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EDITOR: MAGGIE D. BROWN, PhD
Birmingham, UK

S1

ROLE OF CYTOCHROME P450-DERIVED EPOXYEICOSATRIENOIC ACIDS IN THE REGULATION OF VASCULAR TONE

I. Fleming
Institut für Kardiovaskuläre Physiologie, Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

Epoxyeicosatrienoic acids (EETs) are generated from arachidonic acid by cytochrome P450 (CYP) epoxygenases, the expression of which is determined by a number of physical (fluid shear stress and cyclic stretch) and pharmacological (nifedipine and cerivastatin) stimuli as well as by hypoxia. The activation of CYP epoxygenases in endothelial cells is an important step in the nitric oxide and prostacyclin-independent vasodilatation of several vascular beds and EETs have been identified as endothelium-derived hyperpolarizing factors. EETs contribute to the activation of Ca^{2+}-dependent K^+ channels in endothelial cells and the subsequent hyperpolarization, by a mechanism that involves the opening of TRP channels and an increase in transmembraneous Ca^{2+} influx. Moreover, EETs potentiate interendothelial gap junctional communication by a cyclic AMP/protein kinase A-dependent process, and thus contribute to the phenomenon of ascending dilation. Inhibition of the soluble epoxide hydrolase (sEH) which metabolises EETs to their diols, enhances the EDHF-mediated relaxation of porcine coronary arteries and induces a marked decrease in the blood pressure of hypertensive animals. However, in the pulmonary circulation CYP epoxygenase inhibitors attenuate while sEH inhibitors potentiate hypoxia-induced vasoconstriction. These results highlight both the role of EETs in the regulation of endothelial Ca^{2+} signaling and endothelium-dependent responses and the differences in the effects of EETs and inhibition of the sEH in the systemic and pulmonary circulations.

Address correspondence to Dr. Maggie D. Brown, School of Sport and Exercise Sciences, University of Birmingham, Birmingham, B15 2TT, UK. E-mail: m.d.brown@bham.ac.uk

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S2

EPOXYEICOSATRIENOIC ACIDS (EETS) AS ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTORS (EDHFS) IN CORONARY ARTERIES

W. B. Campbell, W. Yang, K. M. Gauthier, P. Li, J. R. Falck
Department of Pharmacology, Medical College of Wisconsin, Milwaukee, WI, USA

EETs are cytochrome P450 metabolites. They are synthesized by the endothelium and relax and hyperpolarize vascular smooth muscle. Acetylcholine and bradykinin cause endothelium-dependent relaxations and hyperpolarizations that are blocked by cytochrome P450 inhibitors and EET antagonists. When 22 analogs of 14,15-EET were tested on arteries, a specific structure was required for relaxation, 14(*S*), 15(*R*)-*cis*-epoxyeicosa-8*Z*-enoic acid (14,15-EE-8Z-E), suggesting a specific binding site. 20-Iodo-14,15-EE-8Z-E relaxed coronary arteries. 20-^{125}I-14,15-EEZE showed time-dependent, specific binding to isolated membranes. The binding was concentration-dependent and saturable (K_d = 8.5 ± 1.5 nM; B_{max} 7.0 ± 0.5 pmol/mg). 14,15-EET and 15-HETE displaced the ligand with a Ki of 33.4 nM and 2 μM, respectively. GTPγS reduced 20-^{125}I-14,15-EE-8Z-E binding, a 53% decrease in B_{max}. EETs open the large conductance calcium-activated potassium (BK_{Ca}) channels in coronary smooth muscle cells. 11,12-EET (1–1000 nM) increased the open state probability of the BK_{Ca} channel in cell-attached patches. In contrast, the EETs were without effect in inside-out patches. When GTP was added to the bath, the EET-induced channel activation was restored. This activity of the EETs was inhibited by GDPβS and anti-Gsα antibodies but not by anti-Giα or anti-G$\beta\gamma$ antibodies or rabbit γ-globulin. These studies implicate a G-protein, possibly Gs, in the BK_{Ca} channel activation by the EETs. These findings indicate that EETs represent EDHFs in the coronary artery and suggest that EETs act through a G protein-coupled receptor or binding site.

S3

ACTIVATION OF ENDOTHELIAL CALCIUM-ACTIVATED POTASSIUM CHANNELS AND EDHF-MEDIATED RESPONSES

M. Félétou
Department of Angiology, Institut De Recherches Servier, Suresnes, 92150, France

Changes in vascular smooth muscle membrane potential are an important regulating mechanism for smooth muscle contractility. The hyperpolarization of the smooth muscle cell can contribute to the mechanisms involved in the relaxation elicited by endothelial-derived NO and prostacyclin and is the predominant mechanism involved in the relaxations elicited by EDHF. Most of the EDHF-mediated responses involve an increase in the endothelial intracellular calcium concentration that activates endothelial potassium channels (K_{Ca}). The opening of small (SK3) and intermediate conductance (IK1) K_{Ca} hyperpolarizes the endothelial cells. Then, direct electrical coupling through myoendothelial junctions can explain the endothelium-dependent hyperpolarization of the smooth muscle cells. Additionally, the activation of endothelial K_{Ca} implies a potassium efflux and a potential accumulation of potassium ions in the intercellular space between the endothelial and smooth muscle cells. Potassium ions evoke the hyperpolarization of the vascular smooth muscle cell by activating the smooth muscle inward rectifying potassium channel (Kir) and/or the Na^+/K^+-ATPase and can therefore contribute to EDHF-mediated responses. These two mechanisms are not necessarily exclusive and can occur simultaneously and even in synergy, and are likely to play an important role in the local control of blood flow especially in the coronary circulation and in the periphery.

S4

GAP JUNCTIONAL COMMUNICATION AND ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION

T. M. Griffith

Department of Diagnostic Radiology, Wales Heart Research Institute, Cardiff University, Cardiff CF14 4XN, UK

Synthetic peptides possessing homology with domains of the 1st and 2nd extracellular loops of the major vascular connexins (Cxs 37, 40, and 43) inhibit direct cell–cell communication in a connexin-specific fashion and have provided evidence that electrotonic signaling via myoendothelial and homocellular smooth muscle gap junctions contributes to the EDHF phenomenon. Thus, in the rabbit iliac artery, a vessel in which endothelial gap junction plaques are constructed from Cx37 and Cx40 and medial plaques are constructed from Cx43, peptides targeted against Cx37 and Cx40 attenuate endothelium-dependent subintimal smooth muscle hyperpolarization, whereas peptides targeted against Cx43 selectively attenuate the subsequent relay of this electrical response across the arterial media. Connexin-mimetic peptides similarly inhibit EDHF-type responses in arteries from other species. Endothelial stimulation also evokes a prostanoid-independent increase in the formation of cAMP, a nucleotide that positively regulates intercellular communication. Inhibition of adenylyl cyclase or cAMP hydrolysis can thus modulate the conduction of endothelial hyperpolarizations and the associated EDHF-type relaxation. Gap junctions are also sensitive to cellular redox status and under conditions where intercellular coupling is depressed, EDHF-type responses are enhanced by 5-methyltetrahydrofolate and tetrahydrobiopterin, but not by their oxidized forms folic acid and 7,8-dihydrobiopterin. Further research is necessary to evaluate how vasoactive agents and intracellular 2nd messengers modulate arterial function via effects on the functionality of gap junction channels.

S5

ROLE OF EDHF IN CONDUCTED DILATIONS IN THE MICROCIRCULATION

C. de Wit

Physiologisches Institut, Universität Lübeck, Ratzburger Allee 160, 23538 Lübeck, Germany

Intercellular communication along the vascular wall contributes to the coordination of arteriolar behavior. This is especially important under conditions of high flow due to downstream vasodilatation because upstream vessels would limit flow increases under these circumstances. Communication along the vessel is reflected by the conduction of vasodilatations along the arterioles after locally confined application of endothelial stimulatory substances (e.g., acetylcholine, bradykinin). The vasodilatation is initiated by a locally induced hyperpolarization in endothelial and smooth muscle cells that involves EDHF and K_{Ca}-channels, but not NO. Conducted responses can also be initiated by adenosine through the activation of K_{ATP}. The hyperpolarization spreads along the vascular wall through intercellular channels, called gap junctions, which are composed of connexins. In vascular cells different connexin proteins are expressed (Cx40, Cx43, Cx37, and Cx45), of which Cx40 is especially important for the conduction of vasodilatations as conducted responses are attenuated in Cx40-deficient animals. The function of Cx40 cannot be taken over by Cx45 expressed instead of Cx40 (Cx40K145 mice), which suggests that specific Cx properties are necessary to support conduction along the endothelium. Interestingly, Cx40-deficient and Cx40K145 mice are hypertensive, suggesting that intercellular communication through Cx40 contributes to the control of peripheral vascular resistance. Since Cx40 is mainly expressed in endothelial cells, the endothelial layer seems to provide the main pathway.

S6

MECHANISM OF RELAXATION OF PHENYLEPHRINE-CONTRACTED RAT ISOLATED MESENTERIC ARTERIES BY ELEVATED POTASSIUM

P. D. Langton, D. X. P. Brochet

Department of Physiology, Medical Sciences Building, University of Bristol, Bristol BS8 1TD, UK

Rat isolated mesenteric arteries do not reliably relax to an increase in extracellular potassium ($[K^+]_o$) and no explanation has general approval. The aim of this study was to identify the conditions that determine the response of rat mesenteric artery to elevated $[K^+]_o$. Male Wistar rats (200–250 g) were killed humanely and sections of third order mesenteric arteries mounted for isometric tension recording. Relaxation

of phenylephrine- (PE) induced force by raised $[K^+]_o$ was depressed by a reduction in extracellular sodium and enhanced, sodium-dependently, by monensin (Brochet et al. 2002 *J Physiol;* 544: 5P). Relaxation was independent of the endothelium and sensitive to ouabain (Brochet & Langton 2003 *Biophys J*; 84: 418A). Relaxation to raised $[K^+]_o$ was independent of the concentration of PE and could not be revealed by inhibition of large conductance calcium-activated K channels by TEA (5 mM), charybdotoxin (100 nM), or iberiotoxin (100 nM). Increasing the duration of exposure to PE enhanced hyperpolarization and relaxation to raised $[K^+]_o$. Time-dependent effects of PE were attenuated in the presence of 2-APB (75 μM) and abolished by SKF96365 (10 μM). In contrast, ryanodine (50 nM) enhanced the relaxation. These data suggest that accumulation of $[Na^+]_i$ following activation of store-operated channels by PE and the depression of Na,K-ATPase activity in low $[K^+]_o$ both serve to potentiate the upturn in the activity of the Na,K-ATPase when $[K^+]_o$ is subsequently increased, resulting in hyperpolarization and relaxation of the arterial smooth muscle.

S7

ENDOTHELIAL FUNCTION IN OMENTAL SMALL ARTERIES ISOLATED FROM WOMEN WITH NORMAL AND PRE-ECLAMPTIC PREGNANCIES

W. R. Dunn,[1] A. M. Mathewson,[1] L. C. Kenny;[2] P. N. Baker[2]
[1]School of Biomedical Sciences, University of Nottingham, Nottingham, NG7 2UH, UK; [2]Maternal & Fetal Health Research Centre, University of Manchester, Manchester M13 0JH, UK

Pre-eclampsia (PE) is a disease of human pregnancy characterized by proteinuria and maternal hypertension. Abnormal endothelial function may underlie these symptoms. In this study, we examined endothelial-dependent responses in omental arteries isolated from normotensive pregnant women or those with PE. Functional experiments were carried out using pressure myography. Responses to the endothelium-dependent vasodilator bradykinin (BK) were obtained in the absence and presence of inhibitors of nitric oxide (NO), gap junction communication, and cyclo-oxygenase, or in the presence of raised extracellular potassium or catalase. Connexin expression in blood vessels was assessed by confocal microscopy. Responses to bradykinin (BK) were of a similar magnitude and sensitivity in omental arteries isolated from women with normal pregnancies or PE. In women with pre-eclampsia, responses to BK were almost abolished by the NO synthase inhibitor, L-NAME (100 μM). In normal pregnancy, responses to BK were abolished by the combination of L-NAME and raised extracellular potassium and sensitive to L-NAME, gap junction inhibitors or catalase (3000 U/mL) in combination. There was no difference in connexin expression in omental arteries from normal or PE pregnancies. The present results indicate that in normal pregnancy, BK causes the release of both NO and EDHF. The EDHF response involves, in part, the production of H_2O_2 and involves gap junction communication. In PE women there is virtually no EDHF response in omental arteries.

OC1

DETERMINATION OF ABIN-2 FUNCTION BY IN VIVO GENE TRANSFER INTO MICROVESSELS

A. Tadros, N. P. J. Brindle
University of Leicester, Department of Cardiovascular Sciences, RKCSB, PO Box 65, Leicester LE2 7LX, UK

Angiopoietin-1 (Ang1) is a ligand for the endothelial receptor tyrosine kinase Tie2. Among other actions, Ang1 inhibits vascular inflammation and suppresses endothelial cell apoptosis. Recently we identified A20 binding inhibitor of NFκB -2 (ABIN-2) as a mediator in Ang1 suppression of the inflammatory transcription factor NFκB in endothelial cells (Hughes et al. 2003 *Circ Res*; 92: 630–636). In addition, ABIN-2 was found to inhibit endothelial apoptosis (Tadros et al. 2003 *Blood*; 102: 4407–4409). In this study we have sought to establish a model for examining ABIN-2 function in vivo. Plasmids encoding wild-type and mutant forms of ABIN-2, or the NF$_\kappa$B inhibitory protein A20, together with green fluorescent protein were introduced into microvessels of chorioallantoic membranes of chicken eggs. The effects of expressed ABIN-2 and A20 on leukocyte rolling and adhesion following inflammatory stimulation was determined using intravital microscopy. TNF-α and lipopolysacharide caused a time- and concentration-dependent stimulation of leukocyte rolling in CAM vessels. Expression of wild-type ABIN-2 as well as A20 significantly inhibited leukocyte rolling in this model. These data provide the first demonstration of the effects of ABIN-2 on leukocyte rolling in vivo. This model is applicable to analysis of in vivo function of other genes.

OC2

LEUKOCYTE ADHESION AND NEOINTIMAL FORMATION ARE ALTERED IN THE PROTEASE ACTIVATED RECPTOR-2 (PAR-2) DELETION MOUSE

G. Tennant, R. M. Wadsworth, S. Kennedy
Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G4 ONR, UK

Recent research suggests PAR-2 may play a role in the development of neointimal hyperplasia. Our aims were to develop a murine model of vascular injury to determine the effect of PAR-2 deficiency on restenosis and the response of isolated injured arteries to autologous lymphocytes and neutrophils. Injury was induced in the left common carotid artery and aorta of anesthetized mice (sodium pentobarbitone 50 mg/kg) by a guidewire. Arteries were harvested after 28 days and lymphocyte adhesion was measured by adding ^{51}Cr-labeled lymphocytes to the lumen of the aorta. Injured and noninjured carotid artery rings were mounted in a wire myograph and subjected to trypsin or leukocytes. Histological staining showed substantial neointimal growth in the injured carotid artery in both PAR-$2^{+/+}$ and PAR-$2^{-/-}$ mice ($n=6$). In PAR-$2^{+/+}$, neointima was cellular with extensive renarrowing. In PAR-$2^{-/-}$ there was also vessel renarrowing but with medial atrophy. Trypsin induced relaxation of non-injured arteries from

PAR-$2^{+/+}$ mice, but not from PAR-$2^{-/-}$ mice. Arterial injury abolished this relaxation. Neutrophils and splenocytes induced a contraction of healthy arteries from PAR-$2^{+/+}$ and PAR-$2^{-/-}$ mice. Injury significantly reduced the constriction to splenocytes in both strains of mice ($n = 4$, $p < .05$). Non-injured arteries from PAR-$2^{+/+}$ mice contracted in response to splenocytes, and this was significantly reduced in injured arterial rings and in PAR-$2^{-/-}$ mice ($n = 5$, $p < .001$). Injury caused a significant increase in splenocyte adhesion in PAR-$2^{+/+}$ mice but not in PAR-$2^{-/-}$ mice ($n = 5$, $p < .05$). These data suggest that PAR-2 modulates leukocyte–vessel interactions, which may determine neointimal formation.

