C10

Selective block of intracellular calcium release and EDHF dilatation in rat isolated resistance arteries

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Although it is generally accepted that the generation of an EDHF (endothelium derived hyperpolarizing factor) response follows calcium dependent endothelial cell hyperpolarization, recent data from rabbit mesenteric arteries has challenged this concept (Vequaud & Thorin, 2001). We have modified a technique for cellular loading of large membrane impermeant molecules for use in pressurized arteries, and selectively loaded heparin into endothelial cells in rat mesenteric artery to block IP_3-receptors. We demonstrate directly that increases in endothelial cell calcium are necessary for EDHF mediated vasodilatation in this vessel.

Male Wistar rats (200–250 g) were killed humanly and sections of a third order branch of the superior mesenteric artery (i.d 250–300 μm) then mounted in a pressure myograph at 50 mmHg and perfused with MOPS at 37 °C. In some experiments, endothelial cells were loaded with fluo-4 AM. The endothelial cells were selectively loaded with low molecular weight heparin (15 kDa) using an adaptation of the osmotic loading protocol developed for cells in culture by Okada & Rechsteiner (1982). Arteries were then contracted with phenylephrine (PE, 3 μM) and vasodilatation stimulated with acetylcholine (ACh, 10 nM–3 μM) or cyclopiazonic acid (CPA, 15 μM) in the presence of the NO synthase inhibitor, 100 μM L-NAME.

Increasing concentrations of ACh evoked a concentration-dependent dilatation (EC_{50} 177 nM; $n = 8$), which was unaltered by the osmotic loading protocol (EC_{50} without heparin present 170 nM; $n = 8$). The incorporation of heparin caused a significant rightward shift in the concentration response curve to ACh (EC_{50} 822 nM; $n = 5$; $P < 0.0001$, Student's t test) and reduced the maximum response. Dilatation to 300 nM ACh was reduced from $76 \pm 4\%$ ($n = 8$) to $22 \pm 5\%$ ($n = 5$; $P < 0.001$) or $14 \pm 5\%$ ($n = 7$; $P < 0.001$) by 10 mg ml^{-1} or 20 mg ml^{-1} external heparin, respectively, while contractions to 3 μM PE were unaltered. The ability of endothelial cells to store and release Ca^{2+} was not modified by loading heparin, as dilatation to CPA (15 μM) was unaltered ($n = 3$). However, heparin (20 mg ml^{-1}) did reduce the rise in intracellular Ca^{2+} normally evoked by 300 nM ACh. Peak increases in cytoplasmic Ca^{2+} after 10 seconds exposure to ACh ($53 \pm 10\%$) were greatly attenuated by the incorporation of heparin ($10 \pm 2\%$) ($n = 4$; $P < 0.05$). These data indicate that selectively loading endothelial cells with heparin significantly reduces the calcium increase normally evoked by ACh. This reduction was associated with marked surpression in EDHF mediated dilatation.

Together, these data provide direct evidence that EDHF evoked smooth muscle relaxation requires Ca^{2+} release, presumably from IP_3 sensitive intracellular stores in endothelial cells in rat mesenteric small arteries.

Okada CY & Rechsteiner M (1982). *Cell* **29**, 33–41.

Vequaud P & Thorin E (2001). *Circ Res* **89, 716–722**, 716–722.

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All procedures accord with current UK legislation

C11

Investigation into hydrogen peroxide-induced relaxation in rat mesenteric arteries

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Endothelial hydrogen peroxide (H_2O_2) generated through eNOS activation or shear stress is believed to play an important role in vasorelaxation as an endothelium-derived hyperpolarising factor (EDHF) in mouse and human mesenteric arteries. In these arteries, exogenous H_2O_2 caused a dose-dependent relaxation which was inhibited by the combination of charybdotoxin and apamin, and blocked by catalase or a high potassium solution (Matoba *et al* (2000); Miura *et al* (2003)). We assessed whether H_2O_2 had similar effects in the rat mesenteric artery.

Male Wistar rats (250–275g) were killed humanely by cervical dislocation and small resistance mesenteric arteries (i.d. 200–300 mm) were isolated and mounted on a Mulvany-Halpern small vessel wire myograph for measurement of isometric contraction. Results are expressed as mean percentage relaxation ± S.E.M. and statistical significance ($P < 0.05$) was performed using unpaired Student's t test.

H_2O_2 (10 nM–1 mM) caused a dose dependent relaxation that was present in both endothelium intact and denuded arteries. 100 μM H_2O_2 consistently gave rise to a full relaxation of the tissue, and this concentration was therefore used for subsequent experiments. This relaxation was almost completely blocked by the combination of charybdotoxin (100 nM) and apamin (100 nM) ($n = 3$), while partial blockage occurred when these toxins were applied separately in endothelium intact arteries (32 % ± 6 and 46 % ± 6 blockage for charybdotoxin and apamin, respectively, $n = 5$). However, the combination of apamin and charybdotoxin had no effect in endothelium denuded arteries. The large conductance calcium-activated potassium (BK_{ca}) channel inhibitor iberiotoxin (100 nM) also inhibited the hydrogen peroxide response in intact arteries ($73\% \pm 5$ inhibition, $n = 4$, $P < 0.001$). The application of pertussis toxin (400 ngml^{-1}) had no effect on the vasodilatation to H_2O_2 indicating that C-type natriuretic peptide (CNP) is not involved (Chauhan *et al* 2003); pertussis toxin did however abolish the response to acetylcholine.

In conclusion, we found that hydrogen peroxide causes vasodilatation which appears to involve multiple types of K_{ca} channels present on both the endothelium and smooth muscle. However, relaxation can also occur in the absence of the endothelium, suggesting that hyperpolarisation of smooth muscle via BK_{ca} channels is the primary mechanism for hydrogen peroxide induced-relaxation.

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Matoba T *et al.* (2000). *Br J Pharmacol* **129**, 1521–30.

Miura H *et al.* (2003). *Circ Res* **92**, 31–40.

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All procedures accord with current UK legislation

C12

IK_{Ca} and SK_{Ca} endothelial cell hyperpolarization underlies vasodilatation to EDHF but does not modify calcium entry in rat isolated mesenteric arteries

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The activation of intermediate (IK_{Ca}) and small conductance-calcium activated potassium channels (SK_{Ca}) in endothelial cells is a fundamental early step in the release of endothelium-derived hyperpolarizing factor (EDHF) (Busse *et al.* 2002). We have used selective inhibitors for these channels to investigate if the endothelial cell hyperpolarization, which is caused by their activation, is a crucial determinant of intracellular calcium concentration in the endothelium.

Male Wistar rats (200–250 g) were killed humanely and segments of a third order branch of the superior mesenteric artery (i.d. 250–300 μm) removed and mounted in a pressure myograph at 50 mmHg, perfused with MOPS at 37°C and visualized by confocal microscopy. Dilatation to acetylcholine (ACh, 1 nM–3 μM) was measured in the presence of 100 μM L-NAME, to block NO synthase, and phenylephrine to stimulate tone (PE, 3 μM). 1-[(2-Chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34, 1 μM: Wulff *et al.* 2000) and apamin (50 nM) were used individually and in combination to block I_{KCa} and SK_{Ca}, respectively. To assess Ca^{2+} levels, endothelial cells were selectively loaded with fluo-4 AM and stimulated with 300 nM ACh, in the presence of L-NAME alone or in combination with TRAM-34 and apamin. The change in fluorescence in individual endothelial cells was then monitored for 2 min.

Increasing concentrations of ACh caused concentration-dependent dilatation (EC_{50} 234 nM, $n = 6$). The concentration response curve to ACh was shifted to the right in the presence of either TRAM-34 (EC_{50} 613 nM, $n = 3$) or apamin (EC_{50} 616 nM, $n = 3$), while in combination these blockers caused a marked rightward shift (EC_{50} 4.03 μM, $n = 6$) and depressed the maximum dilatation (to 300 nM ACh) from 62.4 ± 7.1 % to 2.9 ± 1.3 % ($P < 0.05$, $n = 6$, Student's unpaired t test, mean ± S.E.M.). In the imaging experiments, 300 nM ACh stimulated a sustained rise in endothelial cell Ca^{2+} (increase at t = 10s: 38 ± 13 %; increase at t = 120s: 28 ± 9 %, $n = 4$, average of 16 cells/experiment). The time course and magnitude of these increases in endothelial cell Ca^{2+} was unaltered in the combined presence of TRAM-34 and apamin ($n = 3$), or in the presence of raised extracellular K^+ (35 mM, $n = 3$).

In conclusion, apamin and TRAM-34 inhibited EDHF-mediated vasodilatation in the mesenteric artery. However, these blockers did not modify the sustained increase in endothelial cell Ca^{2+} evoked with ACh. These results suggest that endothelial cell hyperpolarization is not essential for driving Ca^{2+} entry in these cells.

Busse R *et al.* (2002). *Trends Pharmacol Sci* **23**, 374–380.

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All procedures accord with current UK legislation

C13

Gender differences in distensibility and permeability of small mesenteric arteries from the β-estrogen receptor knock-out mouse (βERKO)

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β Estrogen knock out receptor mice (βERKO) have recently been shown to be hypertensive and to have altered contractile function in the aorta (Zhu *et al.* 2002). Since estrogens have also been implicated in vascular compliance and structure, we have evaluated distensibility and permeability of small mesenteric arteries from male and female βERKO mice and their wild-type littermates.

Male and female βERKO mice and their wild-type littermates were humanely killed by a rising concentration of CO_2 and cervical dislocation. Small mesenteric arteries (~185 mm in diameter) were rapidly dissected and mounted on a pressure myograph (Living Systems Instrumentation Inc, USA). Passive distensibility curves were constructed by carrying out stepwise increments in intraluminal pressure (20–100 mmHg) in a calcium free medium and by measurement of lumen diameter. In separate arteries permeability was assessed by infusion of Evans blue dye (0.03 %) into the vessel lumen and pressure maintained at 60 mmHg whilst photographic images were obtained every min for a five min period. Evans blue staining was evaluated on the basis of blue colour distribution within the artery wall. The degree of permeability was calculated in a blinded manner by two independent investigators using an ordered scale from 0 to 3+.

Passive distensibility was similar between mesenteric small arteries from βERKO males and wild-type littermates ($n = 8$ in each group). However arteries from female βERKO mice were less distensible than those of wild-type female littermates ($n = 8$ in both groups, $P = 0.011$ by ANOVA). No differences in permeability were apparent between arteries from βERKO females and wild-type female littermates whereas arteries from βERKO male mice were more permeable than those of male wild-type littermates (arbitrary units, values are median (confidence intervals) 2.5 (0.66–3) $n = 8$ *vs.* 1(0–1) $n = 7$, $P = 0.014$; Mann-Whitney test).

In conclusion, this study suggests that the estrogen receptor β (ERβ) has gender specific effects on distensibility and permeability in small mesenteric arteries of the mouse. Estrogens, through the ERβ may increase distensibility and hence vascular compliance in the resistance vasculature of the wild-type female mouse whereas in the male mouse the same receptor may have an important influence on control of vascular permeability. It may also be concluded that influences of the ERβ on distensibility and permeability are not functionally linked in the mouse mesenteric artery. All procedures were performed in accord with current National guidelines.

Zhu Y *et al.* (2002). *Science* **295**, 505–508.

All procedures accord with current national and local guidelines

C14

Circulating endothelial cells and markers of endothelial damage after cardiac bypass

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Circulating endothelial cell (CEC) numbers rise in many cardiovascular disorders and have been interpreted as reflecting endothelial pathology (Dignat-George & Sampol, 2000). However, CECs also rise after exercise (O'Sullivan, 2003) and there is poor correlation between CECs and two plasma markers of endothelial damage, von Willebrand factor (vWF) and thrombomodulin (TM) (O'Sullivan, 2003; Barry, 2004; Tansey, 2004). It is also unknown whether CECs originate from systemic or pulmonary systems and the extent to which they recirculate. To gain more information on these points, we have measured arterial and venous CEC numbers and viability and plasma vWF and TM, in 15 patients (64 ± 2 years) undergoing cardiopulmonary bypass, which preferentially damages pulmonary endothelium (Kotani *et al.* 2000).

