The Effect of Pulsatile flow on Co-Cultured Retinal Endothelial & Pericyte Cells

A dissertation submitted for the degree of PhD

By

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Declaration:

I hereby certify that this material which I now submit for assessment on the programme of leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

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Abstract

Microvascular cell fate decisions are hallmarks of the microvascular cell response to injury and play a crucial role in the pathogenesis of retinal disease Abnormalities in retinal blood flow play a critical role in remodeling of the retinal vasculature by altering microvascular endothelial and pericyte cell fate (proliferation, apoptosis and migration) Retinal blood flow is controlled locally by vasodilators such as nitric oxide, prostacyclin and the vasoconstrictor endothelin-1, with considerable evidence linking retinal pathologies such as Normal Tension Glaucoma and Diabetic Retinopathy to altered retinal blood flow Shear stress has previously been shown to modulate EC production of these vasoactive agents in macrovascular cells Therefore, using a perfused transcapillary coculture of bovine microvascular retinal endothelial cells (BRECs) and bovine retinal pericytes (BRPs), we examined the acute and chronic effect of pulsatile flow on the release of these vasoactive mediators and their subsequent role in modulating retinal vascular cell fate

Acute exposure to pulsatile flow increased BREC NO, PGI2 & ET-1 formation and release Similarly, chronic exposure to pulsatile flow enhanced NO and PGI2 release while concomitantly inhibiting ET-1 in these cells In parallel studies, there was an increase in BRP apoptosis following exposure to high pulsatile flow, whereas BREC apoptosis decreased Furthermore, the pulsatile flow-induced increases in BRP apoptosis is dependent on increased PGI2, whereas both ET-1 and NO mediate the protective effect of increased flow on BRECs survival

Notch receptor-ligand interactions and the Hedgehog signalling pathway have been strongly implicated in vascular morphogenesis and remodelling of the embryonic vasculature, with Hedgehog acting upstream of Notch signalling during development We therefore tested the hypothesis that Hedgehog (Hh) and Notch pathway interact to promote changes in vascular cell fate in BRECs and BRPs *in vitro* in response to changes in pulsatile flow

There was a potent anti-apoptotic effect of increased Hh signalling in BRECs exposed to increased flow rates, *via* increases in Notch signalling and the Bcl-2 family of apoptosis related proteins, in favour of survival In contrast, BRP Notch and Hh signalling components decreased at high flow, concomitant with an increase in BRP apoptosis, similarly *via* modulation of the Bcl-2 proteins Exogenous addition of recombinant Sonic Hedgehog (Shh) confirmed the potent anti-apoptotic pro-proliferative effect of Shh in BRPs while concomitantly increasing BRP Notch mRNA levels in these cells The importance of these changes was confirmed by validating the presence of Notch and Hh pathway components in the retinal microvasculature of normal and glaucomatous human retina These results demonstrate a significant role for Notch and Hh in controlling microvascular cell fate in response to changes in pulsatile flow

Collectively, these studies suggest that changes in microvascular cell fate in response to flow play a crucial role in the pathogenesis of retinal disease Moreover, changes in retinal blood flow alter microvascular endothelial and pericyte cell fate in part through a Notch/Hh axis and the release of vasoactive substances from the microvascular endothelium The identification of the mechanisms by which pulsatile flow regulates remodelling of the retinal vasculature may lead to possible new drug targets that can promote or inhibit remodelling in disease

Abbreviations

ADMA	Asymmetric dimethylarginine
AGEs	Advanced glycation endproducts
AGS	Alagille syndrome
Ang	Angiotensin
Apaf-1	Apoptosis protease-activating factor-1
ATP	Adenosine tri-phosphate
BCA	Bicinchoninic acid
BH4	Tetrahydrobiopterin
bHLH	Basic helix-loop-helix
BM	Basement membrane
BREC	Bovine Retinal Endothelial Cell
BRP	Bovine Retinal Pericyte
Ca	Calcium
CAD	Caspase-activated DNase
CADASIL	Cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencephalopathy
CaM	Calmodulin
cDNA	Complimentary DNA
cGMP	Cyclic Guanosine monophosphate
COX	Cyclooxygenase
CDK	Cyclin-dependent protein kinase
DAN	2,3-diaminonapthalene
DMSO	Dimethylsulfoxide
DNA	Deoxy nucleic acid
DHh	Desert hedgehog
DR	Diabetic Retinopathy
EC	Endothelial cell
ECE	ET-converting enzyme
ECM	Extracellular matrix
EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIA	Enzyme Immunoassay
ET_A	Endothelin type A receptor
ET_B	Endothelin type B receptor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinase
EtOH	Ethanol
ET-1	Endothelin-1
FAK	Focal adhesion Kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor

GAPDH	Glyceraldehyde phosphate dehydrogenase
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GLUT	Glucose transporter
GON	Glaucomatous optic neuropathy
GTP	Guanosine triphosphate
HBSS	Hanks buffered saline solution
Hes	Hairy/Enhancer of Split
Hh	Hedgehog
HIF	Hypoxia-inducible factor
HIP	Hedgehog interacting protein
HRT	Hairy Related Transcription Factor
HTG	High Tension Glaucoma
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule-1
id	Inhibitor of differentiation/DNA synthesis
Ihh	Indian Hedgehog
iNOS	Inducible nitric oxide synthase
IOP	Intra-ocular pressure
JNK	c-jun N-terminal kinases
LB	Luria Bertram
MAPK	Mitogen activated protein kinases
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
mRNA	Messenger Ribonucleic acid
nNOS	Neuronal nitric oxide synthase
NO	Nitric Oxide
NotchIC	Notch Intracellular-cleavage protein
NOS	Nitric oxide synthase
NTG	Normal Tension Glaucoma
OD	Optical Density
OxLDL	Oxidised Low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
pCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PGI2	Prostacyclin
pH	Log of the reciprocal of the hydrogen ion concentration
PI-3 kinase	Phosphoinositide 3 kinase
PKC	Protein kinase C
POAG	Primary open angle glaucoma
POBF	Pulsatile ocular blood flow

PDR	Proliferative Diabetic retinopathy
PPAR	Peroxisome proliferators activated receptor
PSF	Prostacyclin-stimulating factor
Ptc1	Patched1
PTK	Protein Tyrosine Kinase
PTX	Pertussis toxin
RB	Retinoblastoma
RNA	Ribonucleic Acid
Rnase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Reverse Transcriptase
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of mean
SHh	Sonic Hedgehog
SiRNA	Small interfering RNA
SMC	Smooth muscle cell
Smo	Smoothened
SSRE	Shear stress response element
TAE	Tris Acetate EDTA
Taq	*Thermophilus aquaticus*
TE	Tris EDTA
TEMED	N, N, N, N'-tetramethyl ethylenediamine
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
uPA	urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cell

Units

Bp	Base Pairs
cm	Centimeters
g	Grams
h	Hours
kDa	Kilo Daltons
L	Litre
M	Molar
mg	Milligrams
Min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
°C	Degree Celsius
OD	Optical density
pM	Picomolar
Rpm	Revolution per minute
Sec	Seconds
U	Enzyme units
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
V/v	Volume per volume
W/v	Weight per volume

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Introduction

1 0 Introduction

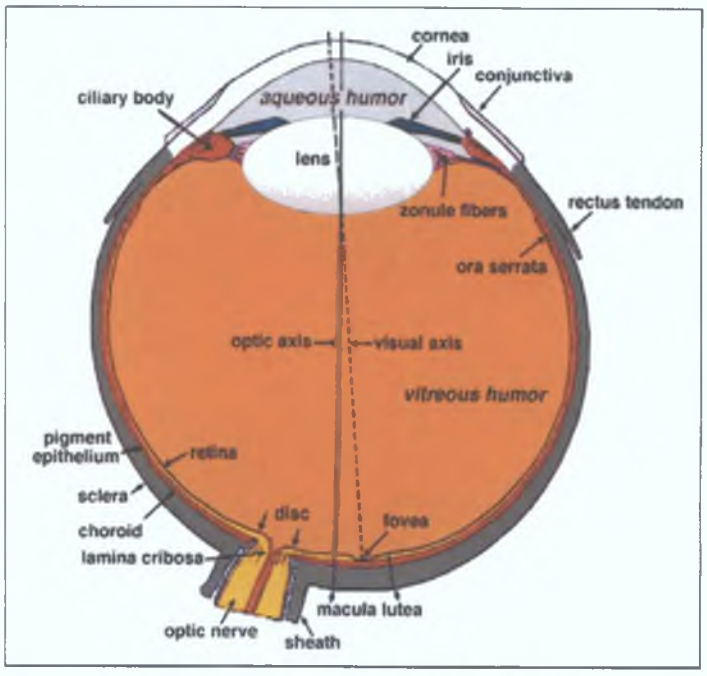
1 1 Retinal blood supply

Blood supply to the eye is through the ophthalmic artery, which leads into the ciliary arteries feeding the choroidal/uveal circulation, and also the central retinal artery which feeds into the retinal circulation (Hayreh, 2001) (Figure 1 1) The uveal vessels include the vascular beds of the iris, the ciliary body, and the choroid The retinal vessels nourish the inner layers of the retina, whereas the outer retinal layers including the photoreceptors are nourished by the choroid

The retinal vasculature is anatomically and physiologically specialized to distribute oxygen and nutrients to areas of metabolic need within a tissue that must also be translucent One adaptation that minimizes interference with passing light is the low density of retinal capillaries (Wu *et al* , 2003) Perfusion of a capillary must be tightly matched to the metabolic needs of nearby retinal neurons Local control of retinal blood flow is unique in three distinct aspects when compared to other microvascular beds firstly, autonomic innervation is absent (Ye *et al*, 1990), secondly, a tight endothelial barrier restricts the effects of circulating vasoactive molecules, and thirdly, precapillary smooth muscle sphincters, which control local perfusion in most other tissues, are absent (Pannarale *et al* , 1996) Taken together, these suggest an essential role in local autoregulation of blood flow, whereby a relatively constant blood flow, capillary pressure, and nutrient supply is maintained in spite of changes in perfusion pressure (Hayreh, 2001)

The retinal circulation has in general, two capillary beds, one feeding into the nerve fibre/ganglion cell layer and the other feeding the middle retinal layers including the inner nuclear layer and plexiform layer (Figure 1 2) The outer layers of the retina, including the photoreceptors of the eye, are avascular and nourished by diffusion from the outer choroidal circulation An avascular zone enabling light to reach the central photoreceptors without encountering a single blood vessel is centrally located in the fovea – the centremost point of the macula (Figure 1 3 & 1 1A)

A



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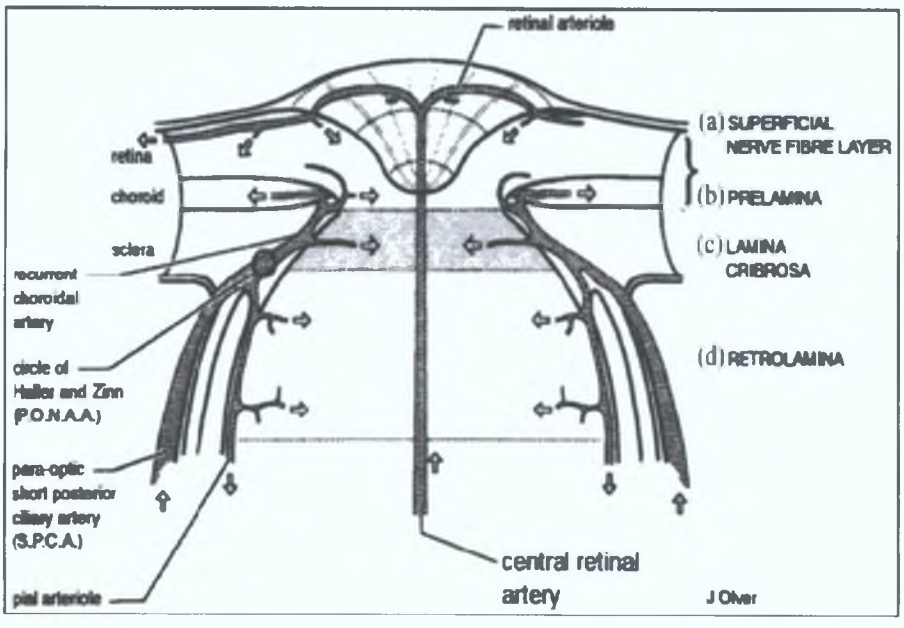


Figure 1.1: Schematic diagram of a horizontal section of the human eye (A) and ONH (B). At the optic nerve head (ONH), retinal vessels enter & exit the eye, and also ganglion cell axons exit to the brain (The impact of ocular blood flow in Glaucoma - J Flammer (2002))

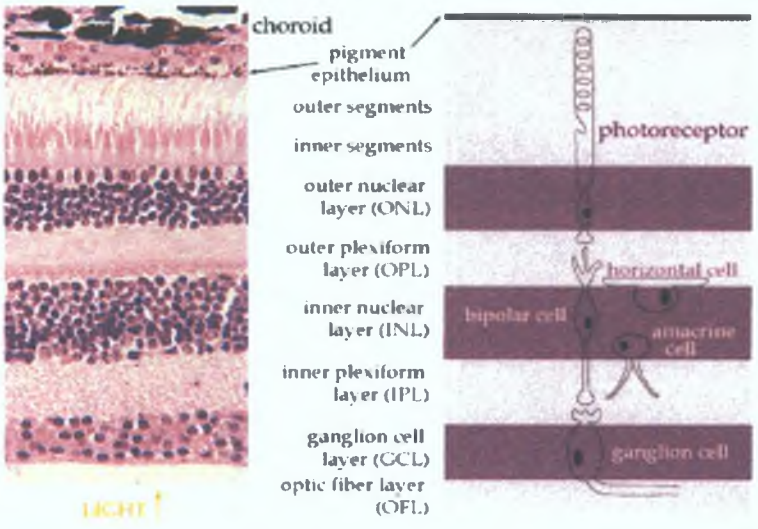


Figure 1.2: The human Retina. Low Power histological picture of the human retina, demonstrating the layered structure of the retinal nerve tissue (left), and a schematic diagram of the cell types in each layer (right) http://thalamus.wustl.edu/course/eyeret.html

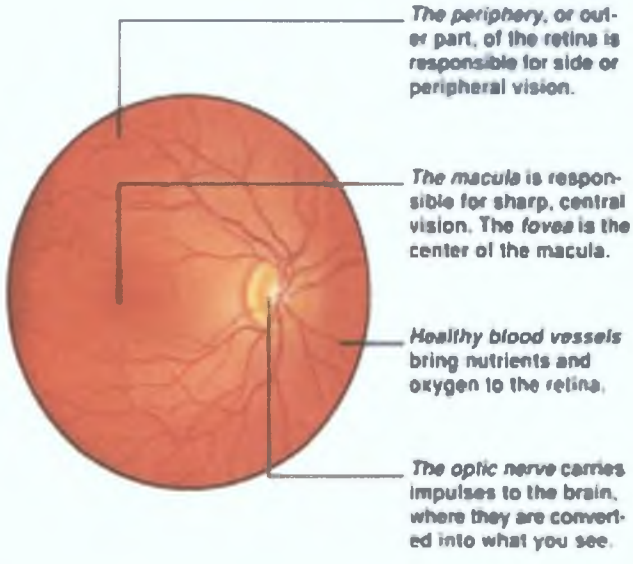


Figure 1.3: Fundus photograph of the human retina taken through the lens. The periphery, macula, fovea and optic nerve are detailed. http://www.stlukeseye.com/images/

1 1 1 Microvasculature

Mature capillaries and postcapillary venules are comprised of two cell types endothelial cells (ECs) and pericytes The ECs are surrounded by a basement membrane, within which there are a large number of intramural pericytes Pericytes exist in intimate association with ECs, forming a single layer that covers varying amounts of the abluminal EC surface This arrangement, which is unique to small vessels, is characterized by frequent sites of contact between the ECs and pericytes (Figure 1 4)

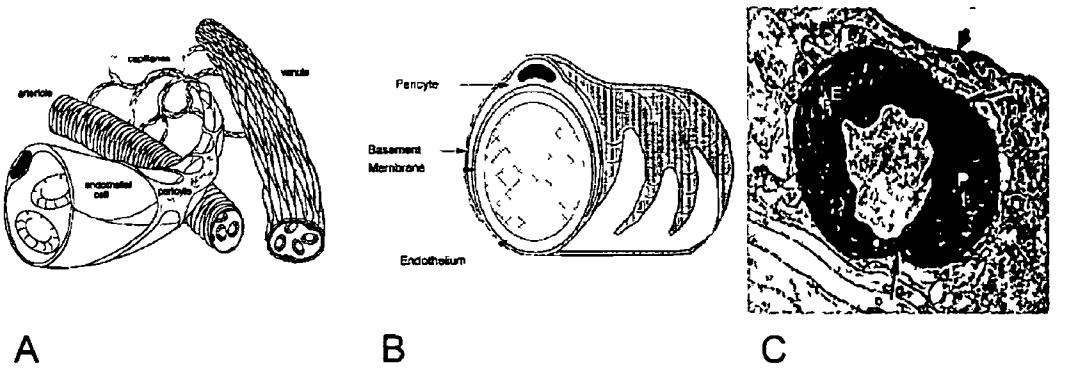


Figure 1 4 (A) Schematic diagram of the sheath of perictyes surrounding the endothelial tube of capillaries (B) Pericytes are embedded in a basement membrane Electron micrograph of the rat retinal capillary adjacent to internal limiting membrane (arrow) **(C)** Endothelial cell nucleus (E) and pericyte cell body and nucleus (P) The pericyte is ensheathed by basal lamina (small arrows) (Chakravarthy *et al* 1999)

1 1 1 1 Endothelial Cells

Endothelial cells (ECs) were once thought to be an inert lining to the vasculature However their strategic location provides the endothelium with the unique opportunity to monitor biochemical and biomechanical stimuli from systemic and local origin As a result, the endothelial cell can adapt its function as required to maintain homeostasis Endothelial cells are thus strategically located between the circulating blood and underlying mural cells, that is vascular smooth muscle cells (VSMCs) in large vessels, and pericytes of the microvasculature

The endothelium provides a semipermeable barrier that allows specific substances to move between the blood and the interstitium With the larger vessels serving as conduits, the exchange of water, gases, solutes and in some instances cells, occurs primarily across the capillaries and postcapillary venules The endothelium also serves to regulate vascular tone and to maintain a relatively constant perfusion pressure in the case of the retinal circulation

To maintain normal blood flow, a balance must exist between the activation and inhibition of coagulation within the circulation There is a dynamic ongoing interaction between the vascular endothelium, blood cells, plasma coagulation factors, fibrinolytic factors, and their inhibitors Under normal conditions, the endothelium slightly favors anticoagulant mechanisms to maintain blood fluidity In times of vascular damage or disease, prothrombotic mechanisms predominate Several *in vivo* and *in vitro* observations also demonstrate biomechanical force modulation of endothelial cell structure and function

1 1 1 2 Pericyte Cells

Microvascular mural cells are referred to as pericytes They can be distinguished from other perivascular cell types by their location within the microvascular basement membrane Pericytes, similar to VSMCs, are mesenchymal cells of presumed mesodermal or neuroectodermal origin (Bondjers *et al*, 2003)

Pericytes are stellar cells which envelop the endothelial cell layer Retinal trypsin digests have shown a ratio of pericytes to ECs equaled to 1, whereas ECs outnumber pericytes in other microvascular beds by as much as 10 to 1 (Stewart and Tuor, 1994) ECs outnumber pericytes by 4 to 1 in brain microvessels, which have similar EC barrier properties to retinal vessels (Stewart and Tuor, 1994) Discontinuities in capillary mural layer enable endothelial cells to contact the glial basement membrane, a distinct morphological difference from VSMCs in small arterioles (Chakravarthy and Gardiner, 1999)

By contracting and relaxing, pericytes may regulate lumen size and thereby control capillary perfusion The high ratio of pericytes to endothelial cells reflects the likely importance of pericytes in regulating retinal blood flow, more so than in any other vascular bed The evidence of a vasoregulatory role is also strongly supported by the presence of the muscle isoforms of actin, myosin, tropomyosin and calponin Although the degree of expression of these contractile proteins is lower than that found in VSMCs, only diminutive changes in the caliber of the capillary are required to modulate blood flow

Poiseuille's equation is a description of how flow is related to perfusion pressure, radius, length, and viscosity

$$\frac{\Delta V}{\Delta t} = \frac{\pi R^4 (P_1 - P_2)}{8\eta L}$$

The relationship clearly shows the dominant influence of vessel radius on resistance and flow, and therefore serves as an important concept to understand how physiological changes (e g vascular tone) and pathological changes (e g vascular stenosis) in vessel radius affect flow For example, a two-fold increase in vessel

radius results in a sixteen-fold increase in blood flow, assuming all other parameters remain equal

1.2 Blood Flow

Blood vessels are exposed to two types of mechanical forces a) *shear stress*, a frictional force acting longitudinally at the blood/endothelium interface, which is related to the velocity of blood flow (Figure 1.5) (Patrick and McIntire, 1995, Davies, 1995) and b) *cyclic circumferential stretch/strain* acting tangentially on the vascular wall, which is directly related to blood pressure and dimensions of the vessel. Both of these factors are essential for the maintenance of a healthy vessel.

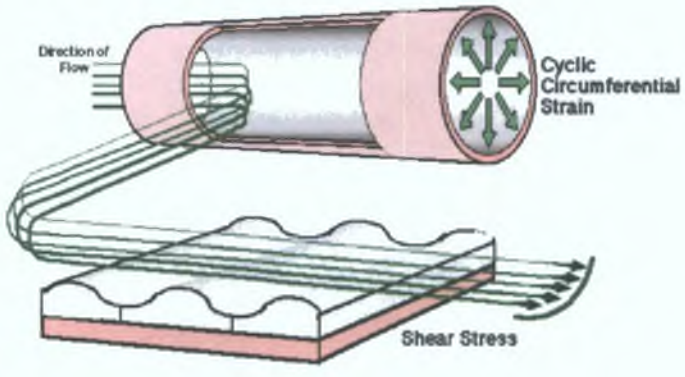


Figure 1.5: Schematic diagram of a blood vessel and the mechanical forces in the vessel wall: Cyclic circumferential strain (stretch) due to the pulsatile nature of blood flow and shear stress due to blood flow.

Blood pressure creates strain on the vessel wall in a direction perpendicular to the endoluminal surface and is the major determinant of vessel stretch via rhythmic distension of the vessel wall. The magnitude of this circumferential force is dependent on vessel geometry, and position within the vessel wall (Lehoux and Tedgui, 2003). The latter is particularly relevant in larger vessels where several layers of VSMCs may be present.

Blood flow also exerts a frictional force on the luminal surface of the endothelium This frictional drag is referred to as shear stress and is defined in terms of blood viscosity and velocity Laminar blood flow within a vessel can be described by the equation $\tau = 4\mu Q / \pi r^3$, where τ is shear stress, μ is blood viscosity, Q is flow rate and r is the vessel radius It is worth noting that the term r is raised to the third power thus where Q is constant a small change in r will result in a large change in τ (Lehoux and Tedgui, 2003)

Ophthalmic circulation is disturbed in many retinal diseases such as proliferative and non-proliferative diabetic retinopathy (Savage *et al*, 2004), primary open angle glaucoma, normal tension glaucoma (Harris et al , 1998), and also in age-related macular degeneration (Grunwald *et al* , 2005) The contribution of variable blood flow in causing each of these conditions is as yet unknown, however the changes in local shear stresses and cyclic strains are certainly contributors to remodelling of the microvasculature

1 2 1 Shear Stress

Under normal physiological conditions the endothelium is continuously exposed to mechanical shear stress due to blood flow Shear stress modulates cellular structure, function and gene expression (Lehoux and Tedgui, 2003, Chiu et al , 2004, Bartling et al , 2000) For example, shear stress regulates gene expression of various proteins, including vasoactive substances (*e g* nitric oxide synthase and endothelin-1) (Kuchan and Frangos, 1993), growth factors (*e g* transforming growth factor-β1 and platelet-derived growth factor) (Negishi et al , 2001, Resnick et al , 1993), adhesion and chemoattractant molecules (*e g* intercellular adhesion molecule-1, vascular cellular adhesion molecule-1, and monocyte chemoattractant protein-1) (Chiu et al , 2004, Cheng et al , 1996), coagulation factors (*e g* tissue factor) (Lin et al , 1997), proto-oncogenes (Bartling et al , 2000), and antioxidant enzymes (*e g* superoxide dismutase) (Wung et al , 2001) Furthermore, shear stress inhibits apoptosis of macrovascular ECs in response to various stimuli, demonstrating a potent atheroprotective effect At certain positions such as bifurcations in the vessel or points of extreme curvature, the vessel may be exposed to turbulent flow, oscillatory shear stress and eddy currents, all of which can abrogate the protective effects of laminar shear, hence atherosclerotic plaques tend to form at these points The mechanisms by which haemodynamic forces such as shear stresses are transduced into cellular signalling are still not fully known

In vitro studies in which endothelial monolayers have been subjected to defined levels of shear stress have been essential to our understanding of shear stress related molecular responses The complexity of the shear stress response is only now being elucidated and some of the best-characterized responses include, reorganization of actin containing stress fibers, alterations in metabolic activities and changes in cell cycle kinetics (Davies, 1995, Davies and Tripathi, 1993) Shear related effects can be broadly categorized into two responses, a) reorganization or regulation of pre-existing proteins and b) *de novo* protein synthesis and gene expression, the latter is usually associated with delayed or chronic shear-mediated responses

As described previously, exposure of the endothelium to fluid mechanical forces may alter the rate of transcription of a specific subset of genes Investigation of the promoter regions of these genes has identified the presence of a cis-element, which is inducible by shear stress The identity of this shear stress response element (SSRE) as GAGACC was achieved by a series of transfections involving deletion mutants of the PDGF-B promoter (Resnick *et al*, 1993) Other examples of SSREs include a divergent transcriptional response element (TRE) in the promoter region of MCP-1 with the sequence TGACTCC, necessary for shear inducibility (Shyy *et al*, 1995) Functional analysis of the tissue factor (TF) gene has identified a GC-rich region containing three copies of the transcription factors Egr-1 and Sp-1 Deletion of the Sp-1 but not the Egr-1 attenuates shear stress activation of this gene (Lin *et al*, 1997) Thus it can be seen that multiple cis-elements may regulate shear stress responsiveness in different genes

EC gene expression in response to shear stress is known to be a function of the magnitude of the force For example, t-PA expression is only increased at shear stresses above 5dynes/cm^2 (Diamond *et al*, 1990), whereas secretion of the vasoconstrictor endothelin-1 (ET-1) is increased in HUVECs at shear stresses less than 5 dynes/cm^2 (Kuchan and Frangos, 1993) This may be explained by the fact that the magnitude of shear stress may vary depending on the location within the vasculature Therefore in situations where shear stresses may be low increases in ET-1 will promote vasoconstriction to increase blood flow rate through that section of the vessel The use of DNA microarray technology has permitted analysis of extensive differential gene expression in response to haemodynamic forces Fifty-two flow sensitive genes have recently been identified in HUVECs with prostaglandin and cytochrome p450 the most strongly up-regulated and ET-1 and MCP-1 the most strongly down-regulated (McCormick *et al*, 2001) 143 genes have been identified in HUVECs, which are differentially expressed in the presence of static, laminar, or turbulent flow (Garcia-Cardena *et al*, 2001) *In vivo*, the expression of a number of genes, such as transforming growth factor-β (TGF-β) (Negishi *et al*, 2001), PDGF-A, PDGF-B (Tulis and Prewitt, 1998), and urokinase plasminogen activator (uPA) have been found to be shear sensitive Further studies

involving microarray technology may lead to a more complete elucidation of cellular responses to mechanical forces by identifying the cellular participants in regulating cell function in haemodynamic environments Although there may be some discrepancies between using vein and artery preparations or between *in vitro* and *in vivo* models these studies clearly indicate the importance of mechanical forces to gene regulation within the vasculature

1 2 2 Cyclic Strain

In 1893, Thoma observed that blood vessel diameter was regulated by the magnitude of blood flow while vessel thickness was dependent on blood pressure (Figure 1 6) This observation was confirmed by comparing the thickness of the pulmonary artery and aorta pre and post birth *In utero*, both vessels experience similar pressures and are almost identical in size, however, after birth the aorta thickens proportionally to increases in systemic pressure while the pulmonary artery undergoes atrophy following the fall in pressure post partum (Leung *et al* , 1977)

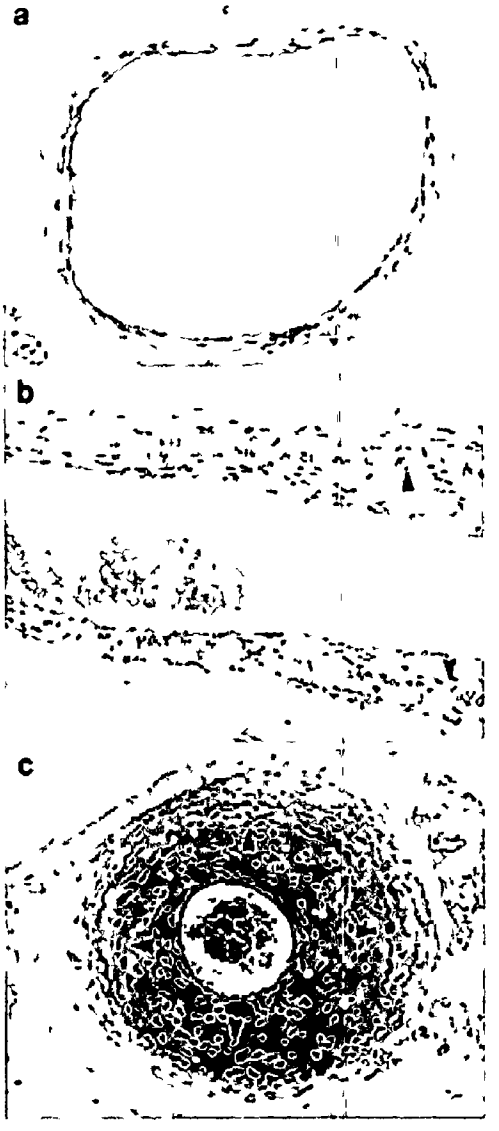


Figure 1 6 Photomicrographs of intimal lesion formation in ligated mouse carotid arteries (A) Cross section of a normal vessel, **(B)** longitudinal section of the proximal mouse carotid artery 4 weeks after ligation near the carotid bifurcation The aortic arch is at the left Note the increased intimal thickening distal to the arch **(C)** Cross section of a central segment of mouse carotid artery 4 weeks after ligation, showing a thick intimal lesion, reduced vessel diameter, and narrowed lumen Arrowheads indicate the Internal elastic lamina Counterstained with hematoxylin (original magnification x200) Kumar *et al* 1997

The relationship between circumferential stress and the structure of the wall has been well established in macrovascular cell types. Increases in arterial pressure are associated with VSMC hypertrophy and increases in extracellular matrix (ECM) production. Conversely decreases in arterial pressure result in vessel atrophy (Bomberger *et al.*, 1980). In a cultured rabbit aorta model of low intraluminal pressure, VSMC markers such as, h-caldesmon and filamin were dramatically decreased compared to aortas maintained under normal intraluminal pressure (Birukov *et al.*, 1998). Continual mechanical stimulation appears to be essential to maintaining a contractile phenotype in SMC. Whilst a certain level of stretching may be essential for VSMC maintenance, over stretching may initiate adaptive processes (Lehoux and Tedgui, 1998). Mechanical stretch is a strong determinant of vascular structure in conjunction with autocrine and paracrine factors (Tedgui *et al.*, 1992). It has also been observed that sustained hypertension leads to thickening of the arterial wall due to SMC hypertrophy, hyperplasia and changes in matrix proteins leading to altered arterial function (Levy *et al.*, 1988) (Figure 1.6).

The fact that endothelial cells are the principal recipients of shear stress does not imply that cyclic strain has no influence on the endothelium. Cyclic stretch increases EC sensitivity to shear stress resulting in a lowered threshold level required to provoke structural changes and ultimately both cyclic stretch and shear stress are required to produce maximal responses in the vessel (Zhao *et al.*, 1995).

Studies from *in vitro* experiments demonstrate that cyclic strain increases EC expression of nitric oxide synthase (NOS), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-14 (MMP-14), monocyte chemotactic protein-1 (MCP-I), platelet derived growth factor-BB (PDGF-BB), endothelin–1 (ET-l), intracellular adhesion molecule-1 (ICAM-I), and plasminogen activator inhibitor-1 (PAI-1) (Awolesi *et al.*, 1995, Cheng *et al.*, 1996, de Jonge *et al.*, 2002, Wang et al., 2003a, Wung *et al.*, 2001). The complexity of these cyclic strain-induced events have not been completely elucidated but the ability of cells to respond to cyclic strain is believed to play a role in a number of pathologies including atherosclerosis, hypertension or restenosis following balloon angioplasty (Li and Xu, 2000, Zou et al., 1998) and retinal pathologies such as diabetic retinopathy (Schmetterer and

Wolzt, 1999) Intracellularly, cyclic strain is responsible for the recruitment of a variety of signalling molecules Yano *et al* reported that cyclic strain increases the tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin in ECs with a concurrent cell elongation and the alignment of F-actin, FAK, and paxillin (Yano et al , 1996) Cyclic strain has also been found to activate extracellular signal-regulated kinase (ERK), c-jun N-terminal kinases (JNK), and p38 (Wung et al , 1999, Kito et al , 2000)

Chien et al demonstrated mechanical strain is a potent stimulator of endothelial cell gene expression (Chien et al , 1998) Following exposure to cyclic strain for half an hour, 237 genes were found to have altered gene expression These genes included cell receptors, protein kinases, cell growth/differentiation factors, extracellular matrix (ECM) proteins, lipid metabolism, protein metabolism, transcription factors, binding proteins and water channels

DNA microarray analysis of human vascular SMC showed that COX-1, PAI-1 and tenascin where all upregulated in response to cyclic strain while MMP-1 and thrombomodulin were down-regulated which suggests a response of defense against excessive deformation (Cui et al , 2004, Feng et al , 1999) The changes in secretion of vasoactive compounds, signalling molecules and gene expression observed in response to cyclic strain result in changes in cell phenotype

Cyclic strain is a powerful stimulus and can also regulate cell fate decisions Exposure of vascular smooth muscle to cyclic strain leads to apoptosis via a p53 dependent pathway, conversely, cyclic strain can suppress EC apoptosis via Akt phosphorylation (Mayr et al , 2002, Persoon-Rothert et al , 2002, Haga et al , 2003) Similarly cyclic strain has been linked to inhibition of proliferation in addition to increases in angiogenesis associated with TGF-β, MMP-2 and VEGF (Rivilis et al , 2002, Zheng et al , 1999, Vailhe and Tranqui, 1996, Banai et al , 1994) These studies clearly demonstrate the importance of cyclic strain in coordinating and regulating cell function by mediating changes in gene transcription, signalling molecules activation and release of vasoactive compounds

1.2.3 Mechanotransduction

Vascular cells respond to mechanical forces namely cyclic strain and shear stress by altering gene expression, which results in changes in cellular structure and function (Okada et al., 1998, Wung et al., 2001, Garcia-Cardena et al., 2001, Awolesi et al., 1995). Before a vascular cell can respond to a haemodynamic stimulus however, it must first of all be able to detect them. This is facilitated by mechanically sensitive receptors. These receptors, which fall into a number of categories, can then elicit a signalling pathway, which culminates in the recruitment of effector molecules to mediate a cellular response (Figure 1.7). Mechanical forces initiate complex signal transduction cascades leading to functional changes in the cell, often triggered by receptors such as G-proteins, integrins, or protein tyrosine kinases (PTKs).

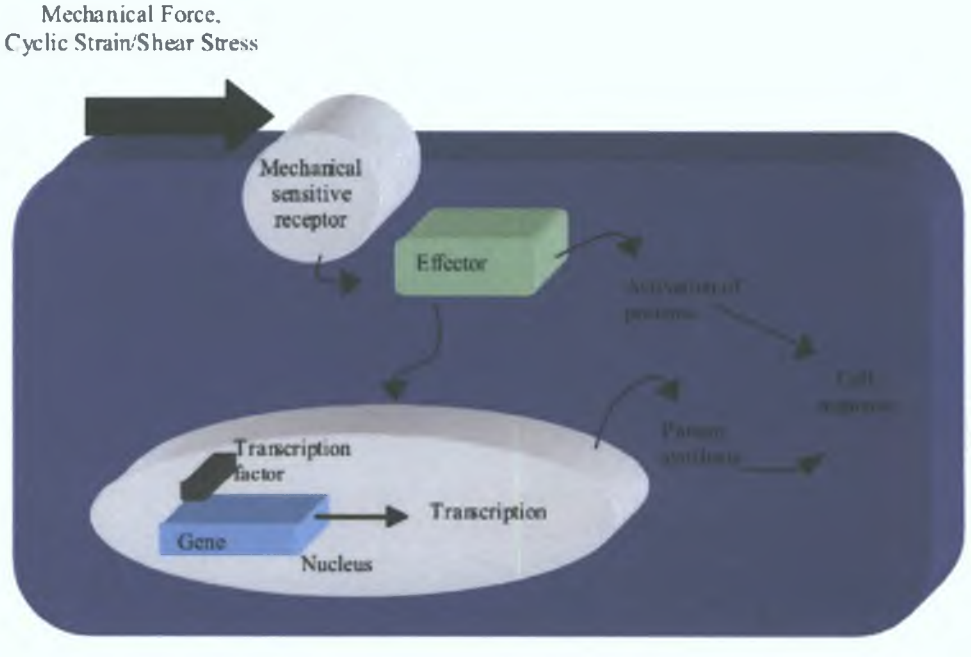


Figure 1.7: An overview of transduction of a mechanical force from cell membrane to nuclear transcription.