OC3

PHOTODYNAMIC THERAPY (PDT) INDUCES BOTH NECROSIS AND APOPTOSIS IN MURINE SVEC4-10 ENDOTHELIAL CELLS IN VITRO

S. L. Hankin,[1,2] M. W. R Reed,[1] A. G. Pockley,[2] N. J. Brown[1]

[1]Microcirculation Research Group and [2]Immunobiology Research Unit, School of Medicine, University of Sheffield, Sheffield S10 2JF, UK

The efficacy of anti-cancer treatments such as PDT might be influenced by the mode of cell death that they induce, as necrosis promotes inflammation, whereas apoptosis can be anti-inflammatory. Tumoricidal effects are further enhanced by the microvascular collapse and hypoxic death that are induced by concurrent damage to endothelial cells. This study evaluated the sensitivity of cultured SVEC4-10 endothelial cells to 5-aminolaevulinic acid (ALA)-PDT. Cells were incubated for 4 h with 1 mM ALA and then illuminated using a 635-nm laser (3 J/cm^2). The effects of PDT on cellular apoptosis and necrosis between 30 min and 24 h after treatment were evaluated using an Annexin V-FITC/PI flow cytometry assay. In the early post-treatment period, ALA-light treatment induced SVEC4-10 cell necrosis ($85.6 \pm 3.1\%$ vs. $63.9 \pm 3.6\%$ in light-treated controls, 30 min after treatment, $p = 0.01$). Although this necrotic response persisted until 8 h after treatment, 24 h after treatment, apoptosis dominated ($41.0 \pm 8.4\%$ vs. $9.2 \pm 5.2\%$ in light-treated controls, $p = 0.01$). If similar responses were to occur in vivo, then although the initial necrotic response would induce an acute inflammatory response and promote the induction of anti-tumor immunity, the subsequent appearance of endothelial cell apoptosis might attenuate this protective response.

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OC4

ELUCIDATION OF THE ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN THE OVARY BY A VEGF TRAP

H. M. Fraser

MRC Human Reproductive Sciences Unit, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

As follicles grow during the ovulatory cycle, they develop a blood supply. Selected follicles undergo further angiogenesis and expansion before ovulation. This is followed by a period of intense angiogenesis throughout the resultant corpus luteum which produces progesterone required for pregnancy. We investigated the role of VEGF during follicular maturation and corpus luteum formation in the marmoset by in vivo by administration of a VEGF Trap (gifted by Regeneron Pharmaceuticals) at the early and midfollicular phase and luteal phases of the cycle ($n = 5$ per group) and compared results with controls. Angiogenesis was quantified by dual staining with bromodeoxyuridine and CD31. Inhibition of VEGF starting at the early follicular phase resulted in a marked decrease in endothelial cell proliferation and inhibition of follicular growth. Delaying treatment until the midfollicular phase also inhibited angiogenesis and ovulation but the largest follicles continued to expand. Treatment at the early luteal phase prevented the intense angiogenesis associated with corpus luteum formation, resulting in a marked restriction in the development of the microvascular tree and suppression of plasma progesterone. Treatment in the midluteal phase led to a rapid decline in plasma progesterone secretion despite angiogenesis being largely complete. It is concluded that VEGF is essential for normal follicular and luteal angiogenesis and function. In addition, VEGF is required for corpus luteum function post angiogenesis, perhaps indicating a role in ovarian vascular permeability. However, VEGF is not essential for follicular expansion.

OC5

THE ANGIOPOIETINS MODULATE THE RESPONSE OF ENDOTHELIAL CELLS TO THE VASCULAR DISRUPTING AGENT COMBRETASTATIN A-4-PHOSPHATE

C. Kanthou, O. Greco, M. Fulker, G. M. Tozer

Tumour Microcirculation Group, Surgical Oncology Unit, University of Sheffield, Floor K, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF, UK

Our previous work has identified important cytoskeleton-dependent pathways for the mechanism of action of the vascular disrupting agent (VDA) combretastatin A-4-phosphate (CA-4-P) (Tozer et al. 2005 *Nature Rev Cancer*; 5: 423–435). The susceptibility of the tumor vasculature to this agent is ascribed to the relative "immaturity" of tumor blood vessels. In the present study, we analyzed the effects of the angiopoietins, a family of angiogenic growth factors implicated to play a central role in angiogenesis and blood vessel maturation, on the cytoskeletal responses of endothelial cells to CA-4-P. Endothelial cells were treated with either single recombinant angiopoietins (Ang1 and Ang2) or were co-cultured in the presence of tumor cells genetically modified to overexpress Ang1 or Ang2. We demonstrate that endothelial cells exposed to Ang 1 show a decrease in microtubule depolymerization and actin remodeling into stress fibres compared to control untreated cells, whereas pretreatment with Ang 2 results in the formation of more prominent actin stress fibers. Analysis

of signal pathways revealed that the angiopoietins interact with the Rho-GTP signal pathway, which regulates actin remodeling in response to CA-4-P in endothelial cells. Our data suggest that the angiopoietins, through their capacity to alter the stability of the cytoskeleton, alter the extent of the response of endothelial cells to CA-4-P.

This work was supported by Cancer Research UK.

OC6

MICROVESSEL REMODELLING IS DEPENDENT ON BLOOD FLOW AND GROWTH FACTORS

A. V. Benest, W. H. Miller,[1] A. H. Baker,[1] S. J. Harper, D. O. Bates
Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK; and [1]BHF Cardiovascular Research Division, University of Glasgow, Glasgow, UK

An adenovirus-driven model of angiogenesis in the adult rat mesentery demonstrated that VEGF + Ang-1 combined are able to generate neovessels with a distinct phenotype to either growth factor alone (Benest et al. 2005 *Microcirculation;* 12: 645). Although neovessel pericyte coverage could be altered, no change in vascular smooth muscle cell (vSMC) recruitment was seen. In comparison, local eNOS+VEGF+Ang1 overexpression increased the presence of vSMC and reduced pericyte recruitment, possibly due to localized vasodilatation. The effect of eNOS alone is reported here. All procedures were carried out as described previously (Wang et al. 2004 *Microcirculation*; 11: 361–374). eNOS+VEGF+Ang-1 induced a significantly higher fractional vessel area (%) (1480 ± 132 vs. 876 ± 77, $p < .003$, $n = 6$) and sprout density (mm^{-2}, 50 ± 5 vs. 2 ± 5, $p < .02$) than eNOS alone, but eNOS induced vessels had a greater degree of pericyte coverage ($26 \pm 1\%$ vs. 19 ± 4, $p < .01$). There was no significant difference in any other standard phenotypical parameter measured: branch point density (mm^{-2}, 256 ± 26 vs. 210 ± 5) vessel density (609 ± 40 vs. 549 ± 18) 16–35 μm vessel density (195 ± 28 vs. 153 ± 13), < 16 μm vessel density (395 ± 50 vs. 397 ± 28), vessel diameter (15.4 ± 0.8 vs. 14.0 ± 0.3 μm). No SMC was present in eNOS-treated mesenteries, but a combination of eNOS+Ang-1+VEGF increased SMC associated vessel density (120 ± 9 vs. 0). In summary, the recruitment of smooth muscle cells requires a combination of growth factors and nitric oxide production (probably to stimulate local vasodilation) but not either stimulus alone.

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OC7

ASSESSMENT OF TELANGIECTASES WITH DUAL WAVELENGTH LASER DOPPLER IMAGING

A. K. Murray, T. L. Moore, C. E. M. Griffiths,[1] A. L. Herrick
Rheumatic Diseases Centre, and [1]Dermatology Centre, University of Manchester, Hope Hospital, Salford M6 8HD, UK

Superficial and deeper telangiectases may respond differently to light treatment. the aim of this study was to determine whether dual-wavelength laser doppler imaging (ldi) was sufficiently sensitive to ascertain the distribution of blood flow within telangiectases and whether distribution relates to their size. the hands of 10 systemic sclerosis patients (2 m, 8 f; median 57 [range 36–72] years) were studied. Green and red LDI wavelengths measure small, superficial microvessels (e.g., capillaries) and larger, deeper microvessels respectively. Blood flow increase in 20 telangiectases, compared to adjacent skin, was measured. Pearson's correlation coefficient was determined for the diameters in grayscale images and of the area of increased perfusion (from LDI). Flow values and diameters are shown below. Grayscale diameters correlated with those of the green but not red wavelength images. Dual wavelength LDI may aid prediction of treatment response. Lack of correlation between grayscale and red wavelength image diameters indicates that the size of the telangiectases at the skin surface does not predict activity of the vessel at deeper levels. Therefore, clinically apparent size is unlikely to predict treatment response.

	Red laser images	Green laser images	Grayscale images
Flux (lesion/adjacent)	1.8 [1.2–6.2]	1.3 [1.1–1.6]	N/A
Diameter (pixels)	9.3 [3.5–19.0]	10.0 [3.7–16.7]	5.0 [3.0–12.0]

Note. Values are expressed as median [range].

OC8

CORONARY FLOW RESERVE AND ITS RELATIONSHIP WITH SKIN MICROVASCULAR FUNCTION

F. Khan, D. Patterson, J. J. F. Belch, A. D. Struthers, C. C. Lang
Division of Medicine & Therapeutics, Ninewells Hospital & Medical School, University of Dundee, Dundee DD1 9SY, UK

Endothelial dysfunction is associated with atherosclerotic risk and future cardiovascular events. Coronary endothelial function can be tested but requires invasive procedures that can be applied only to individuals requiring diagnostic coronary angiography. Recently, a relatively noninvasive assessment of coronary flow reserve (CFR), using transthoracic Doppler echocardiography, has been developed. We compared measurements of CFR with peripheral measurements of microvascular function using iontophoresis of acetylcholine and sodium nitroprusside and laser Doppler imaging in 22 healthy control subjects (mean age 26.9, SD 9.8 years). Imaging of the left anterior descending (LAD) coronary artery and perforating branches and measurement of coronary blood flow were made at rest and after intravenous adenosine (140 μg/kg/min). Mean diastolic velocities were measured at baseline and at peak hyperemic conditions from the Doppler signal recordings. CFR is defined as the ratio of hyperemic to basal mean

diastolic velocities. Skin perfusion was measured in the forearm. Peak skin microvascular responses to acetylcholine were significantly correlated with CFR ($r = .525$, $p = .012$). No correlation was found between CFR and microvascular responses to sodium nitroprusside ($r = .298$, $p = .180$). These results suggest that an estimate of coronary function could potentially be determined from measurements made in peripheral skin microvessels. This would have potential applicability in clinical practice where invasive procedures are not desirable.

OC9

IS MICROVASCULAR DISTENSIBILITY RELATED TO HYPERTENSION OR INSULIN RESISTANCE IN NEWLY DIAGNOSED TYPE 2 DIABETIC PATIENTS?

K. M. Gooding, M. Y. Gorshunska, D. Mawson, J. Piper, J. E. Tooke, A. C. Shore
Diabetes and Vascular Medicine, Institute of Biomedical and Clinical Science, Peninsula Medical School, Exeter, UK

Distensibility of large vessels in type 2 diabetes mellitus predicts future cardiovascular events. Microvascular distensibility is unexplored. The study evaluated the contribution of hypertension and insulin resistance on microcirculation abnormalities, in newly presenting type 2 diabetes mellitus. Sixty type 2 diabetic (T2DM) patients (24–69 years) were recruited. Insulin resistance (IR) was derived from fasting glucose and insulin concentrations (HOMA). Skin perfusion was recorded by laser Doppler fluximetry in a maximally vasodilated area of skin with the limb at heart level and following an increase in perfusion pressure generated by lowering the limb 50 cm below heart level. Distensibility was calculated as perfusion at heart level/perfusion with the limb lowered. Microvascular distensibility was related to both systolic ($r_s = -.405$, $p < .002$, Spearman's correlation coefficient) and diastolic ($r_s = -.385$, $p < .004$) blood pressure, vessels being less distensible the higher the blood pressure ($r_s = -.412$, $p < .002$). There were no correlations between vascular distensibility and markers of IR: fasting insulin ($r_s = -.024$, $p < .860$), HOMA-IR ($r_s = -.160$, $p < .227$), body mass index ($r_s = -.273$, $p < .073$), triglycerides ($r_s = .021$, $p < .894$). This study has demonstrated no association of microvascular distensibility with insulin resistance in newly presenting T2DM patients. In contrast, impaired distensibility against the background of hypertension suggests it may have adverse effect on certain aspects of vascular function, and thus may have implications for the genesis of microangiopathy.

OC10

LYMPHATIC COLLECTOR PUMP PRESSURE IN HEALTHY AND LYMPHOEDEMATOUS ARMS ASSESSED BY A NEW LYMPHOSCINTIGRAPHIC TECHNIQUE

S. Modi,[1,2] A. W. B. Stanton,[1,3] *A. M. Peters,*[2] *W. E. Svensson,*[3] *J. R. Levick,*[4] *P. S. Mortimer*[1]
[1]Cardiac & Vascular Sciences, St George's University of London, London, UK; [2]Nuclear Medicine, Royal Sussex County Hospital, Brighton, UK; [3]Nuclear Medicine, Hammersmith Hospital, London, UK; [4]Physiology, Basic Medical Sciences, St George's University of London, London SW17 0RE, UK

Axillary lymph node removal for breast cancer is thought to increase resistance to lymph outflow from the arm. Lymphatic collector vessels may therefore have to pump against a chronically raised afterload, leading to lymphatic collector pump (LCP) failure and breast cancer related lymphoedema (BCRL). We developed a new noninvasive method to test the hypothesis that pump force is impaired in BCRL. In an Ethics Committee-approved study we first measured the time taken for ^{99m}Tc-human IgG (^{99m}Tc-HIG, 10 MBq, i.d.) injected in the hand to reach axillary lymph nodes ($t_{transit}$, 7 ± 5 [SD] min) in 7 healthy subjects (52 ± 10 y) using a γ camera. LCP force was assessed in 11 healthy (54 ± 5 y) and 9 BCRL subjects with swollen forearms (61 ± 6 y, 12–37% swelling) with a sphygmomanometer cuff at 60 mmHg around the upper arm before injection of ^{99m}Tc-HIG. Retention of ^{99m}Tc-HIG distal to the cuff and absence of axillary counts at $> t_{transit}$ indicated inhibition of LCP function. Cuff pressure was lowered by 10 mmHg steps until ^{99m}Tc-HIG passed under the cuff and reached the axilla, indicating the pressure lymphatics could overcome (P_{pump}). In healthy subjects P_{pump} was 41 ± 4 (SEM) mmHg, but was reduced in BCRL subjects with swollen forearms (24 ± 6 mmHg) ($p = .023$, unpaired t test). We conclude that LCP force can be measured noninvasively in this way. The results support the hypothesis of forearm LCP failure in BCRL.

OC11

VEGF-C SECRETION MEDIATES MIGRATION OF LYMPHATIC ENDOTHELIAL CELLS TO METASTATIC MELANOMA CELLS

M. S. Emmett, D. O. Bates
Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol, BS2 8EJ, UK

Malignant melanoma is the fastest growing cancer in the UK. Initial metastatic spread is via the lymphatic system and it is metastatic deposits that cause death. Animal models of tumors expressing vascular endothelial growth factor C (VEGF-C) showed increased lymph angiogenesis and metastasis (Skobe et al. 2001 *Nat Med;* 7:192–198). Although increased lymphatic vessel density and metastasis have been linked in human tumors (Shields et al. 2004 *Br J Cancer*; 90:693–700), it is unclear whether this is due to lymphangiogenesis or active migration of tumor cells. To determine the role of melanoma-secreted VEGF-C in migration of lymphatic endothelial cells (LEC) toward the melanoma, A375 (metastatic, MM) human melanoma cells, two subclones — A375SM (MM), A375P (nonmetastatic, NM), human dermal endothelial cells (HDEC), and immuno-magnetically isolated (LEC) were grown to confluence in vitro. VEGF-C western blot analysis showed that A375SM cells expressed VEGF-C

at 90% of A375 cells, but A375P cells expressed only 50%, adjusted for α actin levels. Migration was analyzed using a modified Boyden chamber. Depletion of VEGF-C from A375 CM by immuno-precipitation with VEGFR3-Ig, reduced A375 CM-mediated migration of HDECs to $60 \pm 15\%$ of control. A375SM CM, but not A375P CM, significantly increasing migration (13.6 ± 2.0 mean fold increase $\pm$ SEM, $p < .05$, t test) of LECs. These results suggest that MM produces more VEGF-C than NM melanoma and this has a significant migratory effect on LECs. Lymphangiogenesis in response to VEGF-C secreted by melanoma may be crucial to metastasis.