Patients were recruited from the Coronary Care Unit at St James's Hospital. With institutional ethics approval and written informed consent, jugular venous and radial arterial samples were taken immediately before surgery and 1 and 6 h after cessation of bypass. CECs were isolated by differential centrifugation and stained with cresyl violet for cell counts or with trypan blue to detect viability. Plasma vWF and TM were assayed by ELISA. Results were corrected for peri-operative haemodilution and analysed with ANOVA or paired *t* tests.

Pre-operative plasma vWF was 2-fold higher and TM was 3-fold lower than in normal middle-aged individuals (see Barry, 2004). Both these markers rose during the bypass period (vWF pre 390 ± 25, 1 h post 595 ± 35 Units; TM pre 1.1 ± 0.2, 1 h post 3.5 ± 0.5 ng ml^{-1}, means ± S.E.M.) and the increases were sustained over the 6 h post-bypass period ($P < 0.01$). Pre-operative CEC numbers were closely similar in venous and arterial bloods (201 ± 53 and 217 ± 65 cells ml^{-1}) and around 2-fold higher than in healthy controls (see Barry, 2003). By 6 h post-bypass, CECs showed a non-significant downward trend. At the same time point, there was a rise in viable venous CECs (35 ± 7 from 24 ± 5 %, $P < 0.05$) with no change in arterial viability.

Our data demonstrate that in cardiac patients, as in normal individuals, there is lack of correlation between CEC numbers and plasma levels of vWF or TM. The absence of an arteriovenous CEC gradient indicates that these cells recirculate freely. The selective rise in viable cells in venous blood after bypass suggests that they originate preferentially from the systemic rather than the pulmonary circulation.

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Dignat-George F & Sampol J (2000). *Eur J Haematol* **65**, 215–220.

Kotani N *et al.* (2000). *Anesth Analgesia* **90**, 1039–1045.

O'Sullivan SE (2003). *Int J Sports Med* **24**, 404–409.

Tansey E (2004). *J Physiol* **554.P**, C74.

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

C15

Plasma from women with severe pre-eclampsia contains a circulating macromolecule that increases hydraulic conductivity in frog mesenteric microvessels *in vivo*

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Pre-eclampsia is the leading cause of morbidity of pregnant women. It is associated with high blood pressure, intrauterine growth retardation (IUGR), haemolysis, proteinuria and increased oedema, in part due to increased vascular permeability. To determine whether this increase in permeability is stimulated by a circulating macromolecule we have compared the effect of plasma from patients with severe and mild pre-eclampsia with plasma from patients with normal pregnancies on permeability of frog mesenteric microvessels.

With ethical committee and patient consent, plasma was taken from 6 patients with severe pre-eclampsia [3 with IUGR, 3 with haemolysis, elevated liver enzymes and low platelets (HELLP)], 10 patients with mild pre-eclampsia (proteinuria and hypertension but without IUGR or HELLP) and 45 normal pregnancy controls, matched for age and gestation. The samples were dialysed against frog Ringer using a 12 kDa molecular weight dialysis tubing. The colloid osmotic pressure of the dialysed plasma was measured in a modified Hansen oncometer. Frogs were anaesthetised by immersion in MS222 and the mesentery exposed as previously described. A microvessel was cannulated and perfused with a solution of human serum albumin that was matched for colloid osmotic pressure to the plasma to be perfused. A series of measurements of filtration rate were taken using a modification of the Landis Michel technique (Harper *et al.* 2002). The pipette was then refilled with dialysed plasma and filtration rate measured at two pressures (20 and 30cmH_2O) for up to ten min. Hydraulic conductivity (L_p) and oncotic reflection coefficient (σ) were calculated from the slope and *x* intercept respectively of the filtration pressure relationship. The frogs were then humanely killed by destruction of the brain.

Neither control nor mild pre-eclamptic plasma caused a significant change in L_p or σ within 10 min of perfusion. Perfusion of plasma from patients with severe pre-eclampsia, however, resulted in a rapid transient increase in L_p from mean ± S.E.M. 1.6 ± 0.26 to 11.5 ± 2.3 × 10^{-7} cm.s^{-1}.cmH_2O^{-1} ($P < 0.02$, paired *t* test) that returned to control within two min with all six plasma samples. σ reduced from 0.98 ± 0.05 to 0.90 ± 0.05 ($P = 0.1$).

These results show that a macromolecule in pre-eclamptic plasma can increase microvascular permeability. The identity of the molecule is not known.

Harper SJ *et al.* (2002). *Kidney Int* **61**, 1416–1422.

All procedures accord with current local guidelines, the Declaration of Helsinki and UK legislation

C16

Interleukin-1β potentiation of bradykinin-mediated permeability increase via nadph oxidase activation in single pial venular capillaries of anaesthetized rats

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Previous work has shown that pial venular permeability increase mediated by bradykinin is potentiated by IL-1β within 10 min. of its application with further potentiation after 30 min. (Hu & Fraser, 1997). The bradykinin B2 receptor on pial venular capillaries activates PLA_2, and the resulting arachidonic acid forms free radicals following its interaction with cyclooxygenase and lipoxygenase (Sarker *et al.* 2000). The subsequent lipid peroxidation of the plasma membrane is thought to result in calcium entry. IL-1β will activate PLA_2 in bronchial smooth muscle (Schmidlin F. *et al.* 2000), and it is possible that a pathway coincidence in this endothelium results in the observed potentiation.

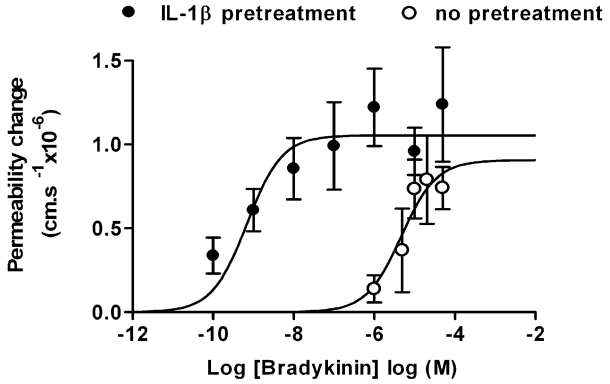

Figure 1. The effect of IL-1β pretreatment on the permeability response to bradykinin. The sensitivity to Bk was increased ($logEC_{50}$ no pretreatment -5.3 ± 0.23, $logEC_{50}$ following IL-1β -9.4 $logEC_{50}$ 0.27, $n = 5$, $P < 0.0001$, 't' test), but there was little change in the maximum (from 0.88 $\pm$ 0.07 to 1.08 $\pm$ 0.12 following).

The permeability (P_{Rh}) to sulforhodamine dye (588 Da) of single venular capillaries of rats (anaesthetized with hypnorm/hypnoval: 10 mg kg^{-1} I.P., and humanely killed at the end of the experiments) was measured as previously described (see Sarker *et al.* 2000). Application of with IL-1β (30 pM) resulted in a potentiation of the PRh response to Bk within 10 min, which remained stable in the presence of cycloheximide (100 μM) for up to 90 min. IL-1β pretreatment had little effect on the maximum P_{Rh}, but shifted the dose-response relationship considerably leftward (see Fig. 1). P_{Rh} was free radical dependent, as combination of superoxide dismutase and catalase (each 100 U ml^{-1}) reduced the response to 5 μM Bk from 0.82 ± 0.134 cm s$^{-1} \times 10^6$ (mean $\pm$ S.E.M.) to 0.20 ± 0.32 ($n = 4$, $P < 0.01$, paired t test). The idea that PLA_2 activation provides a coincidence in the signalling pathways was tested by blocking this with palmitoyl trifluoromethyl ketone (100 μM) in paired experiments. The maximum response was reduced by 62 ± 15% ($n = 8$), but with little change in EC_{50}. The possibility that IL-1β promotes NADPH oxidase assembly was tested by applying diphenylene iodonium chloride (DPI, 100μM), and this reduced PRh by 48 ± 4% ($n = 8$). As DPI had no effect on PRh to Bk without IL-1β pretreatment (Bk alone 1.05 ± 0.125, with DPI 1.10 ± 0.07, $n = 8$), it is likely that IL-1β can cause rapid NADPH oxidase assembly.

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Schmidlin F *et al.* (2000). *Naunyn-Schmiedebergs Arch Pharmacol* **361**, 247–254.

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All procedures accord with current UK legislation

C17

Basement membrane development in long-term endothelial cultures: a major factor regulating migration of neutrophils?

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Neutrophils use an ordered sequence of known adhesive interactions to bind to cytokine-stimulated endothelium. However, the mechanisms regulating migration through and under the endothelial cell monolayer are uncertain. We hypothesised that constituents of the basement membrane might influence neutrophil migration, for instance by providing a reservoir for chemotactic agents or a haptotactic adhesive substrate.

To test this hypothesis, confluent monolayers of first passage human umbilical vein endothelial cells (HUVEC; isolated from umbilical cords obtained with informed consent) were cultured for 1, 3, 5, 10, 15 or 20 days, and then stimulated with 1, 10 or 100 U/ml of the inflammatory cytokine tumour necrosis factor-α (TNF) for 4 h prior to the experiment. Neutrophils were allowed to bind to the endothelial surface, and were directly observed by phase-contrast video-microscopy as they migrated over and under it. Several differences were noted in the responses when, for instance, day 1 and day 20 cultures were compared (all data are means $\pm$ S.E.M. of 3 to 5 experiments): (i) day 20 cultures induced higher levels of neutrophil adhesion at 1 U/ml TNF (67 ± 4% of neutrophils adherent *vs.* 20 ± 5% for day 1 cultures) or 10 U/ml TNF (61 ± 3% of neutrophils adherent *vs.* 35 ± 3% for day 1 cultures). ANOVA showed significant effect of culture time on adhesion ($P < 0.01$); (ii) the percentage of adherent neutrophils transmigrating was higher for day 20 cultures than day 1 (35 ± 3% of adherent neutrophils transmigrated at 1 U/ml *vs.* 7 ± 2% for day 1 cultures). ANOVA showed significant effect of culture time on transmigration ($P < 0.01$); (iii) at 100 U/ml TNF, adhesion and efficiency of transmigration were similar for day 1 and day 20 cultures, but the transmigrated neutrophils moved more slowly for the day 20 cultures (7 ± 0.3 μm/min *vs.* 12 ± 0.1 μm/min for day 1 cultures; $P < 0.05$ by Student's t test). Using similar cultures, enzyme-linked-immuno-sorbent-assay (ELISA) showed increasing deposition of basement membrane components (e.g., collagen type IV and laminin) with culture time. In separate experiments, varying concentrations of purified basement membrane proteins (collagen type IV, laminin and fibronectin) were adsorbed to plastic dishes, and their ability to bind activated neutrophils was tested. Interestingly, at surface concentrations comparable to those found under cultures (verified by ELISA), neutrophil adhesion was markedly augmented by these proteins (e.g., ratio to control adhesion = 4.0 ± 1.0 for laminin; mean $\pm$ S.E.M. from 3 comparisons).

These data support the concept that substances laid down in the basement membrane influence neutrophil behaviour on endothelium, either by directly interacting with migrating cells or indirectly modifying the responses of the endothelial cells growing on them.

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

C18

The phototoxic effects of fluorochromes and high intensity illumination on the migration of neutrophils in a flow-based adhesion assay

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Leukocyte recruitment to sites of inflammation has been studied using a variety of experimental techniques including; intravital microscopy, static adhesion assays and flow-based adhesion assays using whole blood or isolated leukocytes. The development of fluorescent dyes that passively diffuse into cells and bind to intracellular compartments has enabled investigators to visualise interactions between leukocytes and endothelial cells (EC) using real-time imaging. However, there is little information on the detrimental effects that fluorescent dyes have on leukocyte behaviour *in vivo* or *in vitro*, which is an important factor in any study purporting to investigate their detailed adhesive behaviour.