1 2 3 1 G-proteins

G-protein signalling represents a highly sophisticated molecular system with the ability to receive, integrate, and process information from extracellular stimuli The components of the G-protein signalling machinery include a G-protein coupled receptor (GPCR), a heterotrimeric G-protein complex itself, and effector proteins, in addition to the more recently identified regulators of G-protein signalling (RGS-proteins) and activators of G-protein signalling (AGS-proteins) (Offermanns, 2003) G-protein signalling is known to play a pivotal role in cardiovascular signalling

All of these receptors have seven membrane spanning elements that use intracellular loops and their C-terminal tails for interaction with heterotrimeric G-proteins, which consist of α, β and γ subunits The β and γ subunit forms an undissociable complex which represents a functional subunit Ligand activated receptors catalyse the GDP/GTP exchange at the α subunit of a coupled G-protein and promote dissociation of the α and βγ components (Wieland and Mittmann, 2003) The duration of a G-protein activation is controlled by the intrinsic GTPase activity of Gα Following GTP hydrolysis the Gα subunit returns to the GDP-bound conformation and reassociates with the Gβγ subunit

1 2 3 2 Integrins

Integrins comprise a large family of heterodimeric cell surface receptors most widely known for their role as receptors for extracellular matrix proteins The heterodimer comprises of one of eighteen α and one of eight β subunits not including splice variants Each possible combination of subunits has its own binding specificity and signalling properties (Giancotti and Ruoslahti, 1999) These subunits can form twenty-four different integrins Sixteen of the known integrins are reportedly involved in the vasculature, with seven expressed in endothelial cells (Rupp and Little, 2001) The cytoplasmic tail of integrins is generally devoid of enzymatic activity As a result of this, integrins transduce signals via adaptor

proteins which connect the integrin to the cytoskeleton, cytoplasmic kinases and transmembrane growth factors (Giancotti and Ruoslahti, 1999)

1 2 3 3 Protein Tyrosine Kinases (PTKs)

The initiation of signal transduction events by "shear-stress-sensitive receptors" leads to a cascade of downstream signalling events many of which are mediated by protein tyrosine kinases PTKs are crucial in the shear stress regulation of cell shape and stress fibers This can be demonstrated by inhibition of shear stress induced ERK and JNK activation by genistein, a PTK inhibitor Once the kinases are activated they relay signals downstream by phosphorylating other protein kinases and transcription factors These kinases are turned off by activation of specific phosphatases Mitogen activated protein kinases (MAPKs) are the most well studied kinases in response to haemodynamic forces, and they were first identified as microtubule associated kinases, due to their involvement with the cytoskeleton (Berk et al , 1995)

1 2 3 4 Ion channels

In addition to being regulated by G-proteins, some ion channels function as receptor molecules themselves Two different mechano-sensitive channels have been identified in vascular cells shear stress activated potassium channels and stretch activated cationic channels Inhibition of ion channel activation can attenuate strain induced smooth muscle cell proliferation (Sweeney et al , 2002) Stretch activated phospholipase C activity was found to involve the influx of calcium via gadolinium sensitive channels Similarly, Ang II activation of mitogen activated protein kinases is calcium dependent in VSMC (Lehoux and Tedgui, 1998) The exact mechanisms by which mechanical forces regulate ion channel conformation remains vague, though deformation of the cytoskeleton is thought to be an important contributor in this regulation

1 2 3 5 Role of Mitogen activated protein kinases

The mitogen-activated protein kinase (MAPK) signalling cascade is an important pathway whose activation can lead to or stimulate gene transcription and/or protein synthesis The MAPK super-family is comprised of three main and distinct signalling pathways the extracellular signal-regulated protein kinase (ERK), the c-jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK), and the p38 family of kinases Each of the MAPK modules operates as a three-tier system The MAPK module is activated by a MAPK kinase (MAPKK), which is a dual-specific kinase, which phosphorylates ERK, JNK and p38 at both Ser/Thr and Tyr sites The MAPKK is activated by a MAPKK kinase (MAPKKK), which receives its stimulus from receptors on the cell surface MAPK have a key role in the regulation of many genes because the end targets of these cascades are often nuclear proteins or transcription factors (Cowan and Storey, 2003)

1 2 2 Autoregulation of Blood Flow

Blood flow through the human retina varies from ~50 to 80 µl/min (Garcia et al , 2002), with a mean retinal circulation time of 4 to 5 seconds (Hickam et al , 1959) Hayreh described a formula to calculate blood flow in the optic nerve head or retina

Flow = Perfusion pressure / resistance to flow

Perfusion pressure is defined as the difference between arterial and venous pressure, with venous pressure equal to or slightly higher than intra ocular pressure (IOP) in the eye Local resistance in turn is controlled by the size of the vessels, which as is the case in most microvascular beds, are 'autoregulated' Vessels regulate local resistance to flow via alterations in vessel tone and thus, diameter For example, a decrease in perfusion pressure results in vasodilation and a resulting increase in blood flow, whereas an increase in perfusion pressure results in vasconstriction, in order to return blood flow to its 'normal' level (Figure 1 8) ECs can 'detect' local alterations in blood flow via membrane bound 'mechanosensors' and thus alter production of vasoactive mediators such nitric oxide, prostacyclin or endothelin-1

Therefore autoregulation is defined as the ability to maintain a relatively constant blood flow, capillary pressure and nutrient supply, in spite of changes in perfusion pressure (Hayreh, 2001)

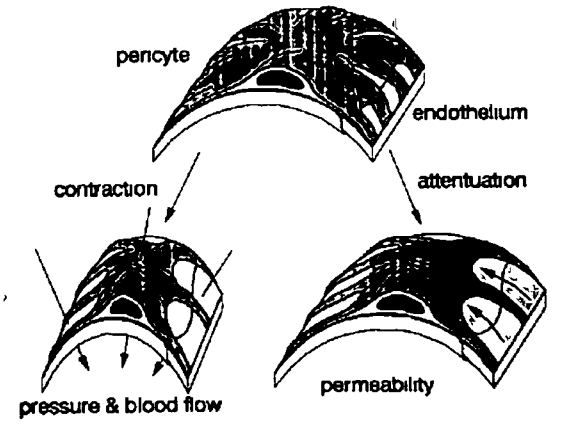


Figure 1 8 Schematic diagram of pericyte contraction (left) or relaxation (right) http //www udel edu/Biology/Wags/histopage/illuspage/

Autoregulation has been demonstrated in the vascular beds of cats, pigs, rabbits and humans However, terminal arterioles are physically limited in terms of how much they can relax or contract, and as such, can only autoregulate within a certain range of perfusion pressure In some studies the upper limit for autoregulation of the retinal blood flow is suggested as 30 mmHg, with a lower limit of 15 mmHg (Hayreh, 2001) Chronic exposure to pressures outside of this critical range (which will have large inter-individual variation) is likely to result in pathological alterations For example, such a mechanism has been observed in the human eye with BP elevations of 40% above baseline, with corresponding increases in retinal blood flow (Robinson et al , 1986) Such increases may also be responsible for the clinical lesions of hypertensive retinopathy and why in conjunction with diabetes mellitus, there exists clinical evidence of accelerated retinopathy It is known that the ability of the diabetic's circulation to distribute blood is affected, especially during increased blood flow In most tissues this causes no serious burden, but three tissues are usually susceptible to disturbance They are the retina, renal cortex, and peripheral nerves (Lam et al , 2003)

Along with impaired autoregulation outside the manageable range of perfusion pressures, endothelial dysfunction is also considered a factor impairing retinal blood flow Endothelial dysfunction can result in altered basal EC secretion

of vasoactive mediators such as NO, PGI_2 and ET-1 or impaired secretion in response to changes in blood flow Interestingly, chronic alterations in blood flow can lead to endothelial dysfunction

1 2 2 1 Vasodilators

1 2 2 1 1 NO

The recognition that nitric oxide (NO) is a critical signalling molecule mediating a broad variety of physiologic and pathophysiologic events has prompted the rapid growth of investigations in NO biology Along with prostacyclin, NO is responsible for endothelium derived tonic relaxation of all types of blood vessels by stimulating soluble guanylate cyclase and increasing cGMP in smooth muscle/pericyte cell, counteracting endothelium derived vasoconstrictor effects of endothelin and thromboxane A2 The increase in intracellular cGMP concentration leads to a relaxation via a decrease in intracellular Ca^{2+} (most likely by increasing Ca^{2+} efflux and reuptake into intracellular stores) and dephosphorylation of myosin light chains (Luscher et al , 1990a) NO also functions as a neurotransmitter in the central and peripheral nervous system, and contributes to the antimicrobial activity of macrophages as well as to hormone release and platelet inhibition Thus, NO is formed within neural, cardiovascular and immune system

NO is a unique bioactive mediator because it is a gas with no known storage mechanism and is the lowest molecular weight of any human cell secretory product Dissolved in cellular fluids, lipophilic NO can easily cross membranes by diffusion NO is highly reactive and extremely labile, with a biological half-life of only a few seconds, and is oxidized to stable nitrite and nitrate Therefore, NO action is very transient and directly controlled by the generation of NO

NO is generated from the metabolism of L-arginine by a family of enzymes, the nitric oxide synthases (NOSs) The NOS enzymes were first identified and described in 1989 and the three major isoforms were cloned and purified between 1991 and 1994 (Alderton et al , 2001) The three isoforms of NOS are neuronal NOS (nNOS), iNOS and eNOS (also called NOS-1, NOS-2 and NOS-3, respectively) iNOS is controlled by inflammatory mediators and cytokines and produces large, unregulated quantities of NO, whereas nNOS and eNOS produce low amounts of NO eNOS and nNOS are generally referred to as constitutively expressed, Ca^{2+}-dependent enzymes, although eNOS can also be activated in a Ca^{2+}-

independent manner (Ayajiki et al , 1997) The enzymes have a bi-domain structure in which the N-terminal, oxygenase domain contains binding sites for heme, tetrahydrobiopterin (BH4) and L-arginine This is linked by a calmodulin(CaM) binding domain to a C-terminal reductase domain that contains binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and NADPH All NOSs have approximately 60% amino acid homology, with very similar structures

The physiological importance of this endothelium-derived vasodilator is reflected by the significant increase in vascular resistance that is induced in animals and humans exposed to pharmacological antagonists of nitric oxide synthase (Vallance et al , 1989) Endothelium-derived nitric oxide also inhibits platelet and leukocyte adherence to the vessel wall (Kubes et al , 1991) This effect of nitric oxide is mediated in part by the activation of cGMP and phosphorylation of intracellular signalling proteins, such as vasodilator stimulated phosphoprotein (Smolenski et al , 1998) In addition, nitric oxide suppresses the expression of adhesion molecules and chemokines regulating endothelial interaction with circulating blood elements (Spiecker et al , 1998) Finally, endothelium-derived nitric oxide also inhibits vascular smooth muscle cell proliferation (Garg and Hassid, 1989), in part mediated by an increase in vascular smooth muscle cell apoptosis (Weidinger et al , 1990) In contrast, nitric oxide is a survival factor for endothelial cells (Dimmeler et al , 1997) These observations are consistent with the view that nitric oxide is an endogenous antiatherogenic molecule Impairment of endothelial NOS contributes to the pathological alterations in vascular reactivity and structure that are observed in atherosclerosis (Cooke and Dzau, 1997) Pharmacological inhibition or genetic deficiency of NOS inhibits endothelium-dependent vasodilation, impairs tissue blood flow, and raises blood pressure (Cooke and Dzau, 1997) Furthermore, nitric oxide deficiency promotes the adherence and intimal accumulation of mononuclear cells and accelerates lesion formation in animal models of atherosclerosis (Cooke and Dzau, 1997, Gimbrone et al , 1995) By contrast, enhancing nitric oxide production in the vessel wall slows or even reverses atherogenesis or restenosis (Tentolouris et al., 2000)

Regulation of NO is reported at several levels transcriptional and post-transcriptional regulation of RNA, enzyme phosphorylation and availability of enzyme substrate (L-arginine) and co-factors (Ca^{++}, calmodulin, NADPH, BH4, FAD, FMN and heme) Additionally, eNOS is activated and deactivated via phosphorylation at serine residue 1177 and threonine residue 495, respectively eNOS phosphorylation at serine^{1179} increases activity of the enzyme, possible via an increase in electron flux through the reductase domain eNOS phosphorylation by protein kinase Akt is promoted by VEGF (Fulton et al , 1999), fluid shear stress and stretch (Dimmeler et al , 1999, Gallis et al , 1999) NO production in response to short-term VEGF exposure is mediated by the activation of tyrosine kinases and PI3K, whereas longterm exposure to VEGF augments NO release through increased expression of eNOS protein Moreover, NO mediates a proliferative and angiogenic response to VEGF *in vitro* (Papapetropoulos et al , 1997) Lastly, several protein-protein interactions with a number of partners can also activate or inhibit the enzyme e g HSP90, Akt, caveolin-1 and protein inhibitor of NOS (Alderton et al , 2001, Balligand, 2002)

1.2 2 1 2 PGI_2

In addition to NO, endothelial and pericyte cells produce prostacyclin (PGI_2), a critical modulator of vascular tone in both physiological and pathophysiological conditions (Davidge, 2001) PGI_2 activates adenylcyclase, causing an increase in intracellular production of cyclic adenosine 3',5'-monophosphate (cAMP), resulting in pericyte vasodilation

Production of PGI_2 is regulated by the availability of arachidonic acid and also the activity of COX enzyme (Smith et al , 2000c) Liberation of arachidonate from membrane phospholipids is mediated through phospholipases Once arachidonate is released, COX converts it to PGI_2 COX is a rate-limiting enzyme that exhibits a cyclooxygenase activity and a peroxidase activity There are two known isoforms, COX-1 and COX-2 COX-1 is generally termed the constitutive isoform, and COX-2 the inducible isoform (Smith et al , 2000b) While this is the case in many tissues, these terms are not exclusive COX-1 can be induced in some situations and COX-2 is constitutive in the brain and in other tissues Also, recent evidence suggests that in addition to constitutive endothelial COX-1, COX-2 is a major source of circulating prostacyclin (McAdam et al , 1999) COX-1 and COX-2 have approximately 60-65% sequence homology within the same species, and 80-95% homology of the individual isoforms across different species The tertiary structures of each are almost identical, with each existing as homodimers

Both the endothelium and smooth muscle cell contain COX, however, endothelial cells contain up to 20 times more enzyme With regard to sub-cellular localization, immunogold-labeling microscopy has demonstrated that both COX1 and COX2 are present in equal proportions in the luminal surface of endoplasmic reticulum and in the inner and outer membranes of the nuclear envelope in human umbilical vein endothelial cells (Spencer et al , 1998) There does not appear to be a different sub cellular localization of COX1 versus COX2

Similar to NOS enzymes, COX enzymes are also regulated by mechanical forces cyclic strain and shear stress Most studies demonstrate COX2 induction with cyclic strain or shear stress (Dancu et al , 2004, Okahara et al , 1998, Inoue et al ,

2002, Hendrickson et al , 1999) and no change in COX1 (Inoue et al , 2002) Some investigators have however demonstrated induction of COX1 with cyclic strain or shear stress (Okahara et al , 1998) These discrepancies can be explained by several variables apparatus type, magnitude of force, duration, culture media, force type (e g laminar, oscillatory or pulsatile shear stress), and finally, whether the cells are derived from micro- or macro-vascular vessels

1 2 2 2 Vasoconstrictors

1 2 2 2 1 Endothelin-1

Endothelin-1 (ET-1), which was initially isolated and identified in 1988 from conditioned medium of cultured porcine endothelial cells, is a potent vasoconstrictive peptide comprising 21 amino acid residues (Masaki, 2004) Analysis of human genomic DNA revealed the existence of three distinct ET-related genomic loci encoding three similar but distinct sequences of ET These three have been designated ET-1, ET-2 and ET-3, though ET-1 is the only isoform transcribed in the vasculature (*i e* endothelial cells)

ET-1 causes an increase in intracellular Ca^{2+} and initiates a signalling cascade that will lead to contraction via actin-myosin interaction The first step in this pathway is activation of calmodulin (CaM), a widely distributed 17kDa intracellular protein CaM can then activate myosin light chain kinase, a serine/threonine kinase, which catalyses the phosphorylation of myosin, allowing the actin-myosin interaction to occur, resulting in force production

The physiological importance of endogenous ET-1 in the maintenance of basal vascular tone and blood pressure in humans has been demonstrated by local and systemic vasodilation in response to inhibitors of the endothelin system (Verhaar et al , 1998) The plasma half-life of ET-1 is 4 to 7 minutes, and as such, vascular endothelial cells can rapidly adjust production as required for the regulation of vascular tone The translation of pre-proendothelin mRNA results in the formation of a 203 amino acid pre-proendothelin peptide, which is cleaved by a furin convertase to a 38 amino acid peptide big ET-1 Once formed, big ET-1 is processed to ET-1 through cleavage of the Try21-Val22 bond by ET-converting enzyme (ECE-1) (Luscher and Barton, 2000) 75% of ET-1 is released on the abluminal side, demonstrating a paracrine rather than endocrine effect Plasma concentrations are nevertheless clinically useful, since plasma concentrations have been found to correlate well with severity of some diseases, such as congestive heart failure (D'Orleans-Juste et al , 2002, Dschietzig et al , 2001)

ET-1 mediates its action via two G-protein coupled receptor subtypes, the endothelin type A receptor (ET_A) and type B receptor (ET_B) (Wackenfors et al , 2004) Both ET_A and ET_B receptors are present on VSMCs and pericytes, with ET_A the predominant subtype, whereas endothelial cells are reported to have ET_B only receptors Activation of endothelin receptors on VSMCs is known to cause vasoconstriction via a transient increase in intracellular Ca^{2+} ions through the phospholipase C pathway, and also via a sustained increase from Ca^{2+} influx from the extracellular space (Masaki, 2004) On the other hand, endothelial cell activation of ET_B promotes the release of nitric oxide and prostacyclin, thereby potentially limiting an excessive ET_A and/or ET_B mediated VSMC stimulation by ET-1 Thus far, it is not clear whether the receptors in endothelial cells and VSMCs represent the same or subtypes of ET_B

1 2 3 Endothelial Dysfunction

Endothelial dysfunction was initially identified as impaired vasodilation in response to specific stimuli such as acetylcholine or bradykinin (Chand and Altura, 1981) A broader understanding of the term would include not only a shift of the actions of the endothelium toward reduced vasodilation, but also an increased proinflammatory and prothrombic state (Endemann and Schiffrin, 2004) Such pathophysiologic alterations in endothelial structure and/or function are associated with most forms of cardiovascular disease, such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure (Endemann and Schiffrin, 2004)

Vascular pressure, exogenous and endogenous vasoactive substances, and metabolic activity each influence blood flow either by acting directly on mural cells or by influencing vascular endothelial cell release of vasodilators or vasoconstrictors Secretion of these vasoactive substances is finely balanced and generally tilted towards vasodilatation The regulatory function of the endothelium is altered by cardiovascular *risk factors* or disorders such as smoking, hypercholesterolemia and hyperglycaemia, thus disrupting the balance of vasodilators/vasoconstrictors A plethora of studies have shown mechanical forces associated with blood flow (shear stress and cyclic strain) modulate both production and release of NO (Gallis et al , 1999, Hendrickson et al , 1999), PGI_2 (Doroudi et al , 2000, Inoue et al , 2002, Hendrickson et al , 1999) and ET-1 (Yoshizumi et al , 1989, Morita et al , 1993, Sharefkin et al , 1991, Malek and Izumo, 1992, Kuchan and Frangos, 1993), however chronic alterations in mechanical forces associated with blood flow may damage this flow-induced response For example, in animal models of disease, poor dilatation in response to flow has been reported in isolated arterioles from hypertensive rats (Koller and Huang, 1994, Koller and Huang, 1999), in the coronary circulation of atherosclerotic pigs (Kuo et al , 1992), and also impaired myocardial contractility in patients with left ventricular hypertension (Palmieri et al , 2005)

Endothelial dysfunction in the retinal circulation results in either inadequate constriction (vasospasm) or dilation when needed, thus reducing the autoregulatory capacity of these vessels Due to the large number of symptoms caused by vasospasms, the term vasospasm or vasospastic syndrome is often used rather than endothelial dysfunction Vasospastic syndrome is often characterized by a tendency towards cold hands and feet, low blood pressure, higher incidences of migraine and Raynauds-like microvascular circulation (Flammer et al , 2001)

Attempts to determine a role of endothelial dysfunction in disease has focused on disturbance of the local and/or circulating balance of vasoactive factors - NO, PGI_2 and ET-1 (Drexler and Hornig, 1999, Nicolela et al , 2003) Healthy circulation maintains a balance tilted towards an excess of vasodilators over vasoconstrictors Several mechanisms may be involved in such a disturbance of this balance and thus cause vasospasm of vessels reduced synthesis or release of vasodilators, enhanced inactivation after their release, or increased amounts of circulating vasoconstrictors For example, plasma levels of asymmetric dimethylarginine (ADMA), an endogenously produced inhibitor of nitric oxide synthase (NOS), are elevated in disorders characterized by endothelial dysfunction - hyperhomocysteinemia(Boger et al , 2000, Boger et al , 2001), hypercholesterolemia (Boger et al , 2000), atherosclerosis (Miyazaki et al , 1999), diabetes (Asagami et al , 2002) and coronary artery disease (Maas et al , 2005) Additionally, in a canine model of microvascular renal failure, endothelial dysfunction was attributed to inhibition of NO production by elevated ADMA (Okubo et al , 2005)

The crucial role of NO in maintaining vascular homeostasis is also apparent with the association of eNOS polymorphisms with disorders that have in common a dysfunctional endothelium coronary heart disease, ischemic stroke, hypertension and Fabry's disease (Wilcox et al , 1997, Heltianu et al , 2005, Abe et al , 2005, Howard et al , 2005) Similarly, systemic factors such as hypercholesterolemia and hyperglycaemia are thought to impair endothelial NO signalling via oxidative stress damage Oxidative stress manifests as an imbalance between the levels of NO and reactive oxygen species (ROS) such as the superoxide anion (SO) (Miller et al , 2005, Napoli et al., 2001) Studies on various animal models of diabetes have

showed that administration of scavengers of ROS such a superoxide dismutase (SOD) and catalase (CAT) improved or normalised the agonist induced endothelium dependent dilation of arteries, suggesting that elevated levels of superoxide and hydrogen peroxide may inactivate NO after its release Clinical studies demonstrated decreased NO synthesis and increased ROS production in patients with essential hypertension, renovascular hypertension and malignant hypertension (Higashi et al , 2002, Lip et al , 2002) Indeed, recent reports targeting ECs with adenovirus expressing NOS prevents elevation of blood pressure in stroke-prone spontaneously hypertensive rats (Miller et al , 2005) Moreover, in diabetes, several studies using different animal models demonstrate a decrease in endothelium-dependent vasodilatation For example, streptozotocin diabetic rats have a decreased EC response to NO stimulators such as acetylcholine In diabetic patients, several reports suggest basal NO release is impaired (Calver et al , 1992, Elliott et al , 1993), and endothelium-dependent responses to methacholine in the forearm resistance vessels are impaired in insulin-dependent (Johnstone et al , 1993) and non-insulin dependent patients (Williams et al , 1996)

Coronary artery disease (CAD) is associated with impaired endothelium-dependent vasodilatation (Thanyasiri et al , 2005), often causing transient myocardial ischemia (Kawano and Ogawa, 2005) In humans, acetylcholine, serotonin, histamine, or ergonovine, which are all endothelium-dependent vasodilators by virtue of the release of nitric oxide, induce coronary dilation in young healthy subjects but cause vasoconstriction in patients with atherosclerosis (Kawano and Ogawa, 2005) In addition, recent experimental and clinical data have shown that endothelial dysfunction *precedes* the formation of arteriosclerotic lesions and is associated with an increased risk for future cardiovascular events (Schachinger and Zeiher, 2001) Endothelial dysfunction is also evident in advanced atherosclerosis, with compromised constitutive eNOS expression in ECs overlying atherosclerotic plaques

The physiological importance of endogenous ET-1 in the maintenance of basal vascular tone and blood pressure in humans has been demonstrated by local and systemic vasodilation in response to inhibitors of the endothelin system (Verhaar et al., 1998). Pulmonary hypertension is associated with increased ET-1 production in both in animal models and in patients (Yoshibayashi et al., 1991). Elevated plasma levels of ET-1 have been associated with CAD, essential hypertension and heart failure (Sainani et al., 2005, Lerman et al., 1991, Stewart et al., 1991), whereas ET-1 tissue concentrations in hypertensive rats correlate with systemic arterial pressure (Verma et al., 1995). Farkas et al. demonstrated an impaired forearm skin microcirculatory response in hypertensive patients due to increased ET-1, and also decreased response to acetylcholine (Farkas et al., 2005), suggesting the ET-1 and NO pathways interact, as has previously been described (Ohkita et al., 2003, Liu et al., 2003a, Lavallee et al., 2001). Interestingly, ET-1 can induce vasodilation via ET_B receptor activation on ECs, resulting in release of NO and PGI_2. The importance of endothelial ET_B is highlighted by the growing trend of pharmaceutical companies utilising ET_A specific antagonists, rather than non-selective ET-receptor antagonists in the treatment of hypertension (D'Orleans-Juste et al., 2002). Early studies demonstrated selective ET_A receptor antagonism preserves relaxations to endothelins (Takase et al., 1995). Reports by Strachan *et al* (1999) demonstrated increased peripheral vascular resistance in normal subjects with systemic administration of specific ET_B receptor antagonist, suggesting that the overall balance of effects of endogenous ET-1 at the vascular ET_B receptor favors vasodilatation (Strachan et al., 1999).

Several laboratories have reported the potential association of polymorphisms in the ET-1 gene and disease. Studies on dilated cardiomyopathy did not detect a role of genetic polymorphisms in the ET-1 gene (Charron et al., 1999, Herrmann et al., 2001). On the other hand, Brugada et al demonstrated a polymorphism of the ET-1 gene might act as a modifier gene in hypertrophic cardiomyopathy (Brugada et al., 1997). Two variants of the ET_A receptor have been studied with promising results, suggesting an association with idiopathic dilated cardiomyopathy (Charron et al., 1999) and nonischemic dilated cardiomyopathy

(Herrmann et al , 2001) A study in 528 never-treated hypertensives demonstrated that variants in the genes encoding ET-1 and the ET_A receptor are not significant determinants of cardiac morphometric parameters (Lajemi et al , 2001)

1 3 Vascular remodelling & Altered mechanical forces

Vascular remodelling can be described as any enduring change in the size or composition of an adult blood vessel Thus, processes such as vascular cell proliferation, apoptosis and/or matrix synthesis or degradation can significantly alter lumen or vessel dimensions Remodelling of a blood vessel may occur to accommodate and adapt to changes in haemodynamic forces or as a response to inflammation or injury

Inappropriate remodelling of the blood vessel is currently thought to be a major contributing factor to a number of pathologies such as those seen in atherosclerosis, restenosis and diabetic retinopathy (Gibbons and Dzau, 1994) In diseased tissue, additional factors are present locally such as inflammatory cytokines, inflammatory cells or modified cholesterol, and systematically, such as altered haemodynamic forces such as blood pressure or flow

A common feature of vascular disease is altered or elevated biomechanical stress This can lead to an alteration in the balance between VSMC proliferation and apoptosis, or an increase in VSMC migration, and result in remodelling of the vasculature (Wang et al , 1999) Spontaneous atherosclerotic lesions, for example, occur preferentially at bifurcations and curvatures of arterial blood vessels, where haemodynamic forces are disturbed (DeBakey et al , 1985, Thubrikar and Robicsek, 1995) In addition, venous vessels do not develop atherosclerosis when maintained in their normal low-pressure environment, however, atherosclerosis can be observed following arterial vein grafts due to the increased biomechanical force on the venous vessel (Xu, 2000) Alterations in VSMC fate decisions have been associated with numerous vascular disease states Proliferation of VSMC contributes to the pathogenesis of hypertension, intimal hyperplasia, atherosclerosis and the arterial response to injury (Vinters and Berliner, 1987, Thubrikar and Robicsek, 1995, Traub and Berk, 1998) Many studies have demonstrated that changes in intravascular forces that occur in disease states such as hypertension, result in a decreased lumen and increased media lumen ratios in arterial blood vessels, compared to normotensive patients (Nordborg et al , 1983)

Apoptosis plays a pivotal role both in normal vasculogenesis and in the pathobiology of the vascular system Apoptosis is virtually absent in normal adult vessels, but is a prominent feature of pathological conditions involving vascular remodelling This suggests a direct relationship between mechanical force and the regulation of apoptosis In recent years, many studies have independently proven the relationship between increased mechanical forces and increased levels of VSMC apoptosis Analysis of atherosclerotic lesions in both human and animal models reveal high levels of VSMC apoptosis (Kockx, 1998, Mayr and Xu, 2001) Isolated human SMC from atherosclerotic plaques display a higher tendency to undergo both spontaneous and induced apoptosis than VSMC isolated from normal vessels (Bennett, 1999) In addition, a study of biomechanical-induced apoptosis in the development of vein graft arteriosclerosis revealed that the number of apoptotic VSMC in the vein wall increased for at least eight weeks following grafting to an artery, but not to any vein, thus proving the effect of increased mechanical forces on apoptosis (Xu, 2000, Mayr et al , 2002)

1.3.1 Proliferation

The cell-cycle consists of an orderly set of phases, during which specific protein subsets are transcribed and assembled. Quiescent (G0) cells enter a gap period (G1), during which the factors necessary to replicate DNA for the subsequent synthetic (S) phase are assembled. After DNA replication is completed, the cells enter another gap phase (G2) in preparation for mitosis (M). Restriction points at the G1–S and G2–M interphases ensure orderly cell cycle progression (Figure 1.9).

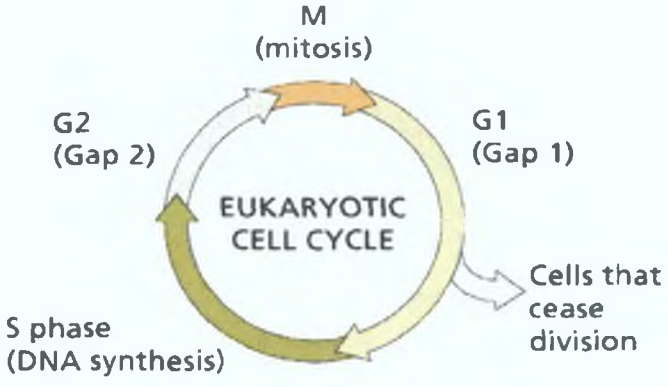


Figure 1.9: Schematic of the eukaryotic cell cycle and the phases G1, S, G2 and M.

The central components of the cell-cycle control system are cyclin-dependent protein kinases (Cdks), whose activity depends on association with regulatory subunits called cyclins. Oscillations in the activities of various cyclin-Cdk complexes lead to the initiation of various cell-cycle events. Thus, activation of S-phase cyclin-Cdk complexes initiates S phase, while activation of M-phase cyclin-Cdk complexes triggers mitosis. The activities of cyclin-Cdk complexes are influenced by several mechanisms, including phosphorylation of the Cdk subunit, the binding of special inhibitory proteins (CKIs), proteolysis of cyclins, and changes in the transcription of genes encoding Cdk regulators. Phase-specific cyclin–CDK complexes confer specificity and orderly progression through the cell cycle. Initially, increasing accumulation of cyclin D–CDK4 and cyclin E–CDK2 complexes, in

cooperation with proliferating cell nuclear antigen (pCNA), coordinate DNA replication by regulating the transition through the G1 and S phases (Sherr, 1995). Antimitogenic signals activate p53, which induces expression of the CKI p21 CIP1 and consequently inhibits the activity of the G1 cyclin–CDK complexes, resulting in G1-phase arrest (Levine, 1997). Conversely, the E2F family of transcription factors controls expression of genes in S-phase (Sherr, 1995). In quiescent conditions, E2F members exist in inactive complexes with retinoblastoma (RB) protein. After mitogenic stimulation, the cyclin D–CDK4 and cyclin E–CDK2 complexes hyperphosphorylate RB, leading to dissociation of E2F, which in turn activates the expression of genes such as those encoding cyclins E and A and CDK1. In addition to p53 and E2F, GAX and GATA-6 are also relevant cell cycle–associated transcription factors in VSMCs. GAX, a homeobox transcription factor that regulates cell differentiation, proliferation and migration, is expressed in quiescent VSMCs (Smith et al., 1997). GATA-6 is a transcription factor involved in tissue-specific gene expression including VSMCs (Narita et al., 1996). Both stimulate the expression of p21 CIP1 and induce subsequent cell cycle arrest (Smith et al., 1997) (Perlman et al., 1998). Both are downregulated by mitogen stimulation *in vitro* and in response to vascular injury *in vivo* (Smith et al., 1997, Narita et al., 1996). Finally, transcription factors are not the only means by which the cell cycle is regulated in VSMCs. NO represses mitogen-stimulated cyclin A promoter activity, resulting in a cell cycle arrest through blockade of cyclin A mRNA and protein upregulation (Guo et al., 1998). In addition NO inhibits proliferation by upregulation of p21 CIP1 (Ishida et al., 1997).

1.3.2 Apoptosis

Cells of multi-cellular organisms generally die in one of two well-characterized ways, depending on the context and cause of death. These two forms of cell death, apoptosis and necrosis, can be defined and contrasted on the basis of their individual mechanisms, biochemistry, and altered cellular morphology (Hetts, 1998) (Figure 1.10). Necrosis is a passive form of cell death, resulting from external noxious stimuli, inducing localized injury and inflammation. A classic example of necrosis is ischemic necrosis of the cardiomyocyte during acute myocardial infarction (Yeh, 1997). The necrotic process is characterized by severe cell swelling, breakdown of the membrane barrier and resulting release of the cellular components into the extracellular space and random degradation of nuclear DNAs. The release of extracellular components such as kinins incites localized inflammation, edema, capillary dilation and macrophage aggregation (Kuan and Passaro, 1998, Hetts, 1998, Yeh, 1997). The inflammatory response is lengthy and unpredictable in its timecourse, often taking hours to days to occur and subside (Kuan and Passaro, 1998). Although necrosis may be important in acute injury and certain acute inflammatory responses, it is not the mechanism whereby cells normally die (Hetts, 1998).

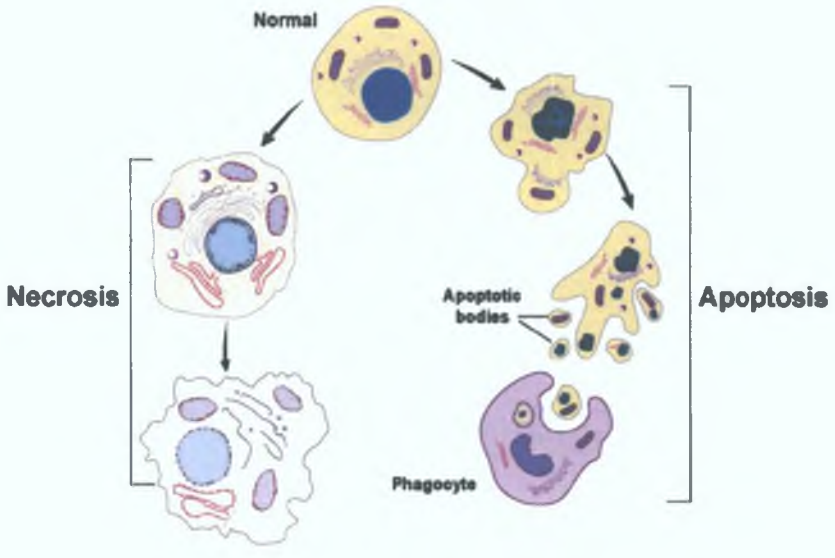


Figure 1.10: Morphology of necrosis and apoptosis. Necrotic cells and their organelles are characteristically swollen. There is early membrane damage with eventual loss of plasma membrane integrity and leakage of cytosol into extracellular space. Despite early clumping, the nuclear chromatin undergoes lysis. In contrast, apoptotic cells are shrunken and develop blebs containing dense cytoplasm. Membrane integrity is not lost until late, after cell death. Nuclear chromatin undergoes striking condensation and fragmentation.The cytoplasm becomes divided to form apoptotic bodies containing organelles and/or nuclear debris. Terminally, apoptotic cells and fragments are engulfed by phagocytes or surrounding cells

http://200.72.204.110/web/images/Biologia/Apoptosis/

In contrast, apoptosis is an active, contained process, resulting from either external or internal stimuli (Kuan and Passaro, 1998). Apoptosis, or programmed cell death, is recognized as an important physiological process, both during development and in the maintenance of homeostasis in the adult. This mode of cell death allows for the removal of damaged, injured, infected or incompetent cells from the body both quickly and efficiently. The morphology of an apoptotic cell is clearly distinct to that of a necrotic cell. The apoptotic process is characterized by cell shrinkage and subsequent membrane blebbing, chromatin condensation around the nuclear membrane, and cleavage of the DNA into regular repeating 180 – 200 base pair units (Steller, 1995, Yeh, 1997). Apoptotic bodies are formed due to cleavage of the membrane, these are phagocytosed and digested by macrophages or

neighbouring cells, or undergoes secondary necrosis. As no cytosolic components are released into the extracellular space, an inflammatory response is not initiated (Kuan and Passaro, 1998, Hetts, 1998). Unlike necrosis, this process is relatively rapid, reaching completion in approximately two hours (Kuan and Passaro, 1998).

The genetics and molecular mechanisms of apoptosis were first characterized during studies in *C. elegans*. Programmed cell death during the development of this nematode is highly precise and predictable, of the 1090 cells produced during development, 131 are destined to die (Ellis et al., 1991). Such studies have identified four sequential steps during the process of apoptosis, commitment to cell death induced by extracellular or intracellular triggers, activation of intracellular proteases, engulfment of the apoptotic bodies by other cells, and degradation of the apoptotic bodies within the lysosomes of the phagoctyotic cells (Steller, 1995). Genetic analysis of factors involved in apoptosis in *C. elegans* implicated three main genes, cell death defective 3 (ced 3), ced 4 and ced 9. As the genetic control of apoptosis is conserved throughout evolution, human homologues of these genes have been identified, these are caspase 8, Apaf-1, and Bcl-2 respectively (Hetts, 1998).

1.3.2.1 Triggers of Apoptosis

Apoptosis-inducing stimuli can be either extrinsic or intrinsic, and can cause apoptosis through the activation of a number of different pathways. In most cases, however, these pathways converge on the caspase system of enzymes to execute their function. Extrinsic triggers of apoptosis include activation of receptor-mediated death-signalling pathways, for example Fas ligand activation, exposure to substances that cause DNA damage including chemotherapeutic agents and ionizing radiation (Hetts, 1998, Rich et al., 2000). Apoptosis can also be induced due to the removal of death-inhibiting (or survival-promoting) ligands, for example, vascular smooth muscle cells undergo apoptosis due to the withdrawal of growth factors, such as insulin-like growth factor and PDGF (Best et al., 1999). In addition, intrinsic signals such as increased intracellular oxidative stress, can cause the initiation of apoptosis within the cell, in which the mitochondria play a pivotal role (Desagher and Martinou, 2000).