Supported by SCaRF and Luff funds.

OC12

EFFECTS OF LIPOPOLYSACCHARIDE ON THE RAT EXTRASPLENIC MICROVASCULATURE AND LYMPHATICS IN VIVO

A. Mansart,[1,2] *N. J. Brown,*[2] *J. J. Ross,*[1] *C. S. Reilly,*[1] *Z. L. S. Brookes*[1,2]

[1]Academic Anaesthesia Unit and [2]Microcirculation Research Group, University of Sheffield, Royal Hallamshire Hospital, Sheffield, UK

Lipopolysaccharide (LPS) does not decrease blood volume following splenectomy in animals. Intrasplenic fluid extravasation may result from extrasplenic hemodynamic changes (Andrew et al. 2000 *Am J Physiol;* 278: R60–R65). The aim of the present study was to determine the effects of low-dose LPS on the extrasplenic microvasculature and lymphatics during the early stage of sepsis in vivo. Male Wistar rats (220–280 g) were anesthetized (sodium thiopental, 20–30 mg/kg/h, i.v.), mean arterial pressure (MAP) measured and fluorescent intravital microscopy used to determine macromolecular leak, extrasplenic vessel and lymphatic diameter for 4 h following FITC-BSA (0.2 mL/100 g body weight, i.v.) administration in (a) control: saline ($n = 6$); (b) LPS: saline+LPS (150 μg/kg/h) ($n = 6$). Four hours after LPS administration ($t = 4$ h), arteriolar diameter remained unchanged ($-5.7 \pm 4.5\%$), venules constricted ($-14.6 \pm 3.7\%$, $p < .05$), lymphatics dilated ($15.8 \pm 2.4\%$, $p < .05$), macromolecular leak increased ($19.8 \pm 2.5\%$, $p < .05$), and MAP decreased ($-53.2 \pm 11\%$, $p < .05$) from baseline ($t = 0$). In the control group, microvascular parameters remained unchanged ($t = 0$–4 h). Venular constriction, i.e., a greater increase in postcapillary resistance could contribute to hypotension and hypovolemia during sepsis, but further studies are necessary to evaluate lymphatic permeability.

Funded by the Anaesthetic Cardiothoracic Research Fund, NGH Sheffield.

OC13

COMMON VARIATION IN THE *PPARG* GENE INFLUENCES SUSCEPTIBILITY TO SIGHT-THREATENING DIABETIC RETINOPATHY IN PATIENTS WITH BOTH TYPE 1 AND TYPE 2 DIABETES

A. S. F. Doney, G. L. Leese, A. M. Morris , C. N. A. Palmer

Department of Medicine and Therapeutics, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK

The Pro12Ala and C1431T variants of *PPARG* have been previously associated with opposing phenotypes in patients with type 2 diabetes. We used a large prospective genetic study of patients with diabetes known as Go-DARTS to investigate the influence of *PPARG* genotype on susceptibility to sight threatening diabetic retinopathy. We identified a study cohort ($n = 1847$) free of either sight-threatening diabetic retinopathy or previous photocoagulation therapy at the time of enrolment into Go-DARTS. Cox's proportional hazards was used to model risk of development of sight threatening disease by *PPARG* genotype separately in patients with type 2 ($n = 1741$) and type 1 ($n = 106$) diabetes and in the combined population. Duration of diabetes and age at enrolment were included as covariates. While both variants were in Hardy-Weinberg equilibrium in both populations, Ala12 was significantly over-represented in the population with type 1 diabetes compared to the population with type 2 diabetes ($p < .0001$). Ala12 was associated with a significantly increased risk of development of sight-threatening retinopathy in both populations independently with a hazard ratio of 2.3 95% CI 1.4–3.8 $p = .001$ for the combined population. The association was dependent on C1431T genotype. *PPARG* genotype may influence susceptibility to sight-threatening diabetic retinopathy.

OC14

VEGF$_{165}$B, AN ENDOGENOUS C-TERMINAL SPLICE VARIANT OF VEGF, INHIBITS RETINAL NEOVASCULARISATION IN MICE

O. Konopatskaya,[1] *S. J. Harper,*[1] *A. J. Churchill,*[3] *D. O. Bates*[1], *T. A. Gardiner*[2]

[1]Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol, UK; [2]Department of Ophthalmology, Bristol Eye Hospital, Bristol, UK; [3] Ophthalmology and Vision Science, Queen's University, Belfast, Northern Ireland

Hypoxia-driven ocular angiogenesis occurs in a range of ischemic retinopathies, including proliferative diabetic retinopathy and retinopathy of prematurity. These conditions are initiated and sustained by vascular endothelial growth factor (VEGF). There are two families of VEGF isoforms formed by differential splicing: the pro-angiogenic VEGF$_{xxx}$ family, known to contribute to ocular neovascularization, and the anti-angiogenic VEGF$_{xxx}$b family, which are downregulated in diabetic retinopathy in humans. To determine whether VEGF$_{165}$b could inhibit hypoxia-driven angiogenesis in the eye, the oxygen–induced retinopathy mouse model of ocular neovascularization was used. C57/BL6 mice were anesthetized with isoflurane (2.5 L/min). Intravitreal injection of 1 ng of VEGF$_{165}$b significantly inhibited the % area of retinal neovascularization from $23 \pm 3\%$ to $12 \pm 3.3\%$, and significantly increased normal vascular areas from $62 \pm 4\%$ to $74 \pm 4\%$. The % area of residual ischemic retina was not affected. These results show that a single injection of VEGF$_{165}$b

can significantly reduce preretinal neovascularization without inhibition of physiological intraretinal angiogenesis. Controlling the balance of $VEGF_{xxx}b$ to $VEGF_{xxx}$ isoforms may therefore be therapeutically valuable in the treatment of proliferative eye diseases such as diabetic retinopathy and age related macular degeneration.

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OC15

DIABETES-ASSOCIATED CHANGES IN THE RETINA ARE ATTENUATED WHEN THE OUTER RETINA IS DEPLETED

A. W. Stitt,[1] T. E. de Gooyer,[1] P. Humphries,[2] D. A. Simpson,[1] T. A. Gardiner[1]
[1]Ophthalmic Research Centre, Queens University, Belfast, Northern Ireland; [2]Trinity College Dublin, Ireland

It has been suggested that diabetic patients who also have retinitis pigmentosa (RP) are protected against retinopathy. Diabetes leads to increased inner retinal hypoxia and this may linked to oxygen usage during the dark adaptation response. Therefore, RP patients with depleted rod photoreceptors may encounter proportionately less retinal hypoxia and when diabetes is also present with less extensive retinopathic lesions. We have tested this hypothesis using rhodopsin knockout mice (Rho−/−) as an RP model in which the diabetic milieu is superimposed. Diabetes was induced in C57Bl6 (WT) and Rho−/− mice. After 5 months, retinal hypoxia was assessed using the bioreductive drug pimonidazole (Hypoxyprobe). The retinal microvasculature was assessed by ADPase reaction and retinal gene expression was also quantified using real-time RT-PCR. Hypoxia was increased in the retina of WT diabetic animals when compared to controls, but this diabetes-induced change was absent in Rho−/− mice. Retinal gene expression of VEGF-A was significantly increased in WT mice with diabetes ($p = .0001$), but was unchanged in Rho−/− mice. Vascular density was significantly reduced in diabetic WT mice compared to nondiabetic controls ($p < .001$), but this diabetes-related pathology was not observed in Rho−/− mice. Loss of the outer retina reduces severity of diabetic retinopathy in a murine model. Oxygen usage by the photoreceptors during dark adaptation may contribute to retinal hypoxia and exacerbate progression of diabetic retinopathy.

OC16

SPLICE FACTOR REGULATION OF VEGF ISOFORM EXPRESSION

J. Woolard, A. Varey, D. G. Nowak, M. Ladomery, S. J. Harper, D. O. Bates
Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK

$VEGF_{xxx}b$, an endogenous inhibitory splice variant of VEGF, is formed by differential distal splice site selection in exon 8. This C terminal alternative splicing event results in both anti-angiogenic and anti-tumourigenic activity (Woolard et al. 2004 *Cancer Res;* 64: 7822–7835). The mechanisms by which the splicing switch from pro- to anti-angiogenic VEGF phenotypes is regulated are largely unknown; therefore, the present study investigated the splice factors involved in this process. Human colon cancer cells, HT-29 cells, were transfected with plasmids expressing specific splicing factors SRp40, 9G8, SRp55, ASF/SF2, sc35, SRp20, SRp30c, or hnRNPA1, under control of a CMV promoter, and conditioned medium and total protein collected and analyzed by ELISA and western blotting. Compared with $pcDNA_3$-transfected cells, SRp40, SRp20, SRp30c, and hnRNPA1 increased the amount of $VEGF_{xxx}b$ protein detected in the medium, while 9G8, ASF/SF2, and SRp55 decreased the amount of secreted $VEGF_{xxx}b$ protein. SC35 did not affect the level of $VEGF_{xxx}$ b. Western blotting showed that although $VEGF_{165}b$ expression was reduced by 9G8, ASF/SF2, and SRp55 consistent with the ELISA, SRp20, SRp30c, and hnRNPA1 also downregulated expression of $VEGF_{165}b$, but upregulated expression of the $VEGF_{145}b$ isoform. These results indicate that splicing factors such as hnRNPA1 and SR proteins (ASF/SF2, SRp20, and SRp30c) are involved in the regulation of alternative splicing of the VEGF gene.

OC17

THE ANTI-TUMOR AGENT PEP005 ACTIVATES ENDOTHELIAL CELLS AND PROMOTES NEUTROPHIL RECRUITMENT IN A PKC-DEPENDENT MANNER

D. P. J. Kavanagh, E. Smith, J. M. Lord, P. Hampson, G. E. Rainger
Centre for Cardiovascular Sciences, The Medical School, Edgbaston, Birmingham B15 2TT, UK

3-Ingenyl angelate (PEP005) is a novel chemotherapeutic agent of plant origin that has shown efficacy in the treatment of squamous and basal cell carcinomas. Recent observations indicate that the effective clearance of the tumor mass involves an initial induction of necrosis in the tumor followed by the intratumoral recruitment of neutrophils (Ogbourne et al., manuscript submitted). However, the molecular mechanisms that regulate this process are currently unknown. Here we show that treatment of endothelial cells (EC) with PEP005 for 4 h promotes the expression of adhesion receptors (ICAM-1, VCAM-1, and E-selectin) and chemokines (IL-8, MIP-1α, and MCP-1) at the mRNA and protein level. PEP005 activated EC could also support the adhesion, activation, and transmigration of flowing neutrophils and neutrophil adhesion was reduced (>80%) by pretreating the EC with antibody against E-selectin. As PEP005 has been reported to be a potent activator of PKC we showed that treatment of EC with this agent led to the translocation of PKC-δ and, further, that inclusion of the PKC inhibitor bisindolylmaleimide 1 completely abolished the EC response to PEP005 as determined by inhibition of neutrophil adhesion in a flow-based assay. Thus, we have demonstrated that the ability of PEP005 to recruit neutrophils to the tumor site may be due to its direct and PKC-dependent effects on the tumor vasculature, which promotes

recruitment of the neutrophils thought to be essential for tumor clearance.

OC18

VEGF$_{xxx}$b LEVELS PREDICT METASTASIS IN MELANOMA

D. B. Dunn,[1] R.O. Pritchard-Jones,[1] Y. Qui,[1] H Rigby,[2] A. Orlando,[2] S. J. Harper,[1] D.O. Bates[1]

[1]Microvascular Research Laboratories, Department of Physiology, University of Bristol, and [2]Department of Plastic Surgery, Frenchay Hospital, Bristol, UK

The most reliable predictive factor for melanoma metastasis is currently Breslow tumor thickness. However, metastasis develops in ~15% of patients with thin melanomas. The VEGF$_{xxx}$b family of VEGF-A splice variants are anti-angiogenic and cause significant growth retardation when overexpressed in cancer cell lines in vivo. To determine whether VEGF$_{xxx}$b expression was altered in malignant melanoma, this was assessed immunohistochemically with a VEGF$_{xxx}$b-specific antibody (MVRL56/1). Section (6 μm) of primary metastatic (MM) and nonmetastatic (NM) melanoma, matched for Breslow thickness, were stained with MVRL56/1 or a control IgG at 4 μg/mL. Following hematoxylin counterstaining sections were analyzed by 3 assessors (blinded to clinical outcome) scoring intensity of staining (0–4) of the melanoma in vertical and horizontal growth phases and surrounding skin. Normal epidermis stained significantly in all samples, and was no different in the MM group (1.6 ± 0.39) compared to NM (2.03 ± 0.27). Significantly weaker expression was seen in MM in vertical (1.7 ± 0.24 NM, 0.48 ± 0.26 MM, $p < .05$) and horizontal (1.5 ± 0.25 NM, $0.37 \pm .21$ MM, $p < .05$) growth phases than in NM. There was no correlation between staining intensity and Breslow thickness. These results suggest that VEGF$_{xxx}$b expression is downregulated in metastatic melanoma but not in nonmetastatic melanoma, and may be a useful aid to identify patients likely to develop metastasis irrespective of tumor thickness.

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OC19

ANGIOPOIETIN PROTEIN AND RECEPTOR EXPRESSION WITH VEGF IN COLORECTAL CANCER

F. Bach,[1] J. Uddin,[1] P. Quirke,[2] P. Jones,[3] D. Burke[1]

[1]Departments of Academic Surgery, [2]Pathology and [3]Molecular Medicine, University of Leeds, Leeds LS1 3EX, UK

The angiopoietins bind to the endothelial cell surface via the tyrosine–kinase receptor, Tie-2, and are crucial to the process of tumor angiogenesis. Little is known about their role in colorectal disease. The aim of this study was to investigate the expression of angiopoietin-1 (Ang1), angiopoietin-2 (Ang2), Tie-2, and vascular endothelial growth factor (VEGF) in colorectal cancer tissue. Tissue microarrays of 636 colorectal cancer specimens with adjacent normal colonic mucosa were constructed. Immunohistochemical studies were performed using antibodies directed against Ang1, Ang2, Tie-2, and VEGF. Tissue staining was quantified using light microscopy (×200). There were significantly more tumor cells that stained for each of the proteins as compared to normal samples with the highest up-regulation seen for Ang2 (1.8×) and VEGF (1.4×). The commonest endothelial staining pattern observed in normal tissue was Angl+Ang2- (43.9 vs. 38.4% in tumor tissue, $p = .001$). There was a 6-fold increase in expression of Ang2+Angl- in tumors (5.2 vs. 0.9% in normal tissue, $p = .001$). Ang2 expression was associated significantly with a higher grade of tumor ($p = .049$) but not with vascular invasion. This study has demonstrated upregulation of Ang2 in colorectal cancer tissue in the presence of VEGF. It may be the switch to increased Ang2 expression that is fundamental to the angiogenic progression of a tumor. Further understanding of the precise nature of this switch might allow appropriate therapeutic interventions to be developed.