In the present study we used two flow-based adhesion assays to investigate effects of high intensity illumination (HI light) on the migration behaviour of neutrophils in the presence or absence of five commonly used fluorochromes; Bisbenzimide (Hoechst 33342), Calcein-AM, Cell^{TM} Tracker Orange (CMTMR), Quinacrine and Rhodamine-6G.

The research was carried out according to local ethical guidelines in collaboration with South Birmingham's Local Research Ethics Committee. Isolated neutrophils, with or without the fluorochrome labelling were either (1) perfused through P-Selectin (1 μg/ml) coated glass capillary tubes to establish a population of rolling cells and then activated by the bacterial peptide analogue formyl-methionyl-leucyl-phenylalanine (fMLP; 10^{-7} M), or (2) perfused through glass capillaries containing Human Umbilical Vein Endothelial Cells (HUVEC; donors gave informed consent) previously activated by the inflammatory cytokine tumour necrosis factor α (TNFα; 1 U/ml), so that the EC could support the adhesion and migration of flowing neutrophils. The research was carried out according to local ethical guidelines in collaboration with South Birmingham's Local Research Ethics Committee.

All experiments were conducted on an Olympus Reflected Fluorescence System microscope and HI light was defined as light emitted from a USH10 100 W Mercury Burner with no barrier filters, and passed through the appropriate filters for Ultra-Violet (UV), blue and green light (excitation wavelengths; 330–385nm, 460–490 nm and 510–550 nm respectively) using a LCPlanFL 20× 0.4 Ph1, CAP-P1.1±0.5 objective lense.

Table 1; Effects of HI light on the migratory and transmigratory behaviour of activated neutrophils.

	P-Selectin						HUVEC					
	Ph.C	UV	Ph.C	B	Ph.C	G	Ph.C	UV	Ph.C	B	Ph.C	G
Trans-migrated (%)	N/A	N/A	N/A	N/A	N/A	N/A	31.88 ±10.61	21.15** ±8.15	26.32 ±4.54	7.30* ±1.52	28.05 ±5.13	18.27* ±3.42
Migration Velocity (μm/min^{-1})	6.99 ±1.07	3.64 ±0.64	6.99 ±1.07	3.77* ±0.21	6.02 ±0.45	3.54** ±0.29	10.34 ±1.23	5.37** ±0.20	8.54 ±0.96	1.70** ±0.18	7.80 ±0.77	4.25** ±0.45

Ph.C; Phase Contrast Light, **UV**; Ultra-Violet Light, **B**; Blue Light. **G**; Green Light.
* (P<0.05), ** (P<0.001) paired Sudent's *t* test. Data are mean ± SEM.

In the absence of HI light fluorochromes did not affect neutrophil behaviour. However, HI light alone caused a ≈50 % decrease in neutrophil migration velocities in both assays and caused a significant decrease in the number of neutrophils transmigrating through the EC monolayer. In the P-Selectin model, Calcein-AM, Cell Tracker Orange and Rhodamine-6G (0.25–4 μg/ml) in combination with HI light caused a decrease in migration velocities synergistic to the effects of HI light alone ($P < 0.01$). In the HUVEC model, the combination of HI light and Bisbenzimide, Calcein-AM and Cell Tracker Orange (1–4 μg/ml) caused a further decrease in the number of transmigrated neutrophils ($P < 0.05$), compared to Quinacrine which caused an increase ($P < 0.05$). Rhodamine-6G caused a decrease in migration velocity synergistic to that of HI light alone ($P < 0.05$).

It is therefore important that investigators examine the full effects that HI light and fluorochromes have on cell behaviour before employing them in experimental systems.

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All procedures accord with current ocal guidelines and the Declaration of Helsinki

C69

Raised activity of glycosylating enzyme core 2 GlcNAc-T in neutrophils and cardiomyocytes of spontaneous diabetic BB-rats

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The mechanisms underlying the pathogenesis of diabetic retinopathy are poorly understood, but studies have suggested that leukocytes may play a major role in its development. Previously, we reported that the activity of UDP-GlcNAc:Galβ1–3GalNAcαRβ-N-acetylglucosaminyltransferase (core 2 GlcNAc-T), a developmentally regulated enzyme of O-linked glycans biosynthesis pathway, is increased in polymorphonuclear leukocytes (PMN) in diabetic patients (Type 1 and Type 2), through PKCβ2-dependent phosphorylation (Chibber *et al.* 2000). The aim of this study was to establish an animal model to provide direct experimental evidence for the role of core 2 GlcNAc-T in increased leukocyte-endothelial cell adhesion in pathogenesis of diabetic retinopathy.

BB-rats and Wistar-rats where maintained by Taconic M & B, Denmark and euthanized according to Danish Animal regulations. Leukocytes suspensions were prepared by density gradient centrifugation. Briefly, 3 ml of blood was layered onto equal volume of Histopaque 1270, and centrifuged (400 g, 30 min), PMN-rich buffy coat was carefully removed, resuspended in phosphate-buffered saline (PBS), centrifuged (250 g, 15 min), and the pellet stored at −20°C until used for the measurement of core 2 GlcNAc-T as described (Chibber *et al.* 2000). Heart tissue was also isolated, rinsed in PBS and homogenized at 4°C in lysis buffer (0.9 % NaCl, 0.4 % Triton X-100, and 0.1 mM PMSF). The data were analysed using unpaired *t* test.

The activity of core 2 GlcNAc-T was significantly higher in PMN leukocytes of BB-rats compared to age-matched control Wistar-rats [2390 ± 439.3 pmol h^{-1} mg protein^{-1} ($n = 6$) *vs.* 244.6 ± 50.2 ($n = 6$), mean ± S.E.M., $P = 0.0007$, unpaired Student's *t* test]. The activity of core 2 GlcNAc-T was also higher in the heart tissue of BB-rats compared to age-matched Wistar-rats

[1622 ± 181 pmol h^{-1} mg $protein^{-1}$ ($n = 6$) *vs.* 125.1 ± 36.04 ($n = 4$), $P = 0.0008$].

These results are consistent with our recent data (Chibber *et al.* 2000) and suggest that BB-rats would be useful as an animal model in Type 1 diabetes to further explore the role of core 2 GlcNAc-T in pathogenesis of capillary occlusion in diabetic retinopathy.

Chibber R *et al.* (2000). *Diabetes* **49**, 1724–1730.

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All procedures accord with national regulations

C70

Temporal sequence of changes in whole-body and vascular insulin resistance in a murine model of diet induced obesity

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Impaired nitric oxide (NO) bioactivity is a prominent feature of the vasculopathy associated with obesity and insulin resistance. Although insulin promotes endothelial NO production, the mechanistic relationship between insulin resistance and endothelial dysfunction is poorly defined. We tested the hypothesis that metabolic insulin resistance precedes vascular insulin resistance in the metabolic syndrome using a model of diet-induced obesity in mice.

Male C57Bl/6 mice receiving an obesogenic diet (35 % fat, 35 % carbohydrate) from weaning were compared with chow-fed controls (8 per group). Body weight, glucocompetence, triglycerides, systolic blood pressure and vasomotor responses in aortic rings *ex vivo* were assessed after 4 and 8 weeks of feeding.Mice were humanely euthanized with pentobarbital (80 microgram/kg i.p) prior to vascular experiments.

Results [mean± SEM]

Parameters	Control 4 weeks	High Fat 4 weeks	p value	Control 8 weeks	High Fat 8 weeks	p value
Weight (grams)	24.1±0.3	29.0±0.4	<0.0001	27.3±0.3	38.4±0.6	<0.0001
Epididymal Fat pad (mg)	11.6±1.1	24.3±1.5	<0.0001	11.7±0.4	33.9±1.0	<0.0001
Systolic BP(mm Hg)	116.1±0.4	122.9±0.5	<0.0001	117±0.8	124.8±0.5	<0.0001
Fasting Blood Sugar(mM)	6.4±0.6	12.0±0.3	<0.0001	7.1±0.4	10.1±0.3	<0.0001
Fasting Triglycerides (mM)	0.7±0.04	5.3±0.07	<0.0001	2.2±0.3	5.4±0.1	<0.0001
% Fall in blood sugar at 30 mins after ITT	70.2±1.9	54.2±0.6	<0.0001	65.6±2.5	48.4±3.3	<0.0001
% Change PE E-max after insulin preincubation	-26.2±5.6	-28.6±11.1	NS	-17.9±11.5	+11.2±15.5	<0.05

The results are summarised in the table [stastitical methods-unpaired Student's *t* test; $P < 0.05$-significant]. Mice receiving an obesogenic diet developed a typical metabolic syndrome; with obesity, hypertension, hyperglycemia and hypertriglyceridemia evident after 4 and 8 weeks feeding. Whole body insulin resistance, demonstrated by a blunted hypoglycaemic response to exogenous insulin (0.75u/kg i.p) was apparent by 4 weeks. In aortic rings from control mice, pre-incubation with insulin (10 mU/ml, 2 hours) significantly blunted the maximal constriction to phenylephrine (PE; 10 μM) – an effect that was blocked by the NO synthase inhibitor L-NMMA (not shown). This vascular effect of insulin was preserved in mice receiving an obesogenic diet for 4 weeks, but was lost after 8 weeks of feeding. Relaxation responses to acetylcholine and nitroprusside were similar in both groups of mice.

In conclusion, these data indicate that the development of whole body insulin resistance in a murine model of obesity precedes the onset of resistance to insulin's NO-dependent vasodilatory effects.

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All procedures accord with current UK legislation

C71

Flow dependence of hyaluronan reflection in synovial joint cavities

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Hyaluronan (HA) in synovial joints is partially reflected by the joint lining and has been reported to assist in the conservation of intra-articular fluid during joint flexion by the creation of a concentration polarization layer which buffers fluid loss (McDonald & Levick, 1995). To examine this hypothesis, a series of experiments has been carried out on joint cavities of rabbit knee using a mixture of HA and fluorescein dextran (FD), which acted as a reference solute (e.g. Sabaratnam *et al.* 2003). Data from these experiments were in agreement with the hypothesis. It was also noted that the reflected fraction of HA by synovial lining decreased at higher trans-synovial flow rate. The authors applied concentration polarization theory to explain the phenomenon in a simplified ultrafiltration model (Coleman *et al.* 1999). However, the steady state assumption used in their analysis restricted its applicability to account for the observed flow dependence of HA reflection in synovial joint cavities. In the current study, we developed a theoretical model considering the unsteady nature of HA filtration in animal experiments, to study the build-up of HA concentration polarization layer near the synovial lining, and to elucidate the mechanism for flow dependence of HA reflection in joint cavities.

Using a one dimensional dead-end filtration model, we derived a differential-integral equation from mass conservation that accommodated unsteady solute filtration near a partially permeable membrane. With model parameters taking values derived from experiments, we solved the equation numerically. Results from the model gave insight into the build up of the HA concentration polarization layer with the time. They revealed how parameters, such as the filtration rate, diffusivity of HA and the reflection coefficient of the synovial lining to HA, affected HA ultrafiltration.

To explain the observed flow dependence of HA reflection in joint cavities, we extracted data from the model at the same time as in experiments. Good agreement between experimental data and model prediction was observed (Fig. 1). In addition, we were able to calculate the time for the system to reach steady state and the thickness of the polarization layer at a given filtration rate. We found that at low trans-synovial filtration rates, the estimated time for the system to reach steady state exceeded experimental time and the thickness of the polarization layer extended >2 mm.

