density in malignant melanoma, basal cell carcinoma and normal skin

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Skin cancer is one of the most common forms of malignancy, and malignant melanoma (MM) is one of the most aggressive types of skin tumour. High mortality rates seen with MM are due to this tumour's ability to metastasise to distant organs possibly via the lymphatic system. Recent studies have shown that MM cells stimulate the growth of lymphatic vessels (lymphangiogenesis) and that this facilitates metastasis in animal models (Mandriota *et al.* 2001; Skobe *et al.* 2001*a, b*). Using the lymphatic marker, LYVE-1, we determined the presence of intra-tumoral and peripheral lymphatics in normal tissue and skin cancers. Normal skin, archival basal cell carcinoma (BCC; non-metastatic) and MM samples were obtained with Local Ethical Committee approval (North Bristol NHS Trust). Sections (5 μm thick) were dewaxed, rehydrated and microwaved in Tris/EDTA buffer, pH 9. Sections were incubated in either 4.2 μg ml^{-1} rabbit anti-human LYVE-1 or 4.2 μg ml^{-1} normal rabbit IgG overnight at 4 °C. Primary antibody was detected with 2 μg ml^{-1} biotinylated goat anti-rabbit, standard avidin-biotin complex and diaminobenzidine (Vector). The number and fractional area and of lymphatics within the tumour and within 350 μm of the tumour edge were calculated using NIH Image 1.62 software. There was a significantly higher peripheral lymphatic area fraction (mean ± S.E.M. 0.83 ± 0.22 %, $n = 6$) and density (13.9 ± 3.6 vessels mm^{-2}) in MM samples than BCC (0.12 ± 0.03 %, and 3.0 ± 0.9 vessels mm^{-2}, $n = 6$; $P < 0.05$, Mann-Whitney *U* test). Moreover, peripheral area fraction and density of lymphatics around MM were significantly greater than in normal tissue ($P < 0.05$), whereas BCC lymphatic area and density were not significantly different from normal tissue controls. BCC samples had a reduced intra-tumoral lymphatic density, consistent with a proliferative, non-lymphangiogenic, non-invasive phenotype. However, the lymphatic density inside the MM was no less than normal tissue. This is not consistent with a lack of lymphangiogenesis, since MM is proliferative as well as invasive. These preliminary results indicate that MM may have the ability to stimulate lymphatic growth and allow metastasis whereas BCC does not.

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

C130

Vascular endothelial growth factor $(VEGF)_{165}$b, a novel and inhibitory VEGF isoform is down-regulated in prostate carcinoma

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$VEGF_{165}$b is an alternatively spliced isoform of VEGF that has been shown to inhibit endothelial cell proliferation, migration and vasodilatation induced by $VEGF_{165}$. It is down-regulated in renal cell carcinoma and it has been hypothesised that it is an endogenous anti-angiogenic isoform of VEGF (Bates *et al.* 2002). To determine whether $VEGF_{165}$b was also expressed in prostate, and whether it was down-regulated in prostate cancer, we measured expression of $VEGF_{165}$b mRNA in prostate using fresh transurethral resection (TUR) chips and archival formalin-fixed paraffin-embedded (FFPE) tissue. TUR chips and archival tissue were obtained from patients who had undergone TUR of the prostate for bladder outlet obstruction and radical prostatectomy for localised prostate cancer, respectively. All tissues were surplus to requirements for histology, and were collected with Ethics Committee approval. Total ribonucleic acid (RNA) was isolated from fresh tissue based on the guanidium thiocynate phenol-chloroform extraction method described by Chomczynski & Saachi (1989). RNA was recovered from FFPE tissue using proteinase K digestion followed by phenol-chloroform extraction (Krafft, 1997). Nineteen prostate carcinoma tissue (9 TUR and 10 FFPE) and 27 benign prostate (17 TUR and 10 FFPE) were analysed. Paired malignant and benign portions were obtained from each FFPE specimen. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using intron spanning primers designed to amplify only those samples containing the novel exon of $VEGF_{165}$b, exon 9. PCR conditions were optimised and products were analysed by agarose gel electrophoresis. Bands corresponding to the amplification of $VEGF_{165}$b were detected in significantly more (82 %) benign TUR chips than the malignant ones (11 %, $P < 0.05$, Fisher's exact test). $VEGF_{165}$ on the other hand was expressed in both normal and malignant tissue. $VEGF_{165}$b was detected in all the FFPE tissues but the mean intensity of the bands from the benign part of the prostate was significantly higher compared with that where prostatic intra-epithelial neoplasia (PIN) was found. $VEGF_{165}$b, a novel VEGF isoform that is potentially anti-angiogenic, can be detected in fresh and even archival prostate tissue. It appears to be down-regulated in carcinoma of the prostate, indicating both transcriptional and splicing control of VEGF is involved in the angiogenic switch in prostate cancer.