OC20

ANGIOGENESIS AND VASCULAR ENDOTHELIAL GROWTH FACTOR IN THE ADENOMA–CARCINOMA SEQUENCE OF COLORECTAL CANCER

A. S. A Chetwood, N. J. Brown, I. C. Cameron, S. S. Cross, M. W. R. Reed, C. A. Staton

Academic Units of Surgical Oncology & Pathology, University of Sheffield, Sheffield S10 2JF, UK

Colorectal cancer (CRC) is the final stage of the adenoma–carcinoma sequence (ACS). Angiogenesis, the formation of blood vessels from an existing vasculature is a fundamental requirement for tumor growth and is stimulated by vascular endothelial growth factor (VEGF) in tumors. This study is the first to assess angiogenesis and VEGF expression in each stage of the ACS. A total of 210 surgical specimens comprising the ACS (45 background mucosa, 49 low-grade and 20 high-grade dysplastic polyps, 30 intrapolyp carcinomas, 10 Dukes' A, 28 Dukes' B, and 28 Dukes' C carcinomas) were immunohistochemically stained for CD31 and VEGF. Angiogenesis was quantified using Chalkley grid analysis (MVD; Fox et al. 1995 *J Pathol*; 177: 275–283) and VEGF expression was graded from 0 (no expression) to 3 (intense staining) and correlated with standard prognostic indicators. A significant increase in MVD across the ACS ($p < .0005$) was identified with significant correlations between MVD and Dukes' stage ($p = .01$) and lymph node involvement ($p = .02$) seen. The greatest increase in MVD was related to the mild dysplasia transformation in the ACS with an associated significant increase in VEGF expression ($p < .0005$). VEGF was expressed by 74% of tumor cells in carcinoma specimens. No correlation was seen between VEGF and MVD, tumor size, Dukes' classification or lymph node involvement. These findings suggest that the "angiogenic switch'' occurs at the onset of the dysplastic transformation in polyp formation and that

VEGF is intimately involved in this process early in the ACS sequence.

OC21

AVASTIN BINDS PRO-ANGIOGENIC VEGF165 AND ANTI-ANGIOGENIC VEGF165b WITH SIMILAR AFFINITIES

A. H. R. Varey, Y. Qiu, A. R. Dixon, S. J. Harper, D. O. Bates
Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK

VEGF-A comprises pro-angiogenic and therefore pro-tumor isoforms, VEGF165, etc. (termed VEGFxxx). A subset of isoforms, e.g., VEGF165b, termed VEGFxxxb, possess a different C-terminal 6 amino acids, resulting in anti-angiogenic and anti-tumor properties (Bates et al. 2002 *Cancer Res*; 62:4123–4131 and Woolard et al. 2004 *Cancer Res*; 64:7822–7835). VEGF165b overexpression by LS174t colon carcinoma cells inhibits tumor growth in subcutaneous murine xenografts (Varey et al. 2006 *MNBWS* www.med.miami.edu/mnbws/06Varey.pdf). Analysis of paired human colon biopsies (normal and carcinomas) by RT-PCR and ELISA revealed the predominant isoforms to be VEGFxxxb in normal epithelium, but VEGFxxx in the carcinomas, due to selective upregulation of VEGFxxx (ratios: 13.2 ± 5.03 vs. 1.03 ± 1.25; $p = .0001$). We investigated the ability of VEGF inhibitor bevacizumab (Avastin) to bind VEGF165b using surface plasmon resonance. Bevacizumab was amine coupled to a sensor chip, then binding kinetics measured at various concentrations of VEGF165 and VEGF165b in solution. The affinity (K_d) for VEGF165 of bevacizumab was 2.5 nM, not significantly different from the K_d for VEGF165b (6.8 nM). Binding of bevacizumab to VEGF165b was confirmed by Western blotting. Thus bevacizumab binds anti-angiogenic VEGFxxxb isoforms such as VEGF165b, as well as inhibiting pro-angiogenic VEGFxxx, although it is not known whether it can inhibit the actions of VEGF165b. The ratio of VEGFxxx to VEGFxxxb in patients' colorectal carcinomas may therefore be a crucial determinant in their response to bevacizumab therapy.

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OC22

OPEN CHANNEL CONDUCTANCE OF THE VEGF-ACTIVATED CATION CURRENT IN HUMAN MICROVASCULAR ENDOTHELIAL CELLS

H. Cheng, A. F. James, J. C. Hancox, D. O. Bates
Microvascular and Cardiovascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK

Vascular endothelial growth factor (VEGF) exerts many of its effects by stimulating endothelial Ca^{2+} influx, but little is known about channels mediating VEGF-induced cation entry. We have previously shown that VEGF activates a gadolinium-sensitive, nonselective cation current in human microvascular endothelial cells (HMVECs). 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), an analogue of the second messenger, diacylglycerol (DAG), activated a very similar current. Superfusion of HMVECs with VEGF or OAG also increased current noise. This increased noise arises from channel gating. The open channel conductance ($g_\circ$) is a function of the variance of the noise, so power spectral analysis yields an estimate of $g_\circ$. We performed a power spectral analysis of the whole cell currents activated by VEGF and OAG. Whole-cell patch-clamp recordings were obtained from HMVECs at 37°C. The power spectra prior to superfusion of VEGF/OAG were subtracted from the spectra in the presence of VEGF/OAG as background noise. Superfusion of the cells with either 1 nM VEGF or 100 μM OAG resulted in the activation of strikingly similar noisy inward currents at the holding potential of –60 mV. The power spectra of the VEGF- and OAG-activated currents were both fitted with a double Lorentzian. There was no significant difference in the mean $g_\circ$ for the VEGF- (26.6 ± 8.9 pS, $n = 3$) and the OAG-activated (23.2 ± 8.5 pS, $n = 5$) channels. These conductance values are concordant with VEGF and OAG activating similar cation channels, possibly of the TRPC family.
Supported by the British Heart Foundation.

OC23

ENDOTHELIAL PROGENITOR CELL ATTACHMENT AND REPAIR IN A NEW MODEL OF RETINAL VASCULAR ENDOTHELIAL INJURY

A. D. Bhatwadekar,[1] T. A. Gardiner,[1] T. M. Curtis,[1] G. Li,[2] A. W. Stitt[1]
[1]Centre for Vision Science, and [2]Orthopaedics, Queen's University, Belfast, Northern Ireland

Early stages of diabetic retinopathy (DR) are associated with progressive retinal microvasculopathy eventually resulting in capillary closure. Bone marrow-derived endothelial progenitor cells (EPCs) contribute to systemic vascular repair, although it is unknown if such a phenomenon occurs in the retina or how it may be altered during diabetes. Using a novel in vitro model, whereby delineated regions of apoptotic death were induced in monolayers of retinal microvascular endothelial cells (RMECs), we assessed the potential for EPCs to contribute to microvascular repair. EPCs were purified from human peripheral blood and characterized by staining with acLDL-DiI, UEA-1, CD31, CD34, and VEGFR2. RMEC monolayers were treated with verteporfin (0.5–1 μg/mL), which was subsequently activated using highly focused red light causing localized apoptosis without damage to the bulk of the monolayer or the underlying matrix. The ability of exogenous EPCs to "endothelialize" these wounds was then assessed. RMEC monolayers exhibited enhanced expression of ICAM and VCAM at the edge of the denuded area and addition of DiI-labeled EPCs to wounded RMEC monolayers resulted in specific targeting, with 5 times more EPCs near the wound site as compared to untreated regions ($p < .001$). This study suggests that EPCs could play a hitherto unrecognized role in retinal capillary endothelial repair and has important implications for progressive vasodegeneration during diseases such as diabetic retinopathy.

OC24

ACETYLCHOLINE VASCULAR RELAXATION IS NO LONGER DEPENDENT ON NITRIC OXIDE IN A GSK3 "KNOCK-IN" MODEL

J. Coleman,[1] *C. D. Sutherland,*[1,2] *D. R. Alessi,*[2] *J. R. Petrie*[1]
[1]Division of Medicine and Therapeutics, Ninewells Hospital, University of Dundee, Dundee, UK; [2]Division of Life Sciences, University of Dundee, Dundee, UK

Insulin phosphorylates and activates endothelial nitric oxide synthase (eNOS) via signaling elements including PI 3-kinase and protein kinase B. Thus, the hormone may contribute to physiological regulation of vascular tone by nitric oxide (NO). Glycogen synthase kinase-3 (GSK3) is an enzyme downstream of PI3K, which is expressed in endothelial cells and has been reported to have elevated activity in muscle tissue in T2DM. In the present study, we have examined endothelial function in mice exhibiting a double "knock-in" (KI) of GSK3. GSK3 double KI animals were generated in which replacement of both alleles of GSK3α and GSK3β—with [S21A] GSK3α and [S9A] GSK3β, respectively—renders the enzyme insensitive to insulin inactivation in all tissues. Tail arteries were mounted on a wire myograph (Danish MyoTech) and preconstricted with U46619 (10nM). Relaxation to acetylcholine (ACh) was measured in the presence and absence of L-NAME (100 μM), indomethacin (100 μM), apamin (100 nM), and charybdotoxin (100 nM). Although ACh relaxation was preserved in GSK3 double KIs, inhibitor studies demonstrated that this response was no longer dependent on NO. Maximal relaxation: (L-NAME/indomethacin) WT $79 \pm 2\%$ vs. KI $38 \pm 7\%$, $p < .01$; (apamin/charybdotoxin) WT $42 \pm 7\%$ vs. KI $77 \pm 3\%$, $p < .05$. These studies implicate GSK3 inactivation as an additional component of insulin signaling in vascular tissue. Further studies are required to determine the molecular basis of this ACh preservation response and the role of endothelium-derived hyperpolarizing factor (EDHF).

P1

THE SUBCHONDRAL MICROCIRCULATION AND NUTRITION IN THE DEEP AND CALCIFIED ZONES OF ARTICULAR CARTILAGE

K. P. Arkill, C. P. Winlove
School of Physics, Exeter University, Exeter EX4 4QL, UK

Articular cartilage is an avasular tissue and its nutrition is derived from microcirculations in the synovial membrane and subchondral bone. The accepted view is that calcified cartilage and subchondral bone are essentially impermeable in mature animals and nutrition derives primarily from the latter source. We have examined these hypotheses using sensitive fluorescent tracer techniques both in excised bone-cartilage plugs mounted in a diffusion chamber and an in vitro-perfused preparation of the equine forelimb (7–10 yrs). Tracer distribution was quantified in frozen histological sections and effective solute diffusivity was determined using custom software (Multiview, Dr CG Phillips). Rhodamine base and sodium fluorescein were used as low molecular weight tracers. The distance between the vessels was 214 ± 10 μm in the subchondal bone approximately 200 μm from the deep cartilage. Calcified cartilage was permeable to both solutes and rhodamine base had an effective diffusivity of $8.2 \pm 0.7 \times 10^{-13}$ m^2s^{-1} compared to $3.7 \pm 0.3 \times 10^{-12}$ m^2s^{-1} in uncalcified cartilage. The tide-mark presented a region of higher reversible binding between cartilage zones. The tracers reached the deep cartilage from the subchondral microcirculation first.

P2

EXTRACELLULAR MATRIX PROTEINS DETERMINE SYNOVIAL PERMEABILITY AND HYALURONAN ULTRAFILTRATION

J. R. Levick, S. Sabaratnam
Physiology, St George's Hospital Medical School, London SW17 0RE, UK

The aim of this study was to test the hypothesis that the network of extracellular matrix proteins in the synovial lining is responsible for retaining the lubricant hyaluronan in the joint cavity by ultrafiltration. Cannulated knee joints of rabbits (anesthetized, IV pentobarbiton-urethane IV) were infused with hyaluronan, with/without pretreatment by chymopapain, a broad-spectrum collagen-sparing protease. Trans-synovial fluid escape rate was measured. Samples of intra-articular fluid and subsynovial fluid were analyzed for hyaluronan to assess trans-synovial ultrafiltration. In controls, hyaluronan ultrafiltration was confirmed by an increase in intra-articular hyaluronan concentration ($259 \pm 17\%$ of infused concentration) and fall in subsynovial concentration ($30 \pm 8\%$) ($n = 11$). The hyaluronan eflected fraction was 0.57–0.75. Chymopapain treatment increased the hydraulic permeability of the lining $\sim 12\times$; almost abolished the trans-synovial difference in hyaluronan concentration; and reduced the reflected fraction to 0.03–0.07 ($n = 6$, $p < .001$, ANOVA). Synovial histology and electron microscopy confirmed proteoglycan depletion and collagen preservation after chymopapain treatment. Synovial hydraulic permeability and molecular sieving of hyaluronan are determined by noncollagen extracellular matrix proteins. Thus, high protease activity in arthritis will promote high rates of fluid escape and hyaluronan loss.

P3

THE BIOMECHANICS OF THE COLLECTING LYMPHATIC PUMP

K. P. Arkill,[2] *A. J. Macdonald,*[1] *G. R. Tabor,*[1] *C. P. Winlove,*[2] *N. G. McHale*[3]
Schools of [1]Engineering and [2]Physics, University of Exeter, Exeter, EX4 4QL, UK; [3]Dundalk Institute of Technology, Dundalk, Eire

The lymphatic system constitutes a complicated, efficient, active drainage network with feedback capabilities and its dysfunction in conditions such as lymphoedema has serious consequences but its fluid mechanics have been less

extensively investigated than other components of the circulation. The conducting elements comprise sequences of pumps, like "mini-hearts," separated by one-way valves and are the subject of an experimental and computational study. Sections of bovine mesenteric collecting lymphatics were mounted in an in vitro perfusion rig on the stage of a microscope. Pressure–radius relationships were determined from digitized video recordings at different stations along the vessels under different conditions of smooth muscle tension. These measurements showed that the valve areas are less compliant than a normal wall segment. In addition, the waveform of spontaneous contraction and velocity of ejection was analyzed and though the velocity of ejection is near sinusoidal in time the contractions are not. These data are now being incorporated into one- and two-dimensional finite element models of flow in a single unit, and through a series of units. These models demonstrate that interplay between the contractility of the segments and the function of the valves is critical in pumping against a pressure gradient. Further to explore the behavior of the valves, a 3D model of flow through the valve is also being implemented.

P4

DIRECTED GROWTH OF MALIGNANT MELANOMA TOWARDS ENDOTHELIAL CELLS IN VIVO

D. B. A. Dunn, M. S. Emmett, A. Orlando,[1] D. O. Bates
MVRL, Department of Physiology, University of Bristol, Bristol BS2 8EJ; [1]Department of Plastic Surgery, Frenchay Hospital, Bristol, UK

Malignant melanoma results in death from metastatic deposits rather than the primary lesion, yet the mechanisms underlying metastasis are unknown. The A375 lymphatic metastatic cell line migrates toward lymphatic endothelial cells (LEC) but not blood endothelial cells (BEC) in vitro and in vivo. Also this migration is absent in the A375-P (nonmetastatic) cell line. The project aimed to determine if a highly metastatic melanoma cell line that spreads by both blood and lymphatic routes has the potential to actively migrate toward both endothelial cell types in vivo. A375-SM human melanoma cells, human dermal endothelial cells (HDEC), immunomagnetically separated into LEC and BEC were grown to confluence in vitro. 1×10^5 LECs or BECs in 100 μL PBS were injected subcutaneously into 6 nude mice with a tattooing needle. A375SM cells were then injected ~10 mm rostral to the EC injection site with a tattooing needle. Tumors were excised at 12 mm in diameter. Measurements were made from macroscopic prosections of the tumor area relative to the endothelial cell injection site. A375SM grew significantly toward LEC ($85 \pm 9\%$ of tumor caudal side, $p < .05$) and BEC ($89 \pm 6\%$, $p < .01$) when compared with expected growth and data for nonmetastatic cells. These results, and those previously presented, show that melanoma cell lines of differing metastatic potential are able to actively migrate to endothelial cells of either blood or lymphatic origin in concordance with their metastatic potential.

Supported by The Healing Foundation, SCaRF, and the Luff Fund.