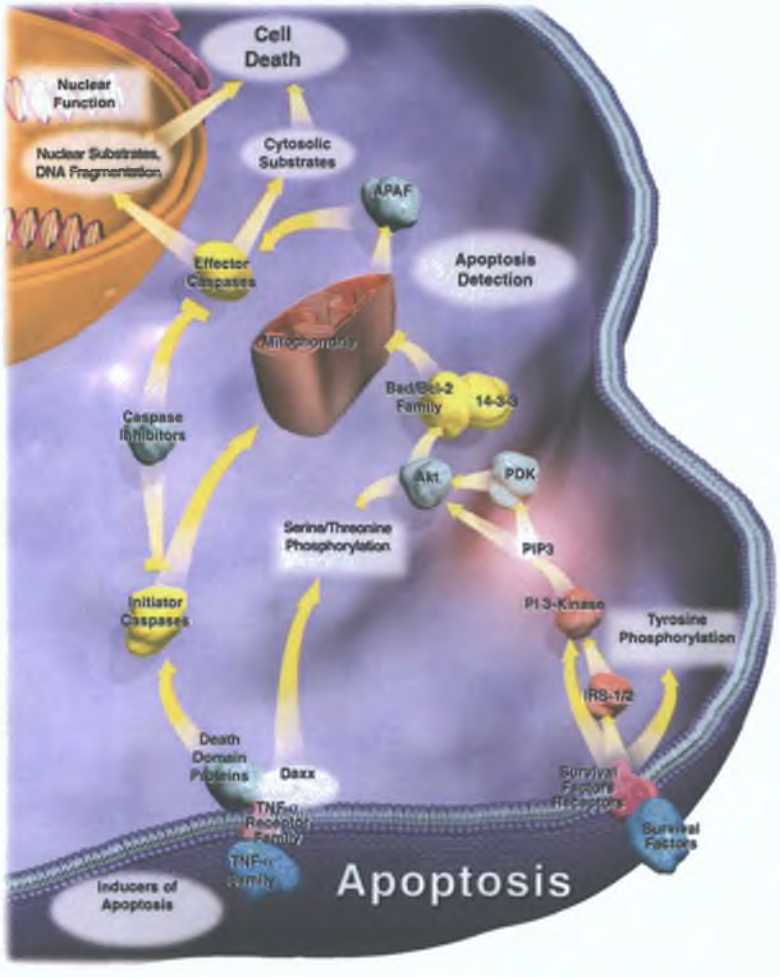


Figure 1.11: Schematic diagram of the multiple pathways involved in inducing, inhibiting, detecting and effecting the apoptotic event http://www.chemicon.com/resource/

1.3.2.1.1 Receptor-Mediated Death Signalling Pathways

Death receptors belong to the tumour necrosis factor (TNF) superfamily, and play a central role in instructive apoptosis (Ashkenazi and Dixit, 1998). Members of the death receptor family contain one to five cysteine-rich repeats in their extracellular domain, and a death domain (DD) in their cytoplasmic tail. This DD is essential for initiation of the apoptotic signal by these receptors. TNF receptor 1 and Fas (CD95) are two such receptors that initiate apoptosis when activated either by their respective ligands, TNF-α and Fas-L, or by agonist-like antibodies (MacLellan and Schneider, 1997, Gupta, 2003) (Figure 1.11). Following receptor-ligand interaction, the receptor oligomerizes, recruits adaptor molecules forming a death inducing signalling complex (DISC), which recruits and activates the caspase cascade and can culminate in apoptosis of the cell (Gupta, 2003, Yeh, 1997).

1.3.2.1.2 Apoptosis due to DNA Damage

Growth arrest, repair and apoptosis are all legitimate cellular responses to DNA damage. The choice of cell fate in each instance will depend on cell type, location, environment, and extent of damage.

p53 is a transcription factor that has been implicated in cell cycle arrest and in some, but not all, forms of apoptosis (MacLellan and Schneider, 1997). The level of p53 activity within the cell is maintained at a low level under normal conditions due to interaction with the Mdm-2 protein, which marks it for ubiquitin-mediated destruction (Mayo et al., 1997). DNA damage-induced phosphorylation of either p53 or Mdm-2 prevents these two proteins from interacting, thus stabilizing and activating p53 (Evan and Littlewood, 1998). p53 levels are reported to increase within minutes of DNA damage, resulting in growth arrest or apoptosis of the cell (Lundberg and Weinberg, 1999).

Several cell-cycle regulators are induced by p53, including the cyclin-dependent kinase inhibitor p21, GADD 45 and members of the 14-3-3 family, resulting in growth arrest, followed by either DNA repair or cell death (Rich et al.,

2000). p53 alone will not induce apoptosis, but acts as a transcription factor, activating the expression of numerous apoptosis-mediating genes (Bennett et al., 1995). In contrast to these findings, it appears that a sustained and moderate level of p53 elevation induced by laminar shear stress causes endothelial cell cycle arrest, enhances DNA repair, and serves a protective function against excessive cell proliferation and cell death caused by increasing GADD 45 (Lin et al., 2000).

1.3.2.1.3 Mitochondrial Pathway of Apoptosis

A number of stimuli, including UV radiation, stress molecules (reactive oxygen and nitrogen species), and growth factor withdrawal, mediate apoptosis via the mitochondrial pathway (Gupta, 2003). During the process of apoptosis the mitochondria undergo morphological and cellular re-distribution changes. The mitochondria undergo a reduction in size and an increase in matrix density known as mitochondrial pyknosis. In addition, the mitochondria which are normally dispersed throughout the entire cell, display perinuclear clustering (Desagher and Martinou, 2000).

Mitochondria are organelles comprising of a matrix surrounded by an inner membrane (IM), an inter-membrane space, and an outer membrane (OM). The IM contains molecules that contribute to the formation of an electrochemical gradient or membrane potential, which include adenosine tri-phosphate (ATP) synthase and adenine nucleotide translocator. The OM contains a voltage-dependent anion channel, whilst the inter-membrane space contains proteins that contribute to apoptosis when activated, including holocytochrome c, some pro-caspases, and apoptosis-inducing factor (AIF) (Gupta, 2003) (Figure 1.12). Several mechanisms contribute to mitochondrial-mediated apoptosis. These include disruption of electron transport, oxidative phosphorylation and ATP production, alteration of the cellular redox potential, and release of proteins, such as cytochrome c, that trigger activation of the caspase family of proteases (Green and Reed, 1998).

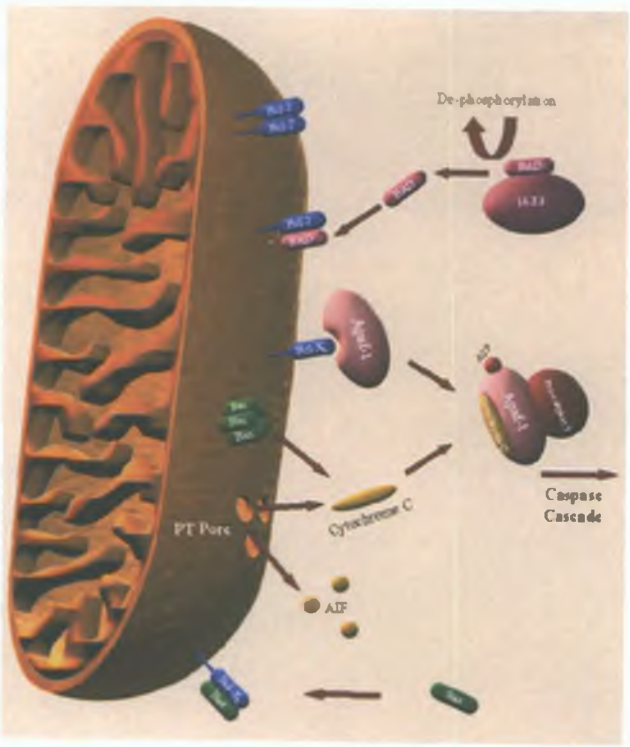


Figure 1.12: Schematic diagram of the mitochondria and its role in apoptosis. (www.sgul.ac.uk/depts/immunology/~dash/)

1.3.2.2 Effectors of Apoptosis

1.3.2.2.1 The Caspase Cascade

Caspases are an evolutionally conserved family of cysteine proteases, which are viewed as the "central executioners" in apoptotic cell death. Caspases are synthesized as enzymatically inert zymogens, requiring proteolytic cleavage at an internal aspartate residue to induce their activation (Gibbons and Pollman, 2000, Hengartner, 2000). These zymogens are composed of three domains, an N-terminal pro-domain, and two domains termed p10 and p20. The activation of caspases generally results in a serial sequence of caspase activation, known as the caspase cascade, which is a common end pathway in apoptosis induced by many different stimuli.

The aggregation of a number of caspase proteins via adaptor proteins renders the caspases capable of auto-proteolytic cleavage, and subsequent activation (Hengartner, 2000). Most caspases are activated by cleavage between the p10 and p20 domains, and between the p20 and N-terminal pro-domain. Activation of caspase 8 and caspase 9, known as initiator caspases, results in subsequent cleavage and activation of downstream effector caspases, such as caspase 3, caspase 6 and caspase 7. The effector caspases are responsible for the induction of the biochemical and morphological changes associated with apoptosis, and are usually more abundant and active than the initiator caspases (Gibbons and Pollman, 2000, Hengartner, 2000).

Caspase 9 is activated through association with a regulatory subunit, known as an apoptosome. The apoptosome consists of cytochrome c, an adapter molecule Apaf-1 (apoptosis protease-activating factor), and pro-caspase 9 (Hengartner, 2000, Gupta, 2003). Cytochrome c is a nuclear DNA encoded protein; its precursor, apocytochrome c, is synthesized on free ribosomes within the cytoplasm, and can spontaneously insert into the mitochondrial outer membrane (Stuart and Neupert, 1990, Gonzales and Neupert, 1990). This protein then incorporates a heme group, the protein re-folds, and is inserted into the inter-membrane space. The release of functional cytochrome c is reported to be an essential component for the formation

of the apoptosome, and subsequent activation of caspases 9 and 3 (Liu et al., 1996). Apaf-1 is another essential component of the apoptosome, and appears to be activated by p53 and adenoviral E1A (Fearnhead et al., 1998, Moroni et al., 2001). As Apaf-1 does not have caspase activity, it is proposed that it facilitates caspase 9 auto-catalysis (Cai et al., 1998).

Caspase 9 subsequently cleaves and activates caspase 3, caspase 6, and a number of other substrates resulting in the biochemical and morphological characteristics of an apoptotic cell. These substrates include caspase-activated DNase (CAD), nuclear laminins, cytoskeletal proteins, and p21-activated kinase 2, among others. Activation of CAD within the cell results from the caspase 3-mediated cleavage of the CAD inhibitory subunit. This active nuclease is subsequently responsible for the characteristic "DNA laddering" of apoptosis. Cleavage of cytoskeletal proteins, such as fodrin and gelsolin, results in an overall loss of cellular shape (Kothakota et al., 1997), whereas nuclear laminin cleavage is responsible for the characteristic nuclear shrinkage and budding seen in apoptosis (Rao et al., 1996). In addition, caspase-mediated cleavage of PAK 2, a member of the p21-activated kinase family, appears to mediate the distinctive blebbing of apoptotic cells (Rudel and Bokoch, 1997).

Whilst caspase activation undoubtedly plays an important role in the initiation and execution of apoptosis, a number of caspase-independent inducers of apoptosis have also been identified, such as reactive oxygen species (ROS) (Suzuki et al., 1997). The generation of oxidants is involved in changes in mitochondrial permeability, and release of molecules, other than cytochrome c involved in the execution of apoptosis. AIF, apoptosis inducing factor, is one such molecule that is released from the mitochondria, and can induce caspase-independent apoptosis. AIF is transported to the nucleus where it causes ATP-independent large DNA fragmentation and chromatin condensation (Susin et al., 1996, Gupta, 2003). In addition, the release of EndoG, a mitochondrion-specific nuclease that translocates to the nucleus, cleaves chromatin DNA during apoptosis (Li et al., 2001).

1 3 2 2 2 The Bcl-2 Family

Bcl-2 was initially identified as a frequent translocation occurring in human B-cell follicular lymphoma, and was found to function by promoting cell survival (Kirshenbaum, 2000) Bcl-2 is now recognized as being part of a large family of homologous proteins that can either promote or suppress apoptosis, known as the Bcl-2 family The Bcl-2 family are considered the primary regulators of mitochondria-induced apoptosis, controlling mitochondrial membrane permeabilization and cytochrome c release (Thompson, 1995, Marsden et al , 2002, Desagher and Martinou, 2000)

At least fifteen members of the Bcl-2 family have been identified, and these can be divided into two functional groups, pro-apoptotic and anti-apoptotic Bcl-2 family members Examples of family members that prevent apoptosis are Bcl-2, Bcl-xL and Bfl-1 among others, whilst Bcl-2 family members that promote apoptosis include Bad, Bax, Bid and Bik, (Table 1 1) (Green and Reed, 1998, Reed, 1994, Sedlak et al , 1995)

Protein	Effect on Apoptosis	Protein-Protein interaction
BCl-2	decrease	Bax, Bak
BCl-XL	decrease	Bax, Bak
BCl-W	decrease	
Bax	increase	BCl-2, BCl-XL
Bad	increase	BCl-2, BCl-XL
Bak	increase	BCl-2, BCl-XL
BCl-XS	increase	Bax, Bak

Table 1 1 Pro- and anti-apoptotic members of the BCl-2 family of proteins

Structural analysis of the Bcl-2 family of proteins has identified four conserved regions within the family, known as the Bcl-2 homology domains (BH1-BH4) All members of the Bcl-2 family contain at least one of these domains, which are formed by α-helices and thus enable different members of the family to form either homo- or heterodimers and regulate each other (Kelekar and Thompson, 1998, Oltvai et al , 1993) The majority of Bcl-2 family members share sequence

homology at the C-terminal region, with a ~20-residue hydrophobic domain, which targets the Bcl-2 family of proteins to intracellular membranes The principal membrane to which the Bcl-2 family members are directed is the outer mitochondrial membrane, therefore, this C-terminal region is critical for the function of both the pro- and anti-apoptotic Bcl-2 family members (Goping et al , 1998, Kirshenbaum, 2000) Variable sequence homology, however, exists between the BH1 to BH4 domains, which implies that this variation in homology may determine whether the given Bcl-2 family member acts to promote or prevent cell death (Kirshenbaum, 2000) The anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, contain at least three BH domains, and all contain the N-terminal BH4 domain The BH4 domain is restricted to Bcl-2 family members with anti-apoptotic properties, therefore, it is postulated that this domain is critical in preventing apoptosis This is supported in a number of studies in which the deletion of the BH4 domain rendered the anti-apoptotic Bcl-2 protein defective in suppressing apoptosis (Hunter et al , 1996, Huang et al , 1998) Pro-apoptotic Bcl-2 family members, such as Bax and Bak, have been identified as closely resembling Bcl-2, containing BH1-BH3 domains Other pro-apoptotic members of this family are described as "BH3 only" as they contain the BH3 domain alone, which is therefore sufficient for the pro-apoptotic activity of these proteins (Kelekar and Thompson, 1998) Cell fate is determined by the ratio of pro- and anti-apoptotic members of the Bcl-2 family within any given cell (Reed, 1997, Sedlak et al , 1995)

The Bcl-2 family can delay or prevent apoptosis by a diverse number of death signals, thus suggesting that it influences a number of signalling factors that can lead to cell death Bcl-2 has been shown, for example, to increase transactivation of the anti-apoptotic NFκB, which can, in turn, up-regulate anti-apoptotic Bfl-1 and Bcl-xL expression (Lee et al , 1999a, Kirshenbaum, 2000)

However, members of the Bcl-2 family primarily exert their pro- or anti-apoptotic influence through regulation of mitochondrial membrane potential and the corresponding cytochrome c release Upon stimulation of apoptosis, many members of the pro-apoptotic Bcl-2 family translocate from the cytoplasm to the mitochondria Following a conformational change, these proteins can insert into the

mitochondrial membranes, disrupting membrane integrity and increasing mitochondrial membrane potential This results in the release of several mitochondrial proteins involved in caspase activation and other apoptotic events (Zamzami and Kroemer, 2001, Goping et al , 1998) The pro-apoptotic protein, Bax, for example, is normally present in the cell cytoplasm Following stimulation of apoptosis Bax migrates to the mitochondria where it inserts into the mitochondrial membrane and forms a homodimer, resulting in an increase in mitochondrial membrane potential, thus facilitating apoptosis Similarly, the pro-apoptotic protein Bid is cleaved by caspase 8, and the resulting C-terminal fragment, tBid, translocates to the mitochondria Bid therefore mediates crosstalk between the death receptor and mitochondrial pathways of apoptosis tBid facilitates insertion of other pro-apoptotic proteins into the mitochondrial membrane, and promotes Bax dimerization (Ferri and Kroemer, 2001, Eskes et al , 1998, Jurgensmeier et al , 1998)

Conversely, many of the anti-apoptotic Bcl-2 family proteins are associated with the mitochondrial membrane, where they act to inhibit increases in mitochondrial membrane potential, and prevent apoptosis by maintaining membrane integrity Both the pro- and anti-apoptotic members of the Bcl-2 family appear, at least in part, to regulate each other Bcl-2, for example, can form a heterodimer with Bax, thus inhibiting the ability of Bax to increase mitochondrial membrane potential Similarly, pro-apoptotic members can exert their effect by binding to their anti-apoptotic counterparts Bad, for example, binds to Bcl-xL thus inhibiting its anti-apoptotic function (Ferri and Kroemer, 2001, Zamzami and Kroemer, 2001, Desagher and Martinou, 2000) In addition, many factors within the cell regulate the level of expression of Bcl-2 family of proteins For example, increased levels of p53 tumour suppressor protein can increase Bax expression (Miyashita and Reed, 1995)

Bcl-2 family members also appear to modulate other cellular processes in addition to apoptosis, however this appears to be restricted to certain family members Bfl-1 is an anti-apoptotic member of the Bcl-2 family that also exhibits proliferative and potent oncogene transforming activities (D'Sa-Eipper and Chinnadurai, 1998) Bfl-1, therefore, communicates with both the apoptotic and

proliferation cell machineries, suggesting a link between these two cellular processes

The importance of the Bcl-2 family of proteins in normal physiology is highlighted by the fact that Bcl-2- and Bcl-xL-deficient mice die either at an embryonic stage or immediately post-natal due to increased apoptosis in multiple organs and tissues of the body (Veis et al , 1993, Motoyama et al , 1995)

1 3.3 Glaucoma

Glaucoma is a group of diseases distinguished by characteristic damage to the bundle of nerve fibers that carries information from the eye to the brain (optic nerve) – termed glaucomatous optic neuropathy (GON) (Figure 1 13A) Typically, there are two classifications of glaucoma open-angle and closed-angle

Closed-angle glaucoma results from a sudden, complete blocking of the fluid flowing out of the eye Symptoms may include severe pain, nausea, vomiting, blurred vision, and seeing a rainbow halo around lights Closed-angle glaucoma is a medical emergency and must be treated immediately or blindness could result in one or two days

Symptoms of open-angle glaucoma include a gradual and often imperceptible failing of peripheral vision, leading to only a small central area of vision (Figure 1 13 B, C, D & E) If the entire optic nerve is destroyed, total blindness will result Worldwide, it is estimated that about 66 8 million people have visual impairment from glaucoma, with 6 7 million suffering from blindness In the United States, approximately 2 2 million people age 40 and older have glaucoma, and of these, as many as 120,000 are blind due the disease The number of Americans with glaucoma is estimated to increase to 3 3 million by the year 2020 (American Health Assistance) Each year, there are more than 300,000 new cases of glaucoma and approximately 5,400 people suffer complete blindness Vision experts estimate that half of those affected may not know they have it because symptoms may not occur during the early stages of the disease

The pathogenesis of optic nerve damage in glaucoma is still not fully understood, however substantial evidence indicates that GON is multifactorial in nature, with elevated intraocular pressure (IOP) being the most common risk factor In fact, glaucoma was initially described as a pressure-related disease and this is still largely the case The realisation that GON often occurs in the absence of elevated intraocular pressure and, conversely, elevated intraocular pressure may occur without associated damage of the optic nerve has encouraged investigators to search for additional factors that may insult the optic nerve It is possible that ischaemia,

hypoxia, disruption of axoplasmic transport or a genetically determined accelerated retinal ganglion cell apoptosis is responsible for the optic neuropathy characteristic of glaucoma, with more than one mechanism contributing to the pathology in some individuals

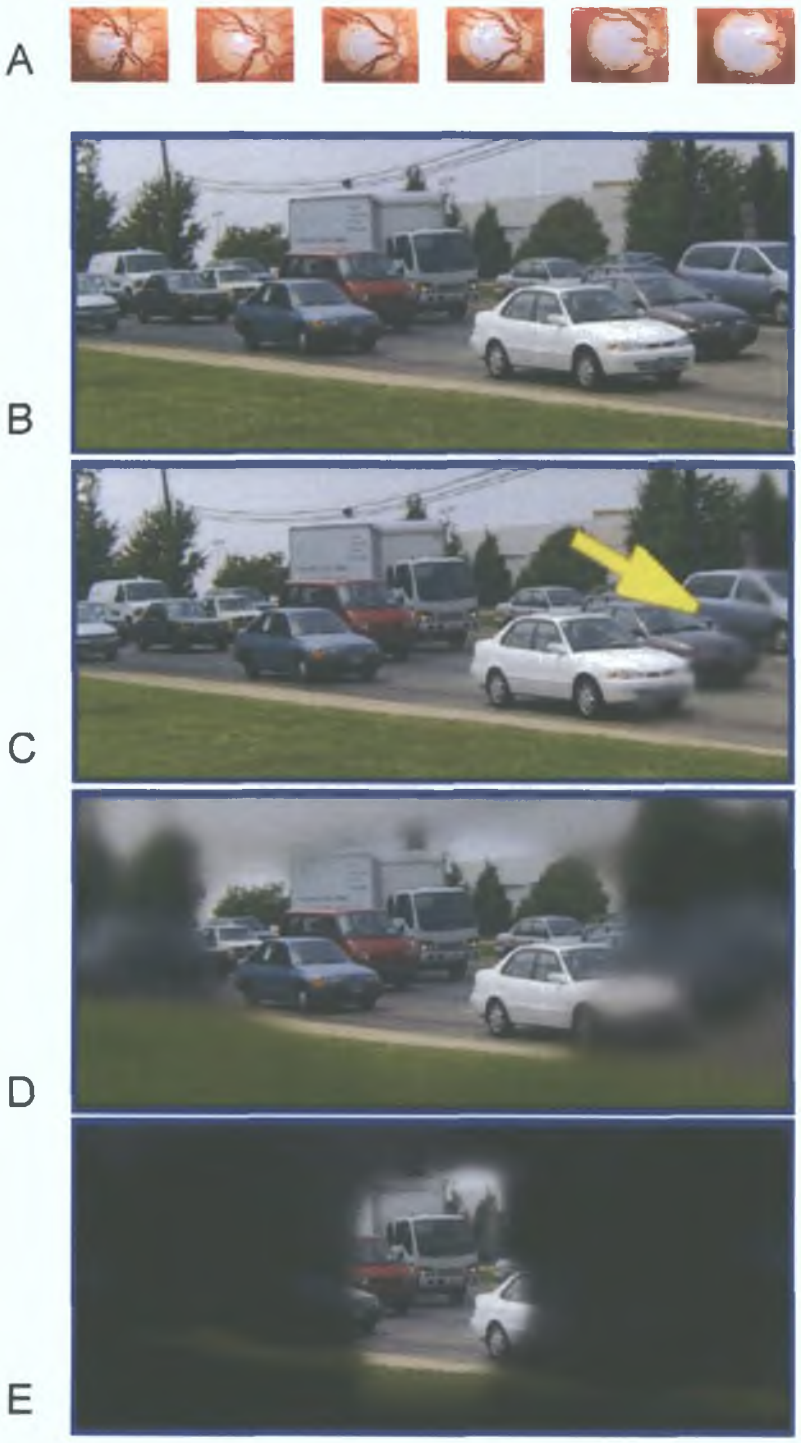


Figure 1.13: Progressive cupping of the optic nerve head causing glaucomatous optic neuropathy - GON (A). Typical vision loss due to GON, **(B)**: Normal; **(C)**: Early (Arrow indicates early blurring of vision); **(D)**: Intermediate; **(E)**: Late Stage GON

In normal tension glaucoma (NTG), where IOP values are within the normal range (i e < 21 mm Hg), IOP is considered to be a risk factor of lesser significance than in high tension glaucoma (HTG) (open-angle glaucoma), and other factors take on greater significance Various vascular and cardiovascular disorders are recognised as being risk factors for NTG since they tend to occur more frequently in these patients They include impaired autoregulation of blood flow in the optic nerve head, systemic hypotension, arterial hypertension, increased blood viscosity, diabetes, migraine and other generalised vasospastic disorders such as cold hands or feet Each of these risk factors tends to support a vascular cause, or at least a vascular component in the cause of glaucoma, where optic nerve perfusion may be affected (Harris et al , 2001) An additional hypotensive factor under investigation is a nocturnal fall in blood pressure, where it has been shown that both POAG and NTG patients with progressive field loss are more likely to have lower nocturnal blood pressure findings than those with stable fields

NTG comprises a significant proportion of the generic grouping of glaucoma, although this proportion varies between samples and possibly between different populations For example, Sommer states that 20 to 25 per cent of GON develops with normal IOP (Sommer, 1996), while in a recent Dutch survey, which found open angle glaucoma in 1 1 per cent of adults 55 years or older, 39 per cent of these cases had statistically normal IOP (Dielemans et al , 1994) Similarly, a 1997 Italian study of a population over 40 years of age found a prevalence of POAG of 1 4 per cent and NTG of 0 6 per cent, showing that 33 per cent of open angle glaucoma cases had NTG (Bonomi et al , 1998) The Swedish Dalby study of 1981 found NTG in 61 per cent of open angle glaucoma cases (Bengtsson, 1981)

The optic nerve head is generally held to be the primary site of pathology in glaucoma (Quigley, 1999, Hernandez, 2000) (Figure 1 13A) GON is characterised by loss of ganglion cell axons, the consequence of which is an excavated appearance of the optic disc and progressive visual field loss, a process which is essentially common to both NTG and HTG The loss of the axons from the optic nerve occurs in a topographic pattern that matches visual field loss in glaucoma This consists of

preferentially greater injury at the upper and lower poles of the optic nerve in a pattern shaped like an hourglass This seems related to the regional structure of the supporting connecting tissues of the optic nerve head, that is, a lower density of connective tissue in the upper and lower poles These areas also show a preponderance of larger ganglion cells that have a greater susceptibility to injury in glaucoma (Schwartz, 2003, Tomita, 2000) As alluded to earlier, glaucomatous axonal damage is hypothesised to occur as a result of one of two general mechanisms (Flammer et al , 2002) First, mechanical disruption of axoplasmic transport may occur because of distortion and kinking of axons during passage through the lamina cribrosa (especially evident in glaucoma associated with high pressure) This mechanical effect results in backward bowing of the lamina cribrosa, axon loss and progressive cupping The second hypothesis argues that axonal transport is detrimentally affected by a primary hypoxia associated with elevated pressure or other causes of decreased optic nerve head perfusion For the primary eye care practitioner, detection of NTG can be especially difficult, since early in glaucoma, there is overlap between normal and abnormal optic disc appearances Disc damage is often difficult to judge, with the assessment requiring careful, and often sequential examination to recognise change (Sack, 2000)

1 3 3 1 Normal Tension Glaucoma Altered Blood Flow & Endothelial Dysfunction

Vasospasm due to endothelial dysfunction or other mechanisms resulting in transient decreases in blood flow to the optic nerve, have been thought of as possible causes of GON particularly when IOP measurements are within the normal range (Flammer et al , 2001) Some evidence of the underlying cause of NTG has emerged through an association between NTG and migraine where some stages of the attack are considered to be due, in part, to vasospasm (Corbett et al , 1985, Phelps and Corbett, 1985, Cursiefen et al , 2000) McKenndrick et al recently demonstrated visual field defects associated with migraine are similar to those reported early in glaucoma (McKendrick et al , 2000) Vasospasm often occur in response to cold-provocation Immersion of the hands in ice-cold water produced vasospasm in the form of more prolonged nailbed capillary spasm in the fingers of patients with NTG when compared to normal subjects (O'Brien and Butt, 1999, Drance et al , 1988) When the NTG subjects were subdivided into those with and without migraine, increased vasospastic response of this kind was seen with greater frequency in the migraine subgroup of NTG subjects Interestingly, HTG and migraine were not associated in either the Blue Mountains (Wang et al , 1997) and Beaver Dam studies (Klein et al , 1993) suggesting raised IOP and vascular insufficiency might act as independent risk factors for GON Measurement of forearm blood flow using venous occlusion plethysmography in NTG, HTG and controls, found an impairment of peripheral endothelium-mediated vasodilatation in NTG, when compared to HTG and controls (Henry et al , 1999) Furthermore, it has been suggested that peripheral vasodilators, such as calcium channel blockers, slow the progression of, or improve visual field damage in selected patients with vasospastic glaucoma

Studies using a measure of ocular vascular perfusion—the pulsatile ocular blood flow (POBF)—have shown that patients with NTG and HTG have a significantly lower POBF than normals (Fontana et al , 1998, Agarwal et al , 2003) Moreover, in patients with unilateral NTG, the eye with glaucomatous damage has a lower POBF than the unaffected eye (Fontana et al , 1998), suggesting that

haemodynamic differences between fellow eyes contribute to determine the side of onset of the disease. In patients with symmetric NTG, duplex sonography showed an elevated resistance to blood flow in the central retinal artery and posterior ciliary artery which are responsible for perfusing the optic nerve head (Akcar et al., 2005). Another implied measure of blood flow to the optic nerve is obtained with colour Doppler imaging, which assesses blood flow in the ophthalmic, ciliary and central retinal arteries. Using this technique, a statistically reduced mean systolic peak flow velocity is found in HTG patients with progressive field loss compared to normals (Butt et al., 1997). Moreover, in both HTG & NTG there is increased resistance to blood flow in the ophthalmic and central retinal arteries compared to control subjects (Butt et al., 1995). Furthermore, retinal arteriovenous passage times are also significantly prolonged in NTG (Arend et al., 2000, Arend et al., 2002) and noctural blood pressure 'dipping' is a distinct risk factor for glaucomatous damage in both HTG and NTG patients (Pache et al., 2003).

Association of altered systemic blood flow responses with NTG led investigators to examine the vasoactive mediators NO, ET-1 & PGI_2 in patients. Buckley et al demonstrated vascular endothelium modulation of contractile responses to both 5-HT and ET-1 in human subcutaneous resistance arteries, but this effect is lost in patients with NTG, indicating a selective defect in agonist mediated release of endothelium derived vasodilators (Buckley et al., 2002). Plasma and aqueous humour levels of cGMP, an indirect indicator of NO are decreased in patients with NTG (Galassi et al., 2000) concomitant with lower systolic and diastolic velocities of the ophthalmic artery when examined with colour Doppler imaging. Similarly, the same laboratory demonstrated decreased concentrations of NO_2^- and cGMP in the plasma and aqueous humour of high tension glaucoma patients (HTG) (Galassi et al., 2004). HTG patients have decreased retinal blood flow velocities, which would impair flow induced EC-NO release. In some conditions, including hypercholesterolaemia (Brandes et al., 1997, Kagota et al., 1999), impairment of the NO component of relaxation may be balanced by an increase in the as yet unidentified vasodilator - endothelium derived hyperpolarising factor (EDHF). EDHFs contribute to the maintenance of endothelium dependent

relaxation in systemic resistance arteries from patients with NTG (Cleary et al , 2005)

eNOS gene variants are associated with ischemic heart disease (Casas et al , 2004), hypertension (Miyamoto et al , 1998), carotid atherosclerosis (Lembo et al , 2001) and diabetic nephropathy (Nagase et al , 2003) A variant in the promoter region of the eNOS gene was found in a significant percentage of familial HTG patients (Tunny et al , 1998) Similarly, Wiggs et al have demonstrated a polymorphism in the eNOS gene in both HTG and NTG patients, with these patients more susceptible to early loss of central vision (arvo 2005) A significant difference was found in the distribution of allele frequencies of the eNOS marker in subjects who had glaucoma with migraine versus control subjects (Logan et al , 2005) No association was found with iNOS, which is normally expressed in response to injury, inflammation, and ischemia in vascular or nonvascular cells However, pharmacalogic inhibition of iNOS provides neuroprotection of retinal ganglion cells in a rat model of chronic glaucoma suggesting a inflammatory response in HTG (Neufeld et al , 1999) C-Reactive protein (CRP) is a reliable measure of underlying systemic inflammation and a strong predictor of future myocardial infarction and stroke Recent evidence has associated CRP levels with NTG (Leibovitch et al , 2005) Indeed, CRP has been shown to decrease eNOS activity (Venugopal et al , 2002) Immunohistochemical studies disclosed an increased presence of eNOS in glaucomatous tissue Such an up-regulation has been suggested to partly prevent neural damage This upregulation is lacking in patients who develop damage at a lower intraocular pressure Furthermore, eNOS gene polymorphisms are a determinant of renal hemodynamic function (Page et al , 2005) and are also associated with coronary vasospasms (Nakayama et al , 1999) Each of these studies demonstrate the critical role of NO and also that eNOS gene polymorphism may act as an additional risk factor in the development of endothelial dysfunction

Several reports suggest NTG patients have increased plasma levels of ET-1 (Sugiyama et al , 1995, Kaiser et al , 1995, Cellini et al , 1997) Though HTG is not associated with elevated plasma ET-1 (Tezel et al , 1997), aqueous humour ET-1 levels are increased in HTG patients (Noske et al , 1997) Kaiser et al reported

increased ET-1 in aqueous humour of NTG patients compared to HTG, however these results were not significant (Kaiser et al , 1995) This work did however demonstrate a significant increase in plasma ET-1 levels when subjects moved from supine to upright position, however this response was absent in NTG patients Similarly, Pournaras demonstrated an increase ONH vascular resistance in NTG patients in response to elevated perfusion pressure compared to HTG or controls (Pournaras et al , 2004) The possibility that ET-1 contributes to vasospasm in NTG is further supported by an abnormal increase in plasma ET-1 when the body cools, suggesting a hyperactive production and/or release of ET-1 (Nicolela et al , 2003) Abnormal release of vasoactive mediators in response to stimuli is a hallmark of endothelial dysfunction leading to vasospasm

The role of ET-1 in maintaining vascular perfusion is complicated by the dual vasoactive effect of the peptide – dependent on receptor subtype binding ET_A receptors are located on mural cells and mediate vasoconstriction, whereas stimulation of endothelial ET_B receptors causes vasodilation through release of nitric oxide and also functions to remove ET-1 from the circulation (Tirapelli et al , 2005) The role of the EC-ET_B mediated vasodilatory response is increasingly recognised Selective ET_B receptor blockade attenuates pulmonary vasodilation at birth in the ovine fetus (Ivy et al , 2004), whereas ET_B receptor knock-out impairs endothelium-dependent ET-1 vasodilation in mice (Quaschning et al , 2005) Infusion of ET-1 in humans reduces forearm blood flow in NTG patients or controls, presumably via SMC ET_A mediated vasoconstriction However, infusion of ET-1 and a selective ET_A inhibitor increased forearm blood flow in both groups (relative to ET-1 infusion) This EC-ET_B mediated vasodilation was lower in NTG patients than in controls (Henry *et al* – IOVS – in press) In contrast, recent incites into vasospams post-SAH demonstrate increased smooth muscle cell ET_B receptor expression causes vasospasms in a rat model of subarachnoid haemorrhage (SAH) (Hansen-Schwartz et al , 2003) and also in vasospastic cerebral arteries isolated from SAH-induced monkeys (Hino et al , 1996) Moreover, the contribution of ET_B receptors to vasoconstriction in human skin is negligible (Lipa et al , 1999) As such, the vasoregulatory effect of ET-1 is no doubt specific to each area of the vasculature and

largely determined by EC-mural cell ratio and ET-receptor expression

A few studies of genetic polymorphisms related to ET-1 have been reported with varying results Genotypic and allelic frequency analysis found no association between alterations in the promoter region of the ET-1 gene and familial HTG (Tunny et al , 1998) Ishikawa et al recently demonstrated an association between NTG and gene polymorphism of ET_A receptor The functional consequence of this polymorphism is unknown Exogenous addition of ET-1 reduces retinal blood flow by ~20% in humans, however ET_A blockade reverses this effect, suggesting the ET_A receptor mediates the vasoconstrictive effects of ET-1 in the retina (Polak et al , 2003) Moreover, selective ET_A receptor blockade reduces the frequency and severity of cerebral vasospasms following SAH (Vajkoczy et al , 2005, Vatter et al , 2005) Under physiological conditions, the ET-1–induced constriction of cerebral blood vessels is mediated by activation of the ET_A receptor (Faraci and Heistad, 1998) Although several studies also investigated the role of ET_B receptor polymorphisms in glaucoma, no significant associations have not yet been found (Charron et al , 1999, Herrmann et al , 2001, Lajemi et al , 2001)

1 3 4 Diabetic Retinopathy

Many of the complications of diabetes mellitus are predominantly vascular in origin These include diabetic retinopathy (DR), the commonest cause of blindness in people of working age in the UK and US (Aiello et al , 1998), diabetic nephropathy, an increasingly common cause of renal failure, and premature peripheral vascular, cerebrovascular and coronary artery disease A person with diabetes is approximately three times as likely to suffer a heart attack, at least twice as likely to suffer stroke, and 20 times as likely to have a limb amputated The determinant of vascular damage in diabetes is chronic hyperglycemia, which has been convincingly shown both in Type 1 and in Type 2 diabetes (The Diabetes Control and Complications Trial Research Group, UK Prospective Diabetes Study (UKPDS) Group, 1998)

DR progresses through two stages of disease pre-proliferative-DR (Pre-PDR, sometimes referred to as non-PDR), and proliferative-DR (PDR) An early characteristic feature of pre-PDR is increased vascular permeability (Sander et al , 1994, Cunha-Vaz et al , 1998) leading to breakdown of the inner blood retinal barrier (BRB) (Patz, 1980) As a result, erythrocytes, blood-borne plasma proteins, and lipids, leak into the subendothelial space causing haemorrhages and hard exudates in the retina (Nathan, 1993) Furthermore, thickening of basement membrane (BM) occurs, which is frequently used as an indicator of disease progression (Engerman and Kern, 1987, Robison, 1988) EC-Pericyte crosstalk in the vessel wall is an important regulator of vascular homeostasis BM thickening may disturb this homeostaic mechanism and contribute to pericyte drop-out as disease progresses (Cogan et al , 1961, Mizutani et al , 1996) Pericyte drop-out is detectable by the presence of "ghosts" in trypsin digests and transmission electron microscopy preparations of the retinal vasculature (Cogan et al , 1961) Pericytes provide the endothelium with an anti-proliferative and also anti-apoptotic stimulus Therefore, loss of pericytes in DR possibly causes EC proliferation and new vessel formation Interestingly, ECs are also lost in DR resulting in acellular capillaries consisting solely of basement membrane