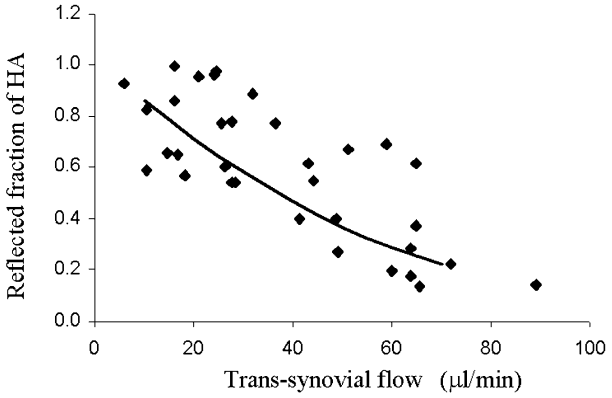

Figure 1. Flow dependence of HA reflection in jointcavities. Experimental data from Sabaratnam *et al.* (2003, *idem*), solid line from model prediction.

In conclusion, results from the model confirmed the concentration polarization layer near the joint lining. Flow dependence of HA reflection could be caused by unsteady HA ultrafiltration in joint cavities before the system reaches steady state.

Coleman PJ *et al.* (1999). *J Physiol* **514**, 265–282.
McDonald JN & Levick JR (1995). *J Physiol* **485**, 179–193.
Sabaratnam S *et al.* (2003). *Microvas Res* **66**, 227–236.

C72

Effects of external dynamic load on the transport of solute in extracellular matrix

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Dynamic compression of soft tissues, such as articular cartilage, affects tissue mechanical properties and metabolic activity. The effect is attributed, in part, to the water and solutes movement in the extracellular matrix, which alters the mechanical (e.g. fluid shear stress) and chemical (e.g. growth factors, cytokines and hormones) microenvironment for cells in the soft tissue (Guilak *et al.* 1997). Experimental studies on cartilages suggest that external dynamic load enhances solute transport to inner regions of specimens, particularly for large sized molecules (O'Hara *et al.* 1990). Few theoretical studies, however, have been carried out to clarify the underlying mechanism and to quantify the enhancement. This study aims to highlight and to quantify the contribution of dynamic loads on solute transport to regions of tissue where cells would, otherwise, remain inactive due to the low solute concentration there.

In our analysis, poroelastic theory was used for the deformation of the solid matrix and the movement of water within (Wang & Parker, 1995). The solid phase represented the matrix of collagens and proteoglycans, and the liquid phase the interstitial fluid. A simplified two-dimensional model was used, that consisted of a deformable matrix embedded with cells immersed in solution in a rigid impermeable well. The top surface of the matrix was in direct contact with the solution with known solute concentration. Solute diffused into the matrix and was consumed by cells. Mechanical cyclic loads were applied in the central region of the top surface of the matrix, causing its deformation and extracellular fluid movement. Resulting cell density in the matrix varied with the time and the location. Solute diffusion coupled with the movement of the extracellular fluid contributed to the solute transport in the matrix.

Governing equations for the matrix deformation, interstitial fluid movement and solute transport were solved numerically. Comparisons on solute transport were made between different loading frequencies and amplitudes. Different sized molecules were also considered in our study. Results from the model confirmed experimental findings that cyclic loads facilitated solute transport in soft tissues and the effect was more significant for large sized molecules. Furthermore, we found that higher loading frequency and bigger loading amplitude introduced better improvement to solute transport. Quantitative analysis of solute concentration distribution in the tissue made it possible to predict regions where cells were activated by the improved solute supply. Activation of cells occurred often without significant elevation of solute concentration in the tissue. The fact that more cells in tissues became metabolically active under dynamic loads exemplified most directly their effects on solute transport in soft tissues.

In conclusion, dynamic loads on extracellular matrix promote solute transport to inner regions of the tissue. Poroelastic theory makes it possible to predict the enhancement quantitatively for different sized solutes under different loading conditions.

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O'Hara BP *et al.* (1990). *Ann Rheum Diseases* **49**, 536–539.
Wang W & Parker KH (1995). *J Fluid Mech* **283**, 287–305.

C73

Flow-dependence of molecular sieving by the joint-to-lymph barrier of rabbit knees supports a concentration polarisation hypothesis

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Synovial fluid hyaluronan (HA) profoundly buffers pressure-driven fluid loss from joints. This may be due to flow-dependent HA accumulation at a partially reflecting interface ('concentration polarisation'). We tested a discriminating prediction of concentration polarisation theory, namely that reflected fraction (= 1-transmitted fraction) should fall at high fluid velocities (cf. rise to a plateau if no concentration polarisation).

The knee joint cavity of anaesthetised rabbits (30 mg kg^{-1} pentobarbitone, 500 mg kg^{-1} urethane, I.V.) was infused with 0.2 mg ml^{-1} rooster HA (2000 kDa, radius of gyration 145 nm) and fluorescein-dextran (FD, reference solute, 20 kDa) at a constant pressure and trans-synovial filtration rate (range 6–89 μl min^{-1}) for 3 h. Joint-derived lymph was harvested from femoral lymphatics. Samples of mixed intra-articular fluid and subsynovial fluid were aspirated at the end. Samples were analysed by gel exclusion chromatography. HA transmitted fraction was calculated from the downstream (filtrate) to upstream (bulk infusate) concentration ratio, using [FD] to adjust for the dilution of joint lymph in femoral lymph. The animal was killed humanely at the end of the experiment.

Lymph flow, lymph [HA] and subsynovial [HA] increased with trans-synovial filtration rate. Lymph [HA] & subsynovial [HA] as fractions of infusate concentration were always lower than FD fractional concentrations in the same samples. The terminal intra-articular HA concentration, mean 0.47 ± 0.02 mg ml^{-1} (mean ± S.E.M., $n = 31$), always exceeded infusate concentration (0.20 mg ml^{-1}), confirming ultrafiltration of HA.

The reflected fraction for the cavity-to-lymph barrier (synovium + lymphatic capillary endothelium), R_{lymph}, was a negative function of trans-synovial flow and fell from 0.93 at the lowest filtration rate (6 μl/min) to 0.14 at 89 μl/min ($P < 0.0001$, regression analysis, $n = 33$). Reflected fractions calculated from intra-articular HA accumulation (R_{asp}) or subsynovial/infusate [HA] ratio (R_{syn}) were almost identical negative functions of filtration rate. Reflection by lymphatic capillary endothelium (R_{endo}), calculated from the HA lymph/subsynovial concentration ratio, was not significantly different from zero (mean -0.03 ± 0.18, $n = 21$). R_{lymph}, R_{asp} and R_{syn} did not differ significantly and all exceeded R_{endo} ($P < 0.001$, ANOVA). Thus synovial interstitial matrix rather than lymphatic capillary endothelium is the chief HA-reflecting membrane.

The negative relation between reflection and filtration rate supports a concentration polarisation hypothesis. Concentration polarisation leads to osmotic buffering of fluid escape, which prevents the joint from wringing itself dry during flexion.

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All procedures accord with current UK legislation

C74

Effects of hormonal status, age and smoking on cardiovascular function in women

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Risk factors for arterial disease and cardiovascular mortality include male sex, ageing and smoking (Bolego *et al.* 2002; Hayward *et al.* 2000). In this study, I have performed some preliminary studies to test the hypothesis that menopause and smoking have synergistically disruptive effects on endothelial function.

The study involved young menstruating women and postmenopausal hormone replacement therapy (HRT) users or non-users. Subjects were either long-term smokers (at least 10/day for 1 year) or non-smoking controls. With institutional ethical approval, young (23 ± 2 years, mean ± S.E.M.) ovulating women in the menstrual phase of their cycle, postmenopausal (56 ± 3 years) women and postmenopausal HRT users (58 ± 2 years) attended the laboratory. Smokers were asked to abstain for at least 1 h prior to testing. A blood sample was taken for plasma analysis of circulating endothelial cells (CECs), von Willebrand factor (vWF) and thrombomodulin (TM) levels. Resting mean arterial pressure (MAP) was measured using applanation tonometry. Forearm reactive hyperaemia following 3 min arterial occlusion was measured by venous occlusion plethysmography as an index of endothelium-dependent vasodilator capacity. Two-tailed paired (longitudinal comparisons) or unpaired (cross-sectional comparisons) *t*-tests were used in analysis. $P < 0.05$ was deemed significant.

The postmenopausal controls ($n = 10$) had elevated MAP when compared with the young group ($n = 10$) and there was no difference in MAP between older controls using (88±2.5 mmHg) or not using (91 ± 4.0 cf 76 ± 1.6 mmHg, $P < 0.05$) HRT. Ageing was not associated with diminution of reactive hyperaemia (area under the curve: HRT 119 ± 26.7, non-HRT 116±25.3, young 185±54 ((ml min^{-1}) 100 ml^{-1}) s) and no age-related changes in plasma levels of CECs, vWF or TM were seen. Among the young women, smokers ($n = 7$) had elevated CECs (117 ± 22.1 cf 59 ± 10.6 cells ml^{-1} blood) but similar levels of vWF (72 ± 34.9 cf 70 ± 25 Units) and TM (8.9 ± 0.74 cf 7.3 ± 0.76 ng ml^{-1}) and similar reactive hyperaemic responses (205 ± 58.6 ((ml min^{-1}) 100ml^{-1}) s) as controls. These characteristics did not alter further with age but postmenopausal smokers ($n = 7$) exhibited marked attenuation of reactive hyperaemia (36 ± 7.0 ((ml min^{-1}) 100 ml^{-1}) s).

In conclusion, moderate ageing is accompanied by an elevation in resting MAP that is not reversed with HRT. This age-dependent rise in MAP is not related to impaired reactive hyperaemia or to altered levels of plasma markers thought to reflect endothelial damage. Long-term smoking is however linked to a functional loss of vasodilator activity that is independent of plasma changes in endothelial markers.

Bolego C *et al.* (2002). *Cardiovasc Res* **53**, 568–576.

Hayward CS *et al.* (2000). *Cardiovasc Res* **46**, 28–49.

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C76

Effects of ageing on forearm reactive hyperaemia and on several circulating markers of endothelial function

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Ageing is a major risk factor for cardiovascular disease and is thought to convey this risk in part by impairing endothelial function (Lakatta, 2002). The purpose of this study was to investigate the hypothesis that ageing alters resting endothelial behaviour and endothelial responses to acute exercise.

With institutional ethical approval, 14 young (mean ± S.E.M., 22 ± 1 yrs) and 13 aged (57 ± 3 yrs) healthy male non-smokers were recruited by local advertisement. Subjects attended the laboratory between 0900 hrs and 1300 hrs, having abstained from caffeine and intense exercise for 12 hours. Forearm reactive hyperaemia following a 3 min period of arterial occlusion was measured using venous occlusion plethysmography. Resting venous blood samples were taken for quantification of circulating endothelial cells (CEC) and for the analysis of plasma thrombomodulin (TM) and von Willebrand factor (vWF). Full blood cell counts were also carried out. Following a medical examination, subjects performed a bout of cycle exercise, in the form of an incremental fitness test to volitional exhaustion. A post-exercise blood sample was taken and blood cell counts were repeated. Inter-group results were compared using unpaired Student's *t* tests and Mann-Whitney non-parametric tests. Intra-group comparisons were carried out using paired Student's *t* tests and Wilcoxon non-parametric tests where appropriate. $P < 0.05$ was considered to be statistically significant.

Resting blood flow was not significantly different between the young and aged groups. Reactive hyperaemia however was significantly greater in young than in aged subjects (percentage increases 457.8 ± 69.9 cf. 177.1 ± 38.4 %; $P < 0.01$). Resting CEC numbers were similar between young and aged subjects (112.5 ± 52.0 *cf.* 100.5 ± 12.0 cells ml^{-1}) and were not altered by acute exercise in either group (post-exercise 106.4 ± 34.2 *cf.* 107.4 ± 21.0 cells ml^{-1}). Resting plasma vWF was significantly higher in aged than in young subjects (115.4 ± 9.1 *cf.* 194.8 ± 27.0 vWF units; $P < 0.05$) and rose significantly during exercise in the young group (115.4 ± 9.1 *cf.* 204.8 ± 26.7 vWF units; $P < 0.01$) but not in the aged group. Plasma TM was not significantly different between groups at rest and rose significantly during exercise in the young group (3.7 ± 0.5 *cf.* 4.1 ± 0.3 ng ml^{-1}; $P < 0.05$) but not in the aged group. Exercise-induced leucocytosis was observed in the young (pre 6.4 ± 0.6 *cf.* post $9.8 \pm 0.9 \times 10^9$.L^{-1}; $P < 0.01$) but not in the aged subjects

(pre 5.8 ± 0.4 *cf.* post 6.8 ± 0.5 × 10^9.L^{-1}).