P5

FREE RADICAL INHIBITION BLOCKS INTRAENDOTHELIAL CALCIUM INCREASE AND EDHF VASODILATATION IN SMALL SINGLE MESENTERIC ARTERIOLES

P. A. Fraser,[1] P. I. Aaronson,[2] M. H. Sarker[1,3]
[1]Cardiovascular and [2]Asthma & Allergy & Lung Biology Divisions, King's College London, Guy's Campus, London SE1 1UL, UK; [3]Chemical & Life Sciences, University of Teesside, Middlesbrough TS1 3BA, UK

Raised endothelial $[Ca^{2+}]_i$ is thought to be a key step in EDHF via the opening sK_{Ca} and IK_{Ca} channels. The formation of free radicals (or H_2O_2) is also thought to play a role and there is evidence that this also depends on a $[Ca^{2+}]_i$ increase. We have examined this in single arterioles (9–22 μm diameter) of the isolated–perfused rat mesentery. The coeliac artery cannulated of freshly killed rats was flushed with heparinized saline, perfused with a Krebs buffer solution at 37°C containing $10\,g \cdot L^{-1}$ bovine serum albumin and phenylephrine (5 μM). The arcade arteries were ligated, the one supplying the selected loop being spared. Fura-2 AM was also included in the perfusate in some experiments. Both bradykinin and carbachol (both 1 μM) resulted in peak fura 380/360 ratio increases from 0.98 ± 0.11 to 2.0 ± 0.32 and 0.91 ± 0.12 to 1.13 ± 0.14 (mean ± SEM; $n = 4$) respectively, and diameter increases of 147 ± 20 and $145 \pm 24\%$. The diameter responses were blocked by apamin and charybdotoxin (both 0.1 μM), and both the fura and diameter responses were blocked by scavenging free radicals using SOD and catalase ($100\,U \cdot mL^{-1}$), which indicated that the $[Ca^{2+}]_i$ increase was ROS dependent. Luminal ouabain (100 μM) resulted in a vasodilatation of $226 \pm 28\%$, while its application to the outside of the vessels produced a much smaller dilatation of $107 \pm 4\%$. We suggest that ouabain may be raising endothelial $[Ca^{2+}]_i$ via raised Na^+.

P6

INVESTIGATING THE RELATIONSHIP BETWEEN LOWER LIMB CUTANEOUS BLOOD FLOW AND MUSCLE FUNCTION IN ASIAN NIDDM (NON-INSULIN-DEPENDENT DIABETES MELLITUS) PATIENTS

M. Rajput, S. Ray
Ninewells Hospital & Medical School, Dundee DD1 9SY, UK

The incidence of non-insulin-dependent diabetes mellitus (NIDDM) is on the increase worldwide, especially in Asian populations. NIDDM is associated with a number of complications that cause lasting morbidity and also mortality. Diabetic patients often report experiencing poor circulation and weakness in their lower limbs. This study's objective was to find a causal relationship with lower limb blood flow and muscle function in a group of NIDDM Asian male diabetics as compared to an age-matched nondiabetic group. Based on a quasi-experimental design, right leg cutaneous blood flow and ankle muscle strength (dorsiflexion and plantarflexion)

were measured using laser Doppler flowmetry and the Cybex Norm dynamometer. Statistical analysis demonstrated a variation in the cutaneous blood flow when comparing the diabetic to control group. Furthermore, the diabetic group had weaker plantar and dorsiflexion mucle strength (Pearson correlation, $p < .01$). These results suggest the co-existence of micro-angiopathy and muscle dysfuction. In conclusion, an intact microcirculation is essential for optimal lower limb muscle function and hereby of clinical relevance to exercise prescription for Asian diabetic patients.

P7

11β-HSD1 ACTIVITY DOES NOT REGULATE GLUCOCORTICOID-MEDIATED INHIBITION OF TUBE FORMATION BY ENDOTHELIAL CELLS

J. J. Logie, B. R. Walker, P. W. F. Hadoke
Endocrinology Unit, Centre for Cardiovascular Sciences, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

Generation of active glucocorticoid (GC) by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) within the vascular wall regulates angiogenesis. This study aimed, using a model of tube-like structure (TLS) development by endothelial cells in vitro, to determine whether generation of GCs within the endothelium itself contributes to the inhbition of new vessel growth. Human umbilical vein endothelial cells (HUVECs) were cultured on Matrigel. The effects of GCs on TLS formation were assessed by counting capillary connections (CCs) after incubation with cortisol (300–1200 nM), cortisone (300–1200 nM), or vehicle for up to 24 h; vascular endothelial growth factor (VEGF; 0.5–500 $ng \cdot mL^{-1}$) was used as a positive control. 11β-HSD1 expression and activity were assessed using RT-PCR and by measuring $^{3}H_{4}$-cortisol generation, respectively. Cortisol induced a concentration-dependent reduction in CCs, which was maximal ($44 \pm 7\%$, $p < .01$) after 22 h in culture. In contrast, the inert metabolite cortisone had no effect on TLS formation. VEGF stimulated a concentration-dependent increase in CCs with a twofold increase ($218 \pm 6\%$) evident after 5 h. 11β-HSD1 expression was not detected, and $^{3}H_{4}$-cortisone was not converted into $^{3}H_{4}$-cortisol, either in quiescent HUVECs or during TLS development. These results indicate that 11β-HSD1 within the vascular wall regulates angiogenesis via a paracrine action of active GC formed in the smooth muscle rather than an autocrine action of steroid produced within the endothelium itself.

P8

EFFECTS OF VEGF165b ON VEGF165-INDUCED TYROSINE PHOSPHORYLATION OF VEGFR-2 IN ENDOTHELIAL CELLS

H. S. Bevan, E. S. Rennel, L. Sage, S. J. Harper, D. O. Bates
Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK

VEGFR-2 is the principal receptor that transduces VEGF-induced signals in endothelial cells. Here we determine the effects of $VEGF_{165}b$ on $VEGF_{165}$-induced VEGFR-2 tyrosine kinase phosphorylation in CHO cells overexpressing VEGFR-2 (CHOR-2) and endothelial cells, in vitro. CHOR-2, human umbilical vein (HUVEC) and dermal microvascular (HMVEC) endothelial cells were incubated with 1 nM $VEGF_{165}$, 1 nM $VEGF_{165}b$, both 1 nM $VEGF_{165}$ and 1 nM $VEGF_{165}b$, or control solution for 5 min at 37°C. Time course experiments were performed to measure VEGFR-2 phosphorylation over a 1-h period. Protein was extracted and subjected to western blot analysis. In CHOR-2 cells $VEGF_{165}$ alone phosphorylated VEGFR-2 at Tyr-996 and Tyre-1175 while $VEGF_{165}b$ did not. $VEGF_{165}$-induced phosphorylation of Tyr-996 and Tyr-1175, however, was inhibited by $VEGF_{165}b$. In HMVEC and HUVEC $VEGF_{165}$, alone or in combination with $VEGF_{165}b$, phosphorylated VEGFR-2 at Tyr-1175, while $VEGF_{165}b$ alone did not. However, we have previously shown a $VEGF_{165}b$-dependent phosphorylation of p44/p42 MAPK and Akt in HMVECs after 20 min stimulation. So we next examined the time course of VEGFR-2 phosphorylation in all cells lines. Maximal phosphorylation of tyr-1175 was observed after 2 min with $VEGF_{165}$, but this was delayed to 20 min upon treatment with $VEGF_{165}b$ in all cell lines. The data show that $VEGF_{165}b$ can phosphorylate VEGFR-2 in endothelial cells, but qualitatively (different tyrosine residues) and quantitatively (different kinetics) differently from $VEGF_{165}$.

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P9

TRANSFORMING GROWTH FACTOR-β (TGF-β) REGULATES THE BALANCE OF ISOFORMS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

D. G. Nowak,[1] J. Woolard,[1] A. M. Hamdollah-Zadeh,[1] M. J. Ladomery,[2] S. J. Harper,[1] D. O. Bates[1]
[1]Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK; [2]Centre for Research in Biomedicine, University of West England, Bristol, BS16 1QY, UK

VEGF pre-mRNA is differentially spliced in exon 8 to form the pro-angiogenic $VEGF_{xxx}$ isoforms by proximal splice site selection and the anti-angiogenic $VEGF_{xxx}b$ isoforms by distal splice site selection. The control of this alternative splicing is unknown. TGF-β has previously been shown to increase total VEGF expression, so to determine whether TGF-β could alter splicing of the VEGF isoforms, proliferating podocytes were treated with TGF-β (0, 0.01, 0.05, 0.1, 0.5, 1 nM) for 24 and 48 h. VEGF protein and conditioned medium were collected from cells after 24 and 48 h and measured by ELISA. VEGF isoforms were determined by western blotting. Incubation with TGF-β at 0.5 and 1 nM for 48 h significantly increased the level of $VEGF_{xxx}b$ protein (4.3 ± 0.2- and 7.9 ± 2.2-fold respectively, $p < .05$, $p < .001$ Dunnet's test) and $VEGF_{xxx}$ protein expression (1.5 ± 0.1- and 1.7 ± 0.1-fold, respectively, $p < .001$ Dunnet's test). The most abundant

isoform of $VEGF_{xxx}b$ family, determined by western blotting, was $VEGF_{165}b$. These results indicate that TGF-β stimulates a dose-dependent increase in $VEGF_{xxx}b$ and $VEGF_{xxx}$ protein expression in podocytes. However, upregulation of $VEGF_{xxx}b$ was greater than upregulation of $VEGF_{xxx}$. These data suggest that TGF-β regulates the balance between pro-angiogenic and anti-angiogenic isoforms of VEGF in podocytes, switching expression to the anti-angiogenic family—$VEGF_{xxx}b$.

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P10

PHARMACOKINETICS AND TISSUE DISTRIBUTION OF ANTI-ANGIOGENIC $VEGF_{165}b$

E. S. Rennel, S. J. Harper, D. O. Bates

Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK

$VEGF_{165}b$, a splice variant of vascular endothelial growth factor (VEGF) inhibits $VEGF_{165}$-mediated angiogenesis and reduces tumor growth (Woolard et al. 2004 *Cancer Res*; 64: 7822–7835). Expression of $VEGF_{165}b$ is downregulated in cancer (Bates et al. 2002 *Cancer Res*; 62: 4123–4131). To investigate the potential of $VEGF_{165}b$ as an anti-angiogenic treatment in cancer, the pharmacokinetics of recombinant protein were studied. Mice were injected with 5 μg $VEGF_{165}b$ either subcutaneously (SC) or intraperitoneal (IP) and blood was collected at different timepoints after injection (1 h up to 24 h, $n = 3$ per time point). Circulating plasma levels of VEGF protein was determined by enzyme-linked immunosorbent assay (ELISA) and rate of clearance and half-lives were calculated. $VEGF_{165}b$ had a relatively short half-life (SO; $k = 2.23 \pm 6.88$ $t_{1/2} = 18.6$ min; IP; $k = 1.11 \pm 0.289$ $t_{1/2} = 37.6$ min) and levels were below detection limit within 8 h. Repeated SC injections every 24 h did not lead to an accumulation of circulating protein. IP injections resulted in accumulation in liver after 24 h by western blotting. Ongoing experiments with repeated IP injections into nude mice bearing xenotransplanted tumors did not lead to accumulation of the injected protein in the tumor but resulted in a small, bit significant increase in tumor size in treated mice compared to mock injected (10 μg vs. PBS, $p < .01$, at day 12, two-way ANOVA Bonferroni post hoc test). $VEGF_{165}b$ has fast clearance, and is not suitable by daily IP or SC injection for tumor treatment.

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P11

TISSUE OXYGEN SATURATION MAY PREDICT HEALING OF SURGICAL SITE WOUNDS

C. L. Ives, D. K. Harrison, G. Stansby

Department of Medical Physics, University Hospital of North Durham, Durham, DH1 5TW, UK

In 2004 we reported results of relationships between oxygen saturation of skin (SSO_2) and muscle (MSO_2) (measured by visible lightguide and near-infrared spectrophotometry, respectively) and physiological characteristics. We now present results of a trial measuring tissue oxygen saturation (StO_2), using the same methods, to predict surgical site infections (SSI). StO_2 was measured at the wound preoperatively and at 12, 24, and 48 h postoperatively in patients undergoing major surgery. Age, body mass index (BMI), and triceps skinfold thickness (TST) were recorded. Signs of infection were assessed for independently at 1 month using the Control of Diseases Centre definition: those who healed were classified into group A and those who developed a wound infection into group B. Of 59 patients (38 M, 21 F), 42 healed and 17 developed SSI. There were no significant differences in sex, age, TST, or BMI between the two groups. Wound SSO_2 was not significantly different between the groups at any stage. At 12 h postoperatively there was a significant difference in wound MSO_2 between the two groups ($A = 58.3 \pm 21.6\%$, $B = 42.2 \pm 16.6\%$, $p = .005$). Chi-squared tables for differing values of wound MSO_2 showed that the test performs best when a value of 53% was chosen as the threshold to classify potential infection (chi-squared test, $p = .002$). This correlated to a sensitivity of 70.6% and a specificity of 76.2%. The use of the near-infrared spectrophotometer as a tool to predict wound infections should be further evaluated and advocated.

P12

DIFFERENTIAL REGULATION OF PRO- AND ANTI-ANGIOGENIC VEGF ISOFORMS BY IGF-1, TNF-α, AND HYPOXIA

O. Konopatskaya,[1] S. J. Harper,[1] A. Churchill,[2] D. O. Bates[1]

[1]Microvascular Research Laboratories, Department of Physiology, and [2]Bristol Eye Hospital, University of Bristol, Bristol BS2 8EJ, UK

Vascular endothelial growth factor-A isoforms ($VEGF_{xxx}$) are key stimulators of pathological retinal neovascularization. VEGF is alternatively spliced to form a family of sister isoforms, $VEGF_{xxx}b$, which are expressed in normal human eye tissues, but downregulated in diabetic vitreous (Perrin et al. 2005 *Diabetol*; 48:2422–2427) and are anti-angiogenic in vivo. We set out to determine how retinal neovascular disease-associated factors, namely IGF-1, TNF-α, and hypoxia, modulate the relative expression of the two families of VEGF isoforms in human retinal pigmented epithelial (RPE) cells. A 24-h incubation of RPE with various concentrations of IGF-1 showed a pronounced dose-dependent increase in the secreted form of $VEGF_{xxx}$, peaking at 1 μM (7.87 ± 2.15-fold over control, $p < .05$, Friedman test), as determined by ELISA. No significant changes in $VEGF_{xxx}b$ levels were seen at any IGF-1 concentrations studied. Similarly, TNF-α produced a 4.13 ± 1.5-fold ($p < .05$) upregulation in $VEGF_{xxx}$ in cell conditioned medium at as low as 0.2 ng/mL, sustainable up to 100 ng/mL, but did not affect $VEGF_{xxx}b$. Finally, while a 24 h exposure of RPE to hypoxia resulted in 4.45 ± 1.73-fold change in $VEGF_{xxx}$, $VEGF_{xxx}b$ was altered only $1.25 \pm .09$-fold ($p < .05$). None of the stimuli studied showed any changes in the intracellular content of either VEGF

isoforms, according to western blotting. In conclusion, the secreted inhibitory $VEGF_{xxx}b$ isoforms are regulated differently to angiogenic isoforms by factors known to be involved in angiogenic eye disease.

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P13

EGF RECEPTOR MEDIATES EGF AND TGF-α MOTOGENIC ACTIVITIES VIA DISTINCT MATRIX-DEPENDENT MECHANISMS

I. R. Ellis, A. M. Schor, S. L. Schor
Unit of Cell and Molecular Biology, Dental School, University of Dundee, Dundee DD1 4HR, UK

The angiogenic factors EGF and TGF-α induce an identical stimulation of cell migration and proliferation in vitro. In spite of their homologous structure and binding to the same receptor (EGFR), we report that their apparently identical motogenic bioactivities on fibroblasts are mediated by distinct matrix-dependent mechanisms. Our data indicate that EGF and TGF-α motogenicity may be resolved into two stages: (a) cell "activation" by a transient exposure to either cytokine, and (b) the subsequent "manifestiation" of elevated migration in the absence of further cytokine. Motogenic activation by EGF requires the concomitant functionality of EGFR and the hyaluronon receptor CD44, whereas activation by TGF-α required EGFR but does not require the above-mentioned matrix receptors, as well as their respective ligands, i.e., hyaluronon (in the case of cell activation by EGF) and vitronection (in the case of activation by TGF-α). In contrast, the mitogenic activites of EGF and TGF-α and independent of CD44 and avb3 functionality. These results highlight the role of the extracellular matrix in modulating the activities of soluble angiogenic factors.

