D4

A demonstration of a method to investigate the effects of Angiopoietin1 on angiogenesis

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Angiopoietin1 (Ang1) is a vascular growth factor, essential for normal vascular development. Its role in adult physiological and pathological angiogenesis is not yet clear and a detailed investigation into its angiogenic phenotype (e.g. sprouting, intussusception, arteriogenesis, pericyte recruitment, etc) is lacking. To characterise Ang1 mediated angiogenesis in more detail, an adenoviral mesenteric angiogenesis assay was used (Wang et al. 2004) Male Wistar rats (300g) were anaesthetised by halothane inhalation and a laparotomy performed. All animals were killed humanely. A region of the mesentery was externalised and a mesenteric panel draped over a quartz pillar. The vasculature was imaged and 25µl of Ad–growth factor was injected into the fat pad. The panel was marked with 0.6% Monastral blue in mammalian Ringer solution, the gut replaced in the animal and the animal allowed to recover. 6 days later the same panel was found, imaged as before, and fixed *in vivo* with 4% paraformalydehyde. The panel was then prepared for immunofluorescent staining, before being imaged with confocal microscopy. 5 images per panel were taken, and measurements of vessel density, branch point density, sprout density, vessel diameter, length, endothelial cell proliferation pericyte coverage. N=5 in all groups. All analyses were carried out by Student's t test or ANOVA as appropriate. Ad–eGFP elicited no angiogenic response, whereas Ad–VEGF and Ad–Ang1 increased the angiogenic index. As previously demonstrated in the mesentery Ad–VEGF induced a 'sprouting angiogenesis ' phenotype, Ang1 displayed a significantly lower vessel density, reduced sprout density, branch point density than Ad–VEGF. However vessels were longer and wider) in the Ang1 compared with the VEGF injected mesenteries. Taken together these results show that the increase in Angiogenic index induced by Ang1 could be attributed to fewer, larger vessels compared with that induced by VEGF.

Wang WY et al. (2004) Microcirculation 11, 361-367.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Simultaneous measurement of hydraulic conductivity, oncotic reflection coefficient and compliance following treatment with high glucose Ringer solution in *Rana* mesenteric microvessels *in vivo*

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Whilst cardiovascular complications represent the major cause of morbidity and mortality in diabetic patients, the pathophysiological mechansims responsible are poorly understood. Development of diabetic angiopathy can be delayed by improved glycaemic control (UKPDS, 1998) and current clincal treatment involves managing serum blood glucose to normal levels.

In *Rana* mesenteric microvessels, exposure to 20mM glucose results in an increase in the microvascular permeability (hydraulic conductivity, L_p), without altering the oncotic reflection coefficient to albumin (σ) or the compliance of the vessel wall *in vivo*. Here we will demonstrate the measurement of L_p, and compliance (the change in vessel radius per unit change in pressure) in a single microvessel under basal conditions (5mM glucose) and in the presence of 20mM glucose.

Rana were anaesthetised by immersion in 1mg ml^{-1} MS222 in amphibian Ringer solution, and anaesthesia was maintained by superfusing the mesentery with 0.25mg ml^{-1} MS222. Frogs were humanely killed by cranial destruction at the end of the experiment. L_p was measured during perfusion and superfusion with 5mM or 20mM glucose Ringer solutions in capillaries or post capillary venules using the Landis-Michel method (Michel, 1974). Each vessel was cannulated with a bevelled glass micropipette connected to a manometer and perfused at controlled pressures with 3% BSA in Ringer solution containing rat erythrocytes as flow markers. Erythrocytes were collected by cardiac puncture from 5% halothane anaesthetised rats (humanely killed by cervical dislocation). The vessel was occluded downstream of the pipette at a known pressure. After approximately 5 s the perfusion pressure was either increased or decreased by 10cmH_2O within the same block.

The regression line taken from a plot of the filtration rate against pressure, allowed L_p to be calculated from the gradient of the line and $\sigma\Delta\Pi$ (and thus σ) from the intercept on the abscissa. Vessel compliance was determined by measuring the distance moved by an erythrocyte away from and then towards the occlusion (to calculate the change in radius) during a pressure change from 35 to 25 cmH_20 and back to 35 cmH_2O (Bates, 1998). The measurements were repeated during perfusion and superfusion of the vessel with 20mM glucose.

We demonstrate that whilst 20mM glucose increases hydraulic conductivity, reflection coefficient and vessel compliance remain unchanged.

UK Prospective Diabetes Study (UKPDS) Group. (1998). Lancet 352, 837-853.

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

Bates DO (1998). J Physiol 513, 225-233.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Simultaneous measurement of apparent solute permeability (P_a) to two differently sized fluorescent molecules in individually perfused microvessels of the frog and rat mesentery

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Previous studies have shown that shear stress, and molecules such as VEGF differentially increase the permeability to large

and small molecules. However, it has not been possible to measure the solute permeability to both large and intermediate sized molecules within a single vessel at the same time. We have therefore designed and built a system to measure solute permeability to two different fluorescently labelled solutes simultaneously in capillaries and postcapillary venules of living animals.