These results indicate that aging is associated with impaired reactive hyperaemia. This is accompanied by resting haemostatic changes and by a marked attenuation of several endothelium-derived responses to exercise.

Lakatta EG (2002). *Heart Fail Rev* **7**, 29–49.

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C77

Stabilisation of endogenous HIF-α induces angiogenesis in mouse ischaemic skeletal muscles

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Hypoxia-inducible factor (HIF-1α) modulates transcriptional control of several genes involved in vascular growth and cellular metabolism. Polypeptide-induced HIF stabilisation leads to tube formation in co-cultured human microvascular endothelial cells, and local angiogenic response in a murine sponge assay (Willam *et al.* 2002). We examined the effect of the peptide dimethyloxaloylglycine (DMOG), which interferes with HIF degradation pathway *via* suppression of propyl hydroxylase activity, on capillary growth in mouse (C57Bl6) ischaemic extensor digitorum longus muscles.

Left femoral artery was ligated under Fluothane inhalation anaesthesia (2% in O_2) in two groups of animals. One group ($n = 10$) received DMOG I.P. every second day (8 mg in 0.5 ml saline) for 11 days while the other was injected with sterile saline (0.5 ml) at the same intervals ($n = 6$). Additionally, a third group was treated with DMOG without ligation ($n = 4$) and four unoperated mice served as controls. Angiogenesis was evaluated by estimation of capillary to fibre ratio (C:F) based on alkaline phosphatase staining. Animals were killed humanely and induction of HIF-dependent genes for vascular endothelial growth factor (VEGF) and its receptor Flk-1 (Elvert *et al.* 2003) was examined by Western blotting, normalised to actin expression.

Table 1. Capillary to fibre ratio (C:F) and HIF-1α, VEGF and Flk-1 protein expression in ischaemic mouse muscles

	C:F	HIF-1α	VEGF	Flk-1
Control	1.95 ± .06	1.17 ± .15	0.98 ± .02	1.10 ± .04
DMOG	1.94 ± .06	1.43 ± .26	1.03 ± .06	1.24 ± .09
DMOG + Lig.	2.71 ± .09 *#	5.40 ± .88 *#	2.50 ± .69 *	3.64 ± .11 *#
Saline + Lig.	2.17 ± .08	2.58 ± .80	2.61 ± .14 *	2.55 ± .46 *

All data are shown as means ± S.E.M. * $P < 0.005$ *vs.* Control, # $P < 0.005$ *vs.* Saline + Lig (ANOVA)

DMOG treatment resulted in 39% increase in C:F ratio in ischaemic muscles ($P < 0.0001$). Inhibition of HIF degradation led to the significant increase of endogenous HIF protein that was only apparent in tissues where HIF production was induced by ischaemia, but not in ligated and saline-treated or DMOG −treated mice without ligation. Ischaemia induced VEGF protein production to a similar degree, irrespective of DMOG treatment. However, production of Flk-1 was enhanced in ischaemic and DMOG-treated muscles, which may explain an intensive growth of capillaries in those muscles. The findings indicate that treatment with DMOG has a potential therapeutic use in enhancing angiogenesis in ischaemic diseases. Furthermore, it can be applied with low risk of side effects since it acts locally by stabilisation of HIF protein only in hypoxic regions of muscles.

Elvert G *et al.* (2003). *J Biol Chem* **278**, 7520–7530.

Willam C *et al.* (2002). *Proc Natl Acad Sci U S A* **99**, 10423–10428.

All procedures accord with current UK legislation

C78

The vascular endothelial growth factor (VEGF) isoform $VEGF_{165}b$ is anti-angiogenic *in vivo*

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VEGF is the dominant angiogenic factor in angiogenesis. Here we describe the effect of a novel splice variant of VEGF, VEGF165b, in an *in vivo* mesenteric adenovirus mediated model of VEGF induced angiogenesis.

Male Wistar rats (250g) were anaesthetised with halothane inhalation at 5% for induction and 3% for maintainence, and the gut exposed after laparotomy. 25 μl (1–3.3 × $10^8$$TCID_{50}$/ml) of adenovirus (AdV) expressing EGFP (Ad-EGFP, control), $VEGF_{165}$ (Ad-VEGF) or $VEGF_{165}b$ (Ad-$VEGF_{165}b$), or 50 μl of both (25 μl each) was injected into fat pad adjacent to a mesenteric connective tissue panel that had few blood vessels and no overt angiogenesis. 0.6% Monastral blue, diluted in saline, was injected into the fats at both sides of the AdV injection site. The mesentery was replaced in the animal and the body wall and skin sutured. The rats were allowed to recover and kept for 6 days under normal housing conditions. The rats were again anaesthetised with halothane, the mesentery exposed and vessels imaged using a digital imaging system (Leica). The rats were then humanely killed by cervical dislocation before recovery from the anaesthetic. The mesentery was dissected and immunofluorescence staining of the whole mount mesenteries with *G simplificolia* isolectin (endothelial cells), anti-Ki67 (proliferating nuclei), phalloidin (actin) and Hoechst 33324 (nuclei), carried out and microvascular parameters associated with angiogenesis measured. Unlike Ad-$VEGF_{165}$ injection, Ad-$VEGF_{165}b$ injection did not cause a significant increase in the relative fractional vessel area (FVA) of the mesentery compared with the control group, and injection of both Ad-$VEGF_{165}$ and Ad-$VEGF_{165}b$ into the same fat pad resulted in a lower FVA compared with the Ad-$VEGF_{165}$ ($P < 0.001$ ANOVA). This was also true for the increase in proliferating endothelial cells density, sprout density, branch point density, microvessel density, and for the decrease in mean vessel length (all $P < 0.01$ ANOVA).

Table 1. Effect of adenovirus expressing VEGF isoforms on angiogenesis parameters.

Mean±SEM(n)	Ad-EGFP	Ad-$VEGF_{165}$	Ad-$VEGF_{165}b$	Ad-$VEGF_{165}b$ +Ad-$VEGF_{165}b$
Fractional Vessel Area (%)	45±7.6(6)	568±110** (10)	26±18 (6)	185±47^{++}(6)
Proliferating endothelial cells density (mm^{-2})	70±14(6)	147 ±15**(10)	81±8.8(5)	67±14^{++}(5)
Sprout density (mm^{-2})	8.4±3.1(6)	29±2.3**(10)	12.1±6.0(5)	15±3.5^{++}(5)
Branch point density (mm^{-2})	94±13(6)	216±22**(10)	112±12(5)	143±14^{+}(5)
Microvessel density (mm^{-2})	221±18(6)	382±30**(10)	225±23(5)	277±31^{++}(5)
Mean vessel length (μm)	107 ±11(6)	72±3.2**(10)	102±6.14(5)	91±5.7^{+}(5)

* = p<0.05, **=p<0.01 compared to Ad-EGFP, $^{+}$=p<0.05, $^{++}$=p<0.01 compared to Ad-$VEGF_{165}$ Student Neumann Keuls post-hoc test.

These results show that $VEGF_{165}b$ does not cause angiogenesis itself but inhibits the angiogenic effect caused by $VEGF_{165}$ *in vivo*.

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All procedures accord with current UK legislation

C79

Inhibition of angiogenesis in mouse skeletal muscle by VEGF sequestration

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Previous work in our laboratory has shown that angiogenesis in skeletal muscle induced by mechanical stimuli can occur via two morphologically distinct pathways (Egginton *et al.* 2001), and that in the rat both these pathways are associated with increased levels of VEGF (Rivilis *et al.* 2002).Recent work suggests that the mouse responds similarly (unpublished data).

To investigate the importance of this increase in VEGF, we pharmacologically blocked circulating VEGF using the novel compound VEGF-trap (Regeneron Inc.), which consists of VEGF receptor portions fused to human immunoglobulin Fc.The m. extensor digitorum longus (EDL) of mice was subjected to two forms of mechanical stimulus 1) increased shear stress caused by oral administration of prazosin 2) muscle overload from surgical extirpation of the synergistic m. tibialis anterior (under hypnorm/hypnoval anaesthesia I.P.). Animals were humanely killed 14 days after the start of treatment.

Without co-administration of VEGF-trap, the capillary to fibre ratio (C:F) increased by 26 % ($P = 0.029$, Student's t test, $n = 3$) after prazosin treatment, and by 28 % ($P = 0.008$, $n = 3$) after extirpation compared to controls.Subcutaneous administration of VEGF-trap completely abolished the increases in C:F seen in both models, with all groups of animals showing no significant difference in C:F from untreated controls ($n = 3$ in all groups).

These data demonstrate that VEGF is essential for angiogenesis *in vivo* in response to two different mechanical stimuli.

Egginton S *et al.* (2001). *Cardiovasc Res* **49**, 634.

Rivilis I *et al.* (2002). *Am J Physiol Heart Circ Physiol* **283**, H1430.

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All procedures accord with current UK legislation

C80

Vascular endothelial growth factor (VEGF) isoform $VEGF_{165}b$ inhibits VEGF-R2 mediated phosphorylation of p44/p42 MAP kinase *in vitro*

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VEGF is essential for vasculogenesis, development of the embryonic vasculture and is a potent angiogenic factor in pathological angiogenesis. Here we determined the effect a novel splice variant of VEGF, $VEGF_{165}b$ on VEGF-R2 receptor tyrosine kinase mediated phosphorylation of mitogen activated protein kinase (p42/p44 MAPK).

Chinese Hamster Ovary cells (CHOs) were transfected with either an empty vector (pcDNA3) or VEGF-R2 containing expression vector. Cells were incubated with media or with media containing 1 nM $VEGF_{165}$, 1 nM $VEGF_{165}b$, or 1 nM $VEGF_{165}$ and 1 nM $VEGF_{165}b$ for 20 min at 37°C. Western blot analysis was used to determine whether $VEGF_{165}b$ inhibits VEGF-R2 induced phosphorylation of MAPK by using specific antibodies against phospho-p44/p42 MAP kinases.

A basal level p44/p42 MAP kinase phosphorylation was detected in pcDNA3 transfected cells, regardless of treatment. A similar p44/p42 MAP kinase phosphorylation level was observed in VEGF-R2 transfected cells that were incubated in the absence of a VEGF isoform (control). As expected, $VEGF_{165}$ increased the intensity of the phosphorylated polypeptide band by mean ± S.E.M. 222 ± 60 % of the untreated cells. In contrast, when VEGF-R2 transfected cells were treated with $VEGF_{165}b$, only a small increase (122 ± 42 %) in the level of p44/p42 MAPK kinase phosphorylation was detected compared to control. This was significantly lower than that of $VEGF_{165}$-induced stimulation (paired t test, $P < 0.05$). Treatment of VEGF-R2 transfected cells with a combination of both $VEGF_{165}$ and $VEGF_{165}b$, resulted in an 190 ± 56 % increase in the level of p44/p42 MAP Kinase phosphorylation compared to the control.

These results show that the intensity of $VEGF_{165}b$-induced phosphorylation of the signalling proteins p44/p42 MAP kinases is significantly less than that detected for $VEGF_{165}$-induced stimulation. Furthermore, combination of $VEGF_{165}$ and $VEGF_{165}b$-induced phosphorylation of p44/p42 MAP kinases was 32 % less than that detected for $VEGF_{165}$-induced stimulation. $VEGF_{165}b$ therefore appears to be able to inhibit VEGF mediated signalling through VEGF-R2.