P14

A KERATINOCYTE-PRODUCED INHIBITOR IS DIFFERENTIALLY ACTIVE AGAINST TWO ISOFORMS OF MIGRATION STIMULATION FACTOR (MSF)

S. L. Jones, M. M. Florence, I. R. Ellis, R. A. Kay, J. Cox, K. Kankiva, S. L. Schor, A. M. Schor
Unit of Cell and Molecular Biology, Dental School, University of Dundee, Dundee DD1 4HR, UK

Migration Stimulating Factor (MSF) is a truncated (70kDa) isoform of fibronectin containing a unique intron-derived 10 amino acid sequence not present in any previously described full-length fibronectin isoform. MSF exhibits a number of bioactivities of relevance to cancer development progression, including stimulation of cell migration and angiogenesis in vivo. These bioactivities are mediated by IGD (ile-gly-asp) amino acid motifs located in modules I 7 and I 9. A second isoform of MSF has been cloned from a transformed foetal fibroblast library. This new isoform differs from its originally reported counterpart solely in terms of a 15 amino acid deletion in module II 1 and is hence designated MSF-aa: for clarity, the originally reported isoform will be referred to as MSF+aa. MSF+aa and MSF-aa contain the same bioactive IGD motifs and exhibit identically potent bioactivities which are neutralised by anti-IGD motif antibodies. Normal keratinocytes in culture secrete MSFI, a potent inhibitor of MSF+aa, but not MSF-aa. Various carcinoma cell lines secrete MSF activity which is either not inhibited or partly inhibited by MSFI. Anti-IGD antibodies completely neutralised MSF activity in all cases.

P15

ADVANCED GLYCATION OF THE EXTRACELLULAR MATRIX INHIBITS ATTACHMENT, SPREADING, AND MIGRATION OF BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELLS

A. D. Bhatwadekar,[1] T. A. Gardiner,[1] T. M. Curtis,[1] G. Li,[2] A. W. Stitt[1]
[1]Centre for Vision Science, and [2]Orthopaedics, Queen's University, Belfast, Northern Ireland

Microvascular repair by marrow-derived endothelial progenitor cells (EPCs) is impaired in diabetes. EPC attachment to fibronectin (FN) is mediated through Arg-Gly-Asp (RGD) and we hypothesized that FN modification by AGE adduct formation, especially on key Arg residues, could disrupt appropriate EPC interaction with the extracellular matrix. EPCs were isolated from human peripheral blood and characterized by acLDL-DiI, UEA-1, CD31, CD34, and VEGFR2 staining. FN-coated cell culture flasks were treated with methylglyoxal (AGE-FN) and adduct formation was quantified. EPC attachment to native or AGE-FN was quantified at 3 h and spreading at 6 h. EPC migration was assessed using a Dunn's chamber chemotaxis assay with stromal cell derived factor-1 (SDF-1) (50 ng/mL) as chemoattractant. Modulation of EPC attachment and spreading was studied by supplementing AGE-FN with a synthetic RGD tripeptide (1 mM) in an attempt to replenish modified recognition motifs. EPC attachment and spreading on AGE-FN were reduced to 25% ($p < .05$) and 32% ($p < .01$) of control values, respectively, but significantly restored by pretreatment of AGE-FN with the RGD tripeptide. EPCs on native FN showed strong chemoattraction for SDF-1, a response that was abolished by attachment to AGE-FN. We conclude that matrix modification by AGEs decreases EPC adhesion, spreading and directed migration, possibly by modification of RGD sequences. AGE-adduct formation on vascular basement membranes may inhibit EPC attachment and participation in microvascular repair during diabetes.

P16

ADVANCED GLYCATION MEDIATES BLOOD–RETINAL BARRIER DYSFUNCTION IN DIABETES

T. A. Gardiner, P. Canning, A. W. Stitt
Centre for Vision Science, Queen's University, Belfast, Northern Ireland

Inner blood–retinal barrier (iBRB) breakdown occurs early in diabetes and may progress to sight-threatening macular edema. We examined the impact of advanced glycation endproducts (AGEs) on vasopermeability-related variables in the diabetic retina, including tight junction (TJ) integrity and VEGF/VEGF-R2 expression; also, the role of the AGE-receptor galectin-3 (G3) in AGE-mediated iBRB dysfunction. Diabetes was induced in C57Bl6 wild-type (WT) and $G3^{-/-}$ mice with streptozotocin. Blood glucose, HbA1c+ serum/tissue-AGE levels were quantified. One group was treated with the AGE inhibitor pyridoxamine (PM). After 2 weeks diabetes, iBRB integrity was assessed with the Evans blue assay and immuno-staining for occludin-1. HbA1c and the AGE adduct carboxy-methyl-lysine (CML) were significantly increased in all diabetic groups but only CML was lower in the PM-treated groups. WT diabetic mice showed greater iBRB breakdown ($p < .03$) and higher VEGF expression than controls $p < .05$ and $p < .01$. PM-treated diabetics showed normal iBRB parameters and reduced VEGF expression. Diabetic retinal vessels showed disrupted TJ integrity compared to controls while TJs in PM-treated diabetics appeared normal. G3−/− mice showed less diabetes-mediated iBRB dysfunction than WT mice. AGEs mediate iBRB dysfunction in diabetic retina, which correlates with increased VEGF/VEGF-R2 expression. Inhibition of AGE formation with pyridoxamine or genetic deletion of the AGE-receptor galectin-3 can prevent diabetes-induced breakdown of the blood–retinal barrier.

P17

EFFECTS OF GESTATIONAL DIABETES (GDM) ON VASCULAR INTEGRITY OF THE HUMAN PLACENTA: THE ROLE OF $VEGF_{165b}$

L. Leach, K. S. Reeve, M.A. Garrioch, D.O. Bates[1]
School of Biomedical Sciences, University of Nottingham, [1]Microvascular Research Laboratories, University of Bristol, Bristol BS2 8EJ, UK

Increased angiogenesis is not a pathological feature of GDM placenta but is in type 1 diabetic (T1D) placenta, possibly mediated by VEGF. The VEGF splice variant, $VEGF_{165}$ b, is anti-angiogenic and putatively pro-permeability. Vascular integrity and relative localization of $VEGF_{165}$b were therefore assessed in normal term placentae ($n = 4$) and those complicated by type 1 ($n = 4$) and GDM ($n = 4$). Leakage of fetoplacental vessels to TRITC-dextran (76 kDa) was assessed by ex vivo perfusion, and placentae subjected to immunohistochemistry, using antibodies against $VEGF_{165}$b and the angiogenic marker, phospho-p44/42 MAPK. Microscopic random sampling determined the percentage of vessels associated with tracer leakage, pMAPK and $VEGF_{165}$b expression. In GDM significantly more blood vessels were leaky to dextran compared to normal placenta ($p < .05$; Mann-Whitney U). $VEGF_{165}$b was localized throughout normal and GDM placentae in endothelial, perivascular cells, and syncytiotrophoblasts but minimally expressed in T1D placentae ($p < .05$; Kruskall-Wallis). Only the latter showed a significant increase in pMAPK positive endothelial nuclei. There was a negative correlation between weight of placenta and extent of $VEGF_{165}$b localization in GDM ($r = 1$; $p > .001$ Spearman's). Increased vascular leakage is a feature of placentae from pregnancies complicated by GDM. No change in $VEGF_{165}$b in GDM compared with normal placenta contradicts a putative pro-permeability role within the placenta. Its prevalence in the GDM and normal but absence from T1D placenta suggests an anti-angiogenic role.

P18

INSULIN-INDUCED PHOSPHOLIPASE D ACTIVATION IN HUMAN ENDOTHELIAL CELLS

S. McHarg, A. C. Shore, J. L. Whatmore
Institute of Biomedical and Clinical Science, Peninsula Medical School, Exeter, UK

Phospholipase D (PLD) is an intracellular signaling enzyme, stimulated by numerous agonists to produce the second messenger phosphatidic acid. PLD is involved in pathophysiological signaling pathways, e.g., the progression of vascular disease. The altered reactivity of the vasculature to insulin may contribute toward the development of vascular pathologies in type II diabetes. It has been previously shown that insulin activates PLD in skeletal muscle cells, but there is no evidence of such activation in the endothelium. The aim of this project is to establish if PLD is activated by physiological doses of insulin in both human macrovascular and microvascular endothelial cells. [^{3}H] Myristic acid-labeled human endothelial cells were treated with varying doses of insulin (100 pM–1 μM) for 20 min in the presence of 0.5% ethanol. To assess the role of hyperinsulinemia, cells were preincubated with 5 nM insulin for 6 h prior to the PLD assay. PLD activity was assessed by quantitation of [^{3}H]phosphatidylethanol. 1 nM insulin significantly activated PLD in both microvascular and macrovascular endothelial cells, causing a mean increase of $203 \pm 60\%$ ($n = 3$, $p < .037$) and $183\% \pm 60\%$ ($n = 9$, $p < .003$) respectively as compared to control values of 100%. Preincubation with 5 nM insulin increased basal PLD activation, although it completely abolished subsequent activation by 1 nM insulin. This shows for the first time that insulin activates PLD in the endothelium. Hyperinsulinemia appears to increase basal PLD activation but reduces cell responsiveness to physiological insulin levels.

P19

ANGIOPOIETINS AND TIE-2 RECEPTOR EXPRESSION IN BREAST CANCER IN VITRO

K. O. Bramford-Hale, C. A. Staton, M. W. R. Reed, N. J. Brown
Microcirculation Research Group, Academic Surgical Oncology Unit, University of Sheffield, Sheffield S10 2JF, UK

Angiopoietin-1, angiopoietin-2, and angiopoietin-4 (Ang-1, 2, and 4) mediate endothelial cell responses through binding to their endothelial cell-specific receptor, Tie-2. Binding of Ang-1 to Tie-2 enhances endothelial cell stability and promotes a leakage-resistant vasculature (Cascone et al. 2005

J Cell Biol, 170, 993–1004). Conversely, Ang-2 activation of Tie-2 leads to endothelial cell instability and promotes vascular leakage (Roviezzo et al. 2005 *J Pharmacol Exp Ther;* 314, 738–744). Both are important in tumor angiogenesis, but the role of Ang-4 has yet to be determined. The aim of the study, therefore, is to determine the basal expression of the angiopoietins and Tie-2 expression in breast cancer cell lines and primary endothelial cells. Western blot analysis of MDA-MB-436, MCF7, and T47D breast cancer cell lines indicate decreased Ang-1 expression when compared to control human umbilical vein endothelial cells (HUVECS). Ang-2 expression is elevated, particularly in the highly metastatic MDA-MB-436 cell line. Ang-4 levels are low for breast cancer cell lines and endothelial cell controls. Flow cytometric analysis of cell surface angiopoietin expression confirms these data. However, an increase of Tie-2 receptor expression is observed in HUVECs (3-fold; $p < .001$) and HuDMECS (2-fold; $p < .05$) when compared to the breast cancer cell lines. These data support the hypothesis that an altered balance between Ang-1 and Ang-2 promotes angiogenesis, but further studies are required to determine the role of Ang-4.

P20

TUMOR NITRIC OXIDE (NO) PRODUCTION FOLLOWING AMINOLAEVULINIC ACID-PHOTODYNAMIC THERAPY (ALA-PDT) IN VIVO

K. J. Reeves, M. W. R. Reed, N. J. Brown
Microcirculation Research Group, Academic Surgical Oncology Unit, University of Sheffield, Sheffield S10 2JF, UK

PDT is a cancer treatment in which cell death occurs as a result of the interaction of light with a photosensitizing drug (e.g., ALA). PDT induces oxidative stress triggering a vascular-mediated response with neutrophil recruitment; all events sensitive to NO. This study aims to assess NO production and nitric oxide synthase (NOS) activity in murine tumors before and after ALA-PDT. Murine tumors expressing different NO levels, EMT6 (low) and RIF1 (high), were injected into the cremaster muscle of Balb/c or C3H mice ($n = 7$/group) respectively, anesthetized with hypnorm: diazemuls: water (1:1:2). Experimental groups: control, L-NAME, ALA-PDT, and L-NAME + ALA-PDT. Tumors were excised into low-nitrate/nitrite media and incubated at 37°C. Samples were analyzed at 6 and 24 h using a NO detection kit (NO levels nmol g^{-1}). At 24 h ex vivo RIF1 tumors produced more NO compared to EMT6 tumors (20.4 ± 1.1 vs. 12.9 ± 1.2) and L-NAME decreased these levels (RIF1: 5.6 ± 3.6 vs. EMT6: 3.9 ± 2.0). ALA-PDT increased NO levels (RIF1: 37.9 ± 0.7 vs. EMT6: 22.6 ± 1.2) and L-NAME + ALA-PDT decreased NO levels (RIF1: 7.1 ± 1.3 vs. EMT6: 3.6 ± 2.1). ALA-PDT enhances endogenous NOS activity in both RIF1 and EMT6 tumors, but decreases NO production following L-NAME + ALA-PDT, compared to relevant controls. The RIF1 tumor response is more pronounced. Administration of NOS inhibitors in combination with ALA-PDT may enhance the response and efficacy observed in vivo.

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P21

VASCULAR ENDOTHELIAL GROWTH FACTOR ISOFORM EXPRESSION IN COLORECTAL CANCER

S. Houlder, J. Uddin,[1] P. Quirke,[2] D. Burke[1]
Departments of [1]Academic Surgery and [2]Pathology, University of Leeds, Leeds LS1 3EX, UK

Tumor growth is dependent on the ability of the tumor to induce angiogenesis and neovascularization. Angiogenesis in mediated by vascular endothelial growth factor (VEGF). This observational study aims to compare the expression and distribution of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR-1, and VEGFR-2 in Dukes' stage B colorectal cancer and normal colonic mucosa. Samples of neoplastic tissue and normal colonic mucosa were obtained from 635 patients with Dukes' stage B colorectal cancer involved in a national chemotherapy trial. Participants were selected from 134 centers between May 1994 and October 1997 and had a median age of 62 (range 15–86) years. VEGF isoform and receptor expression was detected using immunohistochemistry. Staining intensity was semi-quantitatively assessed. Statistical analysis employed the χ^2 test. High-intensity staining was present in a greater percentage of neoplastic tissue compared to normal tissue for VEGF-A (75.3 vs. 29.7%, $p < .001$), VEGF-B (73.8 vs. 58.5%, $p < .001$), VEGF-C (68.6 vs. 36.7%, $p < .001$), VEGF-D (94.8 vs. 84.0%, $p < .001$), VEGFR-1 (72.1 vs. 61.4%, $p = .004$), and VEGFR-2 (78.9 vs. 73.3%, $p = .651$). VEGF-A, VEGF-D, and VEGFR-2 were the most heavily expressed isoforms and receptors in neoplastic tissue. The expression of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR-1, and VEGFR-2 is increased in Dukes' B colorectal cancer. It is postulated that the hypoxic environment of neoplastic tissue induces upregulation of these isoforms, promoting angiogenesis and tumor growth.

P22

TUMOR NECROSIS FACTOR-RELATED APOPTOSIS INDUCING LIGAND (TRAIL) INCREASES FUNCTIONAL EXPRESSION OF INTERCELLULAR ADHESION MOLECULE (ICAM)-1 IN EA.HY926

P. Chen, A. S. Easton
Department of Pathology, Dalhousie University, 11J1 Sir Charles Tupper Medical Building, Halifax, Nova Scotia, B3H 1X5, Canada

This study explores vascular endothelial cell (EC) activation by the tumor necrosis factor-related proteins TRAIL and Fas ligand (FasL). Human blood-derived neutrophils were calcein loaded (calcein-AM, 10 μM, 60 min), then added within 8 h of isolation to 96 well plates (5×10^6/mL, 30 min) coated with human umbilical vein endothelial cells (HUVEC, passage 3) or the HUVEC-derived EC line EA.hy926. Adhesion was assessed from calcein fluorescence. In EA.hy926, wells pre-treated with TRAIL (100 ng/mL, 24 h) showed increased neutrophil adhesion (153.7 ± 4.0% control, mean $\pm$ SEM, $n = 16$, $p < .001$ vs. control) while FasL (10 ng/ml, 24 h) had no effect. TNF (100 U/mL, 24 h) also increased

adhesion (287.4 ± 12.5%, $n = 16$, $p < .001$). Identical exposures to TRAIL and FasL did not increase neutrophil adhesion to HUVEC, while TNF did (837.2 ± 53.6%, $n = 8$, $p < .001$). Cells were assessed for surface expression of ICAM-1 by flow cytometry following these treatments. TRAIL increased ICAM-1 expression in both HUVEC and EA.hy926, but in a higher percentage of cells in EA.hy926, while FasL had no effect. TNF increased ICAM-1 in both cells. These data suggest EC activation by TRAIL but not Fas ligand. TRAIL induces the expression of ICAM-1 in both EC models, but a functional effect in terms of increased neutrophil adhesion was found in EA.hy926 only.