In this system, frogs (Rana temporaria, anaesthetised by immersion in MS222 (0.25g/l) solution) or rats (anaesthetised by 1:1:2 Hypnovel/Hypnorm/water; i.m. 0.1ml/100 g body wt) were laid supine and the limbs lightly secured to a supporting tray. An incision was made in the abdominal cavity and the exteriorised mesentery was gently stretched over a glass coverslip and pinned in place. The tray was placed on an extended microscope stage with the coverslip positioned over a 20x Fluotar objective on an inverted epifluorescence microscope (Leica DMIRB) and the mesentery was continuously superfused with frog Ringer solution, enabling visualisation of the mesenteric microvessels. A capillary or post-capillary venule that was straight, free-flowing, had at least 300µm between side-branches, 15-35µm in diameter and free of white cells adhering to, or rolling along the vessel wall was cannulated using a bevelled glass theta micropipette with a septum dividing it in two. A hole had been drilled in each side of the pipette and a cannula glued in place to allow refilling of either side of the micropipette independently. Blunt metal needles were glued in place at the back of the pipette allowing two pressure lines to be connected and the pressure on each side of the micropipette to be controlled independently. The vessel was perfused initially with 1% bovine serum albumin (BSA) in frog Ringer solution at pH 7.4 at a pressure of approximately 30cmH2O in one side of the pipette, and 1% BSA with 1mg/ml TRITC BSA and 100µg/ml sodium fluorescein on the other side. The pressure in the fluorescent side was then adjusted to balance the driving pressure of the BSA solution. The vessel was illuminated by a xenon light source (Cairn High Intensity Arc lamp, Cairn Research Ltd, UK). A rotating (50Hz) disc containing 480 and 530nm excitation filters was used to control the excitation wavelength. Fluorescence intensity (I_f) was measured within a defined window around the perfused vessel at 480 and 530nm wavelengths (F480 and F530). Fluorescence was measured during perfusion of 1% BSA, and when the perfusate was switched to the 1% BSA containing TRITC BSA and sodium fluorescein in the other side of the micropipette (pressures in either side of the micropipette were swapped). An infrared camera was used to set the window for the photomultiplier tube (PMT), incident white light was passed through an infra red filter (750nm longpass) to visualise the preparation with an IR camera (Watec WAT-902B). A dichroic filter was placed in front of the photometer to reflect light of <700nm wavelength to the PMT. Light of >700nm passed through the dichroic filter to the IR camera. The PMT was controlled by a Cairn spectrophotometer, which was in turn connected to a PowerLab/4SP (AdInstruments) system. Thus, I_f was measured using Chart software, P_a was calculated from the initial increase in fluorescence following switching ($\triangle I_f$), the radius of the vessel, and the rate of increase of fluorescence (dI_f/dt) for each fluorescent solute, $P_a = (dI_f/dt)(1/\triangle I_f)(r/2)$.

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Retinopathy of Prematurity Murine Model to Study Anti-Angiogenic Properties of Vascular Endothelial Growth Factor 165b (VEGF165b)

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Retinal neovascular diseases such as retinopathy of prematurity and proliferative diabetic retinopathy are the major causes of neonatal and adult blindness. During both physiological retinal development and pathological proliferative retinopathy, new blood vessels growth is controlled by VEGF. The inhibitory splice variant $VEGF_{165}$b is down-regulated in angiogenic tumours (1) and in the vitreous of diabetic patients (R. Perrin, unpublished observations), and is anti-angiogenic in vivo (1). The goal of the present investigation is to examine the role of $VEGF_{165}$b in retinal neovascularization using a murine model of proliferative retinopathy (2). In this model mice are exposed to hyperoxia, resulting in obliteration of posterior retinal vessels. The mice are then returned to room air, which causes relative hypoxia of the now non-perfused retina, producing a quantifiable neovascular response.

For demonstration of fluorescein angiography, C57-BL/6J *Mus musculus* will be anesthetised with Hypnorm/midazolam (1 part Hypnorm : 2 parts sterile water : 1 part midazolam at the rate 10 ml/kg i.p.) and perfused through the left ventricle with 1 ml of PBS containing 50 mg of 2 $x10^6$ *molecular weight FITC dextran. The eyes will be enucleated and fixed in 4% paraformaldehyde. The anterior segment-lens complex will be removed and the posterior eye-cups flat-mounted with the aid of 4 radial full-thickness incisions to yield a Maltese-cross conformation. The flat-mounted retinae will be viewed by epifluorescence microscopy. There will also be a sample demonstration of intravitreal injection of 2µl anti-angiogenic solution under the same conditions of anaesthesia. Treatment of the mice throughout the whole procedure will be conducted in accordance with the ARVO regulations on the Use of Animals in Ophthalmic and Vision Research.

Bates et al. (2002). Cancer Research 62, 4123-4131.

Smith at al. (1994). Invest Ophthalmol Vis Sci 35, 101-111.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.