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

C131

The response of the rat brain vasculature to *in vivo* removal of astrocytes

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With the intention of illuminating the *in vivo* role of astrocytes, we have made use of the highly cell-and region-selective toxicity of α-chlorohydrin (*S*-3-chloro-propane-1,2-diol) to study the response of the brain vasculature to selective loss of this cell type. α-Chlorohydrin is a glycolytic inhibitor that is toxic to astrocytes, but did not increase permeability of monocultured endothelial cells (Romero *et al.* 1997). Systemic administration of α-chlorohydrin (140 mg kg^{-1} I.P.) to 180–220 g male F344 rats produced astrocytic death limited to specific deep nuclei (most notably the inferior colliculi, and the red, vestibular, trigeminal motor and occulomotor nuclei). This occurred over the first 2–24 h, and was followed by neuronal loss from 36 h. From 2 to at least 8 days no astrocytes could be detected within damaged areas by confocal immunohistochemistry (glial fibrillary acidic protein) or electron microscopy. Due to the limited and strictly symmetrical nature of the damage, rats showed only a mild ataxia, and little weight loss, and all experiments were conducted within UK Home Office guidelines. Despite the marked early astrocytic pathology, blood flow in the inferior colliculus (measured by hydrogen polarography in conscious animals previously implanted under anaesthesia) showed no significant change until 24 h, but by 48 h had risen to 2.68 ± 0.50 times predose (mean ± S.E.M., $n = 9$), falling thereafter. In contrast, flow in the resistant cerebellar cortex increased only 1.25 ± 0.08-fold at 48 h. In parallel with this late flow increase, the vasculature in damaged areas became permeable to 10 kDa fluorescent labelled dextran tracers, and severely damaged areas developed petechial haemorrhages, both effects peaking at 2–3 days. Extravasation of 0.5 kDa gadolinium-DTPA was visualised by T1 weighted magnetic resonance imaging under isofluorane anaesthesia, and at 2 days in the inferior colliculus post-gadolinium enhancement reached 61.3 ± 6.6 % ($n = 10$) of that seen in the open barrier pineal. Despite the continuing lack of astrocytic contact, by 9 days damaged areas were no longer permeable to 10 kDa dextran, and gadolinium enhancement had fallen to 42.5 ± 2.7 % of its 2 day value ($n = 10$). These results show that recovery of barrier properties is molecular size dependent.

We hypothesise that the brain endothelium undergoes a reversible dedifferentiation in response to loss of astrocyte contact, but is then capable of a surprising degree of recovery of barrier properties, possibly due to distant diffusible astrocyte-derived factors.

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All procedures accord with current UK legislation.

C132

Possible role of Na^+/H^+ exchanger in H^+ transport across the blood–brain barrier of anaesthetized rats

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The rapid removal of H^+ from the brain is important for homeostasis, but the cerebral endothelium forms a barrier that prevents the free diffusion of ions. We are using the single pial microvessel preparation to study the mechanisms by which this H^+ transport occurs. Single venular capillaries of rats (anaesthetized with pentobarbitone: 60 mg kg^{-1} I.P., an overdose of which was administered to kill humanely; experiments accorded with UK legislation), were filled with the fluorescent ratiometric pH indicator dye, 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS, 0.5 mM) by a bolus intracarotid injection. The dye-blood mixture was trapped in a selected vessel by lowering two probes positioned at least 100 μm apart. The ratio of the emitted light at wavelength greater than 510 nm for 440 nm and 380 nm excitation is found to be proportional to pH between 6.8 and 7.6.

During an occlusion the luminal pH fell by 0.039 ± 0.011 pH units (mean ± S.E.M., $n = 21$) when the brain surface was superfused with a bicarbonate-free Hepes-based buffer at pH 7.32, the steady state being reached between 40 and 60 s. Lowering the superfusing pH to 6.8 resulted in luminal acidification by 0.21 ± 0.08 ($n = 4$). The Na^+/H^+ exchange inhibitor EIPA (ethyl isopropyl amiloride; 50 μM) prevented this fall (0.03 ± 0.05; $n = 4$). When superfusate Na^+ was substituted by choline (at pH 7.32) the luminal pH fell by 0.09 ± 003 ($n = 8$). The possibility that this was due to the Na^+ gradient driving an influx of H^+ was tested by applying EIPA, and this resulted in an alkalization to 0.06 ± 0.01 ($n = 12$). EIPA, however, when applied in the presence of Na^+ and with superfusate pH 7.32, resulted in a similar alkalization by 0.15 ± 0.03 ($n = 4$). The Na^+/H^+ exchanger cannot be the sole mechanism by which H^+ move from brain to blood.

All procedures accord with current UK legislation.

C133

Interactions of testosterone, androstenedione, green tea catechins and the anti-androgen Flutamide with the external glucose binding site of the human glucose transporter, GLUT1

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Excess androgen secretion in females results in hirsutism often accompanied by changes in glucose transport, e.g. diabetes. These symptoms improve with treatment of the anti-androgen Flutamide. Many actions of androgens are genomic via a nuclear androgen receptor, but non-genomic actions have also been identified, some of which are sensitive to Flutamide.

Recent studies have shown that green tea polyphenols reduce glycosuria in diabetics. They also reduce circulating levels of testosterone and benign prostatic enlargement. In the present

study we investigated the non-genomic effects of androgens, the anti-androgen Flutamide and green tea catechins on glucose transport in human erythrocytes.

Fresh human red cells (obtained from R.J.N.) were pre-equilibrated with 100 mM glucose and resuspended in phosphate-buffered saline containing the 'androgen' under test. Following resuspension, the rate of glucose efflux was determined from light scattering changes at 650 nm.

Testosterone and 4-androstene-3,17-dione inhibited glucose exit from human erythrocytes with a $K_{i,test} = 39.2 \pm 8.9$ μM and $K_{i,and} = 44.0 \pm 4.1$ μM (mean ± S.E.M., $n = 3$–4 for each estimate). Flutamide competitively relieved the inhibitions of glucose transport by testosterone ($K_{i,test/Flut} = 0.35 \pm 0.10$ μM) and androstenedione ($K_{i,and/Flut} = 0.14 \pm 0.05$ μM). Flutamide also relieved phloretin-dependent inhibition of glucose exit ($K_{i(phloretin/Flut)} = 1.75 \pm 0.22$ μM), suggesting that it binds exclusively to the external (sugar import site) site of GLUT1. This was confirmed as glucose binding to the external site of the glucose transporter was competitively inhibited by testosterone, $K_{(ic\ ext\ test)} = 42.8 \pm 0.8$ μM.

The green tea catechins also inhibited glucose exit. The catechin with the highest affinity was epicatechin 3 gallate (ECG), $K_{i(ECG)} = 0.14 \pm 0.01$ μM. The major tea constituent, epigallocatechin 3-gallate (EGCG) had a $K_{i(EGCG)} = 0.97 \pm 0.13$ μM. Flutamide competitively reversed both the inhibitions of EGC and EGCG. The high affinities of ECG and EGCG for the glucose transporter (GLUT1) suggest that this might be their physiological site of action.