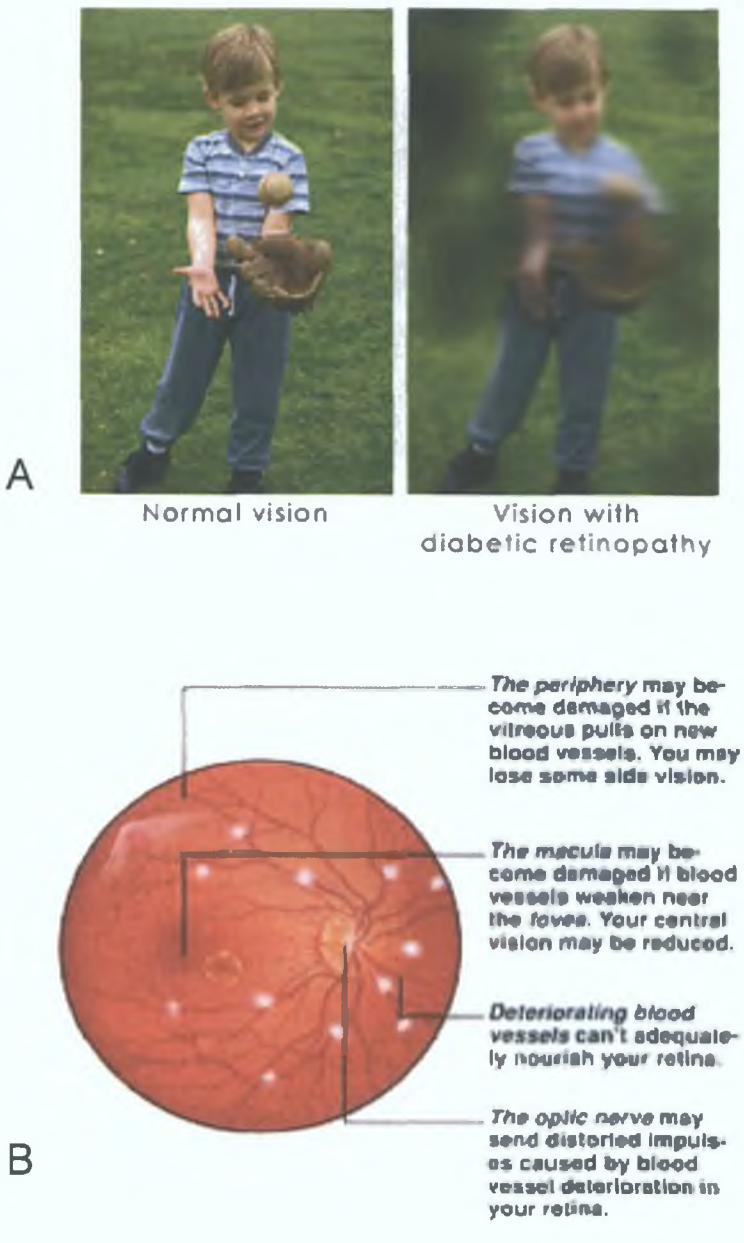


Figure 1.14: Typical vision loss due to Diabetic Retinopathy (A). The location of deteriorating blood vessels may determine what part of your vision is lost and how severe the loss becomes **(B).** http://www.stlukeseye.com/images/

Extensive loss of retinal microvasculature leads to occlusion of vessels, ischaemia and subsequent tissue hypoxia This oxygen deficit provokes nerve fibre layer infarctions creating soft or "cotton wool" exudates (Nathan, 1993), which can be observed as grey or white lesions, as the result of stasis of axoplasmic flow (Aiello et al , 1998) (Figure 1 14 b)

PDR is characterised by a reparative attempt of the retinal vasculature to reperfuse ischaemic/hypoxic areas of the retina Thus, proliferation of new vessels occurs, traversing the internal limiting membrane and into the vitreous As these new vessels are fragile (due to impaired BRB function), they tend to bleed and cause vitreous haemorrhages Furthermore, they can lead to fibrous scar formation with accompanying tractional retinal detachment, resulting in sudden blindness if left untreated (Nathan, 1993) (Figure 1 14)

1 3 4 1 Diabetic Retinopathy Altered Blood Flow & Endothelial Dysfunction

Numerous reports have been published documenting alterations in retinal blood flow in diabetic patients However, due to varying techniques used to measure blood flow, and also the pathological differences at different stages of disease, these results often appear to contradict Perturbations in blood flow are undoubtedly causative factors in disease progression however, with endothelial dysfunction frequently cited in patients

One of the earliest effects of hyperglycaemia is the persistent dilatation of the retinal arteries and arterioles, leading to increases in ocular blood flow (Kohner et al , 1975, McMillan, 1984) Conversely, reports by Savage *et al* (2004) demonstrate little or no changes in pulsatile ocular blood flow (POBF) in early DR, but significant POBF increases in eyes with moderate to severe Pre-PDR POBF is decreased in eyes with laser-treated PDR (Savage et al , 2004) Gracner *et al* demonstrated an increase in the ophthalmic artery blood flow and a decrease in the central retinal & posterior ciliary artery compared to controls with colour Doppler imaging (Gracner, 2004) Furthermore Hudson et al demonstrate reduced macular capillary blood flow in areas of capillary leakage (Hudson et al , 2005)

Obviously there is a lot of discrepancy between various clinical and experimental studies investigating ocular blood flow in diabetes The variety of techniques used for the investigation of haemodynamics may account, in part, for this problem In addition, considerable differences in ocular perfusion may exist between patients with Type I and Type II diabetes, which have not yet been systematically studied In general, there is overwhelming evidence that retinal blood flow is initially increased, followed by an decrease when background retinopathy is present In the late stages of DR, the nature of the ocular perfusion abnormalities appears to strongly depend on glycaemic control as well as on the pathologic features

The development and progression of vascular endothelial cell damage is the basis of diabetic microangiopathy (Cai and Boulton, 2002) Autoregulatory defects in diabetes have their correlates in the central retinal artery, where hyperoxia

significantly reduces central retinal artery end diastolic velocity and significantly increases the resistance index to flow in healthy but not diabetic subjects (Evans et al , 1997) Unlike the central retinal artery whose watershed is exclusively retinal, the ophthalmic artery nourishes the entire orbit Nonetheless, in hyperbaric oxygen conditions, blood flow velocities are reduced in healthy subjects and in diabetics without significant neuropathy, but show increased velocities in diabetics with neuropathy (Okamoto et al , 1998)

The important question therefore, is whether increased levels of circulating glucose can induce endothelial dysfunction, thus causing impaired autoregulation of retinal blood flow The link between chronic hyperglycemia and vascular damage has been established by four independent biochemical abnormalities 1) increased polyol pathway flux, 2) increased formation of advanced glycation endproducts (AGEs), 3) activation of protein kinase C (PKC), and 4) increased hexosamine pathway flux These seemingly unrelated pathways have an underlying common denominator overproduction of superoxide by the mitochondrial electron transport chain Mitochondrial reactive oxygen species (ROS) partially inhibit the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, which diverts increased substrate flux from glycolysis to pathways of glucose over-utilization Moreover, extensive evidence indicates that ROS regulate gene expression by modulating a large number of transcription factors, including NFκB, the peroxisome proliferators activated receptor (PPARγ), and pathways linked to apoptosis It is also increasingly recognized that cell differentiation and proliferation, cytokine expression, and programmed cell death are determined by the interactions between oxidation-sensitive regulatory pathways previously thought to lead to distinct outcomes Endothelial dysfunction is also apparent by increased release of markers of endothelial activation damage into the circulation in diabetic subjects (Tarnow et al , 2004), by several reports of impaired endothelium-dependent vasodilation (Dogra et al , 2001, Maejima et al , 2001) and finally as mentioned previously, by changes in vascular permeability

AGEs play an important role in the pathobiology of Diabetes, and pericytic accumulation of toxic products such as sorbitol or AGEs is a likely causative factor

resulting in pericyte drop-out (Yamagishi et al , 2002) Since pericytes help regulate vessel perfusion, dropout of these cells exacerbates the disturbances associated with retinal haemodynamics Furthermore, loss of pericytes has several 'knock-on' effects on regulation of EC homeostasis Retinal capillary coverage with pericytes is crucial for maintaining a fine balance between promotion of EC survival and inhibition of EC proliferation (Orlidge and D'Amore, 1987, Hirschi and D'Amore, 1996) and regulation of EC production of vasoactive substances such as nitric oxide, prostacyclin and endothelin-1

Several studies implicate nitric oxide in the pathogenesis of diabetes, including increased and decreased basal NO production and altered NO sensitivity For example, several reports demonstrate elevated NO levels which contribute to the vascular dysfunction seen in diabetic rats (Tilton et al , 1993, Do Carmo et al , 1998) A similar alteration is seen in diabetic humans as they respond with significantly less reduction of choroidal blood flow when administered an inhibitor of nitric oxide synthase (Schmetterer and Polak, 2001) Studies by Lakshminarayanan *et al* demonstrated BRECs exposed to increases in shear stress *in vitro* increase hydraulic conductivity via NO, thereby increasing transport of water and proteins across the endothelial barrier Thus NO is implicated in microaneurysm, edema and hard exudate formation in the retina (Lakshminarayanan et al , 2000b) Similarly, glucose transport into the retina may be enhanced by increased EC glucose transporter GLUT1, which is upregulated by NO and also VEGF (Paik et al , 2005, Cai and Boulton, 2002) Furthermore, altered endothelium-dependent vasodilation has been observed in animal models of Type I (Kamata et al , 1989) and Type II diabetes (Sexl et al , 1995), and also in humans with Type I and Type II diabetes (McVeigh et al , 1992, Calver et al , 1992) Interestingly, a direct nitric oxide-mediated vasodilatation action of insulin is seen in experimental animals (Su et al , 1996) as well as in humans (Schmetterer et al , 1997) Lastly, NO is significantly reduced in aortas of spontaneously hypertensive diabetic rats, compared to spontaneously hypertensive non-diabetic rats (Ibrahim et al , 2005)

VEGF is a potent angiogenic and vascular permeabilizing factor which plays a critical role in both physiological and pathological angiogenesis which occurs in DR Similarly, NO is known to mediate angiogenesis and vascular permeability Reports demonstrate that NO modulates VEGF-induced angiogenesis and vascular permeability *in vivo* predominantly *via* eNOS activation (Fukumura et al , 2001) Moreover, intramuscular injection of eNOS plasmid induced therapeutic angiogenesis in a rat ischemic hindlimb model, also *via* VEGF activation (Namba et al , 2003) These studies demonstrate the important role of NO in health and pathological remodelling associated with DR

ET-1 has also been implicated in the pathology of DR Breathing pure oxygen constricts retinal vessels and reduces blood flow, primarily via ET-1 mediated pericyte vasoconstriction (Dallinger et al , 2000) Studies by Grunwald et al (1984) demonstrated a reduction in blood flow of 53% in diabetic eyes without retinopathy, and 38% of those with Pre-PDR, and only 24% in those with PDR Retinal arteriovenous passage times were markedly reduced by breathing 100% pure oxygen in normal subjects, however this is unaffected in diabetics (Harris et al , 1996) Therefore, the capacity to reduce bulk retinal blood flow in response to increased oxygen delivery as mediated primarily by the actions of ET-1 on pericytes is progressively extinguished as the disease severity increases Furthermore, elevated ET-1 plasma concentrations were observed in Type I and Type II diabetic patients (Takahashi et al , 1990, Ferri et al , 1995, Letizia et al , 1997) Moreover, Chakrabarti *et al* demonstrate that retinas from the chronic diabetic BB/W rats show an increase in ET-1, ET-3, ET_A receptor and ET_B receptor mRNA expressions when compared to those from control rats Similar results are noted by them using immunohistochemical methods (Chakrabarti et al , 1998) In the same rat model of DR, Deng *et al* demonstrated increased resistivity index (RI), a marker of retinal vasoconstriction, which is reversible with general ET-receptor blocker bosentan suggesting that the endothelin system is of importance in mediating retinal changes in diabetes (Deng et al , 1999)

Aberrant prostacyclin synthesis has also been reported due to endothelial dysfunction Prostacyclin-stimulating factor (PSF), a factor secreted by pericytes is decreased in early DR, and increased in the latter stages of disease Levels of PSF correlated with retinal blood flow fluctuations, that is a characteristic initial decrease, followed by an increase in blood flow as disease progresses (Hata et al , 2000) Furthermore, Johnson *et al* demonstrated increased prostacyclin in streptozotocin-diabetic rats (Johnson et al , 1999) Recent evidence now suggests a role of VEGF in potentiating EC PGI_2 formation in DR (He et al , 1999) Like PGI_2, NO is also implicated in VEGF mediated vascular permeability and angiogenesis Emerging evidence indicates that COX-2 also interacts with NO and that these two systems have reciprocal effects on each other (Neagoe et al , 2005, Gliki et al , 2001)

1 3 5 Mediators of Cell Fate Decisions

Haemodynamic forces associated with blood flow play a critical role in vasoregulation and vascular remodelling Endothelial and pericyte cell fate decisions arising from transient or chronic alterations of mechanical forces are possibly mediated by one of several pathways, such as the transforming growth factor (TGF), vascular endothelial cell growth factor (VEGF) or platelet-derived growth factor (PDGF) among others We propose two other pathways, namely Notch & Hedgehog (Hh) signalling, which are likely to play a role in remodelling of the retinal vasculature due to altered blood flow

1.3.5.1 Notch Signalling Pathway

1.3.5.1.1 Introduction to Notch Signalling

The Notch gene of *Drosophila melanogaster* was first described by Morgan in 1916, and was so named as haploinsufficiencies of the gene causes "notches" at the wing margin in these flies (Simpson, 1998). Further studies in *Drosophila, C. elegans*, and subsequently in mammals and humans, have identified and characterized a super-family of Notch receptors that show a high degree of evolutionary conservation. Notch was originally identified in humans as a gene involved in the chromosomal translocations in T-cell leukaemias (Kojika and Griffin, 2001). The receptors exhibit a high degree of structural conservation both within, and across species as diverse as flies and humans.

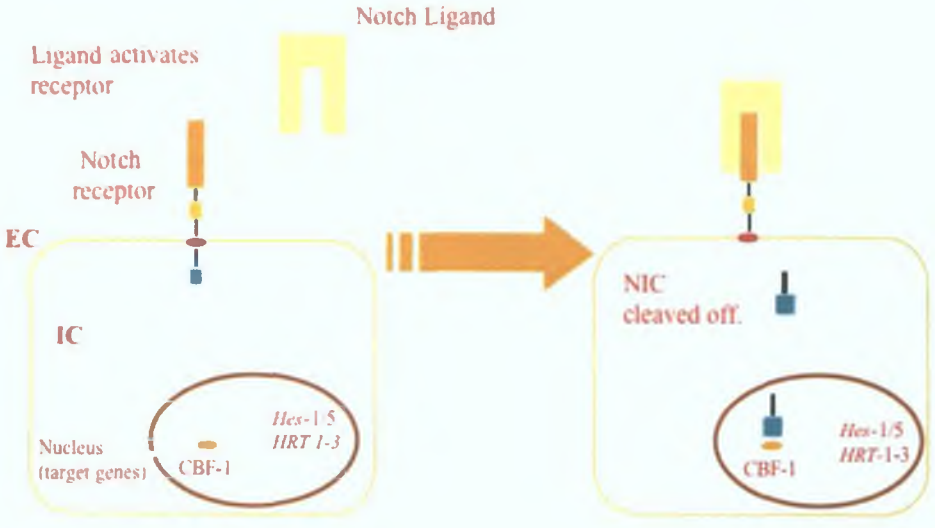


Figure 1.15: Schematic of Notch Signalling pathway: Interaction of Notch receptors (Notch 1 to 4) with their ligands (Delta like 1, -3, -4, Jagged-1 and -2) leads to cleavage of the transmembrane Notch receptor, giving rise to the Notch intracellular domain (NICD) that migrates into the nucleus. In the nucleus, NICD associates with a transcription factor, RBP-Jk (also known as CSL for *C*BF1/*S*u(H)/*L*ag-1) and activates transcription from the RBP-Jk DNA binding site. The NICD–RBP-Jk complex upregulates expression of primary target genes of Notch signaling, such as hairy and enhancer of split (HES)-1, -5, -7 and HES-related repressor protein (HERP)-1 to -3 in mammals.

The main components of the Notch signalling pathway include the Notch receptors (Notch 1-4 in vertebrates) and ligands (Delta 1-4, Jagged 1 and 2 in vertebrates) (Figure 1 15) Analysis of Notch receptor expression within the cell reveals that although Notch is a cell surface protein, the majority of Notch within the cell is found intracellularly (Aster et al , 1994, Fehon et al , 1991), and that a significant portion of Notch is retained in the endoplasmic reticulum (Aster et al , 1994, Weinmaster, 1997)

Notch receptors are single-pass transmembrane proteins, and as such have an extracellular (ExC), transmembrane (TM) and intracellular (IC) domain (Baron et al , 2002) Notch receptors are synthesized as single (300 kDa) polypeptides, and are proteolytically processed into heterodimeric (180 kDa and 120 kDa) forms presented on the cell surface (Blaumueller et al , 1997, Pan and Rubin, 1997, Logeat et al , 1998)

Similar in structure to the Notch receptors, the Notch ligands are also single-pass transmembrane proteins, and as such, also have ExC, TM and IC domains (Artavanis-Tsakonas et al , 1995) Although the general architecture of the ligands is conserved, large variations in specific domain size and composition can be seen both within and among species (Fleming, 1998) Although Notch ligands are primarily transmembrane proteins, proteolytically cleaved, secreted forms of the proteins have been identified (Qi et al , 1999, Klueg et al , 1998) Initial reports indicated that the secreted soluble ligands act as dominant-negative molecules, competing with membrane bound ligands and blocking Notch activation (Sun and Artavams-Tsakonas, 1997) Conflicting reports, however, have been published, describing the soluble ligands as agonists of the Notch signalling pathway (Qi et al , 1999, Han et al , 2000, Wang et al , 1998) A secreted form of Jagged1, for example, can activate Notch1 in hematopoietic cells to inhibit differentiation (Li et al , 1998) The factors controlling whether the soluble form of the ligand is an agonist or antagonist on the Notch signalling pathway remain unclear

NotchIC activates expression of primary target genes of Notch signalling, such as Hairy/Enhancer of Split (Hes) and Hairy Related Transcription Factor (HRT) genes (Bailey and Posakony, 1995, Lecourtois and Schweisguth, 1995)

(Beatus et al , 1999, Lee et al , 1999b, Chen et al , 1997, Ohtsuka et al , 1999, Wang et al , 2002b) These genes are part of a family of basic helix-loop-helix (bHLH) type transcriptional repressors that act as Notch effectors by negatively regulating expression of downstream target genes such as tissue-specific transcription factors (Iso et al , 2003b, Iso et al , 2001a) The Hes family was the first described primary effector of Notch signalling It should be noted that, due to the independent identification of the second family of effectors of Notch signalling by different groups, many different names exist for the HRT family, including HERP (Hes-related repressor protein, (Iso et al , 2001b, Apelqvist et al , 1999)), CHF (Cardiovascular helix-loop-helix factor, (Chin et al , 2000)) and Hey genes (Maier and Gessler, 2000)

1 3 5 1 2 Biological Consequences of Notch Signalling

A number of studies present evidence that Notch signalling acts as an important "switch" controlling cell fate decisions during embryogenesis It is postulated that the Notch pathway contributes to the establishment of two distinct sub-populations at different stages of vasculogenesis and angiogenesis These could include EC versus SMC, artery versus vein, pulmonary versus systemic vessels, and large vessels versus capillaries (Iso et al , 2003a) Indeed, Artavanis-Tsakonas *et al* , (1995) aptly described Notch as a "gatekeeper of cell fate", as Notch signalling critically influences cell proliferation, differentiation and apoptosis

In addition to its role as an arbiter of cell fate decisions in the developing organism, a growing body of evidence has implicated the Notch signalling pathway in determining cell fate decisions in the mature organism (Kojika and Griffin, 2001, Wang et al , 2002c), which may be due to the fact that most tissues are renewed throughout life from reserves of uncommitted stem cells (Artavanis-Tsakonas et al , 1995) Notch, for example, is involved in the control of cell fate decisions during haematopoiesis (Bigas et al , 1998) and inhibits the differentiation of murine myoblast cells (Bush et al , 2001) In addition, the Notch signalling pathway has been shown to affect other cell fate decisions, such as proliferation and apoptosis in the mature organism Notch has been shown to promote proliferation in certain cell types, for example, activation of the receptor and transfection of a constitutively active form of the receptor in bone marrow stem cells and promyelocytic leukaemia cells, respectively, cause accelerated progression through G1 (Carlesso et al , 1999) Activation of Notch signalling in ECs is anti-proliferative and contributes to maintaining the endothelial lining in a quiescent state (Jang et al , 2004, Noseda et al , 2004, Qi et al , 2003)

Notch is reported to have anti-apoptotic properties in many cell types The anti-apoptotic properties of Notch in T-cell systems has, for example, been documented by Deftos *et al* and Jehn *et al,* showing that Notch1 activation inhibits glucocorticoid- and Nur-77-dependent apoptosis respectively (Deftos et al , 1998, Jehn et al , 1999) As human malignant cells display a resistance to both

physiologically- and therapeutically-induced apoptosis, it was postulated that alterations in Notch signalling or expression may contribute to tumourigenesis (Miele and Osborne, 1999) Several lines of evidence support this Increased expression of Notch receptor and ligands is apparent in many malignancies, including cervical carcinomas, leukaemias, neuroblastomas, and pleural mesotheliomas, among others (Zagouras et al , 1995, Daniel et al , 1997) Notch over-expression is, for example, evident in 100% of the cervical cancer specimens studied (Zagouras et al , 1995) In addition, Notch3 signalling has been shown to promote vascular smooth muscle cell survival in response to the pro-apoptotic Fas ligand (Wang et al , 2002c)

It is postulated that the Notch signalling pathway plays a role in determining cell fate decisions following vascular injury Two independent studies have shown changes in the expression of the components of this pathway following balloon catheter denudation of the rat carotid artery Lindner *et al*, (2001) demonstrated that the expression of Notch receptors (Notch 1-4) and ligands (Jagged 1-2) in both endothelial cells and smooth muscle cells of the vasculature was increased following injury This report also suggests that the level of Notch receptor expression may be related to endothelial cell/smooth muscle cell interaction In contrast, Wang *et al*, (2002a) reported that Notch1, Notch2, Notch3, and the effector genes HRT1, HRT2 and HRT3 were coordinately down-regulated following balloon injury The discrepancies between these reports may be accounted for as Lindner *et al*, used *in situ* hybridization thus limiting the study to the inner face of the artery Wang *et al*, however, extracted RNA from the artery following removal of the intimal and adventitial layers, representing the smooth muscle layer of the artery While further work is needed to elucidate the specific role of the Notch signalling pathway in maintaining vascular homeostasis, the importance of this pathway, and its potential for therapeutic intervention is not disputed

Dysfunctions of the Notch signalling pathway are associated with human pathologies involving cardiovascular abnormalities CADASIL (cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencephalopathy) and Alagille syndrome (AGS) are two such disorders (Gridley, 2003) These, coupled

with the altered expression of Notch signalling pathway genes in both vascular smooth muscle and endothelial cells in response to injury *in vivo*, highlight both the presence and the importance of the Notch signalling pathway in both the adult and developing cardiovascular system (Lindner et al , 2001)

1 3 5 1 3 1 CADASIL

The importance of Notch3 in vascular smooth muscle cells was highlighted in 1996 when Joutel *et al*, through positional cloning, found the genetic cause of CADASIL to be point mutations in the human Notch3 gene (Joutel et al , 1996) CADASIL is a cerebral autosomal-dominant adult onset arteriopathy, with the mean onset age being approximately 45 years (Gridley, 1997, Joutel et al , 2000) Affected individuals exhibit a variety of symptoms including recurrent subcortical ischemic strokes, usually in the absence of any vascular risk factors, leading to progressive cognitive decline, dementia and premature death (Joutel and Tournier-Lasserve, 1998, Gridley, 2003)

The vascular lesions underlying CADASIL are non-atherosclerotic, non-amyloid angiopathies preferentially affecting the small arteries and arterioles of the brain (Rubio et al , 1997) However, vascular pathological changes in CADASIL patients are not only confined to the brain, but are also observed in systemic arteries and some veins, as well as in muscle, nerve vessels and skin (Brulin et al , 2002) CADASIL is therefore a systemic vasculopathy Ultrastructural analysis of affected arteries have revealed alterations and eventual loss of vascular smooth muscle cells, and the accumulation of granular osmophilic material within the smooth muscle cell basement membrane and the surrounding extracellular matrix (Gridley, 2003) In addition, endothelial cells appear to shrink, detach from the basal lamina, and the tight and gap junctions appear to be disrupted (Prakash et al , 2002) It has been suggested that the mutant Notch3 molecules present on the cell surface compete with non-mutant Notch proteins for ligand binding, thus dominantly inhibiting the normal signalling pathway (Spinner, 2000)

New emphasis has recently been given to funduscopic examination in patients with cerebral small vessel diseases, including CADASIL, since retinal and optic nerve head arterioles share common anatomical and physiological properties with small cerebral arteries (Rufa et al , 2004b) Haritoglou *et al* (2004) analysed the ocular vasculature of CADASIL patients and made several observations (Haritoglou et al , 2004a, Haritoglou et al , 2004b) Electron microscopy revealed extensive deposits of granular osmophilic material in arterial walls, thickened basement membrane, VSMC loss, and pericyte degeneration Endothelial cells were detached with intracytoplasmatic vacuoles and mild mitochondrial changes were observed Throughout the choroid and choriocapillaris no abnormalities were detected This is of interest with respect to cerebral involvement in CADASIL, as cerebral and retinal blood vessels share similar anatomical and physiological properties Whereas the blood-retinal and blood-brain barriers are maintained by nonfenestrated endothelial cells, choroidal vessels contain a continuous layer of fenestrated endothelial cells This suggests a differential involvement of small blood vessels depending on the angioarchitecture and blood-tissue barriers

Harju et al demonstrated reduced retinal capillary blood flow and a narrowing of the retinal arterioles in CADASIL patients Similar to previous reports, patients had reduced VSMCs in retinal capillaries Furthermore, the pericytes of the central retinal artery were swollen, and focal demyelination of the optic nerve was observed (Harju et al , 2004) Furthermore, arterioles progressively lose their capacity for autoregulation In addition, studies have recently revealed an impaired visual function in these patients by showing abnormal ocular electrophysiological responses in symptomatic and asymptomatic subjects (Parisi et al , 2003) Rufa *et al* (2004) demonstrated a reduced retinal and anterior optic nerve head blood flow in symptomatic and asymptomatic CADASIL patients compared to healthy subjects Each of these studies demonstrate a non-redundant role for Notch signalling in the control of retinal blood flow (Rufa et al , 2004b)

1 3 5 1 5 2 Alagille Syndrome

Alagille Syndrome (AGS) is an autosomal dominant disorder characterized by developmental abnormalities of the heart, liver, eye, skeleton and kidneys (Loomes et al , 1999) The incidence of this disorder is 1 70,000 live births (Joutel and Tournier-Lasserve, 1998), however it is likely to be a cause of death *in utero*, as is evident with homozygous mouse models of AGS (Xue et al , 1999) Congenital heart defects, the majority of which affect the pulmonary circulation, significantly contribute to mortality in AGS patients Most patients (97%) have a heart murmur, and 67% of these have peripheral pulmonary stenosis (Loomes et al , 1999) Two groups independently identified Jagged1 as the defective gene in AGS (Joutel and Tournier-Lasserve, 1998, Henderson et al , 1994), further highlighting the importance of the Notch signalling pathway in the development and maintenance of the cardiovascular system

1 3 5 2 Hedgehog Signalling Pathway

1 3 5 2 1 Introduction to Hedgehog Signalling

Hedgehog (Hh) proteins are secreted morphogenic signalling molecules which play a role during embryonic development (Weed et al , 1997, Pepicelli et al , 1998, Vokes et al , 2004) and in the adult vasculature (Pola et al , 2003, Zeng et al , 2001) In December 1993, Ingham, McMahon, and Tabin reported the isolation of vertebrate homologs of the Drosophila hedgehog (Hh) gene, and named the three mouse Hh genes Sonic hedgehog (SHh), Desert hedgehog (DHh) and Indian hedgehog (IHh) (Nieuwenhuis and Hui, 2005, Fietz et al , 1994)

SHh is synthesised as a 45kDa precursor protein which undergoes autoproteolysis to yield a 20kDa N-terminal domain (SHh-N) and a 25kDa C-terminal domain (SHh-C) (Figure 1 16) The signalling activity of SHh resides only in the N-terminal domain, whilst the C-terminal domain is responsible for the autoprocessing (Bumcrot and McMahon, 1995) SHh-C is thought to act as a cholesterol transferase during the processing, allowing a cholesterol modification of SHh-N at its C terminus Palmitoylation also takes place at the N-terminus of SHh-N to give SHh-Np, in this way, SHh-Np can remain membrane-associated and function as a short range signalling molecule interacting with neighbouring cells (Pepinsky et al , 1998) There is also evidence for a freely diffusible form of SHh-Np (termed s-SHh-Np) that mediates long range signalling The release of the cholesterol modified and therefore hydrophobic SHh-Np from the cell membrane is thought to involve a multimerisation step in order to bury its lipid moieties in the hydrophobic surface of an adjacent SHh protein A transmembrane protein Dispatched (Disp) has been shown to be required for the release of the cholesterol modified SHh-Np (Porter et al , 1995, Burke et al , 1999, Williams et al , 1999)

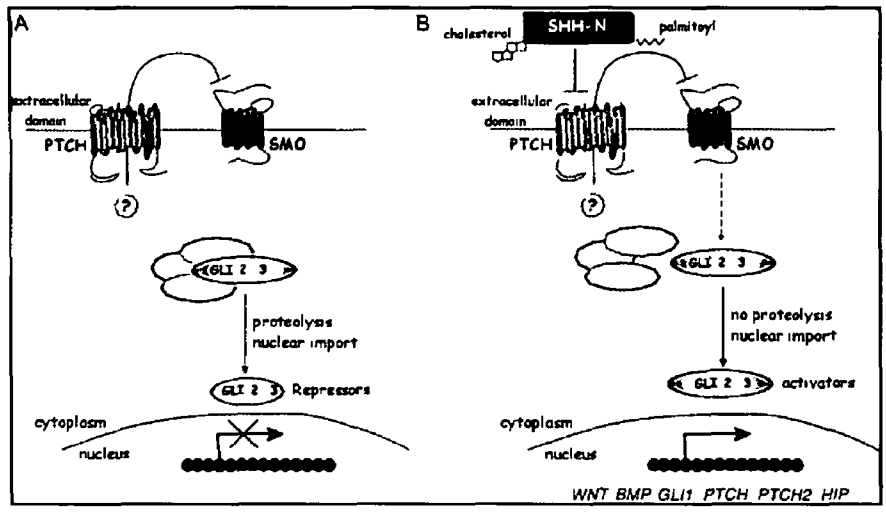


Figure 1 16 Schematic of Sonic Hedgehog Signalling pathway Shh under goes autocatalytic processing prior to secretion The Shh precursor protein is cleaved to yield an ~20 kDa N-terminal domain (signaling domain) and an ~25 kDa C-terminal domain (catalytic domain) Cholesterol modification is important for secretion and activity of the Shh protein **(A)** The Shh signaling path way involves two transmembrane proteins, Patched (Ptc) and Smoothened (Smo) Ptc binds Shh whereas Smo acts as a signal transducer In the absence of ligand, Ptc interacts with and inhibits Smo This inhibition activates a transcriptional repressor (e g Gli invertebrates) In the presence of ligand, the interaction of Ptc and Smo is altered and Smo is no longer inhibited Gli protein may then enter the nucleus and function as a transcriptional activator **(B)** (Sonic hedgehog signaling in basal cell carcinomas, Grosjean *et al* 2005)

The secreted peptide binds to its receptor, the 12-membrane pass protein Patched1 (Ptc1) (Chen and Struhl, 1998), thereby relieving Ptc1-mediated repression of Smoothened (Smo) (Figure 1 16) Hh induces progressive Smo phosphorylation by protein kinase A and casein kinase, leading to elevation of Smo cell-surface levels and signalling activity (Jia et al , 2004, Zhang et al , 2004) Although the precise manner in which Hh binding facilitates activation of the G-protein-like molecule Smo is unknown, downstream events focus on the transcription factor Cubitus interuptus in *Drosophila*, and its homologues, the Gli family, in vertebrates The Gli family of zinc finger transcription factors, then activate transcription of Hedgehog target genes, such as FOXE1 and FOXM1 encoding Forkhead-box transcription factors While Gli1 and Gli2 are transcriptional activators, Gli3 seems to function primarily as a transcriptional repressor Gli2 appears to be the principal effector of SHh signalling since disruption of the Gli2 gene leads to developmental

defects involving several SHh target tissues, while Gli1 null mice are born without detectable abnormalities

Regulation of Gli processing includes the action of Costal2, Fused, and Suppressor of Fused, which form a scaffold that links Gli to microtubules (Sisson et al , 1997) In the absence of the Hh signal, the kinases protein kinase A, glycogen synthase kinase 3, and casein kinase 1, phosphorylate Gli and mediate its degradation to the repressor form Interestingly, one of the genes expressed downstream of Hh signalling encodes the Hh receptor Ptc1, thereby making Ptc1 expression an indicator of Hh responsiveness (Fuse et al , 1999)

The consensus is that the signals encoded by these three Hh genes all activate the same downstream signalling cascade, and that the presence of three genes controlled by separate regulatory elements facilitates the expression of the signal at multiple sites and times during embryogenesis (McMahon et al , 2003) Hh signalling is used throughout embryogenesis in many differentiating tissues to establish cell fate, promote cell proliferation, and mediate programmed cell death (McMahon et al , 2003)

1.3.5.2.2 Biological Consequences of Hedgehog Signalling

Hedgehog has been implicated as an essential signalling molecule in several non-related functions. For example, SHh plays a role in early vasculogenesis, particularly the formation, aggregation and subsequent assembly of angioblasts (endothelial precursors) in endothelial tube formation (Vokes et al., 2004). Furthermore, the embryonic chick has the ability to regenerate its retina after it has been completely removed, one of few organisms with this capacity. This is mediated via SHh signalling of Fibroblast growth factors (Spence et al., 2004). Also the role of Hh signalling in the formation of the proximodistal axis of the eye and the differentiation of retinal pigment epithelium is well characterized (Perron et al., 2003).

In the past decade however, there has been increasing appreciation of the fact that pathways such as Notch and Hh signalling, studied predominantly during embryogenesis and known to be relatively silent during normal adult life may be recruited postnatally in response to *tissue injury* (Pola et al., 2003). For example, expression of DHh and Ptc1 persist in the postnatal and adult peripheral nerves, enabling maturation and maintenance of the peripheral nervous system in normal and in experimental diabetic neuropathy (Calcutt et al., 2003). *In vivo* mouse studies demonstrated postnatal recapitulation of IHh, SHh and Ptc1 in fully differentiated adult muscular tissues and a regulatory role of Hh signalling in angiogenesis during muscle regeneration after ischemia (Pola et al., 2003). Exogenous administration of SHh induces robust angiogenesis, characterized by distinct large-diameter vessels and upregulation of Ptc1 in aortic vessels and heart. SHh can induce corneal neovascularisation, and also blood-flow recovery and limb salvage following operatively induced hind-limb ischemia in aged mice (Pola et al., 2001). Recent results also suggest that reduced expression of Hedgehog interacting protein (HIP), a naturally occurring Hh pathway antagonist, in tumour neo-vasculature may contribute to increased Hh signalling within the tumour and possibly promote angiogenesis (Olsen et al., 2004). In fact, dysregulation of the Hh signalling pathway plays a pivotal role in a variety of human tumours, such as gastric cancer, pancreatic

cancer, colorectal cancer, breast cancer, prostate cancer, basal cell carcinoma and brain tumours (Katoh and Katoh, 2005) – several of which, are now therapeutic targets in the developing field of Hh related drug development

1.3.5.3 Interaction of Notch & Hedgehog Signalling

Identification of a wide range of molecules whose targeted disruption results in defective vascular development, has led to candidate molecules implicated in vascular remodelling in the adult vasculature. For example, mice deficient in flk-1/KDR, flt-1, PDGF-B, PDGF, RTGF1, TGFRII, endoglin, SHh, Notch, tissue factor, neuropilin-1 and -2, ephrinB2, EphB4, tie-1, tie-2, hypoxia-inducible factor, and angiopoietin-1 and -2, to name a few, exhibit defective vascular development (D'Amore and Ng, 2002). The role each of these plays in vessel formation and/or vascular remodelling, and whether their influence is direct or indirect, is not fully understood. In addition to the large number of regulatory molecules and pathways, it is likely that few of these are exclusive and independent of the other pathways.