Supported by the Heart and Stroke Foundation of Canada, and the Nova Scotia Health Research Foundation.

P23

PROTEOMIC ANALYSIS OF INTERSTITIAL FLUID SAMPLED USING MICRODIALYSIS

C. A.Gill,[1] P. J. Skipp,[2] C. D. O'Connor,[2] G. F.Clough[1]
[1]Infection Inflammation and Repair, School of Medicine, and [2]Centre for Proteomic Research, University of Southampton, Southampton, UK

Analysis of the composition of interstitial fluid can provide important markers of tissue homeostasis. The aim of this study is to develop a method by which to identify key proteins in microdialysate recovered from human skin in vivo. Microdialysis probes were constructed using a 3000-kDa molecular mass cutoff polyethylene membrane, dialysis length 20 mm. The probes were placed within the dermis of the volar surface of the forearm of healthy human volunteers ($n = 4$), perfused with Ringer's solution at a rate of 3 μL/min and the dialysate collected for up to 120 min. The performance of the probe was also investigated ex vivo ($n = 6$) using human serum albumin (HSA 10 mg/mL). The dialysis efficiency (E_d) for HSA was 15%. The total protein in the dialysate measured using a BCA protein assay was 420 ± 40 μg/mL. Pooled dialysate samples were depleted of the highly abundant proteins albumin, IgG, antitrypsin, IgA, transferring, and haptoglobin using high-performance chromatography (Agilent Technologies) and the components of the enriched (∼5 mg/mL) low abundance fraction identified using the "shotgun" proteomic analysis technique GeLC-MS/MS. To date, up to 40 protein components have been identified. We conclude that these technologies provide a novel and potentially informative way in which to explore the proteome of the interstitium and to investigate temporal and spatial changes that occur during the inflammatory response in vivo.

CAG is supported by a PhD Studentship from the Gerald Kerkut Charitable Trust.

P24

NITRIC OXIDE RELEASE FROM NO DONORS IS ENHANCED BY STRETCH INJURY TO THE PIG CORONARY ARTERY

S. Kennedy, S. G. Burke
Department of Physiology & Pharmacology, University of Strathclyde, Glasgow G4 0NR, UK

Nitrates and NO donor drugs are widely used in patients with angina and during coronary balloon angioplasty and stent implantation. In a previous study we demonstrated that in vitro balloon inflation enhanced relaxation to the NO/superoxide donor SIN-1 (Kennedy et al. 2003 *Eur J Pharmacol*; 481: 101–107). In this study we have investigated the mechanism underlying this effect. Pig coronary arteries were obtained from an abbatoir and stretch injured with a 3.5-mm balloon catheter. Arteries were mounted in a myograph to measure contraction and relaxation and an amperometric sensor was placed in the vessel lumen to measure NO release. To detect superoxide formation, injured arteries were snap frozen, incubated with dihydroethidium (DHE) and examined using fluorescence microscopy. Balloon injury increased sensitivity to two separate NO donors; SNAP and spermineNONOate. This increased sensitivity following injury was specific to NO donors and not shared by other vasorelaxant agents such as chromokalim and isoprenaline. Nitric oxide release following addition of either SNAP or NONOate was hugely enhanced in contact with injury arteries. Staining with DHE revealed that stretch injury significantly increased superoxide generation within the wall of the vessel. Addition of authentic peroxynitrite was undetected by the sensor but did result in significant vasodilatation. In conclusion, stretch injury enhances NO generation by structurally dissimilar NO donor drugs. The enhanced relaxation response may be due to increased NO generation or by peroxynitrite formation in the organ bath.

P25

DEXAMETHASONE ALTERS THE COMPOSITION OF NEOINTIMAL LESIONS IN MOUSE FEMORAL ARTERY

L. J. Macdonald, B. R. Walker, P. W. F. Hadoke
Centre for Cardiovascular Science, Queen's Medical Research Institute, 47 Little France Crescent, University of Edinburgh, Edinburgh EH16 4TJ, UK

Glucocorticoids, which can directly modulate endothelial cell function and regulate inflammation and proliferation within the arterial wall, inhibit neointimal proliferation in animal models. In clinical trials, however, glucocorticoid administration does not consistently prevent restenosis following intra-luminal injury. We have assessed the ability of dexamethasone to inhibit lesion formation in a murine model of arterial injury/remodeling. A wire was inserted into the femoral artery of male C57Bl6 mice under halothane-induced anesthesia, and arterial structure assessed after 0, 2, 7, 14, 21, and 28 days ($n = 5$–6). Two additional groups ($n = 8$) received dexamethasone (1 mg/kg/day) or vehicle for 21 days following injury. Histology confirmed that injury induced development of fibro-proliferative lesions, first evident at 7 days and peaking in size after 21 days. Lesion development was accompanied by luminal narrowing and medial atrophy. Immunohistochemistry indicated that lesions were smooth muscle rich, with macrophages and fibrinogen also

present. Dexamethasone reduced body weight and the weight of glucocorticoid-sensitive organs but did not reduce lesion size. However, lesions in dexamethasone-treated mice were morphologically distinct from controls, being largely acellular and staining poorly for elastin and smooth muscle but strongly for fibrinogen. These results suggest a thrombotic origin for lesions in dexamethasone-treated mice and may provide insights into the failure of glucocorticoids to prevent remodeling in clinical trials.

P26

THE EXPRESSION OF LARGE CONDUCTANCE CALCIUM-ACTIVATED POTASSIUM CHANNELS (BK_{Ca}) IN RODENT ISOLATED RETINAL MICROVESSELS

I. C. M. Rowe, S. H.-F. MacDonald, J. R. W. Menzies, M. J. Shipston, S. F. Cruickshank,[1] R. Knott.[1]

Centre for Integrative Physiology, College of Medicine & Veterinary Medicine, Hugh Robson Building, University of Edinburgh, Edinburgh, EH8 9XD, Scotland, UK; [1]The School of Pharmacy, Robert Gordon University, Schoolhill, Aberdeen AB10 1FR, Scotland, UK

Large-conductance calcium-activated potassium channels (BK_{Ca}) play an important role in the regulation of vascular tone and are present in both vascular smooth muscle (VSMC) and endothelial cells (Ledoux et al. 2006 *Physiology* 21: 69–78). BK_{Ca} channel pore forming α-subunits are the product of a single gene (*slo* or *KCNMA1*) that are assembled as tetramers to provide functional channels. Extensive splicing of the gene provides a significant mechanism for structural variation and functional diversity. The expression of BK_{Ca} channel α-subunit splice variants was investigated in retinal cDNA produced from both adult and neonatal rodents using RT-PCR and probed with primers designed to reveal splice variation at intracellular splice sites 1–3. ZERO and STREX variants from splice site 2 were observed in adult mouse, adult rat and neonatal rat (postnatal days 5–7). BK_{Ca} channel protein was observed in pericyte, endothelial, and smooth muscle cell types in isolated microvessel networks using fluorescence immunostaining with confocal microscopy. The presence of BK_{Ca} channels and their splice variant expression within retinal microvessels suggest that these channels may have a significant role in microvascular regulation.

Supported by the RGU RDI fund.

P27

THE ROLE OF NITRC OXIDE AND ENDOTHELIAL FUNCTION IN VEIN GRAFT FAILURE

J. Wu, S. Kennedy, R. M. Wadsworth

Department of Physiology & Pharmacology, University of Strathclyde, Glasgow G4 0NY, UK

Vein grafting is the final solution for occlusive vascular diseases. However, grafts have a substantial incidence of subsequent occlusion. Intimal hyperplasia and accelerated atherosclerosis are major causes of vein graft failure. The inferior vena cava from the donor mouse was implanted into the right carotid artery of the recipient mouse, which was anethetized by sodium pentobarbital (5 mg/20 g body weight). Vein grafts were harvested at 1, 3, 7, 14, and 28 days after operation, and were fixed by 4% formalin for histology (hematoxylin & eosin/H&E) and immunocytochemistry (endothelial nitric oxide synthase/eNOS). Other grafts were freshly harvested at 28 days for study of vascular reactivity in a myograph. Significant neointima formation was observed 1 week after grafting, with further growth and maturation up to 28 days. Endothelium was found to be absent up to 1 week after grafting. Endothelial cells subsequently regrew to reach confluence at 14–28 days, staining strongly for eNOS. The grafts had substantial contraction and endothelial-dependent relaxation at 28 days, although much smaller than untreated carotid artery. However, the relaxations induced by a nitric oxide donor were similar in vein graft and artery. Neointima was quickly formed in the vein graft after operation, which was functional for both contraction and relaxation at 4 weeks. The regrown endothelium expressed functional eNOS. However, the bioavailability of NO was impaired.

P28

KV1.5 IS A MAJOR COMPONENT UNDERLYING THE A-TYPE POTASSIUM CURRENT IN MICROVASCULAR SMOOTH MUSCLE

M. K. McGahon, J. M. Dawicki, D. A. Simpson, T. A. Gardiner, A. W. Stitt, C. N. Scholfield, J. G. McGeown, T. M. Curtis

Centre of Vision Sciences & Vascular Biology, Queen's University of Belfast, Belfast, Northern Ireland

Little is known about the molecular characteristics of the K_v channels that underlie the Atype K^+ current in microvascular smooth muscle (MVSM). We investigated the molecular identity of the A-type K^+ current in retinal MVSM cells using patch clamp techniques, RT-PCR, immunohistochemistry, and neutralizing antibody studies. The A-type K^+ current was resistant to the actions of specific inhibitors for K_v3 and K_v4 channels, but was blocked by the K_v1 antagonist correolide. No effects were observed with pharamacological agents directed against $K_v1.1/2/3/6$ and 7 channels, but the current was blocked by riluzole, a $K_v1.4$ and $K_v1.5$ inhibitor. The current was not altered by the removal of extracellular K^+ but was abolished by flecainide, suggesting involvement of $K_v1.5$- rather than $K_v1.4$ channels. Transcripts encoding $K_v1.5$ and not $K_v1.4$ were identified in freshly isolated retinal arterioles. Immunofluorescence labeling confirmed the absence of $K_v1.4$ expression and revealed $K_v1.5$ localized in the plasma membrane of the MVSM cells. Anti-$K_v1.5$ antibody applied intracellularly inhibited the A-type K^+ current, while anti-$K_v1.4$ antibody had no effect. Coexpression of $K_v1.5$ with $K_v\beta1$ or $K_v\beta3$ accessory subunits is known to transform $K_v1.5$ currents from delayed rectifers into A-type currents. $K_v\beta1$ mRNA expression was detected in retinal arterioles, but $K_v\beta3$ was not observed. This study suggests that $K_v1.5$, most likely co-assembled with $K_v\beta1$ subunits, is the major molecular

component underlying the A-type K^+ current in retinal MVSM cells.

P29

GLOMERULI RECONSTRUCTED AFTER FIXATION WITH PHYSIOLOGICAL PRESSURES SHOW RESTRICTIVE URODYNAMIC ROUTES TO THE PROXIMAL CONVOLUTED TUBULE (PCT)

C. R. Neal, P. R. Muston, D. O. Bates, S. J. Harper
Microvascular Research Labs, Bristol University, Bristol BS2 8EJ, UK

We recently described three interconnected urinary spaces in and around the renal glomerulus (Neal et al. 2005 *JASN;* 16:1223). Glomerular filtrate may enter restrictive spaces under the podocyte (subpodocyte spaces, SPS) before passing to glomerular drainage channels (interpodocyte spaces, IPS), the peripheral urinary space (PUS) and the PCT. The previous work used immersion (I) or colloid free perfusion (P) fixation, we have repeated the reconstructions after fixation at physiological pressures (CP). Rat kidneys were perfused with Ringer then glutaraldehyde solutions ($n = 6$, aortic hydrostatic pressure 100 mmHg; oncotic pressure 25 mmHg). Kidney pieces were dehydrated and embedded, and micrographs of serial ultrathin sections of glomeruli were used to reconstruct glomerular regions. The SPS, IPS, and PUS were compared with other data (Neal et al. 2005 *JASN;* 16:1223; mean ± SEMs unpaired t tests unless stated). The SPS height showed CP fixed podocytes more closely opposed to the GFB (i.e., CP, mode 0.3 μm; I, modes 0.5 and 0.9 μm, $p < .001$, Fisher's exact test), IPS narrower (CP, $0.59 \pm 0.05\,\mu$m, $n = 6$; I, $0.74 \pm 0.06\,\mu$m, $n = 29$; $p < .05$), and PUS narrower (P, $8.5 \pm 3.4\,\mu$m; CP, $2.1 \pm 0.35\,\mu$m, $p < .01$). Calculated resistance ratios showed that the exit pores from the SPS had 10-fold greater resistance than the GBM area covered by the SPS. When glomeruli are fixed under in vivo pressure conditions the three urinary spaces are minimised. The urodynamic pathways are more restrictive than described (Neal et al. 2005 *JASN* 16:1223) and incompatible with a low-resistance pathway to the PCT.

P30

GENERATION OF TRANSGENIC MICE OVEREXPRESSING $VEGF_{165}$b IN PODOCYTES

Y. Qiu, D. Murphy, S. J. Harper, D. O. Bates
Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK

Vascular endothelial growth factor (VEGF) is a major regulator of blood vessel biology and is highly expressed in presumptive and mature podocytes within the glomerulus. It has been recognized that dysregulation of this factor occurs in a number of glomerular diseases. Exon splicing of the VEGF pre-mRNA results in different species. Of them, $VEGF_{165}$b, downregulated in renal tumors, inhibits $VEGF_{165}$-mediated proliferation, migration of endothelial cells, vasodilatation of mesenteric arteries, and tumor growth in nude mice. We have described mRNA and protein expression of this inhibitory splice variant of VEGF in normal kidney (Bates et al. 2002 *Cancer Res*; 62: 4123–4131). To identify the biological function of $VEGF_{165}$b in kidney, we have produced transgenic mice specifically overexpressing $VEGF_{165}$b in podocytes. A plasmid composed of murine Nephrin promoter and $VEGF_{165}$b cDNA was generated, purified, and microinjected into fertilized one-cell stage embryos that were transplanted into pseudopregnant mice the next day. Genomic DNA extracted from tail biopsies of pups were screened for the expression of transgene via PCR and Southern blotting. Three lines of mice were generated that express the $VEGF_{165}$b transgene, identified by both PCR and Southern blotting. The mice were viable, and offspring of the F_0 generation were generated in a Mendelian ratio. The transgene expression levels vary, and are present at at least 2 copies in each line. In summary, we successfully produced $VEGF_{165}$b overexpressing transgenic mice. The protein expression and phenotypical analysis are ongoing.