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All procedures accord with current local guidelines.

C134

Rapid activation of p42/p44 MAP kinase and nitric oxide synthase by phytoestrogens in human fetal endothelial cells

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Premenopausal women are known to have a much lower risk of coronary heart disease than age-matched men, with the incidence of risk increasing after the onset of menopause. Accumulating evidence suggests that this protection is due to oestrogen (Guetta *et al.* 1997), and men and women consuming a phytoestrogen-rich diet have a lower risk of cardiovascular disease (Anthony *et al.* 1997). These latter compounds have a similar structure to oestrogen and thus protection may be conferred via similar mechanisms. The present study has compared the actions of 17β-oestradiol and phytoestrogens on mitogen-activated protein kinase (MAPK) and nitric oxide (NO) production in endothelial cells cultured from human umbilical cord veins (Wyatt *et al.* 2002), obtained with the approval of the patient and local ethics committee.

Effects of short (2 min) exposure to 17β-oestradiol (but not 17α-oestradiol), genistein, daidzein and its metabolite equol were studied. Intracellular cGMP levels served as an index of NO production (inhibitable by the NOS inhibitor L-NAME, 100 μM). Low (0.1 μM) concentrations of 17β-oestradiol and phytoestrogens stimulated NO release 1- to 2-fold without elevating intracellular Ca^{2+}. 17β-Oestradiol, daidzein and equol also acutely (2 min) phosphorylated p42/p44^{MAPK} at concentrations of 1–100 nM. Activation of p42/p44^{MAPK} was inhibited by U0126 (1 μM, 30 min pretreatment), an inhibitor of MEK, the upstream activator of p42/p44^{MAPK}, but unaffected by L-NAME. In order to determine whether the actions of phytoestrogens were mediated via the classical oestrogen receptors α and β, cells were pretreated with ICI 182,780 (10 μM, 30 min) or tamoxifen (10 μM, 60 min). Neither of these oestrogen receptor antagonists affected NO production in response to genistein, daidzein, equol or 17β-oestradiol.

The present study establishes that, in addition to 17β-oestradiol, dietary phytoestrogens can acutely stimulate p42/p44^{MAPK} and NO production in human fetal endothelial cells *in vitro*. L-NAME has no effect on MAPK phosphorylation, whereas inhibition of this signalling pathway by U0126 suggests that in these cells activation of MAPK pathway may be upstream of endothelial NO synthase.

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

C136

Gene transfer in isolated human glomeruli

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The glomeruli are unique filtration units. The glomerular endothelium has a high hydraulic conductivity in the context of a high reflection co-efficient. Intact isolated human glomeruli are amenable to both molecular (Whittle *et al.* 1999) and functional (Savin *et al.* 1992) study *ex vivo*. Although gene transfer into rodent glomeruli has been accomplished (Ye *et al.* 2002), gene transfer *ex vivo* in human glomeruli has not been published. We therefore studied gene transfer techniques on isolated human glomeruli with the aim of establishing a method for the manipulation of glomerular gene products for dynamic studies.

Glomeruli were isolated from the normal pole of human nephrectomy specimens removed for renal cell carcinoma. All patients gave informed consent and the study was approved by the North Bristol NHS Trust Ethical committee. Glomeruli were collected by standard sieving techniques. The glomeruli were washed in phosphate-buffered saline and cultured in RPMI medium supplemented with L-glutamine, 10% (v/v) fetal calf serum (FCS), penicillin/streptomycin and Insulin Transferrin Sodium Selenite (Sigma). Fifty glomeruli were transferred to a well of a 24-well cell culture plate as a droplet. 1×10^7 plaque-forming units (PFU) (multiplicity of infection, 100) of Adenovirus expressing red fluorescent protein (Ad-RFP) were added to the droplet and incubated for 60 min at 37°C, 5% CO_2. After incubation the glomeruli were washed three times with RPMI/10% (v/v) FCS, allowing 5 min in between washes for the glomeruli to settle. RPMI/10% (v/v) FCS was added to the

glomeruli, which were incubated overnight at 37°C, 5% CO_2. Areas of infection of the glomeruli were visualised using confocal microscopy, 21 sections of 5 μm thick were imaged.

Glomerular cells infected with Ad-RFP fluoresced red. All cell types within the glomerulus appeared to have been infected and expressed the RFP.

Despite the difficulty of gene transfer into particular glomerular cells this protocol does indicate that exogenous proteins may be expressed in the glomeruli and the local micro-environment of the glomerular filtration barrier. We suggest that this simple and reliable protocol for gene transfer into human glomeruli *ex vivo* allows functional studies to be performed on the same glomeruli (normal or diseased) over time after specific manipulations.

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

PC47

Mechanisms underlying the interaction of adenosine, nitric oxide and cyclo-oxygenase pathways in freshly excised rat aorta

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We recently demonstrated that adenosine increases the synthesis and release of nitric oxide (NO) from aortic endothelium via A_1 and A_{2A} receptors, that A_1- but not A_{2A}-evoked release requires a product of the cyclo-oxygenase (COX) pathway, and that prostacyclin (PGI_2) can also evoke NO release. Further we provided direct evidence that adenosine increases the synthesis of PGI_2 by aortic endothelium (Ray *et al.* 2002). These results suggest an interaction between the adenosine, NO and COX pathways for vasodilatation.