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PC33

The expression of $VEGF_{165}$ and $VEGF_{165}b$ in human malignant melanoma

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Malignant melanoma, as all solid tumours, are dependent on angiogenesis for their growth. Vascular Endothelial growth factor, the predominant angiogenic factor is known to be upregulated in melanoma. We recently described a novel isoform of VEGF -$VEGF_{165}b$ (Bates *et al.* 2002) − that is down-regulated in renal and prostate cancer and inhibits endothelial cell proliferation, migration, vasodilatation and angiogenesis. To determine whether $VEGF_{165}b$ was similarly downregulated in melanoma, we have measured the expression $VEGF_{165}b$ mRNA in aggressively metastatic malignant melanoma.

10–16 serial sections were taken from eight formalin fixed and paraffin embedded primary cutaneous metastatic melanomas (0.88–8.mm thick) from the pathology archives at Frenchay Hospital with local Ethics Committee approval. mRNA was extracted from sections using the technique described by Krafft *et al.* (1997) and reverse transcribed. The cDNA was then subjected to a PCR reaction using primers designed to amplify the region from exon 7 (primer sequence, GTAAGC TTG TAC AAG ATC CGC AGA CG) to the 3'UTR region (ATG GAT CCG TAT CAG TCT TTC CTG G), or with primers specific for $VEGF_{165}b$ (across the exon 7/9 boundary, TTA AGC TTT CAG TCT TTC CTG GTG AGA CTG CA) or $VEGF_{165}$ (in exon 8, TCA CCG CCT CGG CTT GTC ACA T). Agarose gel electrophoresis and ethidium bromide staining showed expression of both $VEGF_{165}$ and VEGF $_{165}$b in all eight melanomas. The intensity of the bands produced by gel electrophoresis was measured using NIH Image, compared to a control band and then subjected to statistical analysis using a paired t test. Although both isoforms appear to be equally expressed in normal skin, the intensity of the $VEGF_{165}b$ band was surprisingly significantly greater than $VEGF_{165}$ ($P < 0.001$) in these metastatic melanomas. ($VEGF_{165}b$-35.6 ± 14.9 % of control cDNA. $VEGF_{165}$ 5.9 ± 16.6 %).

These findings with cutaneous melanoma suggest a mechanism of angiogenic factor expression and regulation that is different in metastatic malignant melanoma than it is in prostate and renal cancer.

Bates DO *et al.* (2002). *Cancer Research* **62**, 4123–31.
Krafft AE *et al.* (1997). *Mol Diagn* **3**, 217–230.

This work was supported by The Skin Cancer Research Fund, and the Wellcome Trust.

All procedures accord with current national and local guidelines

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PC34

Rat skeletal muscle arteries are more sensitive to potassium-induced vasodilatation in a hyperosmotic environment: mechanisms of action

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Exercise hyperaemia is believed to be elicited by vasoactive metabolites released from the active skeletal muscle. In a previous study we found that small increases in extracellular potassium (K^+; 1, 2 or 3 mM) cause profound relaxation of isolated gluteal muscle arteries. Since several vasoactive factors might interact during the hyperaemia response, we investigated the influence of a hyperosmotic environment on K^+ induced relaxations.

Gluteal arteries (mean ± S.E.M.; diameter: 247.51 ± 5.78 μm) from humanely killed female Wistar rats were isolated and mounted in an organ bath filled with Krebs Ringer solution for isometric tension recording. After precontraction with norepinephrine (10^{-6} M), 1, 2 or 3 mM K^+ was added in both control and hyperosmotic (HO, 60 mM sucrose) conditions. Endothelial removal and the addition of ouabain, $BaCl_2$, NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) or glibenclamide were used to study the underlying mechanisms. A two way repeated measures analysis with follow-up paired univariate tests was used to evaluate statistical significance. This study was approved by the local ethics committee of the Faculty of Medicine and Health Science (Ghent University).

The K^+ induced relaxations were significantly ($F = 251.05$, $P < 0.001$, $n = 6$) increased in the presence of 60 mM sucrose (Control: K1: 18.22 ± 6.38; K2: 66.50 ± 8.55; K3: 52.07 ± 10.40; HO: K1: 62.97 ± 9.51, $P < 0.05$; K2: 92.38 ± 2.17, $P < 0.05$; K3: 93.56 ± 3.70, $P < 0.05$). Endothelial removal and the addition of the K_{ATP} blocker glibenclamide (10^{-5} M) or the Na^+/K^+ pump inhibitor ouabain (5×10^{-5} M) did not reduce the HO-induced increased sensitivity to K^+. The application of the K_{IR}-channel blocker $BaCl_2$ (3×10^{-5} M) significantly ($F = 1.02$, ns, $n = 6$) abolished the influence of HO on the K^+-induced relaxations ($BaCl_2$: K1: 14.55 ± 6.10; K2: 14.08 ± 3.46; K3: 36.93 ± 5.87; $BaCl_2$ + HO: K1: 18.84 ± 7.18; K2: 25.91 ± 10.93; K3: 40.22 ± 5.70). NPPB (10 μM), a volume-regulated anion channel (VRAC) -blocker, mimicked the influence of HO ($F = 6.01$, $P < 0.05$, $n = 8$) by increasing the K^+ induced relaxations (Control: K1: 19.81 ± 5.82; K2: 33.39 ± 8.22; K3: 67.34 ± 11.18; NPPB: K1: 54.54 ± 10.62, $P < 0.05$; K2: 74.13 ± 8.57, $P < 0.005$; K3: 67.21 ± 10.60, ns).

In conclusion, HO increases the sensitivity of the rat gluteal skeletal muscle arteries to the vasodilating effect of K^+. This is not due to activation of K_{ATP}-channels or the Na^+/K^+ pump. It is hypothesized that HO inhibits VRAC's causing smooth muscle hyperpolarization. This possibly sensitizes the K_{IR} channels which are known to be involved in the K^+ induced relaxations in this type of artery.

All procedures accord with current national and local guidelines

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PC35

VEGF can stimulate cation entry through VEGF-R2 activation of TRPC3

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Vascular endothelial growth factor (VEGF) is an angiogenic cytokine that stimulates angiogenesis, vasodilatation and increased vascular permeability. All these effects depend on a VEGF-dependent increase in endothelial intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Bates, *et al.* 1999). However it is unclear how VEGF induces the increase of $[Ca^{2+}]_i$. The aim of this study was to determine whether VEGF could stimulate cation entry through a transient receptor potential (TRP) channel.

Chinese hamster ovary (CHO) cells were co-transfected with VEGF receptor 2 (VEGF-R2) and TRPC3 and were used for whole cell recording and analysis within 48–72 h. The bath solution contained (in mM) 140 NaCl, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 Hepes (pH 7.4 with NaOH). The pipette solution contained (in mM) 135 CsCl, 2 $MgCl_2$, 3.62 $CaCl_2$, 10 EGTA, 30 Hepes (pH 7.2 with CsOH) with a calculated free $[Ca^{2+}]$ of 100 nM. Cells were held at a potential of −60 mV, and current-voltage (*I-V*) relations were obtained every 5 s from voltage ramps between 100 and +100 mV with a duration of 400 ms (Jung *et al.* 2002).

VEGF (1 nM) increased net inward and outward current during applied voltage ramps, with a time to peak-response of about 2 min. VEGF-activated current was blocked by gadolinium chloride (100 μM). A similar current was activated in TRPC3-transfected CHO cells, by application of 1-oleoyl-2-acetyl-sn-glycerol (OAG) (100 μM). No VEGF-activated currents could be recorded from control cells transfected with the pcDNA3 vector. VEGF-activated currents were recorded from 3 of 10 cells from VEGF-R2 and TRPC3 doubly-transfected cultures. Of the 7 VEGF-unresponsive cells, 1 responded to OAG whilst 6 responded to neither VEGF nor OAG. Transient transfection efficiency was determined from results of X-gal staining of LacZ-transfected cells, which showed 60–70 % cells were transfected. This is consistent with 30–40 % efficacy for double-transfection of cells and the incidence of VEGF-activated current in our recordings. The amplitude of currents we recorded was similar with that reported by Hofmann (Hofmann, *et al.* 1999) who used TRPC6-microinjected CHO-K1 cells.

Collectively, these findings suggest that VEGF is able to stimulate cation entry through VEGF-R2 mediated activation of TRPC3 channels.This work was funded by the British Heart Foundation.

Bates DO *et al.* (1999). *Microcirculation* **6**, 83–96.
Hofmann T *et al.* (1999). *Nature* **397**, 259–263.
Jung S *et al.* (2002). *Am J Physiol Cell Physiol* **282**, C347–59.

PC36

Effects of 18β-glycyrrhetinic acid upon the electrical properties of pericytes on descending vasa recta isolated from rats

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This abstract assesses electrical coupling between pericytes and other cells in descending vasa recta (DVR), by comparing the input resistances of pericytes on isolated DVR before and during exposure to 18β-glycyrrhetinic acid (βGA), a gap junction blocker (Hill *et al.* 2001). Other microvessels show intercellular coupling via gap junctions (made of connexins 37, 40 and 43) and this may modulate vasoactivity (Hill *et al.* 2001). DVR from rats are vasoactive (Pallone, 1994) and may regulate the distribution of blood flow to the renal medulla (Mattson, 2003), but vasa recta from mice lack immunoreactivity to connexin 40 (Seul & Beyer, 2000).

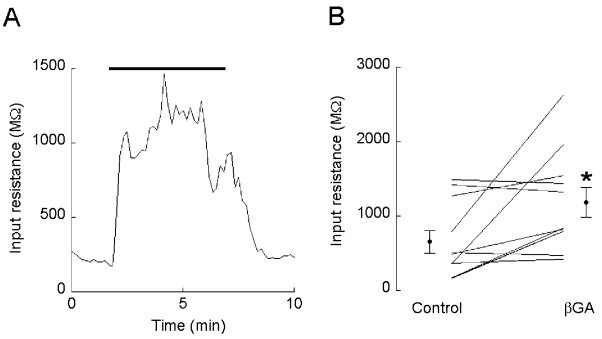

Figure 1. *A,* Input resistance of a pericyte on an isolated descending vas rectum, exposed to βGA during the period marked by a horizontal bar. *B,* Input resistances in control solution immediately before addition of βGA and after 5 min exposure to βGA, for eleven pericytes on different DVR. Mean ± standard error ($n = 11$). *$P < 0.05$, paired t test.

Input resistance was measured by whole cell perforated patch clamp recording. Individual DVR were dissected from renal tissue kept at 4 °C, after removal from rats (Pallone, 1994) 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 control solution at room temperature, containing (mM) Na^+ 150, K^+ 5, Mg^{2+} 1, Ca^{2+} 1, Cl^- 159, Hepes 10 and glucose 10, plus dimethylsulfoxide (0.1 %). Heat polished pipettes containing a solution of (mM) Na^+ 10, K^+ 140, Cl^- 150 and Hepes 10, plus gramicidin (0.4 mg ml^{-1}) and dimethylsulfoxide (0.4 %), were applied to pericytes on isolated DVR, to form gigaohm seals. Pericytes were clamped at −50 mV and exposed every 10 s to a series of steps from this holding potential to test potentials from −80 to +20 mV (at 10 mV intervals), each lasting 100 ms. Input resistance was calculated for each series, from the relationship between test potential −80 to −40 mV) and mean current between 95–99 ms during each step, by linear regression. βGA (40 μM) was added once to each of eleven DVR. Pericytes on isolated DVR displayed a significant increase in mean input resistance on exposure to βGA. The data indicate that pericytes can be electrically coupled to other cells in these DVR, perhaps via gap junctions.

Hill CE *et al.* (2001). *Med Res Rev* **21**, 1–60.

Mattson DL (2003). *Am J Physiol* **284**, R3–R27.

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Seul KH & Beyer EC (2000). *Microvasc Res* **59**, 140–148.

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All procedures accord with current UK legislation

PC37

Lack of active lymphangiogenesis in malignant melanoma despite increased lymphatic density and lymphangiogenic growth factor expression.