How does the Hh signal interact with the other signalling pathways demonstrated to play a role in vascular development? Addition of recombinant SHh to interstitial mesenchymal cells promotes expression of VEGFs and other angiogenic molecules such as angiopoietins (Pola et al., 2001). Recent chimera studies have demonstrated a role for VEGF produced by the visceral endoderm in yolk sac angiogenesis (Damert et al., 2002). Notch signalling is implicated in blood vessel differentiation, and arrest at the capillary plexus stage is observed in embryos deficient in Notch1 (Krebs et al., 2000). Moreover, Notch4 regulates vessel patterning and remodelling in mouse models of vascular development (Krebs et al., 2000, Uyttendaele et al., 2001). Expression of both Notch1 and Dll4 are upregulated by VEGF in human arterial endothelial cells (Liu et al., 2003b). Taken together, these data are consistent with a regulatory cascade for vascular remodelling that begins with Hh promoting Ang1, Ang2, and/or VEGF expression, which in turn promotes Notch expression and signalling. Strong support for this hierarchy comes from work performed in zebrafish, where exogenous VEGF can restore normal arteriogenesis in the absence of SHh, but not in the absence of Notch function. Furthermore, activation of Notch can compensate for the loss of VEGF activity (Lawson et al., 2002). Evidence from a number of sources, however, suggests that this cascade may not be the only means whereby Hh promotes angiogenesis. For

example, zebrafish embryos lacking SHh have a more severely abnormal vascular phenotype, failing to form two distinct trunk axial vessels, than those that lack VEGF or Notch, which have two vessels with the aorta transformed from artery to vein This discrepancy suggests that Hh also promotes vascular development via other signalling cascades (Lawson et al , 2002), possibly the angiopoietin signalling pathway

This angiogenic cascade involving Hh, VEGF, and Notch involves communication between three different cell types the Hh producing cell, the Hh responding cell, and the target endothelial cells However, evidence also suggests that Hh may act directly on endothelial cells, as the Ptc1 receptor is expressed on adult vascular endothelial cells (Pola et al , 2001) In an assay using either murine brain capillary endothelial cells or human umbilical vein endothelial cells, SHh promoted endothelial network and lumen formation in the absence of support cells (Kanda et al , 2003) A vascular network-like structure formed in response to SHh treatment of the bEnd3 endothelial cell line (Vokes et al , 2004) This morphologic transition suggests that Hh may play a direct role in tubulogenesis In both of these *in vitro* experiments, the induction of capillary morphogenesis occurred in the apparent absence of VEGF, suggesting that the direct action of Hh on endothelial cells is independent of VEGF Taken together, the evidence suggests that Hh acts both via the VEGF–Notch cascade, and via an alternate pathway

1 4 Relevance and Objectives of this study

Haemodynamic forces generated by blood flow play an important role in regulating vascular cell fate decisions and are also an important regulator of vasoactive substances such as NO, PGI_2 and ET-1 Aberrant retinal blood flow is associated with numerous retinal pathologies, such as diabetic retinopathy, normal and high tension glaucoma Similarly, endothelial dysfunction, which impairs the ability of vessels to 'autoregulate' blood flow in response to altered perfusion, is also associated with each of these pathologies Precise information on the mechanisms by which haemodynamic forces regulate vasoactive mediators in retinal vessels may aid in understanding the role of altered blood flow and endothelial dysfunction in disease Moreover, our experiments utilised co-culture technology, accounting for reciprocal regulation of cell fate decisions between ECs and pericytes, an important interaction not featured in most other *in vitro* haemodynamic models

Numerous studies have implicated the Notch signalling pathway in the regulation of cell fate decisions, including apoptosis, in many cell types (Artavanis-Tsakonas et al , 1995, Artavanis-Tsakonas et al , 1999, Greenwald, 1998) Whilst the presence and function of the Notch signalling pathway was not established in adult VSMC until recently, several lines of evidence indicated that components of this pathway could be present, and act to regulate apoptosis in adult VSMC Furthermore, recent reports from our laboratory and others suggest a potential crossover or interaction between Notch and Hedgehog signalling pathways To date, the presence or absence of Notch and Hedgehog in the retinal vasculature has not yet been defined Moreover, pulsatile flow regulation of Notch or Hedgehog has not been demonstrated in any vascular cell type

Therefore, the findings of this research project has been divided into four results chapters with the following objectives

Chapter 3

Acute Exposure to Pulsatile Flow Regulates Nitric Oxide, Prostacyclin & Endothelin-1 in BREC mono-culture & BREC/BRP co-culture

- The aim of this chapter was to determine the 'acute' effect (i e 24 hours) of pulsatile flow on cell morphology and production of vasoactive mediators by retinal endothelial cells, either cultured alone or in co-culture with retinal pericytes In addition, I analysed which signalling pathways are activated and the role of the proposed mechanotransducers G-proteins, integrins and protein tyrosine kinases

Chapter 4

BREC/BRP Co-Culture Pulsatile flow regulation of Nitric oxide, Prostacyclin, Endothelin-1 and apoptosis

- The aim of this study was to determine the apoptotic effect of chronic exposure to pulsatile flow on retinal endothelial & pericyte cell co-cultures for 3 days Furthermore, the role of NO, ET-1 and PGI_2 in mediating changes in apoptosis due to pulsatile flow was examined

Chapter 5

Hedgehog signalling is anti-apoptotic and also regulates Notch signalling in static cultures of BRECs and BRPs

- The aim of this study was to identify the presence of Notch & Hedgehog signalling pathway components in retinal endothelial & pericyte cells in static culture and also in human normal and glaucomatous eye sections In addition, the effect of exogenous Sonic Hedgehog addition on Notch signalling and apoptosis was also examined in retinal endothelial & pericyte cells

Chapter 6

Effect of Pulsatile Flow on BREC/BRPs co-culture – Apoptosis, Notch and Hedgehog signalling

- The principal aims of this study were to establish the presence and activity of the Hedgehog/Notch signalling components in co–cultured retinal EC/pericytes exposed to pulsatile flow Furthermore, the role of Notch/Hedgehog signalling in regulating EC/Pericyte cell apoptosis exposed to pulsatile flow was also examined

Chapter 2

Methods

2 0 Material & Methods

2 1 Materials

All general-purpose chemicals and reagents used in experimental work were of analytical grade, and were purchased from the companies listed below

AGB Scientific (Dublin, Ireland)
Whatmann Chromatography paper

Ambion (Cambridgeshire, UK)
Cells to cDNA kit

Amersham Pharmacia Biotech (Buckinghamshire, UK)
Anti-mouse 2^{ry} antibody, HRP conjugated
Anti-rabbit 2^{ry} antibody, HRP conjugated
ECL Hybond nitrocellulose membrane
ECL Hyperfilm
Rainbow molecular weight marker, broad range (6-175kDa)
Amersham Hyperprocessor Automatic Developer

Assay Designs (Ann Arbor, MI, US)
PGI_2 Assay kit

Bachem UK Ltd (St Helens, UK)
Linear RGD peptide
Cyclic RGD peptide

BD Transduction Laboratories (Oxford, UK)
All apoptosis related 1^{ry} antibodies

BDH Laboratory Supplies (Poole, England)

poly-l-lysine-coated glass slides

Bio Sciences Ltd (Dun Laoghaire, Ireland)

DMEM

dNTP's

DEPC-treated water

Trizol® reagent

Calbiochem (Bad Soden, Germany)

Hygromycin

PD142893

Genistein

Pertussis toxin

Anti-ppERK antibody

Anti-ppP38 antibody

Carl Zeiss (Jena, Germany)

Zeiss LSM 510 Confocal Microscope

Cayman Chemical Company (Michigan, USA)

eNOS polyclonal antibody

ET-1 EIA kit

Cell Signaling (Beverly, MA, US)

Phospho-specific eNOS^{1179} 1^{ry} antibodies

Corbett Research Limited (Cambridge, UK)

Rotor-Gene 3000^{TM} lightcycler

Spectrum Laboratories Inc , (Santa Clara, Ca, US)

Cellmax™ Perfused Transcapillary Culture Capillaries

Gibco (Dun Laoghaire, Ireland)

G418

GibcoBRL Horizion 20 25 gel electrophoresis apparatus

Grass Instrument Co (W Warwick, RI)

Grass recorder (Models 7 and 7E)

Hitachi Corp Ltd

Hitachi S 300N SEM

Invitrogen (Groningen, The Netherlands)

Lipofectamine reagent

Vybrant™ Apoptosis Assay Kit #2

Vybrant™ CFDA SE cell tracer kit

Global Medical Instrumentation (Minnesota, US)

Labsystems Luminoskan Luminometer

Laboratory Instruments and Supplies (Dublin, Ireland)

Leica TP 1020 processor

Leica EG 114OH embedder

Leica RM 2135 microtome

MWG Biotech (Milton Keynes, UK)

All Oligonucleotides

National Disease Research Interchange (NDRI, Philadelphia, PA, USA)

Human eyes

Olympus (Singapore)

CK30 phase contrast microscope

Oxford Instruments, Microanalysis Group (UK)

SEM Image Analysis software

Oxoid (Hampshire, UK)

Tryptone

Skim Milk Powder

Yeast Extract

PALL Corporation (Dun Laoghaire, Ireland)

Biotrace nitrocellulose membrane

Perkin Elmer (Turku, Finland)

Luminescence spectrometer LS 50B

Wallac Victor 2 1420 mutilabel counter

Pierce (Northumberland, UK)

Super Signal West Pico Chemiluminescent Reagent

BCA Protein Assay Kit

Promega (UK)

Taq DNA Polymerase

MLV-RT

RNase H

Oligo dT

Luciferase Reporter Reagents

WizardR Plus Midipreps DNA purification kit

Santa Cruz (Heidelberg, Germany)

1^{ry} Antibodies

Notch1, Notch3, Indian & Sonic Hedgehog, Patched1

Sarstedt (Drinagh, Wexford, Ireland)

T25 tissue culture flasks

T75 tissue culture flasks

T175 tissue culture flasks

6-well tissue culture plates

5,10 and 25ml serological pipettes

15 and 50ml falcone tubes

Scientific Imaging Systems (Eastman Kodak Group, Rochester, NY)

Kodak 1D image analysis software

Sigma Chemical Company (Poole, Dorset, England)

Acetone	Acrylamide/bis-Acrylamide
Agarose	Ammonium Persulphate
b-glycerophosphate	2β-mercaptoethanol
Bovine Serum Albumin	Brefeldin A
Brightline Haemocytometer	Bromophenol blue
Calcium Chloride	CHAPSO
Chloroform	DNase/RNase free treated water
Diaminobenzidine	DMEM
DMSO	DTT
EDTA	EGTA
Ethidium Bromide	Foetal Calf Serum
Glycerol	Glycine
Hanks Balanced Salt Solution	Hydrochloric acid
insulin-transferrin-sodium selenit	Isopropanol
Lauryl Sulphate	Leupeptin

Methanol

Monensin

Mineral oil (molecular grade)

N-Acetyl-Asp-Glu-Val-Asp-pNitroanilide

Penicillin-Streptomycin (100x)

p-Nitroaniline

Ponceau S

Potassium Chloride

Potassium Iodide

Potassium Phosphate (Dibasic)

Sodium Acetate

Sodium Chloride

Sodium citrate

Sodium Dodecly Sulphate

Sodium Hydroxide

Sodium Nitrite

Sodium Orthovanadate

Sodium Phosphate

Sodium Pyrophosphate

Streptomycin

Streptomycin

Sulphuric Acid

TEMED

Tetracycline

Tris Acetate

Tris Base

Tris Chloride

Triton X-100

Trypsin-EDTA solution (10x)

Tween 20

Stratagene (La Jolla, Ca, US)

High Sensitivity β-galactosidase Assay Kit

Qiagen (West Sussex, U K)

SYBR Green® PCR Kit

Vector Labs (Burlingame, Ca, US)

Vectastatin Immunohistochemistry kits

2 2 Cell Culture Methods

2.2 1 Culture of microvascular cells

All cell culture techniques were carried out in a clean and sterile environment using a Bio air 2000 MAC laminar flow cabinet Cells were visualized using an Olympus CK30 phase contrast microscope

Bovine retinal endothelial cells (BREC) and bovine retinal pericytes (BRP) were kindly donated by Prof Alan Stitt, Queens University, Belfast BREC were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, and 1 x insulin-transferrin-sodium selenit (ITS) BRP were grown in DMEM supplemented with 20% FBS, 100U/ml penicillin, 100μg/ml streptomycin and 1 x conc ITS Both cell lines were maintained in a humidified atmosphere of 5% CO_2 / 95% air and routinely used between passages 5 to 9

BRECs & BRPs are adherent cell lines As such, trypsinisation was necessary for sub-culturing or harvesting of cells For trypsinisation, growth media was removed from the flask and the cells were gently washed three times in Hanks buffered saline solution (HBSS) to remove α-macroglobulin, a trypsin inhibitor present in FBS A suitable volume of trypsin/ethylenediamine tetracetic acid (EDTA) (10% v/v trypsin EDTA in HBSS) was added to the flask and incubated until all cells detached from the flask surface Trypsin was inactivated by the addition of FBS containing growth medium, and the cells were removed from suspension by centrifugation at 2500g for 2-3 mins Cells were then resuspended in culture medium and typically diluted 1 4 into culture flasks, or cryogemcally preserved

2 2 2 Cryogenic preservation and recovery of cells

For long-term storage of cells, BRECs & BRPs were maintained in liquid nitrogen in a cryofreezer unit Cells to be stored were centrifuged following trypsinisation and the resultant pellet was resuspended in 50% (v/v) FBS containing dimetylsulphoxide (DMSO) at a final concentration of 10% (v/v) 1ml aliquots were transferred to sterile cryovials and frozen in a -80°C freezer at a rate of -1°C/min using a Nalgene cryo freezing container Following overnight freezing at –80°C, the cryovials were transferred to a liquid nitrogen cryofreeze unit (Thermoylen locator jr cryostorage system)

Cells were recovered from long-term storage by rapid thawing at 37°C and resuspension in 5ml of growth medium followed by centrifugation at 1000g for 2-3 mins The resulting cell pellet was resuspended in fresh medium and transferred to culture flasks The following day, the media was removed and the cells were washed in HBSS and fresh culture media added

2 2 3 Perfused Transcapillary Co-Culture System

The perfused transcapillary culture apparatus (Cellmax Quad™ artificial capillary culture system) consists of an enclosed bundle of 50 semi-permeable, Pronectin™ coated polypropylene capillaries (capillary length 13cm, outer diameter 630μm, wall thickness 150μm, luminal area 70cm^2, outer surface area 100cm^2, extra-capillary volume 1 4ml, 95% MWCO 0 5μm) through which medium from a reservoir is pumped at a chosen flow rate via silicone rubber tubing As the gear pump rotates, the motor shaft forces the pump pins to depress the pump tubing on the capillary module, thereby forcing culture media to flow in a pulsatile fashion through the gas-permeable silicone flow path tubing and through the capillary (Figure 2 1) By altering the flow rate using an electronic control unit housed outside the humidified incubator, varying pulsatile flow rates and hence pulse heights (pressure) can be achieved in this system (Table 2 1) To maintain pH, pCO_2, and pO_2 of the culture media at constant levels, the perfused transcapillary culture system was housed in a humidified atmosphere in a standard CO_2 incubator, thereby allowing gaseous exchange to occur through the silicone rubber tubing Prior to the addition of cells, the module is equilibrated for 3 days by circulation of culture media through the capillaries and tubing

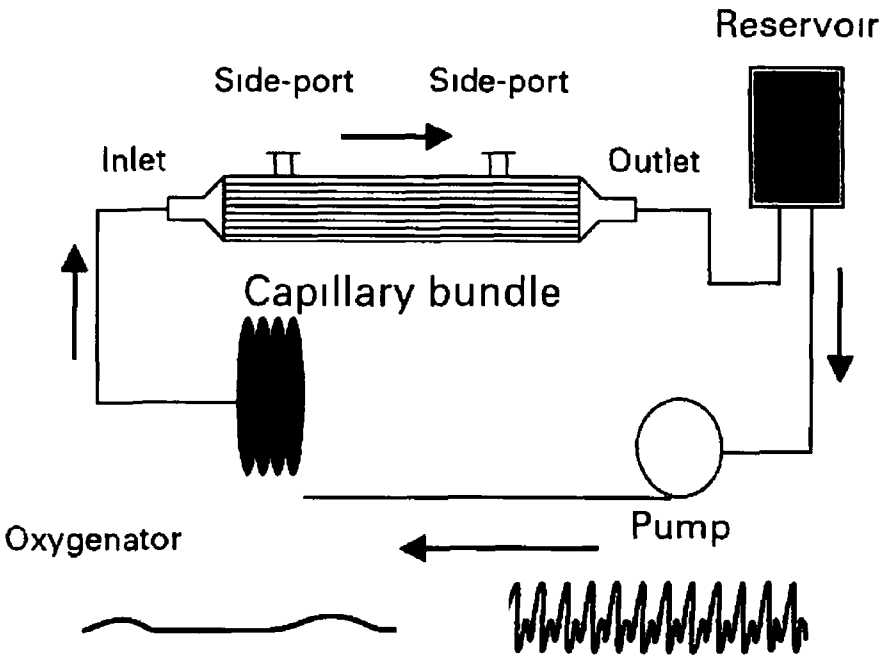


Figure 2 1 Schematic of the Perfused Transcapillary co-culture system demonstrating the normal flow path of the perfusing medium via inlet and outlet ports and through the luminal spaces Below wave forms generated at 'low'(left) and 'high' flow (right)

The outer surface area of the capillaries is 100cm^2 BRP from culture flasks of equivalent or greater surface area were harvested by adding 0 125% trypsin-EDTA and injected into the extracapillary space (ECS) at a density of 2x 10^4/cm^2 using a double syringe method Briefly, BRP cells are introduced with a syringe into one ECS port and the displaced media is withdrawn from the opposite ECS port using another syringe Cells are allowed to adhere for 3 hours, after which the pump is set to low flow (0 3ml/min, pulse pressure of 6mmHg, shear stress of 0 3dynes/cm^2) and returned to the incubator for 3 days BREC are introduced into the luminal compartment, again using the double syringe method at density of 2 x 10^{4}cells/cm^2 and allowed to attach for 3 hours before circulating the media at low flow for a further 3 days Low serum (1%) was used to enhance BREC attachment to the Pronectin-F^{TM}-coated capillaries In addition, to prevent BREC from being flushed out of the capillaries and to promote their adherence immediately following

cell loading, the perfusion media (now also containing 1% FBS) was re-routed for 6 hours, via the extra-capillary space using the side-ports Following this period, the perfusion circuit was returned to its original path, whereby media, containing 20% FBS, was again perfused through the lumen of each capillary that was now lined with BREC The cells were seeded at an appropriate density to guarantee full coverage of all capillaries The number of cells that did not adhere were routinely counted to measure seeding density and adherence after the pump has been turned back on to ensure maximal coverage of each capillary The harvested cells were then routinely counted at the end of each experiment to confirm uniform seeding density and adherence To obtain 'high flow' the flow rate is increased steadily over approximately 5 hours until the desired high flow rate is reached (t = 0) After completion of the experimental time-course, cells are harvested from their separate compartments by first washing the cells with Hanks Balanced Salt (HBSS) solution using the double syringe method, and removing the remaining cells by treatment with 0 125% trypsin-EDTA The circulating media is also harvested at the end of each experiment For BREC mono-cultures, no BRP are seeded into the ECS and the BREC are seeded into the luminal compartment as described above

	Pulse Pressures	Amplitute
ECS Low Flow	Max 24 mmHg	
	Min 18 mmHg	6 mmHg
ECS High Flow	Max 70 mmHg	
	Min 14 mmHg	56 mmHg
Inlet Low Flow	Max 11 mmHg	
	Min 0 mmHg	11 mmHg
Inlet High Flow	Max 80 mmHg	
	Min 10 mmHg	70 mmHg

Table 2 1 Pulse pressures within the luminal and extracapillary space under low and high pulsatile flow conditions

The Cellmax™ system is a valuable tool in vascular biology, especially in its ability to duplicate vascular wall configuration, and its ability for prolonged sustainable growth The positive displacement pump system used to drive the flow in the Cellmax™ generates a pulsatility in the flow depending on the flow regime specified Since blood flow pulsatility is a natural and important component of the mechanical signalling to vascular cells, a model that incorporates pulsatile flow is advantageous Pulse pressures were monitored simultaneously intraluminally at the inlet port and extraluminally (ECS) at the sideport using pressure transducers connected to a Grass recorder (Table 2 1) In the current study, the "low" pulsatile flow rate used was 0 3ml/min, corresponding to a shear stress of 0 5dynes/cm^2 and a pulse pressure of 24/18mm Hg with a frequency of 0 2Hz and an amplitude of 6mm Hg in the extracapillary space The "high" pulsatile flow rate was 23ml/min, corresponding to a shear stress of 23dynes/cm^2, a pulse pressure of 70/14mm Hg with a frequency of 2Hz, and an amplitude of 56mm Hg in the extracapillary space

2 2 4 Immunocytochemistry

BRPs seeded onto capillaries were washed x2 with PBS and fixed by the addition of 3% paraformaldehyde for 15 mins at room temperature The plastic casing housing the capillaries was then removed with a pipe-cutter and the capillaries cut into 1cm sections Cells were permeabilised by the addition of 0 2% (v/v) TritonX-100 in PBS, followed by two PBS washes and incubation for 30 mins at room temperature in blocking solution (1% BSA in PBS) Capillaries were then washed twice in PBS followed by incubation for 2 hours at room temperature with a 1 200 dilution of anti α-smooth muscle cell specific actin primary antibody in blocking solution Following three 5 mins washes in PBS, sections were incubated with a 1 400 dilution of Alexa488-conjugated rabbit anti-mouse IgG in blocking solution for 60 mins at room temperature Alexa488 is a green-fluorescent conjugate (excitation/emission maxima ~495/519 nm) that has spectral characteristics similar to fluorescein conjugates, but exhibits fluorescence that is brighter, much more photostable and less pH dependent Capillary sections were then washed with PBS, prior to being mounted onto microscope slides for fluorescent microscopy analysis

2 2 5 Confocal Microscopy

Visualisation of BRP anti α-smooth muscle cell specific actin was performed on cells grown on capillaries and prepared as per immunocytochemistry above BRP nuclei were stained for 5 mins with 0 2μg/ml propidium iodide Control procedures included unstained cells to allow for autofluorescence and secondary antibody only to control for non-specific binding of the fluorescent secondary antibody Rhodamine fluorescence (propidium iodide) was detected at 546nm and FITC (actin) at 488nm using an argon laser All images were acquired using a Zeiss LSM 510 Confocal Microscope

2 2 6 Scanning Electron Microscopy

Media was removed from the capillary system and replaced with 2 5% glutaraldehyde in 1x PBS and incubated at room temperature for 1 hour Cells were then washed in 0 1M Cacodylate buffer (pH 7 4) and incubated for a further 90 mins in 1% osmium tetroxide in Cacodylate buffer at 4°C After a brief wash with Cacodylate buffer, a graded series of ethanol solutions were perfused to dehydrate the capillaries (50, 60, 70, 80, 90% Ethanol for 10-15mins each) The capillaries are then incubated in 100% Ethanol twice for 10 mins and dried in a standard bell chamber under vacuum overnight Images were acquired using a Hitachi S 300N SEM and analysed with SEM Image Analysis software

2 2 7 Cell counts

Cells counts were performed using a Sigma brightline haemocytometer Trypan blue exclusion dye was routinely used to determine cell viability 20µl of trypan blue was added to 100µl of cell suspension, the mixture was left to incubate for 2 mins 20µl of this mixture was loaded to the counting chamber of the haemocytometer and cells visualized by light microscopy Viable cells excluded the dye while dead cells stained blue The number of cells was calculated using the following equation

Average Cell No x dilution factor x $1x10^4$ (volume under cover slip mm^3) = Viable cells/ml

2 3 Immunohistochemical analysis of tissues

Normal and glaucomatous post-mortem eyes were obtained from the National Disease Research Interchange (NDRI), an organisation which procures and distributes human tissues, cells and organs for research and transplantation The NDRI procures tissue from donors only where informed written consent has been given Ethical approval was obtained from the Mater Misericordiae Hospital's Ethics Committee (Dublin) for the procurement of this tissue Globes were fixed in 10% formalin within 20 hrs of death and were then processed as soon as possible Globes were dissected into two, processed and embedded as described in the following section The average age of normal donors was 69 ± 2 9 (67% male) and the average age of glaucoma donors was 69 ± 5 6 (33% female)

2 3 1 Preparation of tissue samples for histological analysis

Eyes were placed in 4% paraformaldehyde and left to fix overnight The tissues were then processed on a Leica TP 1020 processor using the following programme

	Time (hrs)
Formalin	1
Formalin	2
70% methylated spirit	1
95% methylated spirit	1
Spirit (99% IMS)	1
Spirit	1
Spirit	1
Spirit	1
Xylene	1 5
Xylene	1 5
Paraffin Wax	2
Paraffin Wax	2

The tissue was embedded in paraffin wax on a Leica EG 114OH embedder 5μm sections were cut on a Leica RM 2135 microtome and dried onto poly-L-lysine-coated glass slides The slides were baked overnight at 50°C

2 3 2 Deparaffinisation and rehydration of tissue sections

The slides were deparaffinised and rehydrated as follows

Xylene	5 mm
Xylene	5 mm
Xylene	5 min
100% Methanol	20 sec
100% Methanol	3 min
90% Methanol	3 min
70% Methanol	3 min
50% Methanol	3 min
30% Methanol	3 min

2 3 3 Immunohistochemical staining (peroxidase-diaminobenzidine reaction)

For some proteins, an antigen retrieval step was required prior to staining This was achieved by micro waving the sections in sodium citrate buffer (pH 6) following the dewaxing and rehydration steps outlined in section 2 3 2 Sections were microwaved at a medium heat for approximately 5 mins, without letting the buffer boil and were left in the buffer for a further 30 mins to cool

Endogenous peroxidase activity was quenched in the tissue sections by incubation with 0 3% H_2O_2 in 100% Methanol for 30 mins Slides were then washed 3x 5 mins in PBS at 50rpm Non-specific binding was blocked by the incubation of tissue sections in 1 5% normal serum from the species in which the secondary antibody was raised for 30 mins or 1 hour - normal serum concentrate, provided with the Vectastain Elite kit diluted in PBS The tissue sections were then incubated with the primary antibody in normal serum for the optimal time (typically 1 hour) Sections were washed again, 3x 5 mins in PBS and then incubated with the appropriate biotinylated secondary antibody (Vectastain kit) for 1 hour at room

temperature The tissue was washed 3x 5 mins in PBS and incubated with the ABC complex from the kit for 30 mins at room temperature Following this, slides were washed as before and exposed to diaminobenzidme (DAB) chromogen for up to 10 mins Sections were counterstained with hematoxylin for 30 secs, and washed in tap water for 5 mins The slides were rinsed with methanol and cleared with xylene (3 separate washes) The slides were then mounted using DPX mounting media

2 4 RNA preparation methods

2.4.1 RNA isolation

Trizol is a ready to use reagent for the isolation of total RNA, DNA and/or protein from cells and tissues RNA isolation was developed by Chomczynski *et al* (Chomczynski and Sacchi, 1987) Trizol reagent maintains the integrity of the RNA while disrupting the cells and dissolving the cell components

Cells were lysed in both tissue culture flasks and cartridges by the addition of 1ml of Trizol per $10cm^2$ surface area A volume less than this can result in contamination of the RNA with DNA To ensure complete homogenization, cells were lysed by passing through a pipette a number of times The samples were then incubated for 5 mins at room temperature to allow complete dissociation of nucleoprotein complexes 0 2ml of chloroform was added per ml of Trizol reagent used and was then mixed vigorously for 15 secs before incubation at room temperature for 5 mins Samples were then centrifuged at 12,000g for 15 mins at 4°C The mixture separated into a lower red, phenol-chloroform phase, an interphase and an upper colourless aqueous phase RNA remains exclusively in the aqueous phase

The aqueous phase was carefully removed and transferred to a fresh, sterile tube The RNA was precipitated out of solution by the addition of 0 5ml of isopropanol per 1ml of Trizol used Samples were incubated for 15 mins at room temperature and then centrifuged at 12,000g for 10 mins at 4°C The RNA precipitate forms a gel-like pellet on the side of the tube The supernatant was removed and the pellet washed in 1ml of 75% ethanol per ml of Trizol used, followed by centrifugation at 7,500g for 5 mins at 4°C The resultant pellet was air-dried for 5-10 mins before being resuspended in DNase/RNase-free water The sample was then stored at -80°C until used The concentration of total RNA was determined by UV spectrophotometry as outlined in the following section

2 4 2 Spectrophotometric analysis of nucleic acids

DNA or RNA concentrations were determined by measuring the absorbance at 260nm, the wavelength at which nucleic acids absorb light maximally (λ max) A 50μg/ml solution of DNA or 40μg/ml solution of RNA has an absorbance reading of 1 0 at this wavelength In order to calculate the concentration of DNA/RNA in samples the following calculations were used

For DNA Abs @ 260nm x 50 x dilution factor = μg/ml

For RNA Abs @ 260nm x 40 x dilution factor = μg/ml

The purity of the DNA or RNA samples was established by reading the absorbance at 260nm, the absorbance at 280nm, and then determining the ratio between the two (Abs260/Abs280) using the Shimadzu UV-160A dual-beam spectrophotometer Pure DNA, which has no protein impurities, has a ratio of 1 8, whereas pure RNA has a ratio of 2 0 Lower ratios indicate the presence of proteins, while higher ratios imply the presence of organic reagents

2 4 3 Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription was preformed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) in accordance with manufacturers specifications 0 5μg of total RNA was mixed with 0 125μg oligo dT primers and the reaction mixture brought to a final volume of 12μl with DEPC water This mixture was heated for 10 mins at 70°C to allow annealing of oligo dT primers to polyA tails of mRNA Following this, tubes were immediately cooled on ice and the remaining components of the reaction were added as follows

MLV 5x Reaction Buffer	5μl
10mM dNTP	3μl
MLV-RT	200units

The mixture was then made up to a final volume of 25μl using DNase/RNase-free water and incubated for 60 mins at 42°C Contaminating RNA was subsequently removed by the addition of 1μl of RNaseH (2units/μl) at 37°C for 20 mins cDNA samples were then either used immediately or stored at -80°C

2 4 4 Polymerase Chain Reaction

A 50µl PCR reaction mixture was prepared as follows

RNase free water	36 5µl
10x reaction buffer	5µl
10mM dNTP	1µl
25mM MgCl	3µl
10µM Forward primer	1µl
10µM Reverse primer	1µl
Taq Polymerase	0 5µl
cDNA sample	2µl

The mixture was overlaid with 50µl of mineral oil and then placed in a Hybaid PCR Thermocycler (SPRT 001) Samples were subjected to an initial incubation of 92°C for 2 mins, followed by 30 cycles comprise of the following steps 92°C for 1 min, annealing temperature for 2 mins (variable – dependent on primer set), and 72°C for 3 mins PCR products were removed from beneath the mineral oil and placed in fresh tubes before being subjected to agarose gel electrophoresis PCR was performed in order to test for the presence or absence of specific genes and confirmed via product size on agarose gels

Quantitative PCR was carried out using a Real time Rotor-Gene 3000^{TM} lightcycler The principle of real time amplification detection is that the amount of fluorescence is proportional to the concentration of product in a reaction Higher fluorescence indicates a higher concentration of a product Each PCR reaction was set up typically as follows

SYBR-Green	12 5µl
RNAse free water	8 5µl
cDNA	2µl
Forward primer	1µl
Reverse primer	1µl

Each sample was assayed in triplicate, and the program used for the different primer sets was as follows for 55 cycles

Denaturing Phase	95°C	20 s
Annealing Phase	57°C	45 s
Elongation Phase	72°C	30 s

2 4 5 Agarose gel electrophoresis

Agarose gels were prepared by boiling agarose in TAE buffer Gels were generally 1-4% (w/v) depending on the size of the DNA being visualised, and contained 0 5μg ethidium bromide per 1ml of agarose for visualization of DNA When the gel was hand-hot the gel was poured into a GibcoBRL Horizon 20 25 gel electrophoresis apparatus

Samples were mixed with 6x gel loading buffer 15μl of PCR product was mixed with 3μl of loading buffer and subsequently loaded The gel was run at 100V in TAE buffer until the blue dye front was approximately 0 5cm from the end of the gel DNA was visualized on a transilluminator and photographed for densitometric analysis using the Kodak 1D gel documentation system

2 4 6 Whole-Eye Tissue RNA Preparation

Several 20μM tissue sections were prepared as per section 2 3 2, one of which was dewaxed and counterstained in order to localize the retina in each eye Retinas were then crudely removed from the slides with a blade and pooled in a sterile eppendorf 1 ml of xylene was added per tube, vortexed vigorously for 1 min, and incubated at 58°C for 20mins Samples were then centrifuged @12,000 g for 2 mins The supernatants were aspirated and the step repeated with 1 ml of fresh xylene 500μl of 100% EtOH was then added and tubes were again vortexed and centrifuged @ 12,000 g for 1min 500μl of 70% ethanol was added, centrifuged again for 1 min, and the pellet air dried for 5-10mins Pellets were then washed twice in ice-cold PBS and resuspended in lysis buffer (cells-to-cDNA kit) and incubated @ 75°C for 12mins Samples were then cooled at room temperature for 5mins, after which 2μl Dnase was added Each sample was then gently vortexed and briefly centrifuged to collect samples to bottom of each tube Samples were incubated @ 37°C for 15 mins, then @ 75°C for 5mins The pellet/cell debris was then removed and the samples were ready for reverse transcriptase reaction

2 5 Protein Preparation & electrophoresis

2 5 1 Preparation of whole cell lysates

Following trypsinisation of cells as described in section 2 2 1, pellets were washed in PBS to remove any trace levels of FBS The cell suspension was then centrifuged at 1000g for 5 mins The PBS supernatant was removed and the cells were resuspended in lysis buffer The resulting lysates were frozen and thawed three times followed by three cycles of ultrasonication for 5 secs on ice using a sonic disembrator (Vibra Cell, Sonics and materials Inc) Samples were stored at -20°C for short-term storage or -80°C for long-term storage

2 5 2 Bicinchoninic Acid protein microassay

In this assay, Cu^{++} reacts with protein under alkaline conditions to produce Cu^{+}, which in turn reacts with BCA to produce a coloured product Two separate reagents were supplied in this commercially available assay kit (Pierce Chemicals) A, an alkaline bicarbonate solution and B, a copper sulphate solution 1 part solution B is mixed with 50 parts solution A 200µl of this mixture is added to 10µl of protein lysate or BSA protein standards (standard curve in the range 0-2mg/ml) The plate is incubated at 37°C for 30 mins and the absorbance read at 560nm using a microtitre plate reader

2 5 3 Western Blotting

SDS–PAGE was performed as described by Laemmli using 10% polyacrylamide gels (Laemmli, 1970) 10% resolving and 5% stacking gels were prepared as follows

Resolving Gel	1 5ml	1 5M Tris pH8 8
	1 5ml	40% acrylamide stock
	3 0ml	Distilled water
	60µl	10% (w/v) SDS
	30µl	10% (w/v) ammonium persulphate
	7µl	TEMED
Stacking Gel	0 75ml	0 5M Tris pH6 8
	0 375ml	40% acrylamide stock
	1 85ml	Distilled water
	30µl	10% (w/v) SDS
	15µl	10% (w/v) ammonium persulphate
	7µl	TEMED

Analysis of cell lysate protein concentration was determined by BCA assay and equal amounts of protein were resolved per gel Samples were mixed with loading buffer and boiled at 95°C for 5 mins, then immediately placed on ice The gel was electrophoresed in reservoir buffer at 40 milliamps (mA) per gel using an Atto vertical mini-electrophoresis system until the dye front reached the bottom of the gel

Following electrophoresis, the gel was soaked for 15 mins in transfer buffer Nitrocelluose membrane and 16 sheets of Whatmann filter paper were cut to the same size as the gel and soaked in transfer buffer Proteins were transferred to the membrane for 90 mins at 100V in a wet transfer system Following transfer,

membranes were soaked in Ponceau S solution to confirm transfer of protein to the membrane and also to normalize for variations in protein loading

Membranes were blocked for 1 hour in blocking solution (5% skimmed milk in PBS) Membranes were then incubated either overnight at 4°C or for 3-4 hours at room temperature, with primary antibody diluted according to manufacturers instructions in blocking solution The blots were then washed in three changes of PBS-T (0 1% (v/v) Tween in PBS) and then incubated for 2 hours at room temperature with a suitable Horseradish-peroxidase (HRP) linked secondary antibody diluted in PBS-T Following incubation in secondary antibody, the blots were again washed with three changes of PBS-T

Antibody-antigen complexes were detected by incubation in West Pico Supersignal reagent Briefly, an equal volume of solution A and B were mixed and the blot was incubated for 5 mins at room temperature Blots were exposed to autoradiographic film to visualize bands present on the blot and developed (Amersham Hyperprocessor Automatic Developer) Bands of interest were identified either by use of an antigenic positive control or based on molecular weight markers Exposure times varied depending on the antibody being used but were typically between 1-2 mins

2.6 DNA preparation methods

2.6.1 Transformation of Competent cells

10ng of plasmid DNA of interest was placed in a sterile microfuge tube. To this was added 100µl of competent *E.coli*. The mixture was gently mixed and placed on ice for 30mins. The cells were heat-shocked by placing the tube in a waterbath at 42°C for 45-50 seconds after which they were placed on ice for 2mins.

Cells were grown for 1 hour at 37°C with agitation (200rpm) in 1ml of sterile LB broth. The cells were then centrifuged at 5000g for 1 min and the supernatant removed. The resultant pellet was resuspended in 0.2ml of LB broth and spread plated either 150µl or 50µl on LB agar plus ampicillin (LB medium containing 1.5% (w/v) agar plus 35µg/ml ampicillin). The plates were incubated at 37°C overnight and for no longer than 18 hours to prevent coalescence of colonies. As a control for each transformation a mock transformation was included, in which no DNA was added to the competent cells.

2.6.2 Plasmid DNA Mini-preparation:

Plasmid DNA was isolated as specified by Qiagen Plasmid Kit protocol. Single colonies of transformed cells were removed from plates and grown in 3ml of LB broth supplemented with 35µg/ml ampicillin. These mini-cultures were grown at 37°C for 8 hours with gentle agitation (<200 rpm). 1.5ml of the final culture was used for the generation of glycerol stocks. The remainder was diluted in 100ml of LB broth supplemented with 35µg/ml ampicillin and grown at 37°C overnight at 250rpm. The following day the cells were harvested by centrifugation at 6000 rpm for 15 mins at 4°C.