In the present study on aortae taken from male Wistar rats killed by halothane overdose and cervical dislocation, NO release evoked by 1 mM adenosine and measured with an NO-sensitive electrode (see Ray *et al.* 2002), was attenuated by the adenyl cyclase (AC) inhibitor 5′-dideoxyadenosine (5 μM) in the presence of either the A_1 receptor antagonist DPCPX (100 nM), from 6.75 ± 1.04 nM (mean ± S.E.M., $n = 8$) to 1.31 ± 0.71 nM NO*, or the A_{2A} adenosine receptor antagonist ZM241385, from 14.31 ± 2.92 nM to 0 nM NO* ($^*P < 0.05$, $n = 6$). The phospholipase C (PLC) inhibitor U73122 (1 μM) had no effect on NO release to adenosine after 10 or 30 min ($n = 7$). However, the phospholipase A_2 (PLA_2) inhibitor, $AACOCF_3$ (10 μM), reduced the NO response to 1 mM adenosine, from 66.38 ± 10.16 nM to 34.16 ± 6.47 nM NO*** ($^{***}P < 0.0001$) after 10 min ($n = 6$). The K_{ATP} channel inhibitor glibenclamide attenuated the NO response to 1 mM adenosine from 129.76 ± 9.23 nM to 76.82 ± 5.80 nM NO*** ($n = 8$). However, in the presence of DPCPX, glibenclamide had no further effect on the response to adenosine ($n = 10$), whereas in the presence of ZM241385, glibenclamide attenuated the response to adenosine from 48.85 ± 13.67 nM to 11.16 ± 5.43 nM NO*** ($n = 7$). All statistical analysis was carried out by ANOVA for repeated measures with Fisher's *post-hoc* test.

These results suggest that (i) adenosine-stimulated release of NO evoked via either A_1 or A_{2A} receptors requires an increase in intracellular cyclic AMP (cAMP), (ii) stimulation of PLA_2, but not PLC, is required and (iii) the ability of A_1 receptor stimulation to evoke NO release depends upon activation of K_{ATP} channels. We propose that adenosine acting at A_1 receptors stimulates K_{ATP} channels and that the resulting K^+ efflux leads to a Ca^{2+} influx which stimulates PLA_2. This leads to synthesis and release of PGI_2 which acts on the endothelial cells, stimulating AC, increasing intracellular cAMP and triggering the cascade of phosphorylation, which leads to the activation of NO synthase and NO release.

Ray CJ *et al.* (2002). *J Physiol* (in the Press).

All procedures accord with current UK legislation.

PC48

Effects of cyclopiazonic acid and caffeine upon transient currents in pericytes on descending vasa recta isolated from rat kidneys and exposed to angiotensin II

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Angiotensin II (AngII) constricts isolated descending vasa recta (DVR), apparently by contracting surrounding pericytes, at least partly by activating transient or steady inward chloride currents (Pallone & Huang, 2002; Zhang *et al.* 2001). In smooth muscle, transient chloride currents depend upon Ca^{2+} release from intracellular stores and diminish when stores are depleted by agonists (Large & Wang, 1996). Some intracellular Ca^{2+} stores respond to inositol 1,4,5-triphosphate (IP_3) or cyclopiazonic acid (CPA) and some to ryanodine or caffeine. IP_3- and ryanodine-sensitive stores may overlap or be separate, in different types of vascular smooth muscle (Janiak *et al.* 2001). This study examines the effects of CPA or caffeine upon transient inward currents in pericytes clamped at −50 mV, during whole-cell permeabilised patch-clamp recording from isolated DVR exposed to AngII.

Individual DVR were dissected from renal tissue kept at 4 °C (Pallone & Huang, 2002; Zhang *et al.* 2001), after removal from rats humanely killed by stunning and cervical dislocation. DVR were incubated in collagenase and hyaluronidase (0.4 mg ml^{-1} of each) at room temperature for 8–9 min, stored on ice and transferred at intervals to solution at room temperature, containing (mM): Na^+ 150, K^+ 5, Mg^{2+} 1, Ca^{2+} 1, Cl^- 159, Hepes 10 and glucose 10, plus 18β-glycyrrhetinic acid (40 μM), a gap junction blocker (Yamamoto *et al.* 1998). Heat-polished pipettes containing a solution of (mM): Na^+ 10, K^+ 140, Cl^- 150 and Hepes 10, plus gramicidin (0.4 mg ml^{-1}), were applied to pericytes to form gigaohm seals.

CPA significantly reduced the mean frequency of these transient currents (Fig. 1), but caffeine did not, which suggests that pericytes on DVR contain heterogeneous Ca^{2+} stores. Caffeine significantly increased mean current amplitude, by an unknown mechanism.

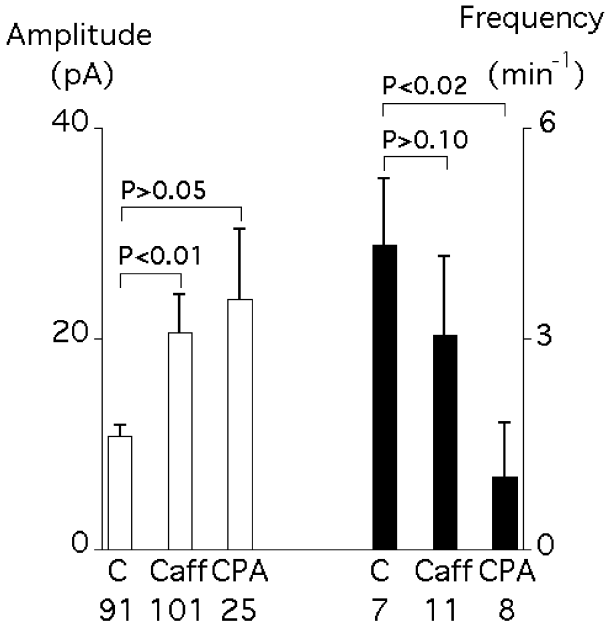

Figure 1. Amplitude (□) and frequency (■) of pericyte transient inward currents recorded during 3 min after addition of AngII (10^{-8} M). AngII was applied alone (controls, C) or 1–3 min after addition of caffeine (Caff; 10 mM) or CPA (10 μM). Means ± standard error. *P* values are from unpaired *t* tests. Numbers of transients (amplitude) or pericytes (frequency) appear below the abscissa.

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All procedures accord with current UK legislation.