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Malignant melanoma (MM) metastasises to distant organs probably via the lymphatic system. Tumours stimulate new lymphatic vessel formation (lymphangiogenesis) via secretion of Vascular Endothelial Growth Factor C (VEGF-C) or D and this facilitates metastasis in animal models (Mandriota *et al.* 2001, Skobe *et al.* 2001). Lymphatic vessel density (LD) can be used to predict MM metastasis (Shields *et al.* 2003, Dadras *et al.* 2003) and that VEGF-C and D expression have prognostic potential. We have determined VEGF-C and D expression in melanoma and assessed their association with lymphatic density, lymphatic endothelial cell proliferation and metastatic potential.

Normal skin ($n = 11$) and 17 archival samples of MM (11 metastatic, 6 non metastatic, obtained with Local Ethical Committee approval, North Bristol NHS Trust). Lymphatics were identified by immunohistochemical staining with rabbit anti-human LYVE-1 (4.2 μg/ml, gift of Dr Jackson), VEGF-C with goat anti-human VEGF-C (1.14 μ/ml, Santa Cruz,), VEGF-D with goat anti-human VEGF-D (10 μg/ml) and Ki67 with mouse anti-human ki67 (4 μg/ml, Oncogene). LD was then calculated for the whole sample.

LD was significantly greater in metastatic MM (12.8 ± 1.6 mm^2) than non metastatic MM (5.4 ± 1.1 mm^2, $P < 0.01$ Mann Whitney), clearly discriminating between tumours that had metastasised with those that had not. 13/17 (76 %) tumours expressed VEGF-C and 9/17 (53 %) expressed VEGF-D. Intensity of staining was assessed blindly by subjective scoring. There was no significant difference in the intensity or frequency of staining in metastatic compared to non-metastatic samples for either VEGF-C or VEGF-D. Furthermore, no evidence of Ki67 expression in lymphatic vessels was seen in the non metastatic or the metastatic group indicating that lymphatic endothelial cell proliferation was not occurring at the time of biopsy. From this small study, we can conclude that although a significantly increased LD is seen in metastatic MM, and lymphangiogenic growth factors VEGF-C and D are expressed, lymphatic endothelial cell proliferation was not actively occurring at the time of melanoma excision. Since LD was increased this suggests that lymphangiogenesis had occurred prior to excision and maybe an early feature of melanomas that have a high probability of forming distant metastasis.

Dadras SS *et al.* (2003). *Am J Pathol* **162**, 1951–60.

Mandriota S *et al.* (2001). *Embo J* **20**, 672–82.

Shields JD *et al.* (2003). *J Physiol* **547.P** C129.

Skobe M *et al.* (2001). *Nat Med* **7**, 192–8.

All procedures accord with current national and local guidelines

PC38

Perfusion and superfusion of hyperosmotic Ringer solution increases microvascular permeability in mesenteric microvessels *in vivo*

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Diabetic patients are often subjected to glucose concentrations that may exert significant osmotic effects on endothelial cells. It is possible that this may contribute to the increase in vascular permeability reported in diabetic patients and animals. Previous work has shown that large osmotic stresses do not increase permeability in mesenteric microvessels unless the attachment of the endothelial cells to the basement membrane are disturbed (Kajimura, *et al.* 1997). To determine whether small but physiologically relevant osmotic stresses due to increased glucose could increase permeability, the effect of 20 mM osmolarity Ringer's solution on the hydraulic conductivity of mesenteric microvessels *in vivo* was investigated.

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}). Animals were humanely killed after each experiment by destruction of the brain. Lp (mean ± S.E.M. × 10^{-7}cm.s^{-1} .cmH_2O^{-1}) was measured during perfusion and superfusion with normal or hyperosmotic Ringer solutions in 20–30 μm diameter mesenteric microvessels, using a modification of the Landis Michel method (Michel, *et al.* 1974). A mesenteric microvessel was cannulated with a bevelled glass micropipette connected to a manometer and perfused with 1% bovine serum albumin (BSA) in frog ringer (5 mM D-glucose) containing rat red cells as flow markers. The vessel was subsequently perfused and superfused with either 20 mM D-glucose or 20 mM mannitol Ringer's solution. A glass rod was used downstream from the cannulation site to occlude the vessel for 5–10 seconds. Hydraulic conductivity, Lp was then calculated from the radius of the vessel (r), velocity of the marker cells (dl/dt) and the length (l) between the marker cell and the occlusion site. The hyperosmotic Ringer's solution significantly increased the hydraulic conductivity 4.0 ± 2.9 fold above baseline values (from 2.3 ± 2.2 to 13.4 ± 11.6). This was true for both hyperosmotic glucose (3.9 ± 4.3 fold, from 1.3 ± 0.6 to 6.1 ± 7.7), and mannitol (4.2 ± 2.0 fold, from 3.7 ± 3.4 to 11.9 ± 6.7 x$10^{-7}$$cms^{-1}$.$cmH_2O^{-1}$).

These results show that hyperosmotic solutions applied to both sides of the vessel wall can increase hydraulic conductivity in mesenteric microvessels *in vivo*.

Kajimura *et al.* M (1997). *J Physiol* **503**, 413–25.

Michel *et al.* CC (1974). *Quart J Exp Phys Cog Med Sci* **59**, 283–309.

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All procedures accord with current UK legislation

PC40

Differential blood flow responses in the human lower limb and skin to small cumulative increases in venous congestion pressure

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When venous pressure in the human lower limb was raised by application of small cumulative pressure increases to a venous congestion cuff, Gamble *et al.* (1998) observed, using strain gauge plethysmography and arterial Doppler ultrasound, that arterial inflow to the limb remained constant even at congestion pressures approaching mean arterial pressure. They attributed this to a progressive reduction in pre-capillary resistance due to retrograde veni-arteriolar transmission of vasodilatory signals via the endothelium. This idea was supported by their subsequent finding that in pre-eclampsia, a condition characterised by dysfunction of the endothelium, calf blood flow decreased during venous congestion (Anim-Nyame *et al.* 2003). Since vascular control mechanisms in skin and skeletal muscle within a whole limb differ, we studied whether retrograde transmission is evident in the former during a venous congestion protocol.

With approval from the University of Birmingham Local Ethics committee, 8 healthy males, aged 26 ± 1 years (mean ± S.E.M.), with resting mean arterial pressures (MAP) of 82 ± 2 mmHg and heart rates 56 ± 2 beats per min, participated in the study. Calf blood flow was measured by strain gauge plethysmography (Filtrass 2000, DOMED) and skin perfusion by Laser Doppler probes on the shin and foot dorsum during supine rest and at the end of 5 min periods of venous congestion by a thigh cuff inflated successively to 10, 20, 30, 40 and 50 mmHg. Blood flow measurements were made by brief (10 s cuff inflations to 40–50 mmHg above pre-existing cuff pressure. Blood flows and averaged arbitrary perfusion values for shin and foot during venous congestion were expressed relative to rest values.

Skin perfusion declined steadily as venous congestion increased, reaching 39 ± 3% of resting at 50 mmHg cuff pressure. The decrease was no different than predicted by Darcy's Law based on the rise in venous pressure. Calf blood flow at rest was 2.17 ± 0.83 mls min^{-1} 100 ml^{-1} and as venous congestion increased in steps, it remained unchanged up to 30 mmHg (105 ± 12% of resting). At 40 and 50 mmHg congestion pressures, calf flows were reduced to 75 ± 10% and 60 ± 6% of resting ($P < 0.05$, paired t test), most likely because the thigh cuff pressure used to measure flow exceeded MAP.

We conclude that a protocol of small cumulative increases in venous pressure does not invoke retrograde transmission of vasodilatory signals in skin. Where such a protocol shows whole limb blood flow to be reduced, impairment of signal transmission is more likely to reside in other tissues such as skeletal muscle.

Anim-Nyame *et al.* (2003). *Clin Sci* doi:10.1042/CS20030045.

Gamble *et al.* (1998). *J Physiol* **507**, 611–617.

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

PC41

The release of different endothelium-derived hyperpolarisation factors (EDHF) by propionate and acetylcholine (ACh)

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We have previously shown that the short chain fatty acid propionate gives rise to an EDHF-like response in the rat mesenteric artery. Propionate-induced relaxation is insensitive to L-NAME and indomethacin, yet is abolished in high K^+ solution and by the combination of charybdotoxin and apamin (Knock *et al*, 2002). Many candidates for the identity of EDHF(s) have been published, including K^+, cytochrome P450 metabolites e.g. EETs, H_2O_2, C-type natriuretic peptide (CNP), and electrical transmission through gap junctions linking endothelial and smooth muscle cells. We sought to investigate the potential identity of the relaxing compound released by propionate by using inhibitors of these potential candidates.

Male Wistar rats (250–275g) were killed humanely by cervical dislocation. Small mesenteric resistance arteries (200–300μm) were mounted on a small vessel wire myograph for measurement of isometric contraction. Data were analysed using Student's *t* test and results are expressed as mean ± S.E.M.

Inhibitors of cytochrome P450, 17-ODYA (3 μM) and sulfaphenazole (10 μM), had no effect on either propionate-induced relaxation or acetylcholine (1μM)-induced relaxation indicating that this proposed EDHF is not active in these arteries. Catalase (3000U), which metabolises H_2O_2, inhibited ACh relaxation by ~35 % but had no effect on propionate-induced relaxation (propionate relaxation 52 % ± 2, in the presence of catalase 48 % ± 2, $n = 4$). Pertussis toxin (400 ngml^{-1}), which has been previously shown to block CNP-induced relaxation in these arteries (Chauhan *et al* 2003), similarly had no effect on propionate relaxation (propionate 57 % ± 3, with pertussis toxin 49 % ± 6 ns, $n = 4$) but completely abolished the ACh-induced relaxation (ACh relaxation 88 % ± 3, with pertussis toxin −6 % ± 6 $P < 0.001$ $n = 4$).

In conclusion, we suggest that the EDHFs released by ACh and propionate have different identities in rat mesenteric resistance arteries, and that these may work through separate mechanisms. The mechanism by which propionate causes an EDHF-like relaxation remains unknown.

Chauhan SD *et al.* (2003). *Proc Natl Acad Sci U S A* **100**, 1426–31.

Knock G *et al.* (2002). *J Physiol* **538(3)**, 879–90.

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All procedures accord with current UK legislation

PC42

Mechanism of calcium extrusion in microvascular smooth muscle in the rat retina

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Regional blood flow is influenced by the contractile behaviour of the smooth muscle of microvessels and altered $[Ca^{2+}]_i$ is an important determinant of this contractile behaviour. $[Ca^{2+}]_i$ is determined by the balance between influx, extrusion and redistribution. The extrusion mechanism is well studied in cardiac muscle where the Na^+/Ca^{2+} exchanger contributes to cell pathology. Vascular smooth muscle also expresses Na^+/Ca^{2+} exchanger and its inhibition can have a profound effect on renal blood flow as well as influence large artery function. The present study aims to assess the relative contributions of Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase pump in Ca^{2+} homeostasis of microvascular smooth muscle (arterioles/metarterioles).