P31

ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA) INCREASE NEUTROPHIL–ENDOTHELIAL CELL INTERACTIONS IN VIVO

S. L. Nolan, N. Kalia, G. B. Nash, P. Heeringa, C. O. S. Savage
Renal Immunobiology, University of Birmingham, Edgbaston, Birmingham, UK

Antibodies against neutrophil myeloperoxidase (MPO-ANCA) are implicated in small vessel vasculitis (SVV). It has been suggested that vascular lesions associated with SVV may arise from direct neutrophil activation by ANCA. Therefore, this study aimed to determine the effects of MPO-ANCA on leukocyte activation in the mouse cremaster microcirculation using a newly developed murine MPO-ANCA IgG. Antibodies were generated following the immunization of MPO deficient ($MPO^{-/-}$) mice with MPO purified from a murine myeloid cell line (Xiao et al. 2002 *J Clin Invest*; 110: 955–963). Wild-type (WT) C57BL/6 or $MPO^{-/-}$ mice, pre-treated with an intrascrotal injection of TNF-α (500 ng), were anesthetized with ketamine and xylazine (IP) and the cremaster muscle was prepared for intravital microscopy. MPO-ANCA IgG (or control IgG) was injected intravenously (7 μL/g bwt) 2 h post-TNF-α. Leukocyte–endothelial cell interactions were observed every 5 min for a period of 1 h in postcapillary venules ($20 - 50\,\mu$m diameter). TNF-α induced significant leukocyte rolling, adhesion and emigration in WT mice. However, within minutes of MPO-ANCA infusion, rolling was significantly reduced and a concomitant augmentation in the adhesion and emigration of leukocytes was observed. These inflammatory responses were sustained throughout the 1-h duration of the experiment. TNF-α also induced significant leukocyte rolling, adhesion, and emigration in $MPO^{-/-}$ mice. However, MPO-ANCA infusion did not convert leukocyte rolling to adhesion or further increase their emigration into the interstitium. Novel data from this in vivo study supports the concept that ANCA can bind MPO and directly activate neutrophils to become

adherent which could subsequently initiate tissue damage and thus contribute to SVV pathogenesis.

P32

MEASUREMENT OF FLUORESCENTLY LABELED RED BLOOD CELL VELOCITY USING SELF-ORGANIZING MAPS

C. C. Reyes-Aldasoro, S. Akerman, G. M. Tozer
University of Sheffield, K Floor, Royal Hallamshire Hospital, Sheffield S102JF, UK

Two main image-based techniques for measuring red blood cell (RBC) velocity have been used, both based on the mathematical technique of cross-correlation. Particle image velocimetry (PIV) is a 2D cross-correlation in which an investigating window determines the probable displacement of the investigated region between two consecutive images. The size of the window is a limitation and most studies are limited to single vessels or at most a bifurcation. Kymograph methods require user intervention to determine a line of analysis over which 1D correlation or slope analysis determines the velocity in a single straight vessel. Self-organising maps (SOM) is an algorithm with self-organising properties for a network of adaptive elements (often termed neurons). These receive an input signal and automatically map a set of output signals that acquire the same topological order as the input signal. We propose using SOMs to trace the movements of individual RBCs in intravital microscopy of tumor blood vessels. The final traces identify each moving RBC, not general regions, no manual tracing is required, and by obtaining their initial and final positions, the measurement of the velocity can be obtained. RBCs were obtained by cardiac puncture from donor anesthetized BDIX rats into a heparinized syringe and fluorescently labeled with DiI (D-282 Molecular Probes; Cambridge Bioscience). Intravital microscopy was carried out on tumors growing in transparent "window chambers" implanted into the dorsal skin flap of male BDIX rats under Hypnorm and midazolam anesthesia. Fluorescently labeled RBCs (~0.2 mL/200 g) were injected into a cannulated tail vein. Intravital microscopy was performed with an inverted Nikon Diaphot 200.

Cancer Research UK funded this work.

P33

DIVERSE SIGNALING PATHWAYS IN EQUINE PLATELET ACTIVATION

A. C. Brooks, C. P. D. Wheeler-Jones, S. R. Bailey, F. M. Cunningham, J. Elliott
Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London, UK

Platelet-activating factor (PAF) is capable of inducing aggregation, 5-HT release and thromboxane (Tx) production in equine platelets (Bailey et al. 2000 *Res Vet Sci*; 68:175–180). In contrast, stimulation with ADP causes only aggregation and 5-HT release, suggesting differential activation of intracellular signaling pathways regulating these events. The aim of this study was to investigate the role of signaling pathways in PAF-induced platelet activation. Washed equine platelets were preincubated with signal transduction inhibitors for 1 h prior to stimulation with PAF and subsequent Tx and 5-HT release or aggregation were measured ($n = 6$). PAF (0.2–50 nM) induced concentration-dependent release of Tx (from 4 ± 1 ng/mL to a maximum of 11.6 ± 2 ng/mL Tx at 50 nM) and 5-HT (from 0.643 ± 0.12% total 5-HT to a maximum of 41.6 ± 9% total 5-HT at 50 nM), and aggregation (maximum of 51.6 ± 3 mV at 50 nM). The p38 MAPK inhibitor SB203580 (0.1 μM) and the rho kinase inhibitor, HA1077 (10 μM) had no significant effect upon platelet activation. The PI3 kinase inhibitors, wortmannin (4 nM) and LY249002 (250 μM) significantly inhibited PAF-induced aggregation only. The non-isoenzyme-specific PKC inhibitor Ro31-8220 (10 μM) and the novel PKC isoform inhibitor Rottlerin significantly inhibited PAF-induced 5-HT release and aggregation. PAF-induced Tx release was inhibited by LY249002 (500 μM) and the ROS scavenger, Trolox (100 μM). The present study demonstrates that diverse signaling pathways mediate PAF-induced equine platelet activation, and these may play an important role in platelet-mediated pathophysiology in the horse.

P34

PHYSIOLOGICAL MECHANISMS BY WHICH ANGIOPOIETIN-1 (ANG1) MODIFIES MICROVESSEL PERMEABILITY COEFFICIENTS

A. H. J. Salmon, S. J. Harper, D. O. Bates
Microvascular Research Laboratories, Department of Physiology, University of Bristol, Bristol BS2 8EJ, UK

Ang1 reduces solute flux in inflamed vessels (e.g., Baffert et al. 2006 *Am J Physiol* 290; H107). The effect of Ang1 on microvessel permeability coefficients of noninflamed vessels was studied with the Landis-Michel technique (Michel et al. 1974 *Q J Exp Physiol;* 59: 283). Mesenteric microvessels of MS222-anesthetized *Rana temporaria* were studied. 3% bovine serum albumin perfusion (baseline) was followed by 30 min perfusion with 200 $ng \cdot mL^{-1}$ recombinant human Ang1 (rhAng1). Transvascular fluid filtration rate (j_v) was determined at different luminal hydrostatic pressures (p_c). The slope of this relation describes hydraulic conductivity (L_p; $\times 10^{-7}$ $cm \cdot s^{-1} \cdot cmH_2O^{-1}$), the abscissal intercept describes effective oncotic pressure ($\Sigma\Delta\Pi$; cmH_2O). Ang1 reduced L_p in 8 of 10 vessels studied (median ± semi-IQR baseline: 2.33 ± 1.12; rhAng 1 : 2.08 ± 1.20; $p < 0.05$, Wilcoxon). Ang1 elevated $\Sigma\Delta\Pi$ in all 10 vessels (mean ± SEM baseline 8.69 ± 0.67; rhAng 111.62 ± 0.65; $p < 0.001$, paired t test). The ordinate intercept of the relation between measured $\Sigma\Delta\Pi(\sigma\delta\pi)$ and the ratio of L_p in unmodified portions of the vessel wall (L_{pU}) to measured $L_p(\bar{L}_p)$ (effective oncotic pressure applied across modified portions of the vessel wall, $\Sigma_M\Delta\Pi_M$) was 3.20 ± 3.68), significantly different from both baseline $\Sigma\Delta\Pi(p < 0.005$, unpaired t test) and zero ($p < .05$, one-sample t test). Ang1 affects microvessel water permeability and reflection coefficient to albumin by modifying a portion of the vessel wall that imposes a low

but significant reflection of albumin, the identity of which is unknown.

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P35

MATERNAL INSULIN INDUCES VASCULAR LEAKAGE IN THE PERFUSED HUMAN PLACENTA

L. Leach, M. Uppal, D. B. J. Hughes
School of Biomedical Sciences, University of Nottingham, Nottingham NG7 2UH, UK

Increased vascular leakage and altered junctional molecular profiles are features of feto-placental vessels in pregnancies complicated by type1 insulin-dependent diabetes. It remains unclear if this is solely attributable to the complications of the diabetic milieu or if exogenous insulin therapy may paradoxically contribute to vascular disturbances. The aim of this study was to investigate whether presence of exogenous insulin in the maternal perfusate would result in changes in vascular leakage, alteration in localization of the key adherens junctional molecule, vascular endothelial (VE-) cadherin and transplacental transfer of insulin. Microvascular beds of normal term placentae were perfused in a dual independent closed circuit system, in the presence ($n = 3$) and absence ($n = 3$) of human recombinant insulin (1 U/mL) introduced to the maternal reservoir. After 20 min perfusion, a $76M_r$ dextran tracer (0.5 mg/mL) was introduced to the fetal circuit for a further 10 min. The beds were perfusion fixed and duly processed for imaging. Perfusate (maternal and fetal) were sampled at 1 and 20 min by radioimmunoassay. Insulin-treated placenta demonstrated a threefold increase in tracer leakage from the fetal vascular compartment, with >60% of vessels showing leaks. These vessels also exhibited loss of junctional VE-cadherin. Within the 20-min duration, 0.08% of insulin was transferred from the maternal to the fetal venous outflow. Thus, high concentrations of insulin in the maternal blood bathing the placenta may disrupt adherens junctions and increase tracer leakage in feto-placental vessels; moreover, transplacental transfer of insulin may complicate the fetal homeostasis in diabetes.

P36

THE INHIBITORY EFFECT OF LOCAL ANESTHETICS ON THE MICROVASCULAR FLARE RESPONSE TO BRADYKININ AND SUBSTANCE P

M. Dias[1], D. Newton[1], F. Khan[1], J. J. F. Belch[1], G. A. McLeod[2]
[1]Vascular Diseases Research Unit, The Institute of Cardiovascular Research, and [2]Department of Anaesthetics, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK

Clinical and experimental observation indicates that local anesthetics, in addition to their analgesic properties, might play a role in reducing inflammation after surgery. Furthermore, there is evidence from animal studies that local anesthetics have an inhibitory effect on the action of inflammatory mediators such as bradykinin and substance P. The aim of this study was to investigate the inhibitory effects of lidocaine (0.5 and 2%), levobupivacaine (0.125 and 0.75%), and ropivacaine (0.2 and 0.75%) on the inflammatory flare in skin blood flow produced by intradermal injection of bradykinin (20 μg/mL) and substance P (1.5 μg/mL) in 10 healthy, young male participants. Blood flow responses were assessed using laser Doppler imaging. The vascular effects of both inflammatory mediators were attenuated significantly by both analgesic and anesthetic doses of all 3 local anesthetics ($p = .001$). The bradykinin response was reduced by 50–70%, and there was no difference between the drugs or between higher and lower doses. The substance P response was reduced by 20–70%, and higher doses of anesthetics had a bigger effect ($p < .001$), although there was no significant difference between the drugs. The timely administration of local anesthetics may help to control postoperative inflammation and pain.

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Author Index for BMS 2006

Author Abstract

Aaronson, PI P5
Akerman, S P32
Alessi, DR OC24
Arkill, KP P1, P3
Bach, F OC19
Bailey, SR P33
Baker, AH OC6
Baker, PN S7
Bates, DO OC6, OC11, OC14, OC16, OC18, OC21, OC22, P4, P8, P9, P10, P12, P17, P29, P30, P34
Belch, JJF OC8, P36
Benest, AV OC6
Bevan, HS P8
Bhatwadekar, AD OC23, P15
Bramford-Hale, KO P19
Brindle, NPJ OC1
Brochet, DXP S6
Brookes, ZLS OC12
Brooks, AC P33
Brown, NJ OC3, OC12, OC20, P19, P20
Burke, D OC19, P21
Burke, SG P24
Cameron, IC OC20
Campbell, WB S2
Canning, P P16
Chen, P P22
Cheng, H OC22
Chetwood, ASA OC20
Churchill, AJ OC14, P12
Clough, GF P23
Coleman, J OC24
Cox, J P14
Cross, SS OC20
Cruickshank, SF P26
Cunningham, FM P33
Curtis, TM OC23, P15, P28
Dawicki, JM P28
de Gooyer, TE OC15
de Wit, C S5
Dias, M P36
Dixon, AR OC21
Doney, ASF OC13
Dunn, DB OC18
Dunn, DBA P4
Dunn, WR S7
Easton, AS P22
Elliott, J P33
Ellis, IR P13, P14
Emmett, MS OC11, P4
Falck, JR S2
Félétou, M S3
Fleming, I S1
Florence, MM P14
Fraser, HM OC4
Fraser, PA P5
Fulker, M OC5
Gardiner, TA OC14, OC15, OC23, P15, P16, P28
Garrioch, MA P17
Gauthier, KM S2
Gill, CA P23
Gooding, KM OC9
Gorshunska, MY OC9
Greco, O OC5
Griffith, TM S4
Griffiths, CEM OC7
Hadoke, PWF P7, P25
Hamdollah-Zadeh, AM P9
Hampson, P OC17
Hancox, JC OC22
Hankin, SL OC3
Harper, SJ OC6, OC14, OC16, OC18, OC21, P8, P9, P10, P12, P29, P30, P34
Harrison, DK P11
Heeringa, P P31
Herrick, AL OC7
Houlder, S. P21
Hughes, DBJ P35
Humphries, P OC15
Ives, CL P11
James, AF OC22
Jones, P OC19
Jones, SL P14
Kalia, N P31
Kankiva, K P14
Kanthou, C OC5
Kavanagh, DPJ OC17
Kay, RA P14
Kennedy, S OC2, P24, P27
Kenny, LC S7
Khan, F OC8, P36
Knott, R P26
Konopatskaya, O OC14, P12
Ladomery, M OC16
Ladomery, MJ P9
Lang, CC OC8
Langton, PD S6
Leach, L P17, P35
Leese, GL OC13
Levick, JR OC10, P2
Li, G OC23, P15
Li, P S2
Logie, JJ P7
Lord, JM OC17
Macdonald, AJ P3
Macdonald, LJ P25, P25
MacDonald, SH-F P26

Mansart, A OC12
Mathewson, AM S7
Mawson, D OC9
McGahon, MK P28
McGeown, JG P28
McHale, NG P3
McHarg, S P18
McLeod, GA P36
Menzies, JRW P26
Miller, WH OC6
Modi, S OC10
Moore, TL OC7
Morris, AM OC13
Mortimer, PS OC10
Murphy, D P30
Murray, AK OC7
Muston, PR P29
Nash, GB P31
Neal, CR P29
Newton, D P36
Nolan, SL P31
Nowak, DG OC16, P9
O'Connor, CD P23
Orlando, A OC18, P4
Palmer, CAN OC13
Patterson, D OC8
Peters, AM OC10
Petrie, JR OC24
Piper, J OC9
Pockley, AG OC3
Pritchard-Jones, RO OC18
Qui, Y OC18, OC21, P30
Quirke, P PC19, P21
Rainger, GE OC17
Rajput, M P6
Ray, S P6
Reed, MWR OC3, OC20, P19, P20
Reeve, KS P17
Reever, KJ P20
Reilly, CS OC12
Rennel, ES P8, P10
Reyes-Aldasoro, CC P32
Rigby, H OC18
Ross, JJ OC12
Rowe, ICM P26
Sabaratnam, S P2
Sage, L P8
Salmon, AHJ P34
Sarker, MH P5
Savage, COS P31
Scholfield, CN P28
Schor, AM P13, P14
Schor, SL P13, P14
Shipston, MJ P26
Shore, AC OC9, P18
Simpson, DA OC15, P28
Skipp, PJ P23
Smith, E OC17
Stansby, G P11
Stanton, AWB OC10
Staton, CA OC20, P19
Stitt, AW OC15, OC23, P15, P16, P28
Struthers, AD OC8
Sutherland, CD OC24
Svensson, WE OC10
Tabor, GR P3
Tadros, A OC1
Tennant, G OC2
Tooke, JE OC9
Tozer, GM OC5, P32
Uddin, J OC19, P21
Uppal, M P35
Varey, A OC16
Varey, AHR OC21
Wadsworth, RM OC2, P27
Walker, BR P7, P25
Whatmore, JL P18
Wheeler-Jones, CPD P33
Winlove, CP P1, P3
Woolard, J OC16, P9
Wu, J P27
Yang, W S2