PC49

Caffeine transiently increases permeability of microvessels without extracellular Ca^{2+} influx

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The role of IP_3 receptors (IP_3R) and/or ryanodine receptors (RyR) in the regulation of microvascular permeability by endothelial cell Ca^{2+} has not yet been defined. Our aim was to determine if activating the IP_3R/RyR was sufficient to increase the permeability of microvessels without extracellular Ca^{2+} influx. The Landis-Michel method (Michel, 1974) was used to measure hydraulic conductivity (L_p) ($\times 10^{-7}$ cm s^{-1} cmH_2O^{-1}) in single mesenteric microvessels of frog. Erythrocytes were collected by cardiac puncture from 5% halothane-anaesthetised rats (killed by cervical dislocation). Frogs were anaesthetised by immersion in 1 mg ml^{-1} MS222 in frog Ringer (FR) and maintained by superfusing the mesentery with 0.25 mg ml^{-1} MS222. They were killed by cranial destruction. All experiments conformed with the national guidelines for the usage of animals. Caffeine (100 μM), an IP_3R/RyR agonist, was perfused to determine if Ca^{2+} store release affects permeability. Nine vessels were perfused with 1% BSA in FR and erythrocytes to determine the baseline L_p. Due to the non-normally distributed L_p measurements, actual values are expressed as median ± interquartile range and fold increases normalised as means ± S.E.M. The Wilcoxon signed ranks test was used in all statistical analyses unless otherwise stated. Vessels were then perfused with caffeine for 10 min. L_p transiently increased on average 2.0 ± 0.5-fold from 1.5 ± 0.5 to 2.8 ± 0.5 within 2 min ($P < 0.02$). To ensure that the effects seen with caffeine were due directly to store release and not Ca^{2+} influx induced by store release the experiments were repeated with the Ca^{2+} influx inhibitor 100 μM SK&F96365 (SK&F). Nine vessels were perfused with 1% BSA followed by SK&F for 10 min then SK&F with caffeine for another 10 min. SK&F decreased the baseline L_p from 3.2 ± 0.8 to 1.5 ± 0.2 ($P < 0.05$). When SK&F and caffeine were perfused the L_p transiently increased 11.1 ± 9.2-fold to 6.5 ± 1.0 ($P < 0.02$ *vs.* BSA, $P < 0.005$ *vs.* SK&F). There was no significant difference between the fold increase in L_p measured with caffeine compared with that with SK&F and caffeine ($P > 0.1$, Mann-Whitney), indicating that caffeine increases permeability by releasing Ca^{2+} from stores and not by inducing Ca^{2+} influx *in vivo*.

Michel (1974). *QJEPCMS* **59**, 283.

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All procedures accord with current UK legislation.

PC50

Protein restriction in pregnancy induces gender-related vascular dysfunction in small arteries of adult rat offspring

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Maternal undernutrition in rat pregnancy induces vascular dysfunction in isolated arteries of their offspring, which are more pronounced in the male (Ozaki *et al.* 2001). Conversely, high fat during gestation resulted in elevated systolic blood pressure in female adult rat offspring only (Khan *et al.* 2002). We have previously shown endothelium-dependent relaxation is impaired in isolated resistance arteries from male rats exposed to maternal low protein diet *in utero* (Brawley *et al.* 2002). This study investigates vascular function of female offspring using the low protein model and compares isolated resistance artery function from female and male adult offspring of control and protein-restricted dams.

Female Wistar rats were fed either a control (C, 18% casein) or low protein (PR, 9% casein) diet throughout pregnancy. Both diets were replaced with standard laboratory chow at term. Male (M-C, M-PR) and female (F-C, F-PR) offspring of C and PR dams were fed standard chow until 130 ± 5 days of age. They were humanely killed by CO_2 inhalation and cervical dislocation. Small mesenteric arteries (internal diameter ~300 μm) were mounted on a wire myograph. Phenylephrine (PE) (10 nM–100 μM) concentration–response curves were constructed. Endothelium-dependent relaxation was assessed by acetylcholine (ACh, 1 nM–10 μM)-induced relaxation in PE (EC_{80}) pre-

constricted arteries. Data are expressed as means ± S.E.M. of 7–8 observations. Differences between groups are determined by Student's unpaired *t* test and ANOVA.

PE-induced vasoconstriction was not different between dietary groups or genders (% maximum constriction: M-C, 106 ± 3; M-PR, 109 ± 2; F-C, 110 ± 3; F-PR, 113 ± 2, $n = 7$–8, $P > 0.05$). In male offspring, relaxation induced by ACh was significantly shifted to the right in the PR group ($-\log EC_{50}$: M-C, 7.80 ± 0.02, $n = 7$; M-PR, 7.32 ± 0.03, $n = 8$, $P < 0.001$) with no change in maximum relaxation. In females, the maximum relaxation induced by ACh was significantly attenuated in the PR group compared with the C group (% maximum relaxation: F-C, 88 ± 2; F-PR, 44 ± 12, $n = 8$, $P < 0.01$). Moreover, the maximum ACh responses of the M-PR and F-PR were significantly different (% maximum relaxation: M-PR, 92 ± 4, $n = 8$, $P < 0.01$).

Dietary protein restriction in pregnancy programmes development of attenuated endothelium-dependant vasorelaxation in female and male offspring. These vascular defects appear to be gender-related with effects being more pronounced in PR-F offspring.

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All procedures accord with current UK legislation.

PC51

The effect of graded muscle overload on angiogenesis in the rat

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Angiogenesis may be induced in skeletal muscle by metabolic factors, or mechanical factors including overload. In the latter case it is thought to be initiated by muscle stretch, rather than the more usual increase in blood flow (Egginton *et al.* 1998). We therefore sought to regulate the degree of angiogenesis by varying the degree of overload, and determined the extent of neovascular formation. Male Sprague-Dawley rats underwent unilateral surgery (2 % Fluothane in oxygen), randomised with respect to side, and treated post-operatively with analgesics and antibiotics (Temgesic, Duplocillin), in accordance with the Animals (Scientific Procedures) Act, 1986. In one group the m. tibialis anterior (TA) was extirpated under aseptic conditions, and animals allowed to recover for 4, 7 or 14 days ($n = 3$, 6 and 5, respectively) before the m. extensor digitorum longus was sampled. In a second group the distal tendon of the TA was sectioned and EDL samples taken at the same time points ($n = 4$, 7 and 14, respectively). A third group of unoperated animals acted as controls ($n = 8$). All animals were humanely killed. Serial 8 μm cryostat sections were immunolabelled for proliferating cell nuclear antigen (PCNA) located at the position of capillaries, identified by histochemical staining for alkaline phosphatase by the indoxyl tetrazolium method.