The pellet was resuspended in 4ml of Buffer P1. The resuspended cells were lysed by gentle inversion with 4ml of Buffer P2 and incubated at room temperature for 10 mins. Protein was precipitated by the addition of 4ml of pre-chilled Buffer P3, gentle mixing and incubation on ice for 5 mins. Protein precipitates were removed by high-speed centrifugation @ 13000rpm for 30 mins at 4°C. The supernatant was

removed and centrifuged @ 13,000rpm for 30 mins at 4°C to ensure complete removal of all protein

Once the supernatant had been removed it was applied to a Qiagen tip-100, which had been equilibrated with Buffer QBT (commercial kit) The sample was allowed to enter the column by gravity flow and the column washed with 2x 10 ml washes of Buffer QC (commercial kit) Finally, DNA was eluted from the column using 5 ml of Buffer QF (commercial kit)

DNA was precipitated from the eluate by the addition of 3 5ml of isopropanol at room temperature The mixture was centrifuged at 12,000rpm for 30 mins at 4°C, to yield a glassy pellet The supernatant was removed carefully so as not to disturb the isopropanol pellet The pellet was then washed in 2ml of 70% ethanol, to remove precipitated salts and to make reconstitution of the pellet easier and then centrifuged at 12,000rpm for 10 mins The pellet was air-dried for 5–10 mins after removal of the supernatant and then redissolved in sterile Tris-EDTA (TE) buffer

2 6 3 Transient Transfections

BREC were transiently transfected in the perfused transcapillary system using Lipofectamine^{TM} reagent according to the manufacturers specifications (Invitrogen) Briefly, 1500µl of DMEM containing 33 75µg Luc reporter plasmid and 11 25µg LacZ (a plasmid encoding β-galactosidase activity) was mixed with 750µl DMEM containing 50µl lipofectamine The DNA/lipofectamine mix was added to cells harvested from 3x 75cm^2 flasks resuspended in 12 5mls DMEM Cells were left to incubate for 15-20 mins at room temperature after which, the volume was split evenly and seeded onto two separate capillary modules Following exposure to increased fluid flow using the apparatus described above, the cells were trypsinised from the capillaries Transactivation of reporter genes was evaluated by the luciferase assay and normalised to the β-galactosidase activity

2 6 4 Luciferase Assay

To analyse promoter transactivation of the COX-2 gene in BRECs exposed to pulsatile flow, cells were transfected with a luciferase tagged reporter gene construct Cells were pelleted by centrifugation at 1,000g for 5min at room temperature, washed once in sterile PBS and then transferred to a microfuge tube in 1ml of sterile PBS After centrifugation at 5,000g for 5 min at room temperature, the supernatant was completely removed and the cell pellet resuspended in 100µl of Reporter lysis buffer The tubes were vortexed for 10-15secs and lysis allowed to proceed for 15min on ice The lysates were clarified by centrifugation at 12,000g for 5min and the supernatant saved in a fresh tube Samples were stored at -80°C until required, when 20µl was used per assay Samples are stable in lysis buffer over several freeze-thaw cycles At the time of assay, it was important to allow sufficient time for the detection reagent (stored at -80°C) to equilibrate to room temperature 100µl of detection reagent was added to the sample, mixed by repetitive pipetting (3 times) and light emission integrated over a period of 60sec after lag period of 10sec Briefly, the enzyme firefly luciferase, generated due to promoter activation on a luciferase tagged plasmid, catalyses the conversion of D-luciferin to oxyluciferin, with a concominant production of a photon of light, with is measured by the luminometer

2 6 5 β-galactosidase assay

LacZ, a plasmid encoding β-galactosidase was used to monitor transfection levels Increased β-galactosidase activity was attributed to successful transfection of the gene of interest Following transfection and cell lysis, a 30µl sample was added to 3µl of 100x Mg solution (0 1M $MgCl_2$ and 4 5M β-mercaptoethanol), 66µl of ONPG (o-nitrophenyl-β-D-galactopyranoside) (4mg/ml ONPG in 0 1M sodium phosphate, pH 7 5) and 20µl of 0 1M sodium phosphate The reaction was incubated for 4-6 hours at 37°C until a yellow colour developed The reaction was subsequently stopped with 500µl of Na_2CO_3, and optical density read at 420nm

2 7 Media Assays

2 7 1 Nitrate Assay

Measurement of nitrate in culture media was carried out by fluorometric assay as described previously (Kleinhenz et al , 2003) In brief, the reaction of 2, 3-diaminonaphthalene (DAN) with nitrate results in the formation of 1-(H)-naphthotriazole, a fluorescent product The reaction is initiated by the addition of 10µl of DAN solution (0 05mg/ml in 0 62M HCl) to 100µl of standard or media sample and allowed to continue for 10 mins The reaction is terminated by the addition of 5µl of 2 8M NaOH Samples were read using a Luminescence spectrometer at excitation 365nm, emission 450nm and nitrate activity recorded as pmoles nitrate/mg protein

2 7 2 Prostacyclin Assay

An enzyme immunoassay (EIA) kit was used to determine the level of prostacyclin in media from cultured cells This kit uses a competitive binding technique The prostaglandin in the sample competes with a fixed amount of alkaline-phosphatase-labelled PG for the binding of a polyclonal antibody, which is immobilised to the wells of the 96-well plate 100µl of sample or standard was placed into each antibody-coated well of the 96-well plate 50µl of blue conjugate (alkaline phosphate conjugated with PG) and 50µl of antibody solution (containing a monoclonal antibody to the PG) was incubated with each of the standards and samples for 2 hours at room temperature at 50rpm All wells were washed 3 times with washing buffer (Tris-buffered saline (TBS) containing detergents and sodium azide) 200µl of p-nitrophenyl phosphate (pNPP substrate) was added to each well and incubated for an additional 45mins at room temperature 50µl of assay buffer (TBS containing proteins and sodium azide) was used to assess non-specific binding and maximum binding The optical density of each well was read at 405nm, with wavelength correction between 600 and 690nm, using the Wallac 1420 Manager on

the Wallac Victor 2 1420 multilabel counter PG concentration was then calculated using the optical density of the samples

2 7 3 Endothelin-1 Assay

This immunometric assay is based on a double antibody 'sandwich' technique Each well of the 96-well plate is coated with a monoclonal antobody specific for Endothelin-1 (capture antibody) An acetylcholinesterase Fab' Conjugate (AchE Fab'), which binds to a different epitope on the endothelin molecule is also added to the well This allows the two antibodies to form a 'sandwich' by binding on opposite sides of the molecule The 'sandwiches' are immobilised on the plate so the excess reagents can be washed away The concentration of the analyte is then determined by measuring the enzymatic activity of the AchE by adding Ellman's reagent (which contains the substrate for AchE) to each well The product of the AchE- catalyzed reaction has a distinct yellow colour which absorbs strongly at 412nm The intensity of this colour, determined spectrophotometrically, is directly proportional to the concentration of the endothelin

2 8 Fluorescense Actıvated Cell Sortıng Analysıs

Flow Cytometry technology ıs utılized to measure properties of cells as they move, or flow, ın lıquıd suspensıon Most flow cytometers can measure two kınds of lıght from cells lıght scatter and fluorescence, consıderıng all materıals, ıncludıng cells, wıll scatter lıght In a flow cytometer, lıght scatter detectors are located opposıte the laser (relatıve to the cell), and to one sıde of the laser, ın-lıne wıth the fluıd-flow/laser beam ıntersectıon The measurements made by these detectors are called forward lıght scatter and sıde lıght scatter, respectıvely Forward lıght scatter provıdes some ınformatıon on the relatıve sıze of ındıvıdual cells, whereas sıde lıght scatter provıdes some ınformatıon on the relatıve granularıty of ındıvıdual cells In thıs case these two are combıned to ıdentıfy the characterıstıc lıght scatter pattern emıtted by condensed, shrunken apoptotıc cells, presented as dot plots

Fluorescence ıs the abılıty of a molecule to absorb lıght of a partıcular wavelength and re-emıt lıght of a longer wavelength The wavelength change relates to an energy loss that takes place ın the process

2 8 1 Apoptosis Assay

Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development Apoptosis is distinguished from necrosis, or accidental cell death, by characteristic morphological and biochemical changes, including compaction and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm and loss of membrane asymmetry In normal viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane However in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment The human anticoagulant, annexin V, is a 35-36kD Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet

We utilised the commercially available 'Vybrant™ Apoptosis Assay Kit #2' containing a recombinant annexin V conjugated to the Alexa Fluor488 dye Alexa Fluor488 dye is an almost perfect spectral match to fluorescein (FITC), but it creates brighter and more photostable conjugates In addition, the kit includes a ready-to use solution of the red-fluorescent propidium iodide (PI) nucleic acid binding dye PI is impermeant to live cells and apoptotic cells, but stains necrotic cells with red fluorescence, binding tightly to the nucleic acids in the cell After staining a cell population with Alexa Fluor488 annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence These populations can easily be distinguished using a flow cytometer with the 488nm line of an argon-ion laser for excitation

BRECs and BRPs were trypsinised from capillaries, washed in ice cold-PBS and resuspended in 400ul Annexin-Binding Buffer (ABB) From this, 50µl cells were mixed with 2 5µl Alexa Fluor488 Annexin V and 1µl of PI and incubated at room temperature for 15 mins A further 200µl of ABB is then added and placed on ice till read on FACs machine

2 8 2 Proliferation Assay

The succinimidyl ester of carboxyfluorescein diacetate (CFDA, SE) is currently the most widely used probe for generation analysis of cells CFDA SE spontaneously and irreversibly couples to both intracellular and cell-surface proteins by reaction with lysine side chains and other available amine groups When cells divide, CFDA SE labeling is distributed equally between the daughter cells, which are therefore half as fluorescent as the parents As a result, each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity, that is readily detected by flow cytometry

BRECs and BRPs were labelled immediately prior to seeding into the compartments of the perfused transcapillary system, utilising the Vybrant™ CFDA SE cell tracer kit A stock solution of 5mM CFDA SE was diluted in DMSO and further diluted with HBSS to a working concentration of 5μM After three washes in HBSS, cells were treated with dye for 15 mins at 37°C Cells were then centrifuged and resuspended in regular media and introduced into the capillary compartment

2 9 Statistical analysis

Results are expressed as mean ±SEM of a minimum of three independent experiments (n=3) unless otherwise stated Statistical comparisons between groups of normalized densitometric data were performed using both unpaired Student's t-test and Wilcoxon signed rank test A value of $P< 0\ 05$ was deemed significant

Chapter 3

Acute Exposure to Pulsatile Flow Regulates Nitric Oxide, Prostacyclin & Endothelin-1 in BREC mono-culture & BREC/BRP co-culture

3.1 Introduction

3.2 Results

3.3 Discussion

3.4 Conclusion

3 1 Introduction

Perfusion of retinal vessels is dependent upon the complex interaction of opposing vasodilatory and vasoconstrictive forces in the retinal blood supply (Flammer et al , 2002) Retinal pericyte and endothelial cell (EC) cross-talk in these vessels is largely influenced by mechanical stimuli due to the pulsatile nature of blood flow *i e* pulse pressure (cyclic strain) and shear stress (Chakravarthy and Gardiner, 1999, Haefliger et al , 2001, Traub and Berk, 1998, Lehoux and Tedgui, 1998) These mechanical forces result in cellular signalling events mediated *via* growth factors and intracellular secondary messengers, G-proteins, small GTPases and kinases (Malek et al , 1999a, Redmond et al , 1998) The response to fluid shear stress, the frictional tangential force imposed on the vessel wall due to blood flow, results in abluminal release of factors from ECs, resulting in dilation or constriction of the underlying pericyte layer (Patrick and McIntire, 1995) While ECs are the major recipient of shear stress, cyclic strain exerts its effect on both the endothelium and the pericyte cell layer and both forces have been shown to modulate local autoregulation of vessel tone

A number of retinal pathologies are associated with disturbed retinal blood flow Vascular dysregulation or impaired autoregulation, leads to vasospasm of retinal vessels and has been implicated in normal tension glaucoma, where decreased retinal blood flow correlates with increasing optic nerve head damage (Prasanna et al , 2003) and reduced pulsatile ocular blood flow (Frank, 2004) Similarly, hyperglycaemic retinas exhibit impaired flicker-induced vasodilation and also lack of an appropriate vasoconstrictor upon breathing of pure oxygen, each implicating vascular dysregulation in diabetic retinopathy

ECs modulate vessel tone *via* release of the dilators nitric oxide (NO) and prostacyclin (PGI_2), and vasoconstrictors such as endothelin-1 (ET-1) Shear stress has already been shown to modulate EC production of these vasoactive agents NO, PGI_2 & ET-1 in macrovascular cells However, a large proportion of our knowledge and understanding of the retinal microvascular system has been obtained by drawing

comparisons with the more extensively researched macrovasculature As such, we examined the interaction between retinal endothelial and pericyte cells exposed to pulsatile fluid flow, that is shear stress and pulse pressure combined, utilising a perfused transcapillary co-culture system

The aim of this chapter was to determine the 'acute' effect (i e 24 hours) of pulsatile flow on cell morphology and production of vasoactive mediators by retinal endothelial cells, either cultured alone or in co-culture with retinal pericytes In addition, we analysed which signalling pathways are activated and the role of the proposed mechanotransducers G-proteins, integrins and protein tyrosine kinases

3 2 Results

3 2 1 Characterisation of Bovine Retinal Pericytes & Endothelial Cells

Vascular cells derived from bovine retina were routinely characterised to confirm a pure culture of either pericyte or endothelial cells Similar to vascular smooth muscle cells (SMCs), pericytes stain positively for contractile proteins such as α-smooth muscle cell specific actin (α-smc-actin), a cytoskeletal marker of pericyte cells, and also other cytoskeletal components such as calponin and myosin (Fig 3 1 (a) (b) and (c)) Pericytes were identified and distinguished from ECs by their size and irregular morphology, by their noncontact-inhibited growth pattern, and by the lack of staining with antisera to von Willebrand factor, endothelial nitric oxide synthase (eNOS) and the astrocyte-specific marker glial fibrillary acidic protein (GFAP) (data not shown) Pericytes were also distinguished from SMCs by their irregular morphology and their lack of "hill and valley" growth pattern

Endothelial cells exhibited a typical 'cobblestone' growth morphology (Fig 3 2 (A)) and also stained positive with anti sera to von Willebrand factor and eNOS (Fig 3 2 (B) & (C)) Endothelial cells were negative for α-smc-actin, calponin, myosin and GFAP (data not shown)

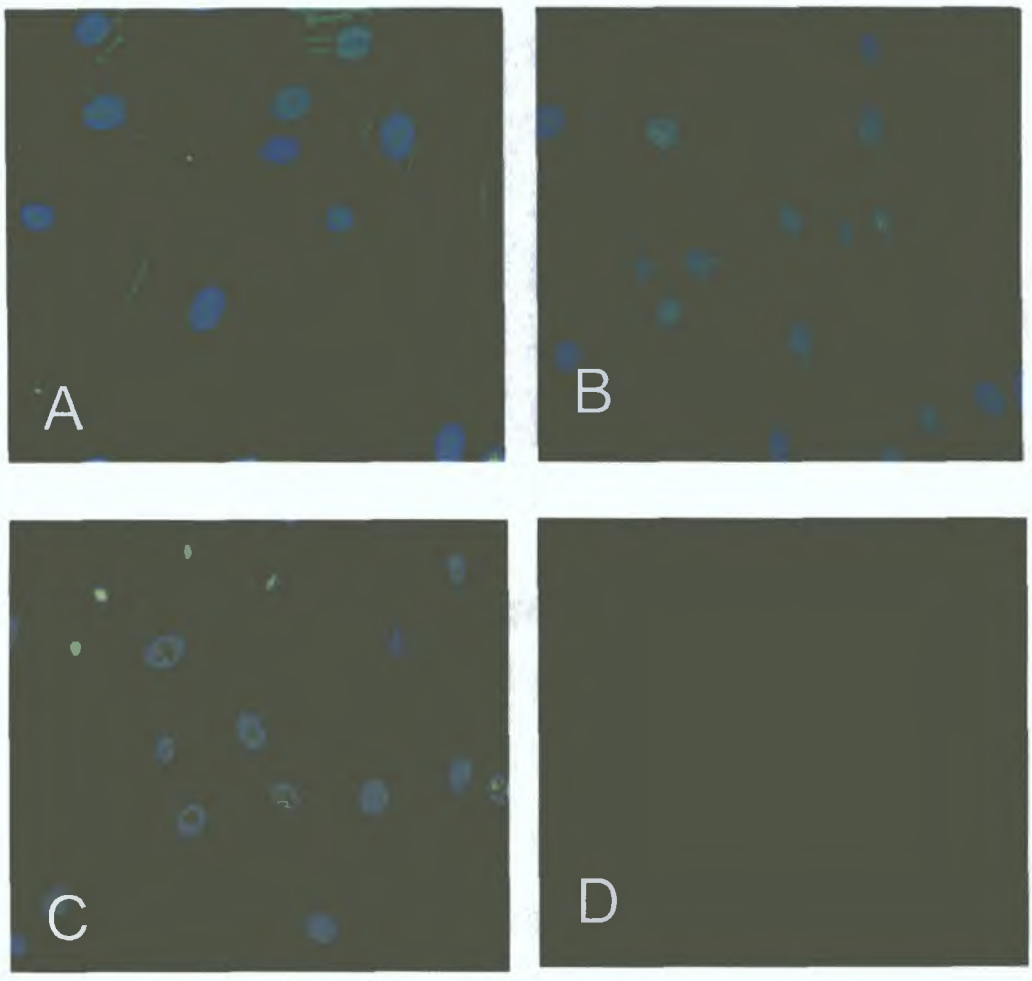


Figure 3.1 Immunocytochemistry staining for pericyte cytoskeletal components (green): **(A)** α-smc-actin; **(B)** Calponin; **(C)** Myosin; **(D)** No primary antibody control. Nuclei are stained with dapi (Blue). Magnification x20.

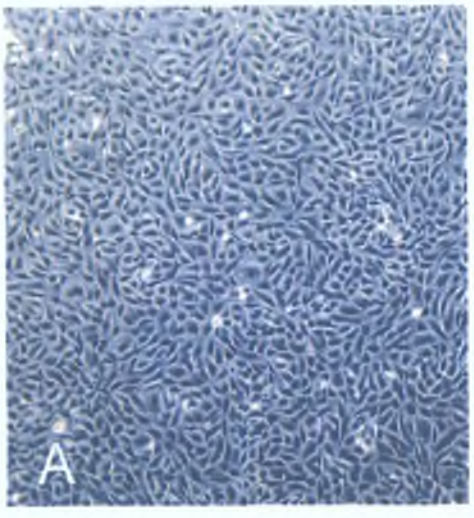
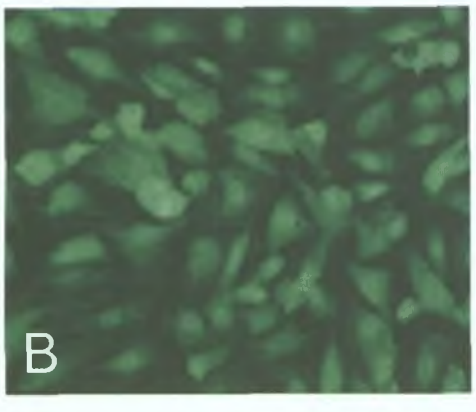


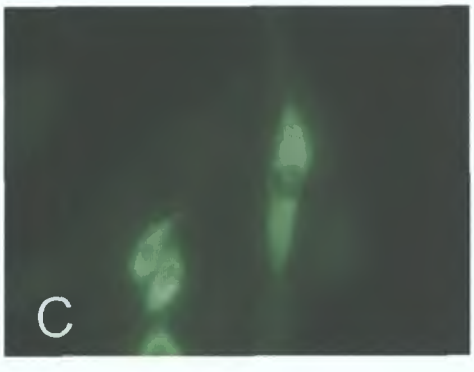
Figure 3.2

Characterisation of Bovine Retinal Endothelial Cells

(A) Phase contrast microscopy of confluent endothelial cells. Magnification x4.



(B) Immunocytochemistry staining for endothelial cell specific marker von Willebrand Factor (green) Magnification x20.



(C) Immunocytochemistry staining for endothelial cell specific marker endothelial nitric oxide synthase (green). Magnification x40.

3 2 2 Characterisation of Pericytes & Endothelial Cells Exposed to Pulsatile Flow

To date, most *in vitro* experiments have been performed on monocultured EC exposed to laminar flow The potential modulatory role of a neighbouring cell type and the pulsatile nature of blood flow have been largely ignored Therefore, using a perfused transcapillary co-culture system we have investigated the effects of acute pulsatile flow (*i e* 24 hours) on monocultured BRECs or co-cultured BREC and BRPs

The presence of BRP cells on the surface of the capillaries was determined by immunocytochemistry and scanning, electron and confocal microscopy of individual capillaries removed from the bundle Staining for the presence of α-smc-actin, showed BRP to be positive for this marker (Fig 3 3 (a) and 3 4) and negative for endothelial nitric oxide synthase and GFAP, (data not shown) A fluorescent stain, 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining (Blue Fig 3 3 (b) and (c)) DAPI binds selectively to double-stranded DNA, with little or no background staining of the cytoplasm Visualisation of BRP with anti α-smc-actin was performed on sub-confluent cells using confocal microscopy and confirmed BRP were present on the capillaries Moreover, actin staining appeared more punctate under high flow as the cells begin to orientate in the direction of flow (Fig 3 4) confirming that these changes in pulsatile flow induce a clear reorganization of the cytoskeleton Scanning electron microscopy revealed that the pericyte cell layer at confluency maintained a stellar morphology under low flow conditions (Fig 3 5 (a)), similar to that observed in cells grown in static cultures (Fig 3 1 (a)) Increases in media flow rate led to orientation of the BRP along the direction of flow, forming elongated striated cells (Fig 3 5) The presence of functional microvascular endothelial cells was further confirmed by western blot of eNOS proteins in these cells (Fig 3 6)

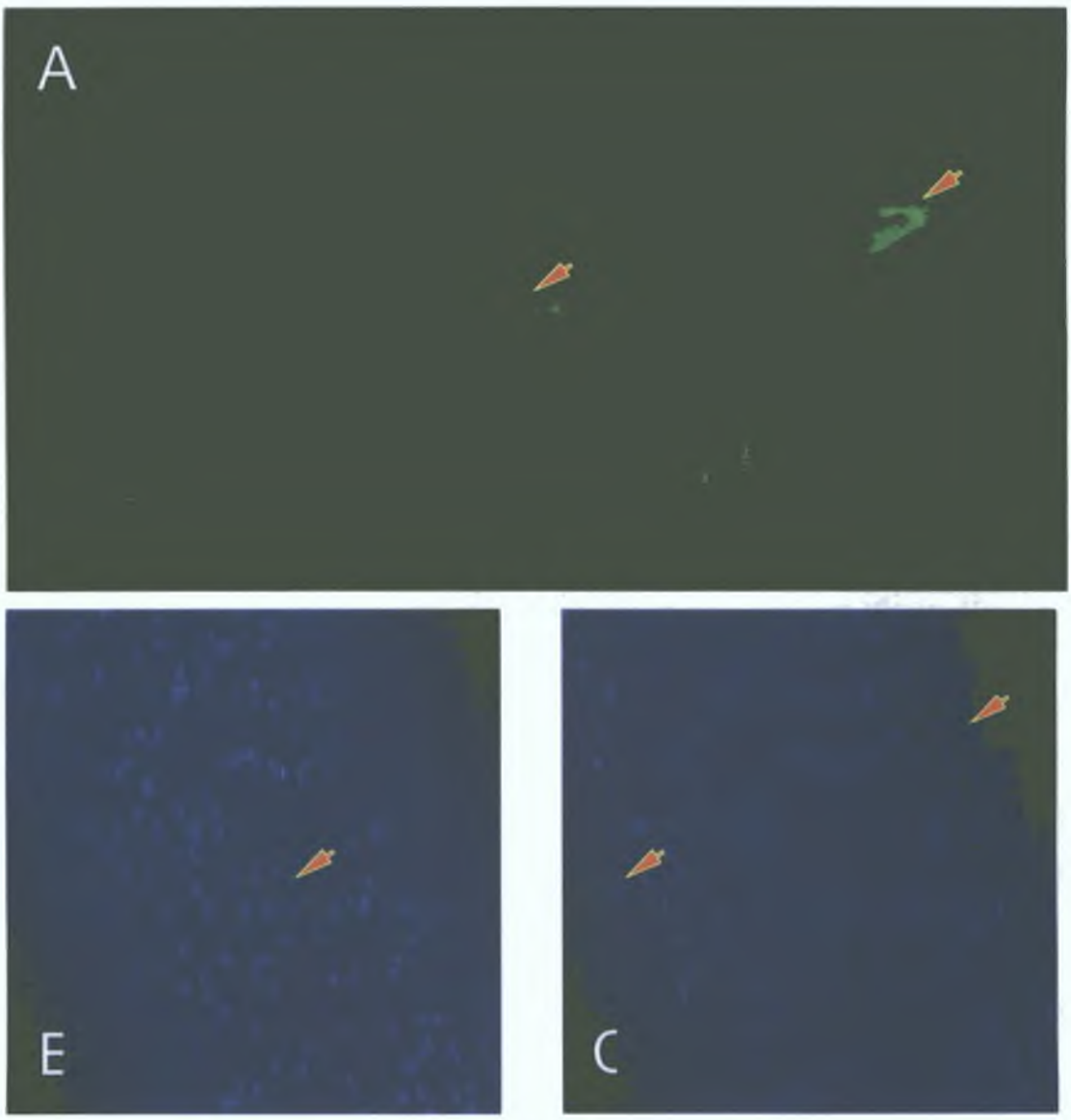


Figure 3.3 Immunocytochemistry analysis of pericytes cultured on CELLMAX™ capillaries. (**A**) Immunocytochemistry staining for pericyte α-smc-actin (arrows); (**B**) & (**C**) Nuclei stained with dapi (Blue) in one field at two depths of focus. Arrows denote top (B) and side (C) of capillaries. Magnification x20.

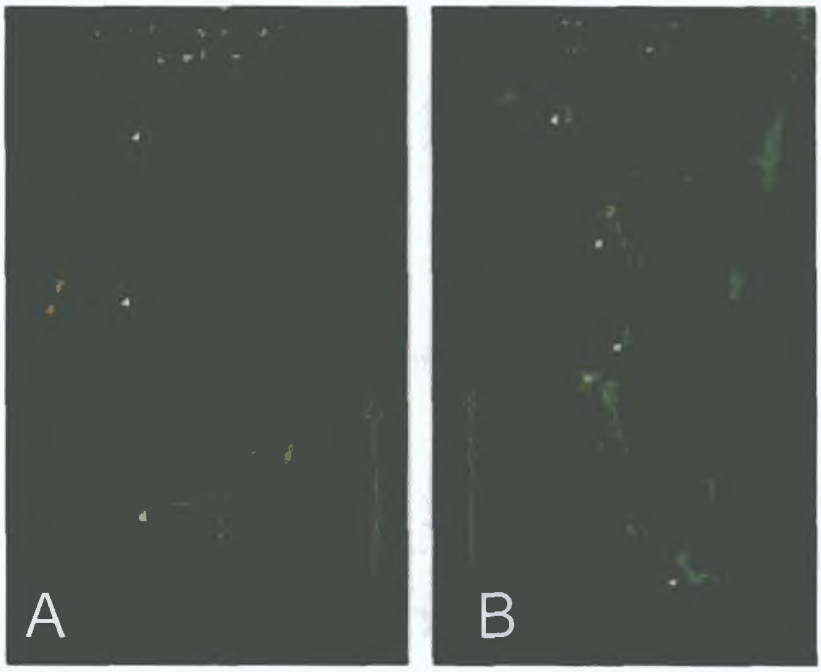


Figure 3.4 Confocal Imaging of BRPs on perfused capillary tubing stained for α-actin at (A) Low Flow & (B) High Flow. White arrows: diffuse staining during low flow and clustering of α-smc-actin with punctate staining in cells exposed to high flow. Green arrow: direction of flow. Insets: punctate staining of α-smc-actin under high flow at higher magnification. Magnification x20; insets, x100.

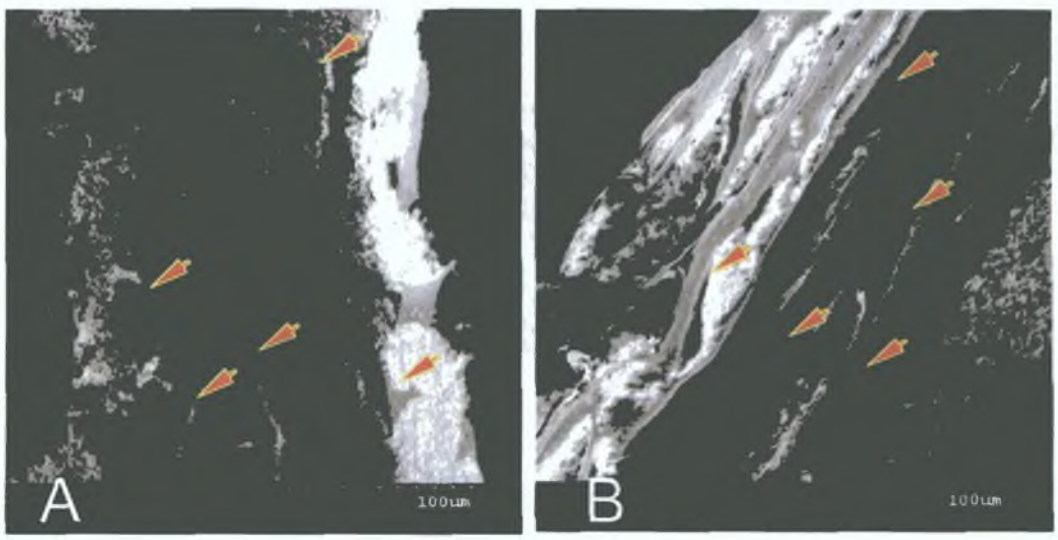


Figure 3.5 Scanning electron micrographs of BRPs cultured on capillary tubing and subjected to (A) low- and (B) high-flow conditions. In low flow conditions, pericytes were more stellar/rounded in their morphology. Arrows: bovine retinal pericytes. In high-flow conditions, the pericytes changed their morphology and oriented themselves along the direction of flow, giving a more striated appearance. Cell contacts became tighter, and the capillary was no longer visible. Magnification, x450.

3 2 3 Pulsatile Flow-induced Activation of Endothelial Nitric Oxide Synthase (eNOS)

We investigated the effect of low (0 3mls/min, 6mm Hg, 0 5 dynes/cm^2) and high (25mls/min, 56 mmHg, 23 dynes/cm^2) pulsatile flow on the total eNOS protein levels and the phosphorylation state of eNOS in BREC cultured alone or in co-culture with BRP over a 24 hour period Under these conditions, no changes in total BREC eNOS expression were observed in either mono-cultures, or co-cultures with BRPs (Fig 3 6 (a) and (b)) as determined by Western blotting However, a significant increase in the phosphorylation state of the eNOS protein was detected in both mono-cultured (1 903 ± 0 332 fold, n=6) and co-cultured (1 932 ± 0 199 fold, n=6) BRECs exposed to high flow (Fig 3 6 (c) and (d)) Metabolism of nitric oxide and release of nitrate into the circulating media was further confirmed by a fluorometric DAN assay There was a significant increase in nitrate levels in mono- and co-cultures of BREC, respectively (2 306 ± 0 276 and 2 404 ± 0 38 fold, n=5) (Fig 3 7 (a) and (b))

Real-time PCR analysis of eNOS expression in mono-cultured BRECs at 2, 8 5 and 24 hours, revealed no changes in eNOS mRNA levels (Fig 3 7 (c)) This suggest that changes in pulsatile flow does not affect eNOS transcription within 24 hours

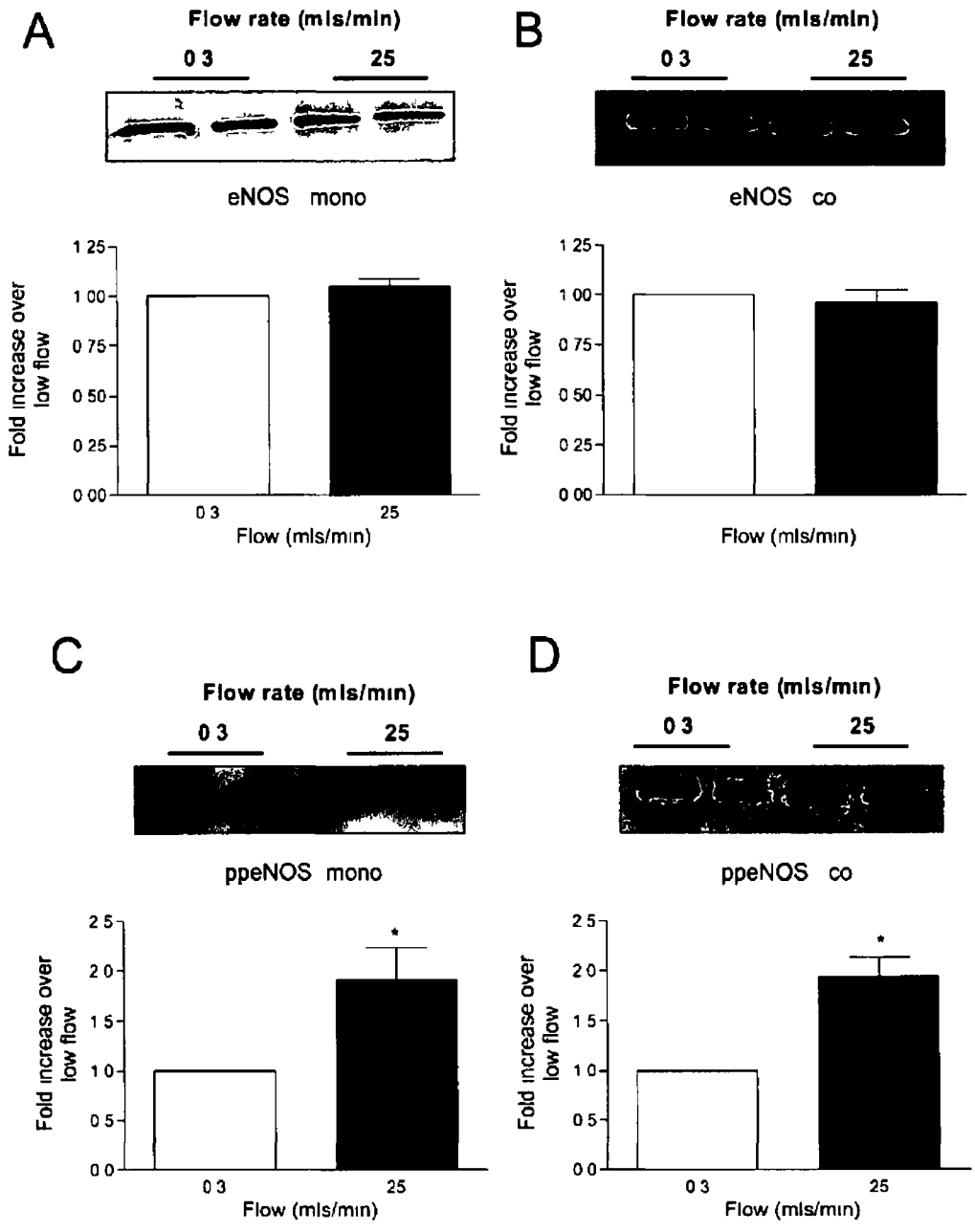


Figure 3 6 The effects of Pulsatile Flow on BREC total and phosphorylated eNOS
Western Blot Analysis of (A) BREC eNOS in mono-culture (B) BREC eNOS in co-culture (C) BREC phosphorylated-eNOS (ppeNOS) in mono-culture (D) BREC ppeNOS in co-culture Histogram represents mean values ± SEM (n=6) *P<0 05 compared to low flow control

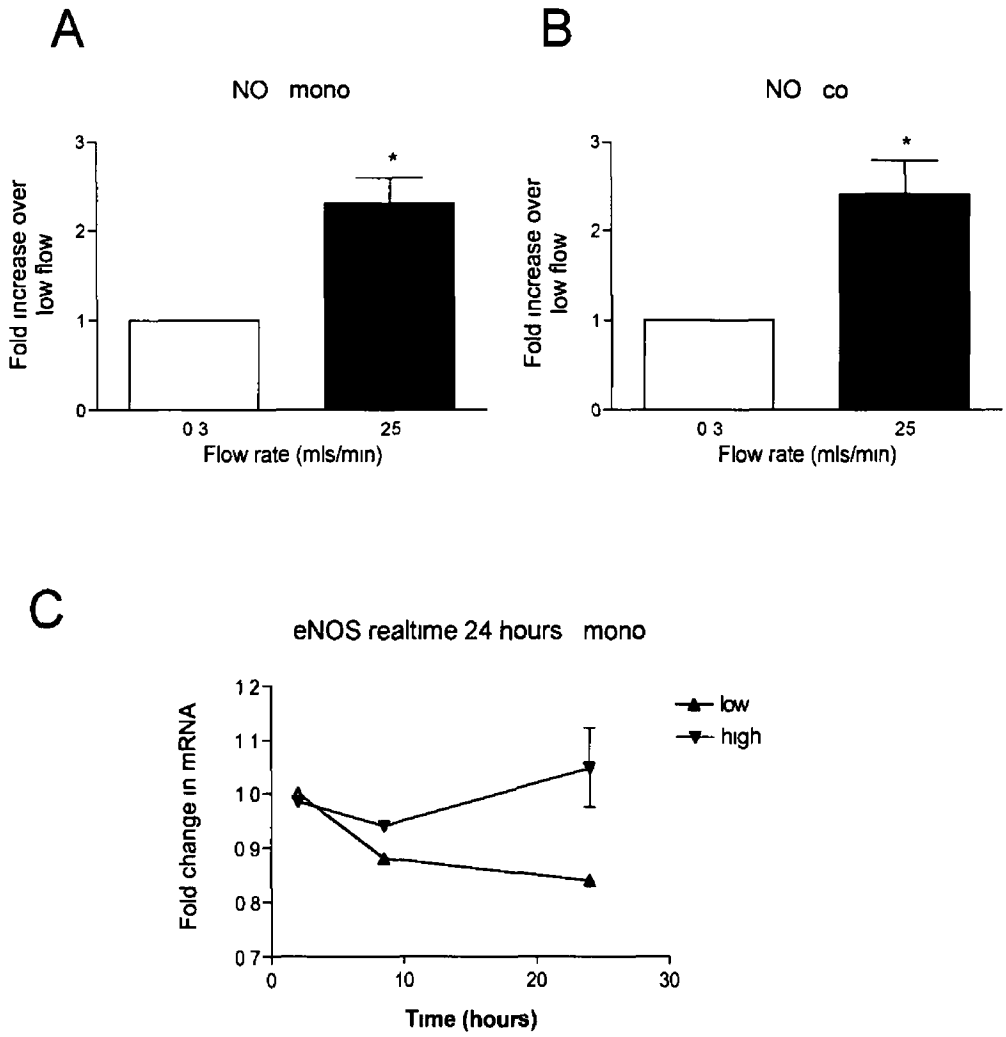


Figure 3 7 The effects of Pulsatile Flow on NO release and BREC eNOS mRNA **(A)** mono-culture ECs nitrate assay after 24 hours (n=6) **(B)** co-cultured EC/Pericyte nitrate assay after 24 hours (n=6) **(C)** Real-time PCR analysis of *eNOS* mRNA in mono-cultured endothelial cells after 2 hours (n=1), 8 5 hours (n=1) & 24 hours hours (n=3) exposed to low or high flow Histogram represents mean values ± SEM, *P<0 05 compared to low flow control

3 2 4 Pulsatile Flow induced Prostacyclin release by BREC

In order to examine the effect of pulsatile flow on the production of PGI_2, we exposed mono- and co-cultures of BREC and BRP to low and high flow rates as previously described The levels of prostacyclin present in the circulating media were determined by enzyme immunoassay There was a significant increase in PGI_2 levels in cells exposed to high flow as compared to low flow (2 260 ± 0 257 fold, n=6, and 2 015 ± 0 372 fold, n=7) in both mono- and co-cultures respectively (Fig 3 8 (a) and (b))