Sprague Dawley rats (200–300 g) were humanely killed and microvessels mechanically dispersed from fresh retinae using light titruration with a Pasteur pipette. Microvessels were loaded in 10 μM Fura-2/AM for 2 h and global cell Ca^{2+} measured by microflourometry in sections of microvessels comprising 6–20 cells (endothelial cells do not load with fura 2). Solutions and drugs were applied to superfusate local to the microvessel and switched within 1 s. $[Ca^{2+}]_i$ redistribution was blocked throughout by inhibiting uptake into the sarcoplasmic reticulum (SR), with cyclopiazionic acid (20 μM, CPA). It was shown using CCCP and oligomycin that mitochondrial uptake was not detectable. Ca^{2+} extrusion was measured by a challenge with 10 mM caffeine, which rapidly releases Ca^{2+} into the cytosol. The rate at which $[Ca^{2+}]_i$ declines is then a measure of Ca^{2+} extrusion. The Ca^{2+}-ATPase extruder was blocked by pre-incubation with the membrane permeant carboxyeosin diacetate (5 μM) for 10 min. The Na^+/Ca^{2+} exchanger was blocked by reducing $[Na^+]_i$ to 10 mM (equimolar Li^+-replacement). Carboxyeosin slowed by 48 % Ca^{2+} extrusion (half time for decline, mean ± S.E.M., 7.8 ± 1.2 to 16.1 ± 2.4 s, paired *t* test, $P = 0.0006$, $n = 14$) whereas low Na^+ alone had no effect (6.8 ± 0.7 to 8.5 ± 0.8 s, $n = 21$) on the decline in the caffeine induced $[Ca^{2+}]_i$ transient nor did they have any effect on resting cytosolic Ca^{2+}. These agents together induced a rise in resting $[Ca^{2+}]_i$ with no recovery after caffeine application ($[Ca^{2+}]_i$ continued to rise). Similarly, the blocker of Na^+/Ca^{2+} exchange KB-R7943 was only effective when applied with carboxyeosin.

These results show that for intact microvascular smooth muscle cells, cell Ca^{2+} is not highly dependent on the Na^+/Ca^{2+} exchanger and this and the Ca^{2+}-ATPase transporter independently handle cell Ca^{2+}. Thus pathologies or pharmacological therapies which block one or other will have little adverse effect on retinal blood flow.

We thank the Wellcome Trust and Dublin Docks Development Authority for financial support.

All procedures accord with current UK legislation

PC43

N-Ethylmaleimide increases cerebrospinal fluid secretion in young but not old sheep

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Cerebrospinal fluid (CSF) secretion by the choroid plexuses (CP) decreases during normal ageing in rat, human and sheep (Preston, 2001). The ability to stimulate CSF secretion during ageing has potential clinical benefits, to prevent accumulation of toxins (e.g. amyloid-β) and facilitate hormone and bio-active peptide distribution in the brain. We studied the effect of N-ethylmaleimide (NEM), a K^+, Cl^- co-transport stimulator (Kirk, 1991), on CSF secretion in the *in vitro* perfused ovine lateral cerebral ventricle CPs.

Six pairs of young (1–2 years) and old (7–8 years) sheep were anaesthetised with I.V. thiopentone sodium (20 mg kg^{-1}), killed by cervical dislocation, their brains removed and CPs perfused *in*

situ via the choroidal artery with albumin Ringer. Procedures were in accordance with Animals (Scientific Procedures) Act 1986 and animals humanely killed. Secretion rates were measured by colourimetric analysis of perfusate and effluent (Preston and Segal, 1990). Ion extraction (^{36}Cl 0.1 MBq 100 ml^{-1} or ^{22}Na 0.05 MBq 100 ml^{-1}) was calculated from the arterio-venous difference in activity, accounting for the extracellular marker ^{14}C-mannitol (0.2 MBq 100 ml^{-1}). NEM (0.5 mM) was added to perfusate and CSF after 30 min perfusion, for an additional 60 min. NEM had significant effects on young CP, increasing CSF secretion rates from 47.2 ± 13 to 91.3 ± 26 ml min^{-1}.g^{-1}, (mean ± S.E.M., $P < 0.05$ paired student t test). In addition, there was an increase in extraction of ^{36}Cl from 8.8 % ± 4 % to 18 % ± 3 %, ($n = 3$, $P < 0.05$) however, there was no effect of NEM on ^{22}Na extraction in young CP. In the old CP, neither CSF secretion rate nor ion extraction (^{36}Cl or ^{22}Na) was altered after adding NEM.

This study has demonstrated that in the young sheep there is a functional correlation between CSF secretion rate and Cl^- extraction in this model, however, the lack of response in old CP indicates an inability to up-regulate Cl^- transport and may help explain the reduced CSF secretory ability in old animals.

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All procedures accord with current UK legislation

PC44

Blood-brain barrier transfer of corticosterone in normal and protein deprived rats. Role of multi-drug resistance protein efflux

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Reduced early growth is a risk factor for cardiovascular disease, stroke, hypertension and type 2 diabetes. Changes in the hypothalamic-pituitary adrenal axis (HPAA) activity are involved, and regulation in part by glucocorticoid feedback to brain. One determinant of glucocorticoid activity in brain is transfer of hormone across the blood-brain barrier (BBB). This study investigated glucocorticoid (in rat, corticosterone) delivery from blood to brain in normal and protein deprived rats.

Brain penetration of ^{3}H-corticosterone was investigated by using whole body perfusion followed by brain capillary depletion (Preston *et al.* 1995) in 3-week-old Wistar rats anaesthetised I.P. with hypnorm (0.4ml kg^{-1}) and hypnovel (0.4ml kg^{-1}) and humanely killed by cervical dislocation. Rats born of mothers fed a low protein (8 %) diet (LPD rats) or a normal protein (20 %) diet (NPD rats) beginning 2 weeks prior to mating and continuing through out gestation and lactation were perfused with a dextran ringer containing ^{3}H-corticosterone (0.3 Mbq 100 ml^{-1}) and vascular marker ^{14}C-mannitol (0.15 Mbq 100 ml^{-1}) for up to 30 min.

Brain uptake of ^{3}H-corticosterone was linear over this time course in both NPD ($n = 9$) and LPD rats ($n = 9$), with greatest uptake in the pituitary and choroid plexuses, which lack the characteristically tight BBB. Lower, but significant uptake was also seen in post-capillary brain samples, with corticosterone uptake rate, 12 times greater than mannitol. After 15 min perfusion, ^{3}H-corticosterone brain uptake of LPD rats was 1.3 times higher than that of NPD rats (Table 1). Interestingly, the brain mannitol space was lower in LPD rats. Potential interaction of corticosterone with the multi-drug resistance protein (MRP) efflux pumps P-glycoprotein and ABCG2 was explored by adding either the P-gp substrate, colchicine (100 μM) or the ABCG2 and P-gp substrate daunorubicin (150 μM) into ringer. Both increased brain uptake confirming that corticosterone is a substrate for MRP efflux in NPD rats. This effect was not observed in LPD pups where uptake was reduced, suggesting a defect in MRP mediated efflux in offspring of protein deprived mother. The long term effects of dysregulation of glucocorticoid feedback to brain remains to be determined, but may contribute to altered hypothalamic-pituitary control of blood pressure as observed in humans with early growth reduction.

Table 1. The 15 min uptake of ^{3}H-corticosterone (dpm.ml^{-1}/dpm.g^{-1}) in post-capillary brain.

	Corticosterone brain uptake (ml/g) n= 9		Corticosterone brain uptake (ml/g) n= 7	
	NPD rat	% of control	LPD rat	% of control
Control	1.39 ± 0.11		1.79 ± 0.09*	
With 100 μM Colchicine	3.56 ± 0.14^{aa}	256.1%	1.17 ± 0.17^{b}	65.4%
With 150 μM Daunorubicin	1.79 ± 0.10^{a}	128.8%	0.86 ± 0.15^{bb}	48.0%

Values are mean ± SEM. *P< 0.05 compared to NPD using unpaired t-test. ^{a}p<0.05, ^{aa}p<0.01 compared to NPD control; ^{b}p<0.05, ^{bb}p<0.01 compared to LPD control using ANOVA

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PC46

Vascular endothelial growth factor (VEGF) signals through nephrin and AKT in human conditionally immortalised podocytes (hCIPs)

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VEGF reduces cytotoxicity and promotes survival in hCIPs, but the signalling pathway through which VEGF acts is unknown (Foster *et al.* 2003). In endothelial cells VEGF promotes survival by stimulating a pathway, which results in the phosphorylation of AKT (Nishino *et al.* 2002). Tyrosine phosphorylation of nephrin, a component of the slit diaphragm, can induce the serine/threonine phosphorylation of AKT (Huber *et al.* 2003). We investigated whether VEGF could interact with nephrin to induce the phosphorylation of AKT and hence induce a survival pathway in podocytes.

hCIPs were serum starved overnight, then treated with 1 nM VEGF for 20 mins. Protein was extracted and quantified. 60 μg of the protein was boiled and then run on an SDS PAGE gel, transferred to a PVDF membrane and probed with 5 μg/ml anti-phospho-AKT, stripped and reprobed with 5 μg/ml anti-AKT. Bands were then visualised using chemiluminescence and analysed using densitometry. 100 μl of the same protein samples

were incubated overnight using 1 μg/ml phosphotyrosine antibody, then immunoprecipitated using protein A/G agarose beads. The immunoprecipitate and supernatant were then analysed by Western blotting as above. The membrane was then probed using a nephrin antibody (1:500). HCIPs and nephrin mutated hCIPs (NMhCIPs) were also serum starved for 16hrs and left in a cell suspension for 4hrs. Apoptosis was assayed using Annexin V/propidium iodide staining and quantified using flow cytometry.

1 nM VEGF significantly reduced phosphorylation of total AKT by 32.7 ± (S.E.M.);14.8 % compared to serum starvation, which induced phosphorylation of total AKT by 78.5 ± 23.4 % in hCIPS (paired Ttest, p≤0.05, $n = 3$). A 180 kDa band corresponding to nephrin was seen in protein samples that had been treated with VEGF and immunoprecipitated with anti-phosphotyrosine, whereas in untreated samples the band was found in the supernatant. Serum starvation and cell suspension significantly induced apoptosis in NMhCIPs, 25.3 ± 2.0 % compared to hCIPs, 2.5 ± 0.6 % (p≤0.0001, unpaired Ttest, $n = 4$), however it reduced necrosis in NMhCIPs, 4.4 ± 0.5 % compared to ± 2.3 % (p≤0.005, unpaired Ttest, $n = 4$).

These results show that VEGF can induce the phosphorylation of nephrin, which may significantly influence podocyte apoptosis. However, this does not appear to be through phosphorylation of AKT.

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PC46a

The effect of age on the transient hyperaemic response of forearm skin

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The hyperaemic response of forearm skin to prolonged ischaemia, heating and pharmacological vasodilatation has been extensively studied and impaired endothelium dependent vasodilator function has been associated with disease such as diabetes (Caballero *et al.* 1999) and hypertension (Panza *et al.* 1995). Such tests are largely impractical for research into acutely changing conditions such as sepsis. We have therefore developed a modification of the technique using brief arterial occlusion to provoke a small hyperaemic response which is predominantly of myogenic rather than ischaemic origin. It is non-invasive and repeatable. We have previously shown this technique to be sensitive to changes in vascular tone (Webster *et al.* 2002) and to inhibition of prostaglandin synthesis (Moppett *et al.* 2003). In this study we investigated whether there was a significant difference in the transient hyperaemic response between young and old human subjects.

Following ethics committee approval and written informed consent, 33 young subjects (21–35 years) and 33 older subjects (65–85 years) were studied. All subjects were healthy and with blood pressure $< 140/90$, non-smoking and not taking any cardiovascular medication Forearm skin blood flow-flux was measured using two laser Doppler probes attached to the volar aspect of the outstretched forearm. Following a period of acclimatisation the axillary artery was compressed digitally for 20 seconds and then released. This was then repeated twice more. The transient hyperaemic response (THR) was recorded using custom designed software (Moor, Axminster, UK) and analysed offline. Values for net baseline flux and the THR ratio (peak hyperaemic flux divided by baseline flow flux) were recorded for each THR test and the mean of the three tests used for analysis. Data were analysed using Student's unpaired t test, taking $P < 0.05$ overall as significant.

Baseline flow-flux was reduced in the older group compared to the younger (10.6 (0.98) *vs.* 15.2 (1.2) but THRR was not significantly different (3.51 (0.24) *vs.* 2.96 (0.11) (mean (S.E.M.)).The reduction in baseline flux is consistent with previous work.

Our data suggest that the healthy elderly have a preserved vasodilator response compared to young adults when using a myogenic stimulus. This is in contradistinction to their reduced response to prolonged ischaemia or pharmacological vasodilatation (Celermajer *et al.* 1994).

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