The transient increase in density of PCNA positive capillaries following extirpation is similar to that described elsewhere (Rivilis *et al.* 2002), with the continued increase in total capillary density reflecting the translation of cell turnover into new vessels. A similar pattern was observed for labelling indices of both capillaries and muscle fibres, supporting the contention that overload-induced angiogenesis is primarily a mechanical response. The reduced and delayed response following tenotomy is consistent with a smaller degree of overload when the agonist muscle was maintained *in situ*.

Table 1. Capillary density (mm^{-2}) in EDL after labelling for cell proliferation (PCNA) or alkaline phosphatase (Total)

	PCNA	Total
Control	7.3 ± 2.9	835 ± 19
4 days tenotomy	18.1 ± 4.7	875 ± 47
7 days tenotomy	15.7 ± 3.5	843 ± 38
14 days tenotomy	32.7 ± 5.5*	869 ± 54
4 days overload	16.4 ± 5.9	844 ± 28
7 days overload	60.1 ± 10.3**	949 ± 51*
14 days overload	16.4 ± 2.7	1038 ± 60**

Means ± S.E.M. *$P < 0.05$, **$P < 0.005$ *vs.* control (ANOVA).

Egginton S *et al.* (1998). *J Appl Physiol* **85**, 2025–2032.
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All procedures accord with current UK legislation.

PC52

Vascular endothelial growth factor-C (VEGF-C) protein is upregulated in the skin of patients with breast cancer-related lymphoedema

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Breast cancer-related lymphoedema (BCRL) is a chronic swelling of the arm that occurs in approximately 25 % of patients treated for breast cancer. Previous studies have shown that the interstitial protein concentration in the swollen arm is reduced (Bates *et al.* 1993). There is also evidence to suggest that lymphangiogenesis may occur (Mellor *et al.* 2000) and that dermal angiogenesis occurs in the swollen arm (Mellor *et al.* 2002). One hypothesis that would be consistent with these findings is upregulation of VEGF-C. This ligand for VEGFR-2 and VEGFR-3 has been shown to be angiogenic and lymphangiogenic *in vivo* and *in vitro*. VEGF-C also increases the hydraulic conductivity (L_p) of single capillaries measured *in vivo* (Hillman *et al.* 2001). To test this hypothesis, 5 mm biopsies were excised from the arms of BCRL patients ($n = 7$) and normal subjects ($n = 5$) under local anaesthesia (2 % lignocaine, intradermal injection). Local ethics committee approval was obtained and informed, written consent was given by all subjects. Biopsies were fixed in 10 % buffered formalin for 36 h before being dehydrated, cleared and embedded in paraffin wax. Sections (5 μm) were dewaxed, rehydrated and subjected to microwave treatment (800 W) for 10 min in Tris-EDTA buffer, pH 9. Sections were incubated with either 1.14 μg ml^{-1} VEGF-C antibody (Santa Cruz SC-7133) or normal goat IgG (Zymed, negative controls) overnight at 4 °C before detection with 2 μg ml^{-1} biotinylated anti-goat secondary antibody using 'Elite' avidin-biotin enzyme complex and diaminobenzidine substrate (VectorLabs). The primary antibody recognised the carboxyl terminus of the VEGF-C propeptide, which is cleaved before

secretion. Only the cellular form is therefore detected. Analysis was performed using NIH Image 1.62. Images were converted using a 'Gold' look-up table to allow densitometric analysis of positive brown staining. The VEGF-C intensity was determined from the difference between the gold scale densities of the VEGF-C antibody and non-specific IgG. The density of VEGF-C staining observed in the dermis and epidermis of the skin from patients with lymphoedema was significantly greater than in skin from normal subjects ($P < 0.0005$ and $P < 0.02$, respectively, Mann-Whitney U test). These results indicate that VEGF-C is upregulated in the skin of patients with BCRL.

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PC53

Knockdown of $VEGF_{165b}$ expression by RNA interference technique

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RNA interference (RNAi) is a sequence-specific, post-transcriptional gene silencing mechanism, initiated by the presence or introduction of double-stranded RNA, known as small interfering RNA (siRNA). The siRNA can serve as a guide for enzymatic and sequence-specific degradation of mRNAs, probably by associating with a multi-component nuclease. We have recently discovered and cloned a novel inhibitory isoform of vascular endothelial growth factor (VEGF), $VEGF_{165b}$ (Bates *et al.* 2002). In the absence of a $VEGF_{165b}$-specific antibody, we investigated if RNAi can be used to reduce production of this new isoform. A $VEGF_{165b}$-specific sequence of 21 nucleotides located across exon 7 and exon 9 of VEGF gene plus an additional 17 nucleotides of universal T7 promoter sequence and a complementary T7 promoter primer were synthesized separately, annealed at 95 °C for 5 min, and allowed to cool down to room temperature to obtain DNA template. Both sense and anti-sense template were constructed in the same way. The template was incubated in 50 μl reaction volume with T7 RNA polymerase at 37 °C for 2 h, then RNase-free DNase was added to remove the DNA template. The crude reaction cocktails of both sense and anti-sense were mixed and heated at 95 °C for 5 min, then slowly cooled down by incubation at 37 °C for 1 h to obtain double-stranded RNA. A different double-stranded RNA with a two nucleotides mismatch from the $VEGF_{165b}$-specific siRNA was also designed and produced as above (mismatch $VEGF_{165b}$ siRNA). Both purified double-stranded RNAs (phenol : chloroform extraction) were transfected with pcDNA3-$VEGF_{165b}$ vector into HEK 293 cells using LIPOfectamine PLUS; pcDNA-$VEGF_{165b}$ vector alone was transfected into cells as control. The VEGF concentration in cell culture media was determined by ELISA 2 days after the transfection. VEGF concentration (mean ± S.E.M.) was reduced 8.32 ± 3.41-fold by co-transfection of $VEGF_{165b}$-specific siRNA compared with pcDNA3-$VEGF_{165b}$ vector transfection alone. Co-transfection of mismatch $VEGF_{165b}$ siRNA decreased VEGF concentration 1.91-fold ($P < 0.05$, ANOVA, compared with $VEGF_{165b}$ siRNA). We have demonstrated that $VEGF_{165b}$ siRNA can be used to specifically reduce expression of $VEGF_{165b}$ protein. We suggest that siRNA transfection is a useful method to study the effect of reduced $VEGF_{165b}$ production *in vitro*.