To determine if the increase in PGI_2 levels was a direct consequence of the induction of COX-2 expression, we examined the effect of pulsatile flow on COX-2 promoter activity in BREC In cells transiently transfected with a plasmid encoding the COX-2 promoter, there was a significant increase in COX-2 transactivation (5 066 ± 1 07 fold, n=5) in monocultured BREC after 24 h exposure to pulsatile flow (Fig 3 8 (c)), demonstrating the presence of elements within the COX-2 gene which can be activated by flow

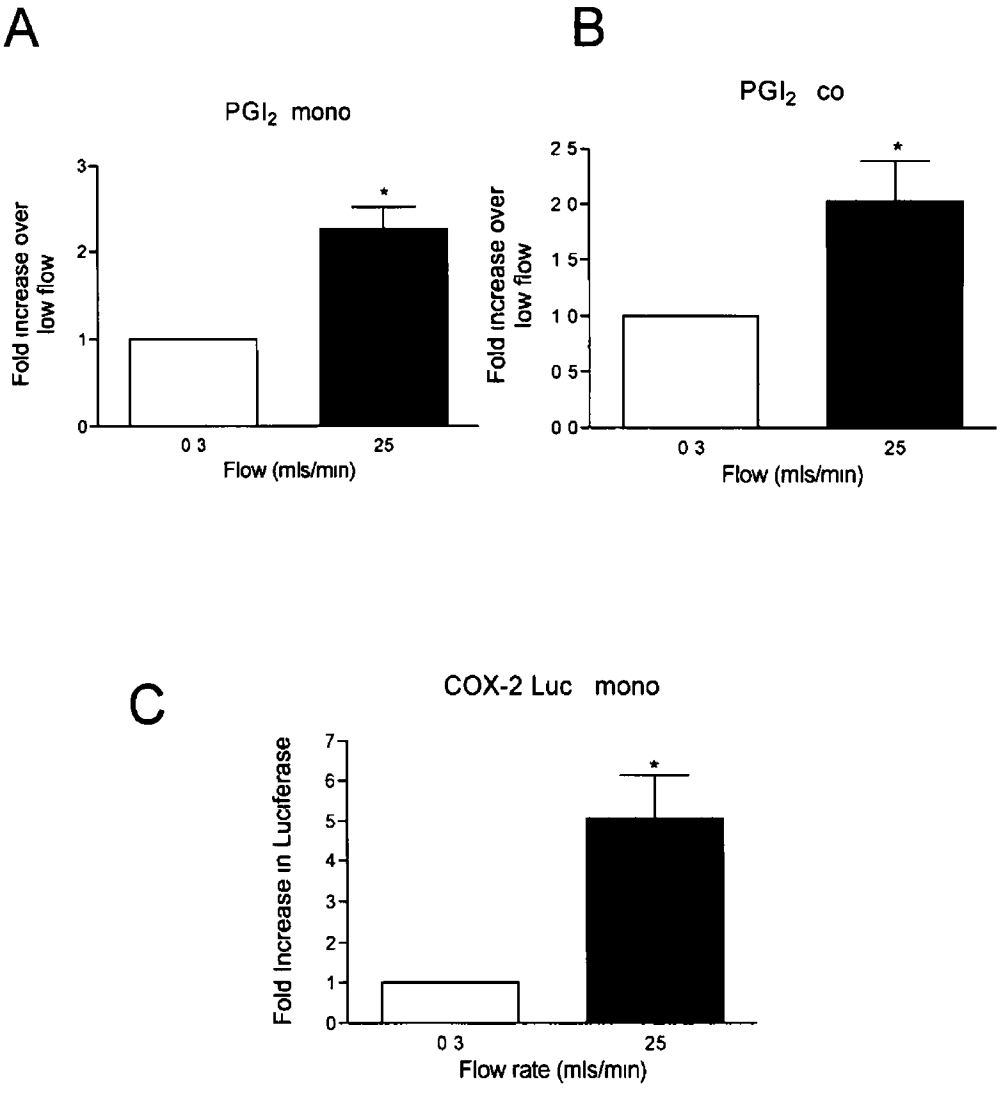


Figure 3 8 The effects of Pulsatile Flow on PGI_2 release and BREC COX-2 protein (A) mono-culture ECs prostacyclin assay after 24 hours **(B)** co-cultured EC/Pericyte prostacyclin assay after 24 hours **(C)** Transactivation of the COX-2-Luc reporter construct Luciferase activity was normalised to b-gal activity and expressed as fold increase over low flow Histogram represents mean values ± SEM, (n=6) *P<0 05 compared to low flow control

3 2 5 Pulsatile Flow-induced Changes in Endothelin-1 (ET-1) Release

To examine the relationship between pulsatile flow and ET-1 peptide release, we exposed BREC and BRP to low and high pulsatile flow rates as previously described Exposure of cells to high pulsatile flow resulted in a 1 850 ± 0 338 and 1 961 ± 0 205 fold (n=5) increase in media perfusate ET-1 levels in mono- and co-cultured BREC, respectively, as compared to low flow (Fig 3 9)

Real-time PCR analysis of ET-1 mRNA expression in mono-cultured BRECs at 2, 8 5 and 24 hours, revealed a slight, but insignificant increase in ET-1 mRNA levels after 2 hours exposure to high flow, with a significant decrease after 8 5 hours, which is maintained at 24 hours high flow (Fig 3 9 (c))

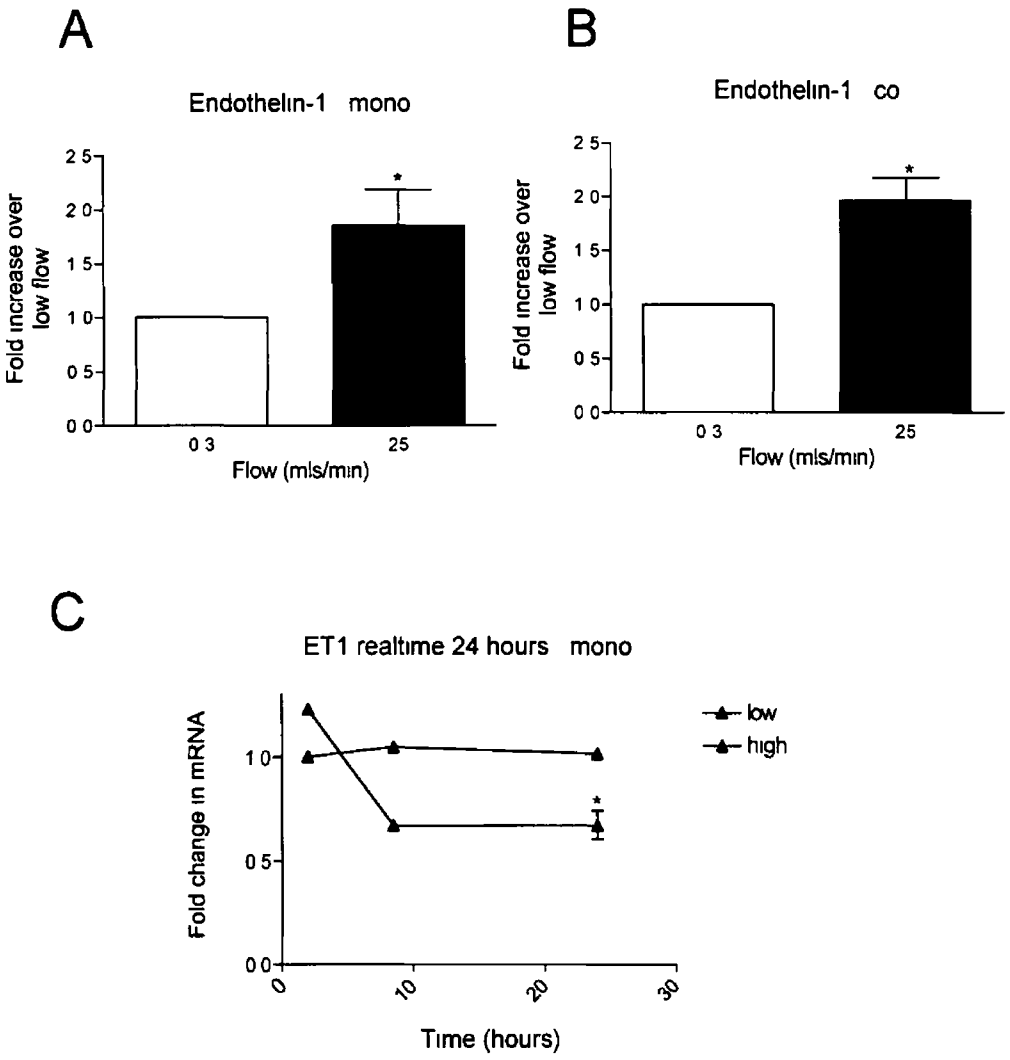


Figure 3 9 The effects of Pulsatile Flow on ET-1 release and BREC ET-1 mRNA (A) mono-culture ECs endothelin-1 assay after 24 hours (n=6) **(B)** co-cultured EC/Pericyte endothelin-1 assay after 24 hours (n=6) **(C)** Real-time PCR analysis of *ET-1* mRNA in mono-cultured endothelial cells after 2 hours (n=1), 8 5 hours (n=1) & 24 hours (n=3) exposed to low or high flow Histogram represents mean values ± SEM, *P<0 05 compared to low flow control

3 2 6 Pulsatile Flow regulation of mitogen activated protein kinases (MAPKs)

To determine if increased nitric oxide, prostacyclin or endothelin-1 is due to an overall change in MAPK signalling, we examined the effect of pulsatile flow on total ERK and total p38, and also the effect on the phosphorylation state of these enzymes (phospho-ERK and phospho-p38) In BRECs analysed from both mono-cultures and co-cultures with BRPs, there was no change in total ERK, total p38, phospho-ERK or phospho-p38 (Fig 3 10 and 3 11) after 24 hours exposure to low or high pulsatile flow

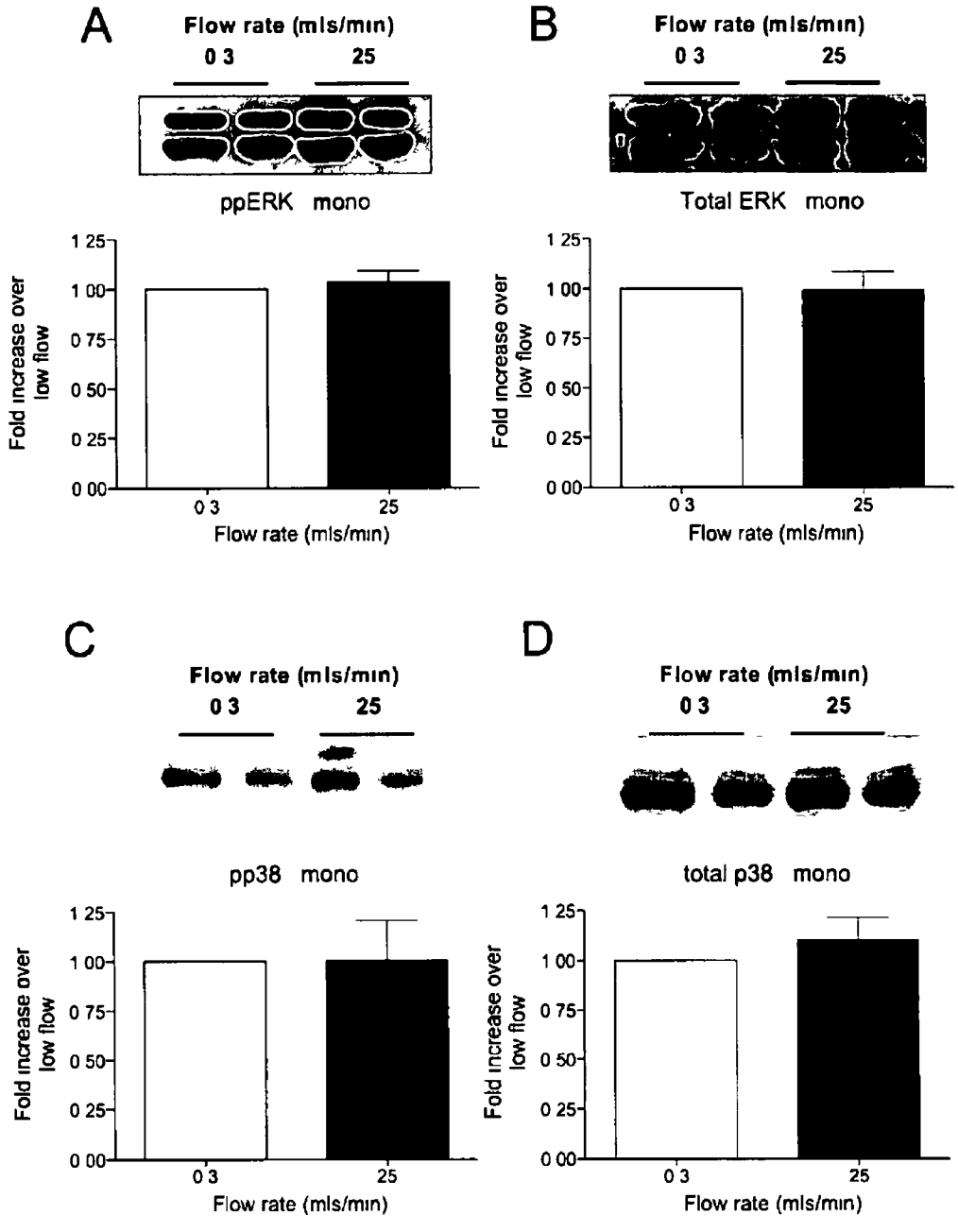


Figure 3 10 The effects of Pulsatile Flow on mono-cultured BREC s total and phosphorylated FRK & p38 Western Blot Analysis of **(A)** ppERK **(B)** total ERK **(C)** pp-p38 **(D)** total p38 Histogram represents mean values ± SEM (n=3)

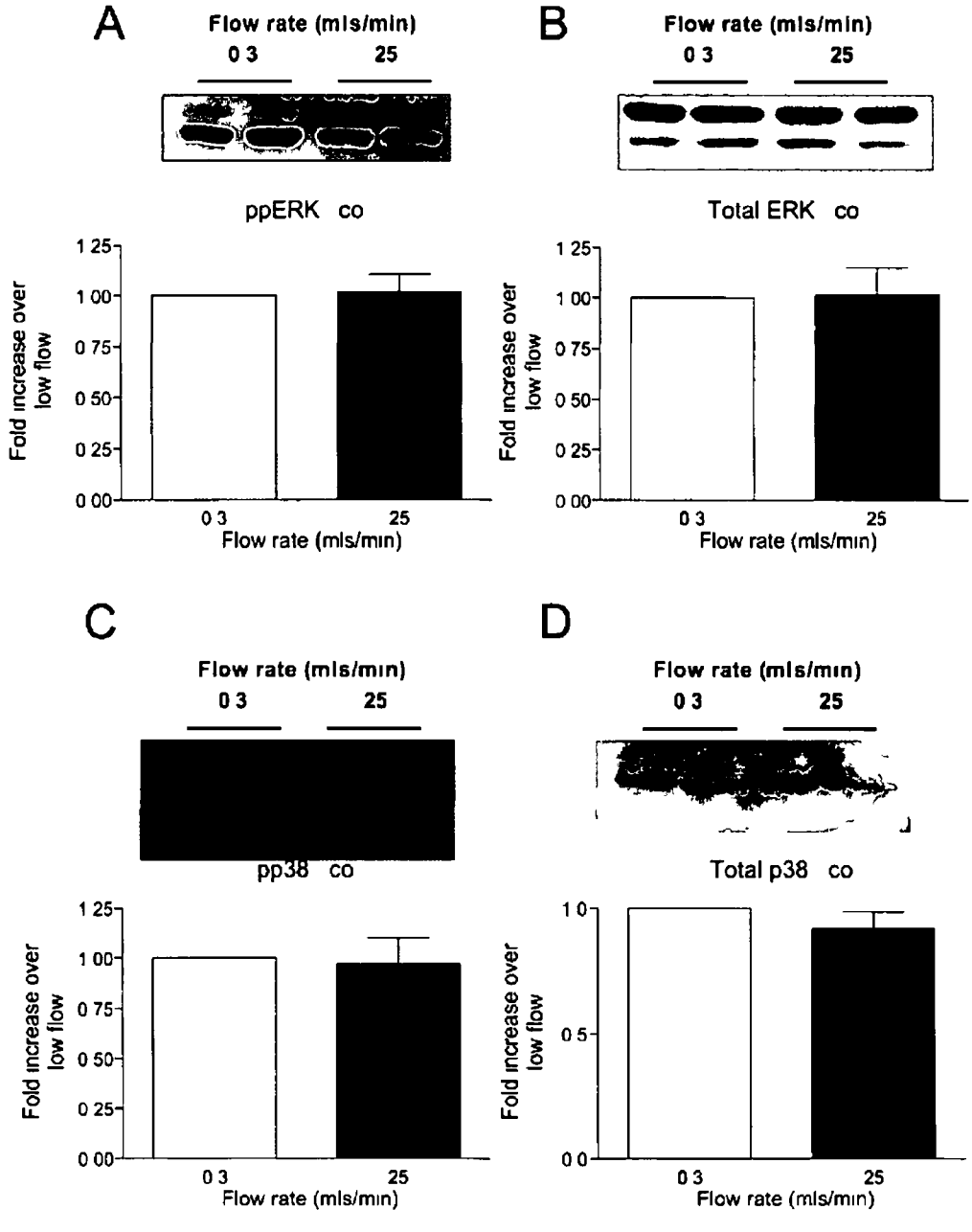


Figure 3 11 The effects of Pulsatile Flow on BREC total and phosphorylated ERK & p38 in co-culture with BRPs Western Blot Analysis of **(A)** ppERK **(B)** total ERK **(C)** pp-p38 **(D)** total p38 Histogram represents mean values ± SEM (n=3)

3 2 7 Mechanotransduction of Pulsatile Flow-induced changes in Nitric oxide, Prostacyclin & Endothelin-1

Endothelial cell transduction of a mechanical force into a biochemical signal is typically mediated via integrins, protein tyrosine kinases (PTKs) or G-proteins In order to determine the role of each of these 'mechanotransducers' in mediating increased nitric oxide, prostacyclin or ET-1 due at high pulsatile flow, we exposed mono-cultures of BRECs to low and high pulsatile flow, in the presense of specific inhibitors of integrins (0 5mM linear RGD peptide), PTKs (40μM Genistein) or G-proteins (25 ng/ml Pertussis toxin (PTX))

Increased endothelial nitric oxide production due to high pulsatile flow is attenuated by 50% when G-protein signalling is impaired with pertussis toxin, while no change was observed with blockage of integrin or PTK signalling (Fig 3 12 (a)) These results demonstrate the importance in G-protein signalling mediating increased nitric oxide in BRECs exposed to high pulsatile flow

Similar to nitric oxide, prostacyclin release is also increased after 24 hours exposure to high flow This increase is partially inhibited with blockage of integrin signalling using RGD peptide (Fig 3 12 (b)) Addition of both genistein and PTX did not inhibit prostacyclin release Endothelin-1 release was inhibited by addition of PTX only, suggesting a role of G-protein signalling in high flow induced release of ET-1 peptide (Fig 3 12(c))

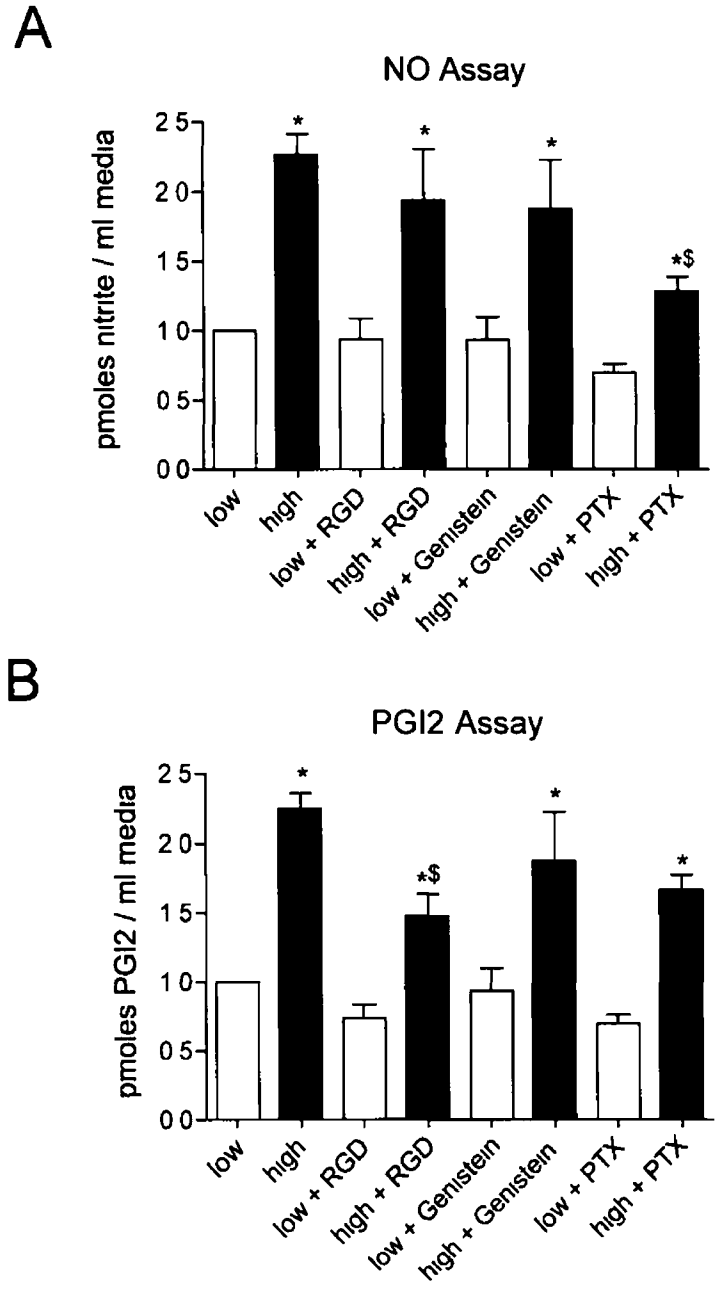


Figure 3 12 Inhibition of Pulsatile-Flow induced (A) NO & (B) Prostacyclin release in mono-cultured BRECs Integrin inhibition with linear RGD peptide, Protein tyrosine kinase inhibition with Genistein, G-protein inhibition with Pertussis toxin (PTX) Histogram represents mean values ± SEM (n=3), *P<0 05 compared to low flow control, $ P<0 05 compared to high flow control

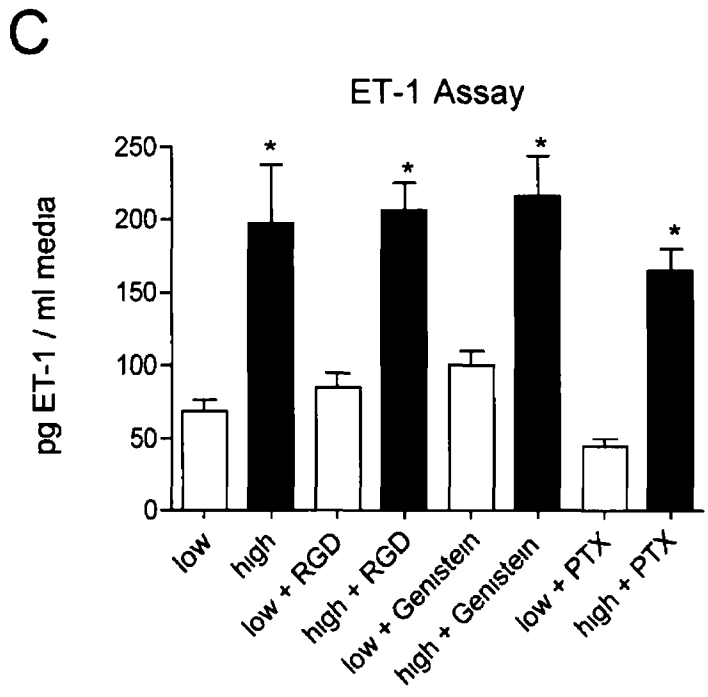


Figure 3 12 (cont) Inhibition of Pulsatile-Flow induced (C) ET-1 release in mono-cultured BRECs P<0 05 compared to low flow control

3.3 Discussion

This study describes the use of a novel transcapillary co-culture system as a basis for mimicking the effects of haemodynamic forces on microvascular endothelial and pericyte cells cultured *in vitro* Blood vessels are continually exposed to haemodynamic forces generated in the vasculature due to fluid flow induced shear stress and cyclic strain Changes in these mechanical forces result in alterations in the signal-transduction pathways and release of vasoactive substances from vascular ECs that are ultimately responsible for autoregulation of retinal vascular tone (Traub and Berk, 1998, Malek et al , 1999a, Redmond et al , 1998)

Retinal blow flow, as measured by various non-invasive techniques, ranges from 60 to 100 μL/min in larger retinal vessels, corresponding to a shear stress level of ~50 dynes/cm^2 (Garcia et al , 2002, Wong et al , 2003) If one assumes smaller ocular vessels receive ~10% of retinal blood flow, the levels of shear stress for microvascular ECs are within the range of 0 5 to 23 dynes/cm^2 used in the present study Changes in haemodynamics can result in endothelial dysfunction, a process that has been described in macrovascular disease states including the pathogenesis of atherosclerosis and thrombosis (Davies et al , 2002), and is also of particular interest in the microvasculature in conditions such as proliferative diabetic retinopathy (PDR) (Kohner et al , 1975, De La Cruz et al , 2004, Caldwell et al , 2003) and normal-tension glaucoma (Orgul et al , 1998, Harris et al , 2001), in which there is growing evidence of endothelial dysfunction as a major risk factor (Flammer et al , 2002) Boehm *et al* demonstrated an alteration of the perfusion of the optic nerve head with increasing age, suggesting that the blood supply is reduced in elderly subjects (Boehm et al , 2005) With glaucoma and PDR more common in elderly than in younger individuals, it is possible that changes in retinal blood flow initiate such pathologies

The present study combined the relevant physiological forces of pulse pressure and shear stress with co-culture technology to evaluate their effects on the expression and activity of the vasoactive substances NO, PGI_2, and ET-1 Immunocytochemistry, scanning electron microscopy, and confocal microscopy, in combination with Western blot analysis, confirmed the presence and growth of microvascular retinal ECs and pericytes in the perfused transcapillary culture system Moreover, there was a single layer of pericytes oriented along the direction of flow on the abluminal side of the capillaries BRP were exposed to increases in pulsatile flow rates and hence pulse pressure from a 'low' flow rate of 0 3 ml/min to a 'high' flow rate of 25 ml/min, corresponding to pulse pressures of 6 and 56 mm Hg, respectively The pressure at the intimal surface of vessels *in vivo* is equal to the intravascular blood pressure, which acts as a compressive force perpendicular to the endothelial lining The distinct compartmentalization of actin observed in pericytes after exposure to high pulsatile flow, with actin less evenly distributed throughout the cytoplasm to accommodate altered cell function, suggests that phenotypic modulation may involve not only quantitative changes in contractile proteins, but also reorganization of these proteins Because the cytoskeleton acts as a spatial regulator of intracellular signalling, reorganization of the cytoskeleton may lead to realignment of signalling molecules, which, in turn, may mediate the changes in function associated with phenotypic modulation after exposure to flow Culturing of the BRECs alone or as cocultures with pericytes did not significantly enhance or diminish the effects of pulsatile flow on eNOS and COX activity or ET-1 levels, suggesting that the major source of pulsatile flow–induced changes in these vasoactive substances was the retinal microvascular EC

ECs are known to modulate vessel tone via the production and release of the vasodilators NO and PGI_2 and the vasoconstrictor ET-1 One of the earliest events occurring in ECs placed under increased haemodynamic constraints is the activation of eNOS through phosphorylation at several sites and the subsequent release of the vasodilator NO (Gallis et al , 1999) A large number of *in vitro* and *in vivo* studies have demonstrated that NO plays an important role in regulation of regional ocular blood flow (Davis et al , 2001, Schmetterer and Polak, 2001) Indeed, NO has been

implicated in the etiology of several ocular diseases that result in altered ocular blood flow (Koss, 1999) Because phosphorylation of eNOS has been recognized as a critical regulatory mechanism controlling its activity, we examined the expression of pp-eNOS in mono- and cocultured BRECs after exposure to pulsatile flow In agreement with previous studies using macrovascular ECs, the present study demonstrated that pulsatile flow can activate eNOS through the increased phosphorylation of eNOS in microvascular BRECs (Hendrickson et al , 1999) No change in total eNOS protein or mRNA levels was found, demonstrating the acute response of BRECs exposed to high pulsatile flow is activation via phosphorylation and not an overall increase in gene expression or transcription It is reported that flow induced phosphorylation of eNOS in ECs is controlled by Akt, a serine/threonine protein kinase As shear stress alone has been shown to stimulate NO release in retinal microvascular ECs, these experiments further suggest that NO may be a key signalling molecule in elevating vascular transport in ocular diseases such as diabetic retinopathy (Davis et al , 2001), where retinal blood flow is increased (Candido and Allen, 2002, Gracner, 2004, Johnson et al , 1999, Kohner et al , 1975, Schmetterer and Polak, 2001, Schmetterer and Wolzt, 1999)

Previous studies have suggested that prostaglandins, synthesized by the enzymes COX-1 or -2, may contribute to normal physiological and homeostatic functions in the retina Doroudi *et al* perfused human umbilical veins at high/low shear stress (25/<4 dyn/cm^2) at identical intraluminal pressure (20 mmHg) for 1 5, 3, or 6 hours COX-1 and COX-2 mRNA showed a biphasic response with peaks at 1 5 and 6 hours, and a decrease at 3 hours (Doroudi et al , 2000) Inoue *et al* demonstrated a rapid and sustained expression of COX-2 in response to shear stress in human umbilical vein endothelial cells, via transcriptional activation and posttranscriptional mRNA stabilization (Inoue et al , 2002) On the other hand, Dancu *et al* demonstrated asynchronous shear stress and circumferential strain reduction in COX-2 gene expression in bovine aortic endothelial cells (Dancu et al , 2004) Furthermore, Hendrickson *et al* demonstrated that sustained increases in pulsatile flow maintain elevated COX-1 & -2 protein expression and activity in EC

while decreasing COX-1 & -2 expression in co-cultured smooth muscle cells (Hendrickson et al , 1999) We therefore investigated the role of pulsatile flow in modulating microvascular retinal endothelial PGI_2 release and the contributory role of the COX-2 isoform in mediating this response As previously observed in macrovascular ECs, pulsatile flow increased the levels of PGI_2, concomitant with a significant increase in the transactivation of the COX-2 promoter in these cells The human COX-2 promoter region contains 3 cis-acting elements, namely, an NF-κB binding site, an NF-IL6 binding site, and a cAMP responsive element (CRE), all of which have been shown to be involved in the regulation of COX-2 gene transcription Inoue *et al* (2002) demonstrated that the CRE site of the COX-2 gene is largely responsible for shear stress induced promoter activity

The data regarding the regulation of ET-1 synthesis and release by shear forces in ECs are controversial Initial reports described a shear stress-dependent induction of ET-1 production (Yoshizumi et al , 1989, Morita et al , 1993) Other groups found no significant changes in ET-1 release (Noris et al , 1995), or a downregulation of prepro-ET-1 (ppET-1) mRNA and endothelin release by shear stress in human and bovine ECs (Sharefkin et al , 1991, Malek and Izumo, 1992, Kuchan and Frangos, 1993) Cyclic stretch has been reported to enhance ET-1 peptide synthesis and pp-ET-1 mRNA expression in cultured endothelial cells (Cattaruzza et al , 2000) In situations in which the pressure-induced distension of the vessel wall is more pronounced and/or chronically elevated, as in aortocoronary venous bypass grafts, endothelial cell ET-1 production may be elevated (Lauth et al , 2000) Exposure of human glomerular microvascular ECs (HGMECs) to low levels of laminar shear stress have shown an initial increase in ET-1 followed by a decrease at 24 hours (Wang et al , 2002a) In contrast, the present study demonstrated a sustained increase in ET-1 levels secreted by BRECs in response to high pulsatile flow conditions in both monoculture and in coculture with pericytes after 24 hours Several studies have suggested that patients with glaucoma have increased circulating plasma ET-1 levels, which may be the source of retinal vessel vasoconstriction and ischemia of the optic nerve head vessels Indeed, a strong

immunoreactivity for ET-1 converting enzyme (ECE)-1 can be found in the blood vessels of the retina, optic nerve, and choroids, suggesting an important role for ET-1 during autoregulation within the eye Because ocular blood flow alterations in patients with glaucoma seem, at least partly, to be related to a systemic vascular dysregulation and are exhibited as altered responsiveness to ET-1, it is possible that flow-induced changes in ET-1 production within the eye contributes to the pathogenesis of glaucomatous damage

The sensitivity of the MAPKs (ERK 1/2, p38 and JNKs) to mechanical forces is well established in both *in vivo* and *in vitro* models Kito *et al* found that cyclic strain activated ERK1/2, JNK and p38 in pulmonary ECs with subsequent activation of transcription factors (Kito et al , 2000) Using rabbit facial vein segments Loufrani *et al* demonstrated stretch-induced ERK1/2 activation via a calcium-dependent pathway involving a tyrosine kinase (Loufrani et al , 1999) A shear stress of 12 dynes/cm^2 was found to activate ERK1/2 and p38 but to reduce activity of JNK (Surapisitchat et al , 2001) Furthermore, Jo *et al* reported differential shear stress regulation of ERK1/2 and JNK with regards to duration of activation and levels of shear required to elicit a response (Jo et al , 1997) The ability of cyclic stretch to activate MAPK is dependent on the substrate on which the cells are seeded, e g ERK1/2 and JNK are activated in cyclically stretched smooth muscle cells grown on pronectin but not on laminin (Reusch et al , 1996) Paradoxically, we found no change in total or phosphorylated BREC p38 or ERK 1/2 in response to increased pulsatile flow cultured on our pronectin™ coated capillaries after 24 hours exposure Further experiments are required to determine if increased pulsatile flow induces MAPK phosphorylation and/or new protein synthesis pre-24 hour Shear related effects can be broadly categorized into two responses acute and chronic As MAPKs are typically activated within minutes to many stimuli, activation may have occurred at an earlier timepoint and plateaued after 24 hours exposure to pulsatile flow (Lehoux and Tedgui, 1998, Cowan and Storey, 2003) Moreover, corroborating studies suggest a role of preconditioning ECs to flow before initiating any flow increases (Rizzo et al , 2003) Exposure of rat

lung microvascular cells to increases in flow exhibit no changes in ERK1/2 activity if ECs have been preconditioned to flow (Rizzo et al , 2003) Our BRECs/BRP pulsatile flow studies each include a period of preconditioning to flow of ~4 days, possibly accounting for no change in ERK1/2 or p38 levels or activation

There appears to be several possible mechanisms by which endothelial cells detect mechanical forces and act as a shear transducer e g G-proteins, integrins and PTKs Following the initial mechanosensing, cell surfaces and cell membranes may be deformed, ions may be translocated, local biochemical responses may be activated, downstream intracellular signalling pathways may be activated and endothelial genes may then be expressed, all to modulate shear-induced endothelial function as well as important shear-induced alterations in endothelial cell morphology G-proteins have been implicated in the transduction of a number of flow-induced responses in vascular cells, with activation by mechanical forces one of the earliest mechanotransduction events reported Cyclic strain activation of G-proteins has been found to be dependent on the magnitude and rate of the strain (Clark et al , 2002, Gudi et al , 1998) G-proteins may detect mechanical forces via G-protein coupled receptor or may be stimulated directly by the deformation of either the actin cytoskeleton or the membrane phospholipid bilayer during exposure to such stimuli

Shear stress and cyclic strain-induced activation of G-proteins results in several flow-initiated responses which function in the regulation of vascular tone, including release of NO, PGI_2 and ET-1 (Liu et al , 2003a, Pirotton et al , 1987) Changes in G-protein expression have been observed within the physiological range of cyclic strain and shear stress, correlated with enhanced NO and PGI_2 release as well as increased G-protein functionality (Redmond et al , 1998) PTX, which inhibits G-protein signalling via ADP ribosylation of the $G\alpha$ subunit, is routinely used to elucidate the role of G-proteins in flow mediated EC responses Pulsatile flow activation of NO and ET-1 was PTX sensitive in our retinal microvascular ECs Thus G-protein activation may be an early event in flow mediated vasoactive

response, representing an important pathway by which ECs adapt to changes in retinal blood flow.

In the current study, BRECs/BRP cultures were grown on polyethylene capillaries coated in Pronectin™, an extra-cellular matrix (ECM) protein. The ECM is an important contributor to mechanotransduction, containing components which interact with integrins, which are displaced by cyclic stretch and shear stress. Mechanical stresses can stimulate conformational activation of integrins and increase cell binding to the ECM. Evidence for mechanical activation of integrins is provided by both direct assessment of integrin conformational changes in response to these forces and blockade of the induced responses by antibodies or blocking peptides such as the Arg-Gly-Asp (RGD) peptide (Lehoux and Tedgui, 1998). Blocking integrins with RGD peptide abolishes the shear stress–induced secretion of basic fibroblast growth factor (Gloe et al., 2002) and the anti-apoptotic effect of shear stress on ECs (Urbich et al., 2000). In addition to modulating the avidity and affinity of integrins, shear stress also increases the mRNA and protein levels of the $\alpha 5$ and $\beta 1$ integrins in ECs (Urbich et al., 2000).