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PC54

Vascular endothelial growth factor mRNA and protein in rat skeletal muscle during chronic ischaemia

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Under conditions of insufficient vascular supply, compensatory neovascularisation is thought to be mediated by hypoxia-induced upregulation of vascular endothelial growth factor (VEGF). In skeletal muscles made chronically ischaemic by a precipitous reduction in blood supply, both VEGF protein and mRNA (*in situ*) were rapidly induced and marked capillary proliferation ensued (Couffinhal *et al.* 1998) but in other animal models of muscle ischaemia, VEGF protein was upregulated without capillary growth (Cherwek *et al.* 2000; Brown *et al.* 2002). This study investigated whether the time course of VEGF protein and mRNA expression is similarly co-ordinated in the latter situation.

One common iliac artery was ligated under Fluothane inhalation anaesthesia (2 % in O_2) in thirteen male Sprague-Dawley rats. Animals were killed 1 ($n = 3$), 2 ($n = 4$) or 5 weeks ($n = 6$) later, together with three unoperated control rats, by pentobarbitone overdose I.P., and extensor digitorum longus muscles removed and frozen for analysis.

Table 1. Time course of expression of VEGF protein and mRNA and capillary:fibre ratio (C:F) in ischaemic rat muscles

	VEGF protein	VEGF mRNA	C:F ratio
Control	1.00 ± 0.12	1.00 ± 0.11	1.41 ± 0.04
Ischaemic 1 day	—	1.65 ± 0.10*	—
Ischaemic 3 days	—	1.52 ± 0.01*	—
Ischaemic 1 week	1.12 ± 0.012	0.77 ± 0.05	1.30 ± 0.03
Ischaemic 2 weeks	1.59 ± 0.15*	0.63 ± 0.13*	1.37 ± 0.03
Ischaemic 5 weeks	1.79 ± 0.20*	0.38 ± 0.06*	1.70 ± 0.10*

Protein and mRNA were estimated by Western and Northern blotting, quantified by densitometry relative to β actin and normalised to values in control muscles = 1.0. C:F ratio was based on alkaline phosphatase staining of cryostat sections. All data are shown as means ± S.E.M. *$P < 0.05$ *vs.* control (ANOVA).

VEGF protein and mRNA are differentially altered during chronic muscle ischaemia, the former increasing while the latter decreases. Inhibition of VEGF mRNA expression has also been observed in cell cultures stressed by hypoxia (Stein *et al.* 1998). Whereas the increase in protein may arise through stabilisation of mRNA, it may also represent enhanced translation through internal ribosomal entry sites (IRES) in a cap-independent manner (Stein *et al.* 1998). These data indicate the variation in growth factor regulation in a realistic setting of progressive ischaemia but do not explain why capillary growth is so limited in this model.

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All procedures accord with current UK legislation.

PC55

Relaxatory actions of oestrogen receptor agonists and 17β-oestradiol on pre-constricted rat mesenteric arteries

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As premenopausal women have a lower incidence of cardiovascular disease than age-matched males or postmenopausal women, ovarian hormones have been thought to exert a vascular protective effect. Hormone replacement therapy (HRT) may confer similar risk reduction in postmenopausal women (Austin, 2000). However, this remains controversial with a recent study reporting increased adverse vascular events in postmenopausal women prescribed combined HRT (The Writing Group for WHI Investigators, 2002). If the positive or negative health effects of HRT are related to oestrogen receptor (ER) subtype-specific actions, it may be beneficial to utilise ER subtype-specific agonists in future HRT therapy. Thus we examined the effects of two such compounds, 4,4′,4″-(4propyl-[^{1}H]-pyrazole-1,3,5triyl)tris-phenol (PPT) and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), putative agonists of ER-α and ER-β, respectively (Tocris Cookson Ltd, UK), on vascular contractility and compared these with previously described endothelial-independent relaxant actions of acute 17β-oestradiol application (Shaw *et al.* 2000). Mesenteric resistance arteries (262–417 μm diameter), were isolated from male Wistar rats ($n = 16$) humanely killed by stunning followed by cervical dislocation in accordance with national guidelines and mounted on a wire myograph in HCO_3^--buffered physiological saline solution (37 °C, 95 % air and 5 % CO_2). Vessels were preconstricted with high K^+ solution (60 mM), and exposed to cumulative concentrations of 17β-oestradiol, PPT or DPN (1 nM–30 μM). PPT induced relaxation of preconstricted arteries in the concentration range 0.3–30 μM. The magnitude of vasodilatation to PPT was larger than for 17β-oestradiol across this range (ANOVA, $P = 0.002$). Alternatively, the magnitude of relaxation to DPN was significantly less than to 17β-oestradiol across similar concentrations ($P < 0.002$). Maximal relaxations (30 μM) were 101 ± 0.52 % (mean ± S.D.), 117 ± 0.37 % and 41 ± 0.29 % for 17β-oestradiol, PPT and DPN, respectively. Thus this pilot study demonstrates that acute relaxation of preconstricted mesenteric arteries with ER agonists or 17β-oestradiol occurs with the relative potency of PPT > 17β-oestradiol > DPN.

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Shaw L *et al.* (2000). *Brit J Pharm* **129**, 555–565.
The Writing Group for the Women's Health Initiative Investigators (2002). *J Am Med Ass* **288**, 321–333.

All procedures accord with current UK legislation.

PC56

Homocysteine increases proliferation and expression of peroxiredoxin I in normal but not pre-eclamptic vascular smooth muscle cells

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Elevated plasma homocysteine (Hcy) levels are associated with cardiovascular diseases such as atherosclerosis and pre-eclampsia (Rajokvic *et al.* 1999). Although Hcy is cytotoxic to endothelial cells, it induces vascular smooth muscle cell (SMC) prolifereration, which contributes to atherosclerotic plaque formation. Endothelial cell dysfunction induced by Hcy may be mediated by elevated oxidative stress, although its effects on SMC function remain to be elucidated. Peroxiredoxin I (PrxI) contributes to cellular anti-oxidant defences by acting as a thioredoxin-dependent peroxidase and heme binding protein (Ishii *et al.* 1993). PrxI also is involved in the regulation of cell proliferation (Fuji *et al.* 2002). In the present study, we have investigated whether induction of PrxI by Hcy is associated with proliferation in human umbilical artery vascular SMC (HUASMC) from normal and pre-eclamptic (PE) umbilical cords (collected with ethics committee approval). Cells were grown from explants in MCDB131 (Clonetics) containing 10 % fetal calf serum as described previously. Thymidine incorporation in G_0/G_1 synchronized cells was used as an index of SMC proliferation induced by Hcy (100 μM, 24 h). In parallel experiments, confocal images of PrxI immunofluorescent staining were obtained using a polyclonal antibody to PrxI. Hcy induced a significant ($n = 4$, $P < 0.05$, Student's t test) increase in thymidine incorporation in SMC isolated from normal (mean ± S.E.M., control *vs.* Hcy: 32.5 ± 5.7 *vs.* 82.5 ± 6.3 cpm (mg protein)$^{-1}$) but not from PE (70.1 ± 11.1 *vs.* 64.1 ± 10.2 cpm (mg protein)$^{-1}$) umbilical arteries. Confocal image analysis revealed that Hcy markedly enhanced expression of PrxI in normal SMC but not from PE arteries. The MEK inhibitor PD98059 attenuated the expression PrxI and proliferation induced by Hcy in normal SMC. These results show that Hcy induces PrxI expression and modulates SMC proliferation in normal SMC while impaired SMC function in PE may arise from the diminished induction of this protective anti-oxidant gene.

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PC57

Vasa vasorum: a system for transporting vasoactive factors in human saphenous vein?

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The vasa vasorum (i.e. 'vessels of the vessels', VV) is a network of nourishing small vessels in the walls of large blood vessels giving rise to a capillary network within the tunica adventitia, which may extend into the adjacent tunica media (Wheater *et al.* 1987). Veins are supplied much more abundantly with VV than arteries. Retrograde filling of VV in saphenous veins (SV) used in coronary artery bypass surgery has been described, suggesting that in certain cases VV may empty into the lumen of this vessel (Souza, 2002). We have used a combination of immunohistochemistry and light microscopy (LM) and transmission (TEM) and scanning (SEM) electron microscopy to identify VV in SV used as bypass conduits.

Following local ethics committee approval and patients' informed consent, segments of human SV ($n = 10$) were collected from patients undergoing coronary artery bypass surgery. For the LM study, frozen transverse sections were used for the immunohistochemical identification of endothelial cells (CD31) and vascular smooth muscle cells (anti-alpha smooth muscle actin) using the ABC peroxidase method. Microscopic examination revealed the distribution of endothelial and smooth muscle cells. Standard EM procedures (JEOL-1010 TEM and JEOL-4010LV SEM) were also used on the same tissue.

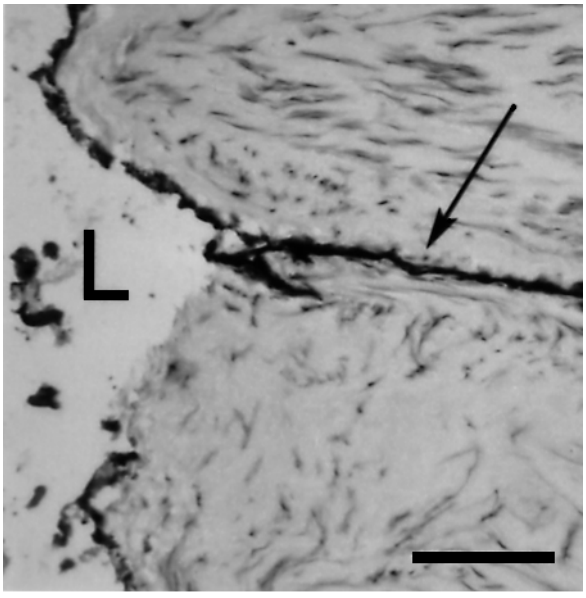

Figure 1. CD31 staining (arrow) of VV terminating in the lumen (L) of a section of human SV (scale bar = 100 μm).

Endothelial cells lining the lumen were observed in all vessels as well as clusters that were scattered throughout the vessel wall. Although the majority of these were located in the adventitia and media, in five of the vessels, endothelial cells were located within 20 μm of the intima, and in two cases the VV terminated in the vessel lumen (Fig. 1). Endothelial cells often co-localised with vascular smooth muscle cells in microvessels measuring approximately 25 μm in diameter. TEM and SEM of SV confirmed the presence of VV-like profiles at the adventitia-media border as well as in the intimal layer.

The VV are situated in close proximity to nerves in the SV. Furthermore, these microvessels are embedded within cells that are potential sources of numerous factors possessing both vasoactive and proliferative properties. The observation that VV empty into the lumen of the SV suggests that this microvessel network may represent a transport system for the local delivery of tissue- or nerve-derived factors within this vessel.

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