Cell surface matrix receptors of the integrin family may in part be responsible for some of the changes occurring in diabetic retinopathy, since topical application of integrin antagonist peptides (RGD) inhibited proliferative retinopathy in a mouse model by 50% (Riecke et al., 2001). The integrin heterodimer most actively involved in retinal neo-angiogenesis is the $\alpha v \beta 3$ integrin, since in a mouse model of proliferative retinopathy induced by hypoxia the expression of this integrin was an early phenomenon and application of RGD-containing pentapeptides was highly effective in inhibiting angiogenesis (Chavakis et al., 2002). Increased plasma concentrations of the vasoactive peptide endothelin have also been found in diabetic retinopathy (Itoh et al., 2002). Blocking of the effects of endothelin by bosentan, a non-specific endothelin receptor blocker, prevented thickening of retinal capillary basement membranes, and also the increased expression of the $\alpha 1$ (IV) chain and fibronectin in streptozotocin-diabetic rats (Itoh et al., 2002). Blockage of integrin signalling in our model of retinal blood flow with RGD peptide inhibited PGI_2 secretion into the media perfusate. No inhibitory effect could be attributed to RGD

with respect to NO or ET-1, which increased independent of integrin activation at high pulsatile flow

Protein tyrosine kinases (PTKs) have been implicated in haemodynamic force-induced changes in EC function (Ravichandran, 2001) Clinically, PTKs such as insulin growth factor receptor, platelet-derived growth factor receptor and fibroblast growth factor receptor have been linked to multiple vascular pathologies, including atherosclerosis, hypertension, restenosis, angiogenesis, arteriogenesis and diabetic vascular disease (Okura et al , 2001, Patel et al , 2001, Rajkumar et al , 1996, Fath et al , 1993, Grant et al , 1996) In the current study, high pulsatile flow induction of NO, PGI_2 or ET-1 could not be inhibited by pre-treatment with genistein, a PTK inhibitor Taken together, these data imply different roles of the proposed mechanosensors G-proteins, integrins and PTKs in mediating pulsatile flow induction of retinal microvascular EC vasoactive response

When considering all these factors it can be concluded that the cell in its entirety may be considered as a mechanosensor, which alters its cytoskeleton, the composition of the ECM and cross talk between receptors in response to mechanical stimuli to maintain the homeostasis within the vascular wall

3 4 Conclusion

In conclusion, these studies demonstrate a pulsatile flow induced vasoactive response by EC in co-culture with pericytes and the potential mechanisms by which retinal endothelial cells autoregulate local blood These results correlate well with characterised physiological responses of EC from other vascular beds exposed to mechanical forces and demonstrate an important role of G-proteins and integrins in mediating this response

Chapter 4

Pulsatile Flow alters microvascular endothelial & pericyte cell apoptosis *via* vasoactive mediators Nitric oxide, Prostacyclin, Endothelin-1

4.1 Introduction

4.2 Results

4.3 Discussion

4.4 Conclusion

4.1 Introduction

Mechanical forces associated with retinal blood flow play an important role in maintaining vessel structure and function Aberrant retinal blood flow has been reported in high tension glaucoma (Bathija, 2000, Hafez et al , 2003), normal tension glaucoma (Butt et al , 1995, Fontana et al , 1998, Henry et al , 1999, Arend et al , 2002) and diabetic retinopathy (Schmetterer and Wolzt, 1999, Kohner et al , 1975, Savage et al , 2004, Candido and Allen, 2002, Gracner, 2004), and may be a causative factor of the vascular remodelling occuring in each of these pathologies Glaucoma for example, is characterised by regression of vessels (Quigley, 1999) and is frequently associated with reduced retinal blood flow and/or systemic hypertension/hypotension (Folkow, 1995, Hayreh, 2001) DR is associated with an initial increase, followed by decreased retinal blood flow (Schmetterer and Wolzt, 1999), and is exacerbated by systemic hypertension In addition, DR is characterised by unstable or leaky vessels, and angiogenic proliferation of new vessels during the later stages of disease Adaptive vascular remodelling such as regression or proliferation, implys an alteration in EC and pericyte cell fate decisions such as apoptosis, proliferation, migration and/or differentiation

We have previously demonstrated an increase in EC production of vasoactive mediators NO, PGI_2 and ET-1 upon exposure to increases in pulsatile flow up to 24 hours (Chapter 3) This EC response to increased pulsatile flow occurred independent of the presence or absence of pericytes In the following experiments, we examined the effect of *chronic* exposure of EC/pericyte co-cultures to low and high pulsatile flow for 3 days

Numerous reports have implicated NO, PGI_2 and ET-1 as important molecules mediating apoptosis in both ECs and VSMCs (Bennett, 1999, Boyle et al , 2002, Chae et al , 2004, Hata et al , 2001, Lau, 2003, Li et al , 2004a, Shichiri et al , 1997, Cattaruzza et al , 2000) As such, we hypothesize that vascular remodelling may occur, in part, as a result of alterations in vasoactive molecules within the vessel wall, generated by chronic alterations in blood flow Therefore, we inhibited NO (L-NAME), PGI_2 (indomethacin) and ET-1 (PD142893) to determine their role in

regulating EC and pericyte cell apoptosis In addition, the apoptotic profile of static EC and pericyte cultures were also examined upon exogenous addition of NO, PGI_2 and ET-1

The aim of this study was to determine the apoptotic effect of chronic exposure to pulsatile flow on retinal endothelial & pericyte cell co-cultures for 3 days Furthermore, the role of NO, ET-1 and PGI_2 in mediating changes in apoptosis due to pulsatile flow was examined

4.2 Results

4 2 1 Pulsatile Flow induced Activation of Endothelial Nitric Oxide Synthase (eNOS)

We investigated the effects of 'low' (0 3ml/min, 6 mmHg, 0 5 dynes/cm^2) and 'high' (25ml/min, 56 mmHg, 23 dynes/ cm^2) pulsatile flow on eNOS mRNA and protein levels in BRECs co-cultured with BRPs over a 72-hour period Under these conditions, both eNOS mRNA (3 190 ± 0 718 fold, n=6) and protein (2 405 ± 0 353 fold, n=5) levels were seen to increase significantly as determined by real time PCR and Western blot analysis respectively (Fig 4 1 (a) and (b)) Increased EC eNOS resulted in an increase in media perfusate nitrate levels in high compared to low pulsatile flow (2 609 ± 0 256 fold, n=6), as determined by fluorometric DAN assay (Fig 4 1 (c))

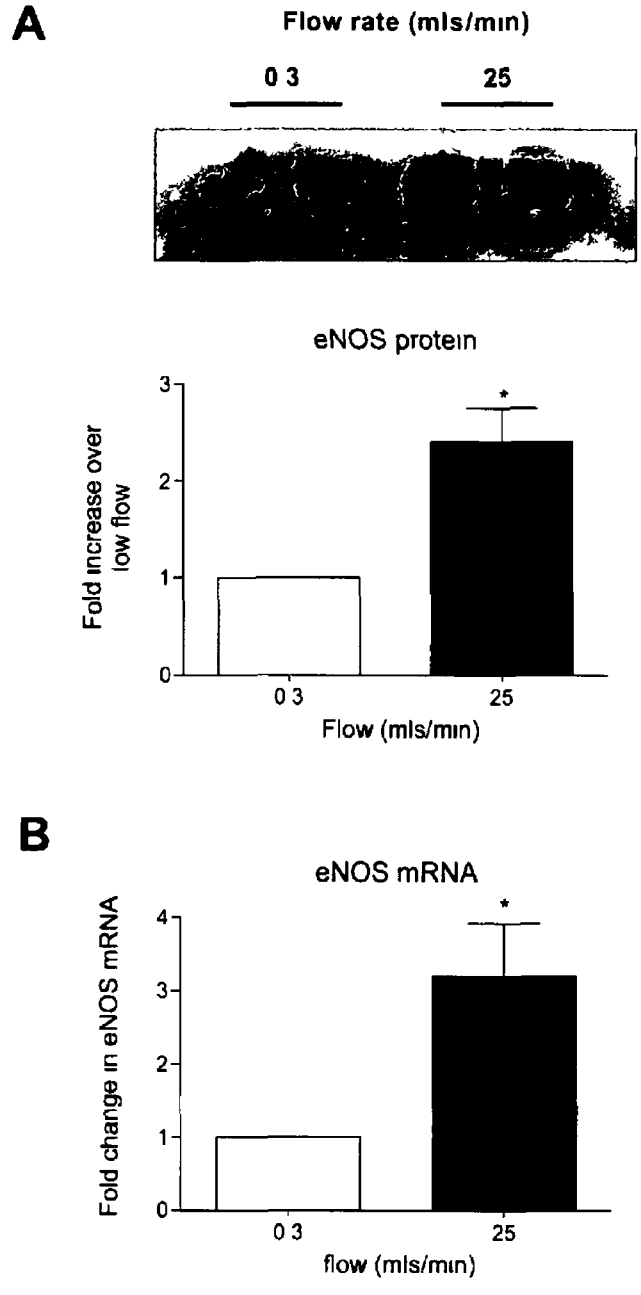


Figure 4 1 The effects of Pulsatile Flow on BREC eNOS in co-culture with BRPs for 72 hours Western Blot analysis **(A)** and realtime PCR analysis **(B)** of *eNOS* Histogram represents mean values ± SEM (n=6), *P<0 05 compared to low flow control

C

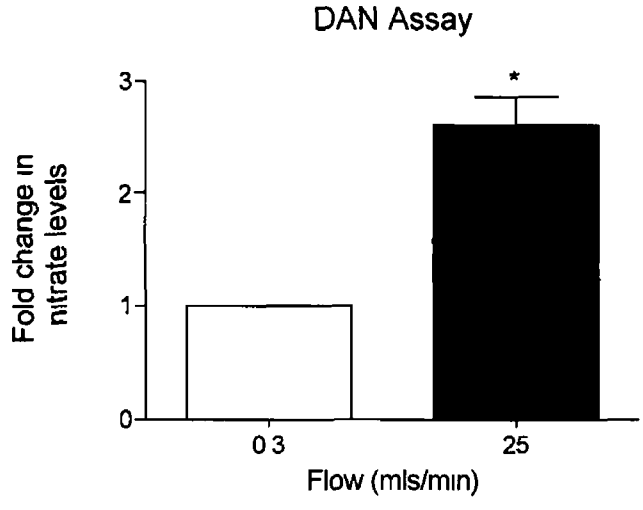


Figure 4 1 The effects of Pulsatile Flow on NO release from 72 hour BREC/BRP co-culture (C) DAN assay of media nitrate levels harvested from BRP/BREC co-culture exposed to low or high flow pulsatile flow for 72 hours Histogram represents mean values ± SEM (n=6), *P<0 05 compared to low flow control

4 2 2 Pulsatile Flow induced changes in Prostacyclin release

To examine the effects of pulsatile flow on the production of PGI_2, we exposed co-cultured BRECs and BRPs to low and high flow rates as described previously Levels of PGI_2 in the circulating media were determined by enzyme immunoassay, which revealed a significant increase in PGI_2 levels in cells exposed to high flow conditions for 72 hrs (2 167 ± 0 088 fold, n=6) compared to low flow (Fig 4 2 (a))

To determine if the increase in PGI_2 levels was a direct consequence of the induction of COX-1 or COX-2 expression in BRECs or BRPs, we carried out western blot analysis on lysates from each cell type (Fig 4 2 (b)) BRECs exhibited a significant increase in COX-2 levels under high flow conditions (3 920 ± 0 573 fold, n=5) whereas COX-1 remained unchanged (n=3)

Western blot analysis of BRPs was also performed, as previous reports suggest COX-2 is mechanically regulated by flow in smooth muscle cells (Hendrickson et al , 1999) COX-2 levels in BRPs remained unchanged however from low to high flow (Fig 4 2) (n=3), suggesting increased PGI_2 levels in the circulating media perfusate is a direct consequence of high flow-mediated induction of COX-2 expression in BRECs Furthermore, PGI_2 levels were undetectable in media from BRPs exposed to low or high pulsatile flow for 72 hours in the absence of BRECs (data not shown)

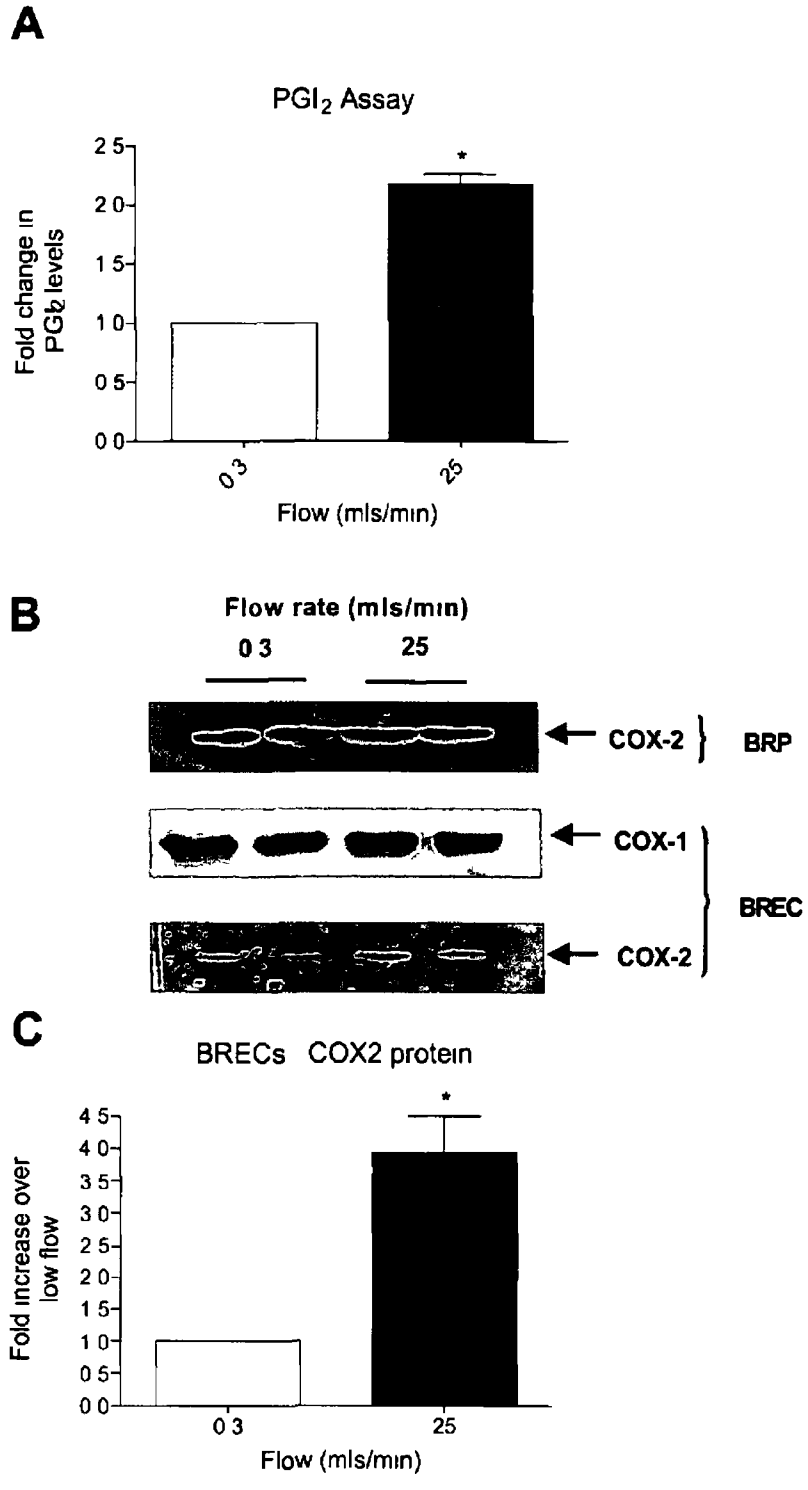


Figure 4 2 The effects of Pulsatile Flow on PGI_2 release, and COX-1 & -2 protein expression in co-cultured BREC/BRPs BRP/BREC co-culture exposed to low or high flow pulsatile flow for 72 hours **(A)** Media assay for PGI_2 levels **(B)** Western Blot analysis of COX enzymes in BREC & BRP lysates **(C)** Graph of BREC COX-2 levels Histogram represents mean values ± SEM (n=6) *P<0 05 compared to low flow control

4.2.3 Pulsatile Flow-Induced Changes in ET-1 peptide and ET_A & ET_B receptor mRNA

Circulating media from co-cultured BRECs and BRPs exposed to low and high- pulsatile flow conditions for 72 hrs was assayed for ET-1 levels by enzyme immunoassay Exposure of the cells to high flow resulted in a 0 534 ± 0 054 fold, (n=7) reduction in media ET-1 levels, compared with low flow (Fig 4 3 (a)) In addition, BREC ET-1 mRNA levels were significantly reduced at high compared to low flow as examined by realtime PCR (0 576 ± 0 071 fold, n=6) (Fig 4 3 (b))

Both ECs and pericytes are target cells for ET-1 peptide signalling, with ECs expressing ET_B receptor, whereas SMCs/pericytes express both ET_A and ET_B receptors (Masaki, 2004) Activation of ET_B on ECs increases release of NO and prostacyclin (Quaschning et al , 2005) Conversely, activation of both ET_A and ET_B on SMCs/pericytes causes vasoconstriction (Masaki, 2004) Alteration of receptor numbers on the cell surface can thus alter the local vasoactive response and chronic alterations can alter the structure of a vessel Several investigators have found increased SMC ET_A and ET_B receptor levels in hypertensive vessel thickening (Cahill et al , 1998) We investigated the mRNA levels of ET_A and ET_B receptors in co-cultured BRECs/BRPs using realtime PCR ET_B mRNA levels in both BRECs and BRPs increased significantly at high compared to low flow (9 964 ± 4 150 fold, n=6 and 2 235 ± 0 531 fold, n=7 respectively) (Fig 4 4 and 4 5), whereas ET_A mRNA levels decreased in BRPs (0 549 ± 0 087 fold, n=7) (Fig 4 5 (b)) ET_A receptor mRNA was not detected in BRECs exposed to low or high pulsatile flow (Fig 3(b))

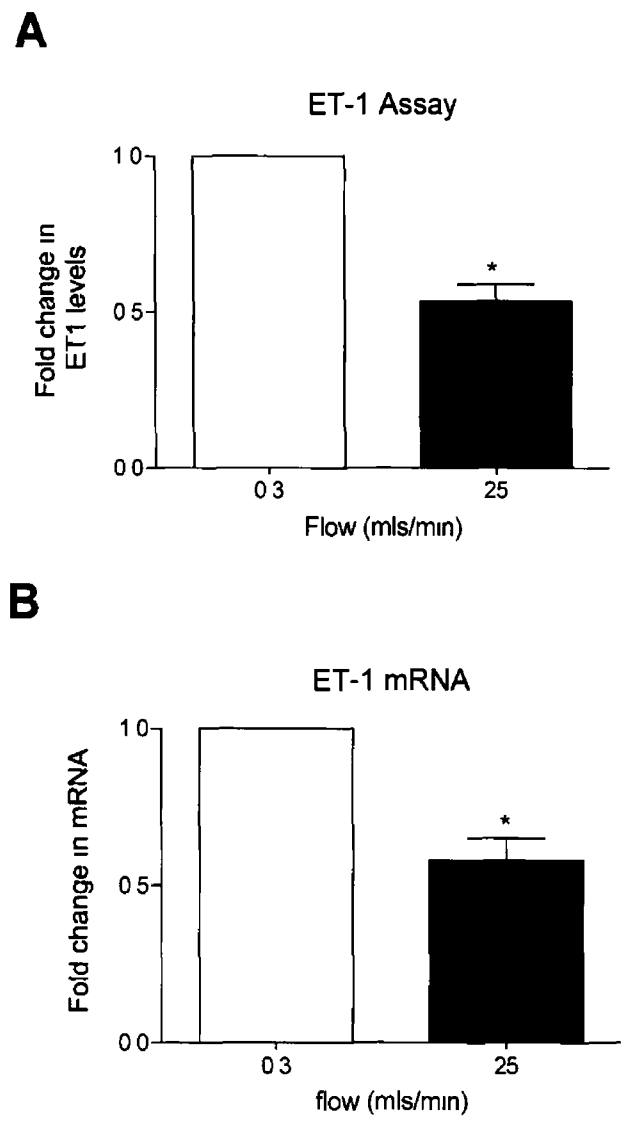


Figure 4 3 The effects of Pulsatile Flow on NO release and BREC *ET-1* mRNA. BRP/BREC co-culture exposed to low or high flow pulsatile flow for 72 hours **(A)** Media assay for ET-1 levels **(B)** Realtime PCR analysis of BREC *ET-1* mRNA levels Histogram represents mean values ± SEM (n=6), *P<0 05 compared to low flow control

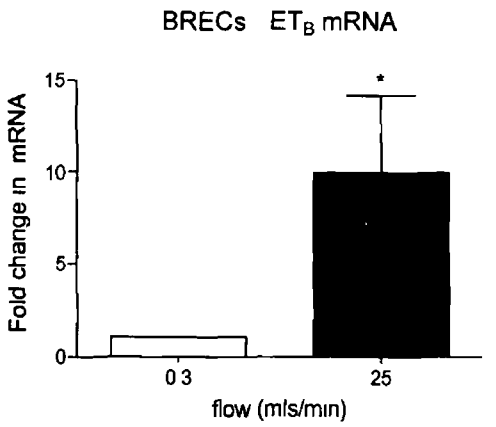


Figure 4 4 The effects of Pulsatile Flow on BREC ET$_B$ mRNA. BRP/BREC co-culture exposed to low or high flow pulsatile flow for 72 hours Realtime PCR analysis of BREC *Endothelin-B* Receptor mRNA levels Histogram represents mean values ± SEM (n=6), *P<0 05 compared to low flow control

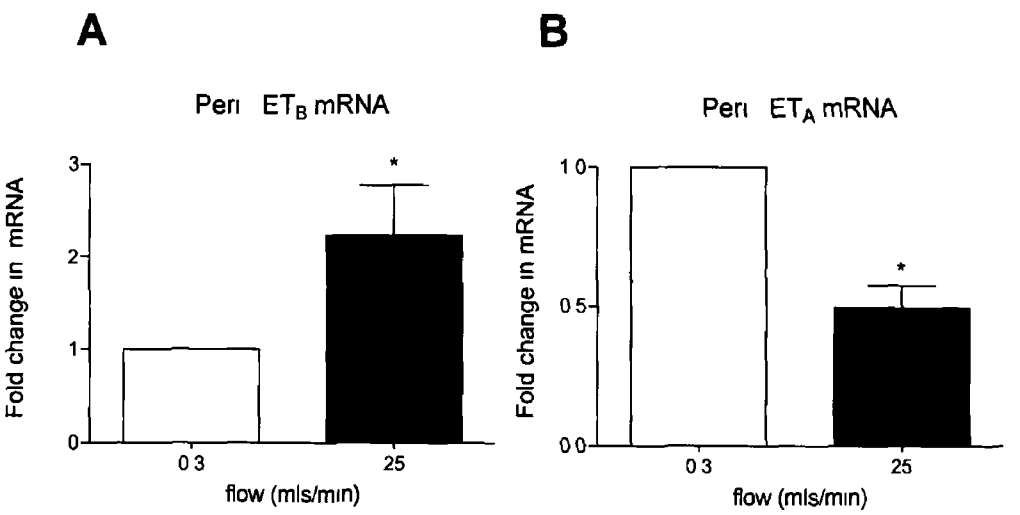


Figure 4 5 The effects of Pulsatile Flow on BRP ET$_A$ & ET$_B$ mRNA. BRP/BREC co-culture exposed to low or high flow pulsatile flow for 72 hours Realtime PCR analysis of BRPs *Endothelin-B* Receptor mRNA levels **(A)**, and BRPs *Endothelin-A* Receptor mRNA levels **(B)** Histogram represents mean values ± SEM (n=6), *P<0 05 compared to low flow control

4.2.4 Effect of L-NAME, Indomethacin and PD142893 on the Flow-Induced changes in NO, PGI_2 and ET-1

BRECs/BRPs co-cultures were exposed to L-NAME (500μM), Indomethacin (1μM) or PD142893 (1μM) to determine the compensatory or competitive interaction between NO, PGI_2 and ET-1, respectively. L-NAME treatment had no effect on eNOS activity under low flow conditions (0.867 ± 0.164 fold, n=6), as detected by DAN assay. However, L-NAME treatment significantly inhibited flow-induced nitrate release at high flow (1.425 ± 0.095 fold, n=6) (Fig. 4.6 (a)). Inhibition of cyclooxygenase with indomethacin resulted in a significant increase in nitrate release at low (1.517 ± 0.148 fold, n=6) but not high pulsatile flow (3.157 ± 0.413 fold, n=6), demonstrating that increased release of nitric oxide compensates for reduced PGI_2 release at low flow. Inhibition of ET-1 did not affect nitric oxide release at either low or high pulsatile flow (0.957 ± 0.081 fold and 2.383 ± 0.224 fold, n=6, respectively) (Fig. 4.6 (a)).

Treatment of BRECs/BRPs with indomethacin significantly decreased PGI_2 release at both low and high pulsatile flow compared to controls (2.703 ± 0.472 fold and 3.200 ± 0.609 fold, n=6, respectively). In order to determine the dual role of nitric oxide and PGI_2 in mediating the vasodilator response, we examined PGI_2 levels upon nitric oxide inhibition with L-NAME treatment. Nitric oxide inhibition at both low and high pulsatile flow resulted in significant increases in PGI_2 circulating in media perfusate compared to controls (2.703 ± 0.472 fold and 3.200 ± 0.609 fold, n=6, respectively) (Fig. 4.6 (b)). PD142893 inhibition of ET-1 binding had no effect on PGI_2 release at either low or high pulsatile flow rates (1.133 ± 0.170 fold and 1.967 ± 0.07 fold, n=6, respectively) (Fig 4.6 (b)).

Nitric oxide inhibits ET-1 at both low and high flow, as determined by increased ET-1 in the presence of L-NAME (1.470 ± 0.070 fold and 0.782 ± 0.132 fold, n=6, respectively) (Fig 4.6 (c)). Similarly, addition of indomethacin also demonstrated the potency of PGI_2 inhibiting ET-1 at both low and high flow (1.315 ± 0.015 fold and 0.839 ± 0.108 fold, n=6, respectively) (Fig 4.6 (c)). ET-1 also decreased in the presence of PD142893 at low and high flow (0.273 ± 0.043 fold and

0 253 ± 0 057 fold, n=6, respectively) (Fig 4 6 (c)), however this is potentially a result of assay interference with samples containing PD142893 compound

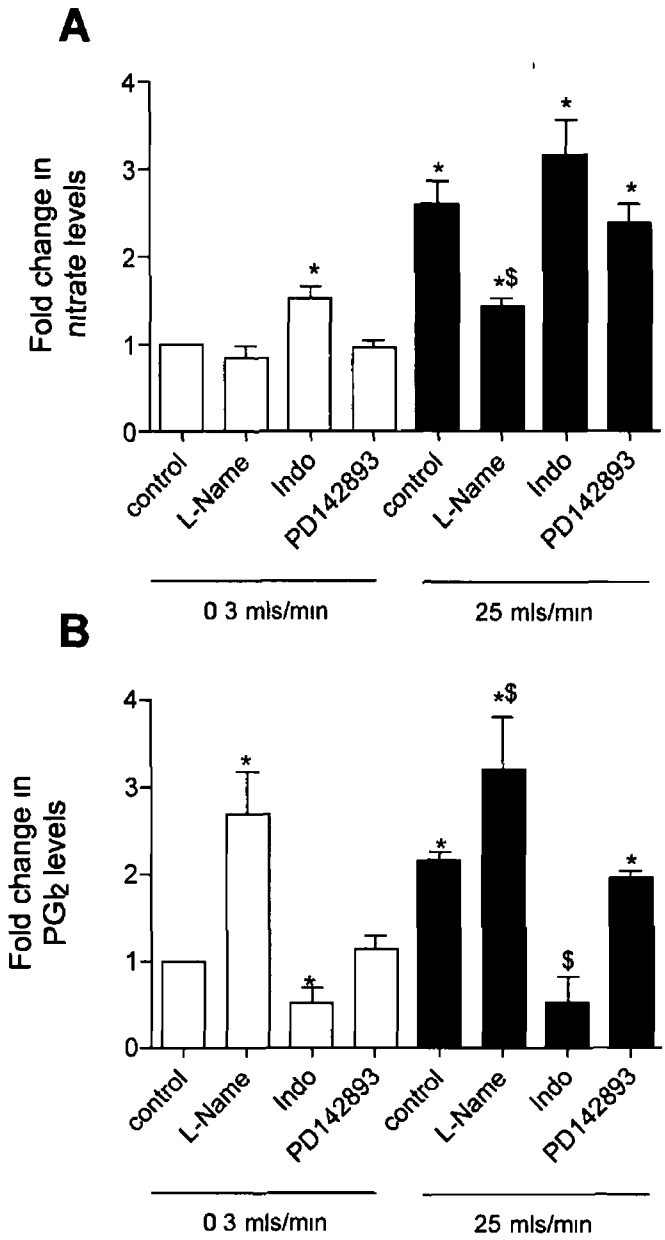


Figure 4 6 The effect of L-Name, Indomethacin & PD142893 on Pulsatile Flow induced release of NO, PGI_2 or ET-1 BRP/BREC co-culture exposed to low or high flow pulsatile flow for 72 hours with non-selective NOS inhibitor, (L-Name), a non-selective COX inhibitor, (Indomethacin), or ET-receptor antagonist (PD142893) **(A)** DAN assay of media nitrate levels, **(B)** media assay for PGI_2 levels Histogram represents mean values ± SEM (n=6), *P<0 05 compared to low flow control, $P<0 05 compared to high flow control

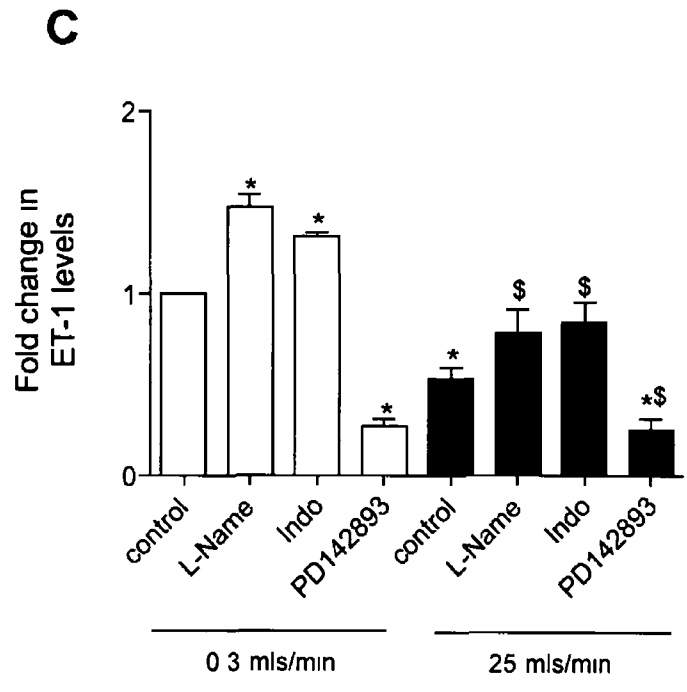


Figure 4 6 continued. **(C)** media assay for ET-1 levels Histogram represents mean values ± SEM (n=6), *P<0 05 compared to low flow control, $P<0 05 compared to high flow control

4 2 5 Effect of L-NAME, Indomethacin and PD142893 on Cell Viability

EC apoptosis was significantly reduced at high compared to low flow (0 626 ± 0 024 fold, n=6), as determined by Annexin V FACs analysis (Fig 4 7 (a)) Inhibition of NO, PGI_2 and ET-1 did not significantly effect EC apoptosis at low flow (0 917 ± 0 055 fold, 0 903 ± 0 044 fold, 1 01 ± 0 048 fold, n=6, respectively) (Fig 4 7 (b)) At high pulsatile flow however, inhibition of both NO and ET-1, but not PGI_2, significantly abrogated the protective effect of high pulsatile flow on EC apoptosis (0 944 ± 0 049 fold, 1 01 ± 0 049 fold, 0 761 ± 0 029 fold, n=6, respectively) (Fig 4 7 (b))

In contrast to BRECs, BRPs harvested from the same co-cultures had a significant *increase* in apoptosis at high compared to low pulsatile flow rates (1 48 ± 0 07 fold, n=6) (Fig 4 7 (c)) Inhibition of PGI_2 and ET-1 did not alter the protective effects of low pulsatile flow rates, however inhibition of NO release significantly increased BRP apoptosis at low pulsatile flow (0 833 ± 0 038 fold, 0 972 ± 0 055 fold, 1 278 ± 0 038 fold, n=6, respectively) (Fig 4 7 (c)) Interestingly, inhibition of NO at high pulsatile flow did not exacerbate the apoptotic BRP response (1 417 ± 0 038 fold, n=6) (Fig 4 7 (c))

Indomethacin and PD142893 treatment at high flow reversed the increase in BRP apoptosis (0 848 ± 0 064 fold and 1 167 ± 0 046 fold, n=6, respectively) (Fig 4 7 (b)), demonstrating an important role of both PGI_2 and ET-1 in mediating pericyte apoptosis at high flow rates

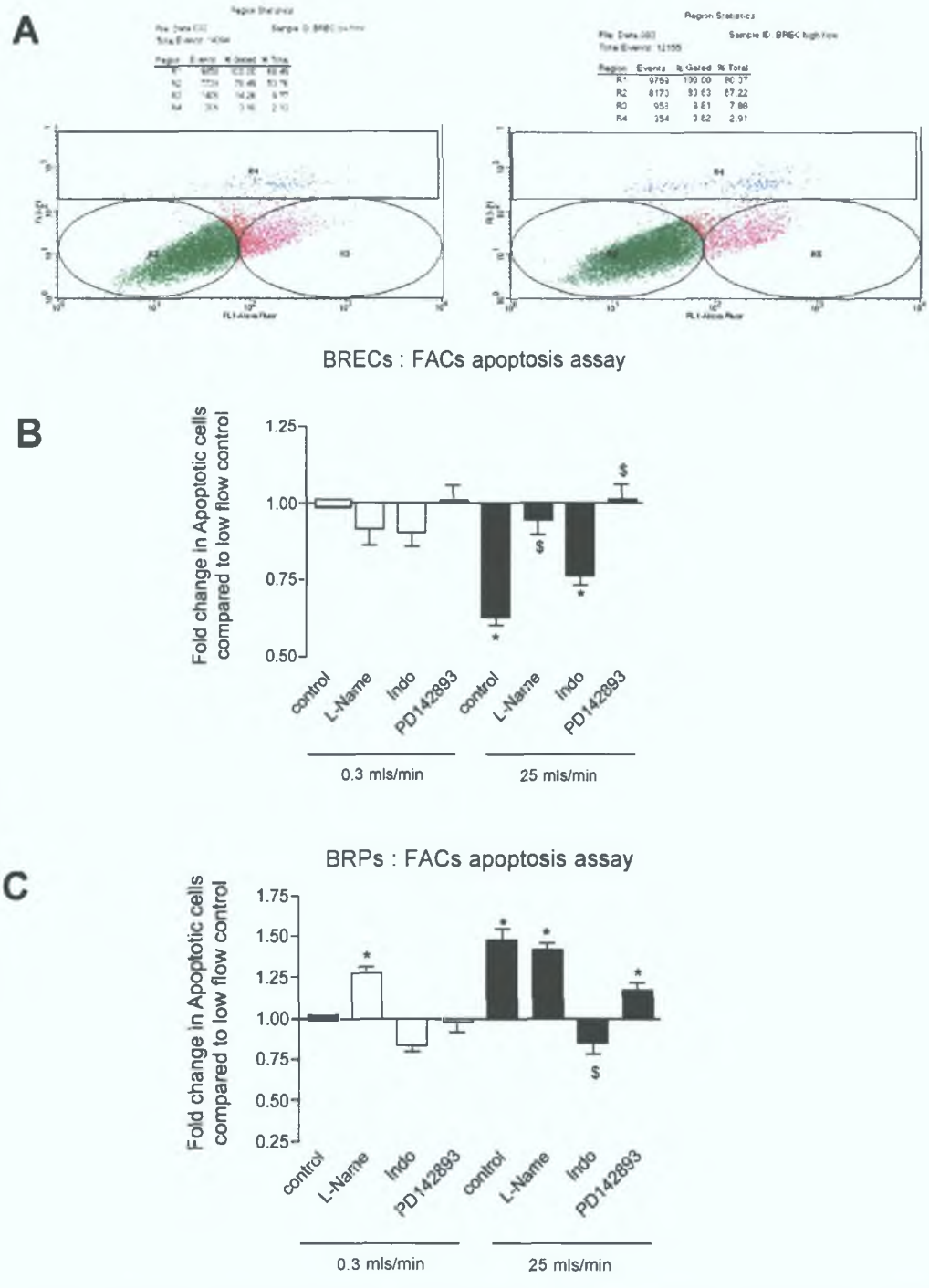


Figure 4.7 The effect of L-Name, Indomethacin & PD142893 on BREC and BRP apoptosis exposed to Pulsatile Flow. BRP/BREC co-culture exposed to low or high pulsatile flow for 72 hours with non-selective NOS inhibitor, L-Name, or non-selective COX inhibitor, Indomethicin. **(A)** Representative AnnexinV FACs scatter graphs of BRECs at low (left) or high (right) pulsatile flow. **(B)** BRECs **(C)** Pericytes. Histogram represents mean values ± SEM (n=3), *P<0.05 compared to low flow control; $P<0.05 compared to high flow control

4 2 6 Effect of SNAP and Iloprost on Cell Viability

To investigate the direct effects of NO, PGI_2 and ET-1 on BREC and BRP apoptotic profiles, static cultures of each cell type were supplemented for 72hrs with a nitric oxide generator, S-Nitroso-N-Acetylpenicillamine (SNAP - 50μM and 500μM), a prostacyclin analogue, iloprost (50nM and 500nM) or ET-1 peptide (10nM and 100nM) in media containing low serum The number of apoptotic cells in both BRECs and BRPs was significantly increased under low serum conditions, compared to normal serum controls (6 000 ± 0 572 fold and 5 100 ± 0 330 fold, n=6, respectively) (Fig 4 8)

Treatment of BRECs with ET-1 or iloprost resulted in a dose-dependent decrease in apoptosis (ET-1 [10nM 1 500 ± 0 535 fold, 100nM 6 000 ± 0 476 fold, n=6] iloprost [50nM 1 500 ± 0 535 fold, 500nM 6 000 ± 0 476 fold, n=6]) (Fig 4 8 (a)) Treatment with SNAP resulted in a significant decrease in apoptosis at 50μM (2 500 ± 0 330 fold, n=6), but not at 500μM (7 000 ± 0 373 fold, n=6), suggesting the concentration of NO is critical in regulating BREC apoptosis

Treatment of BRPs with ET-1 or iloprost did not significantly affect apoptosis in low serum conditions (ET-1 [100nM 4 500 ± 0 386 fold, 10nM 4 800 ± 0 425 fold, n=6] iloprost [500nM 6 300 ± 0 330 fold, 50nM 6 500 ± 0 353 fold, n=6]) (Fig 4 8 (b)) Similar to the concentration dependent effect of SNAP on EC apoptosis, SNAP increased BRP apoptosis at 500μM (7 300 ± 0 341 fold, n=6), whereas 50μM protected against low serum induced apoptosis (1 800 ± 0 353 fold, n=6) (Fig 4 8 (b))

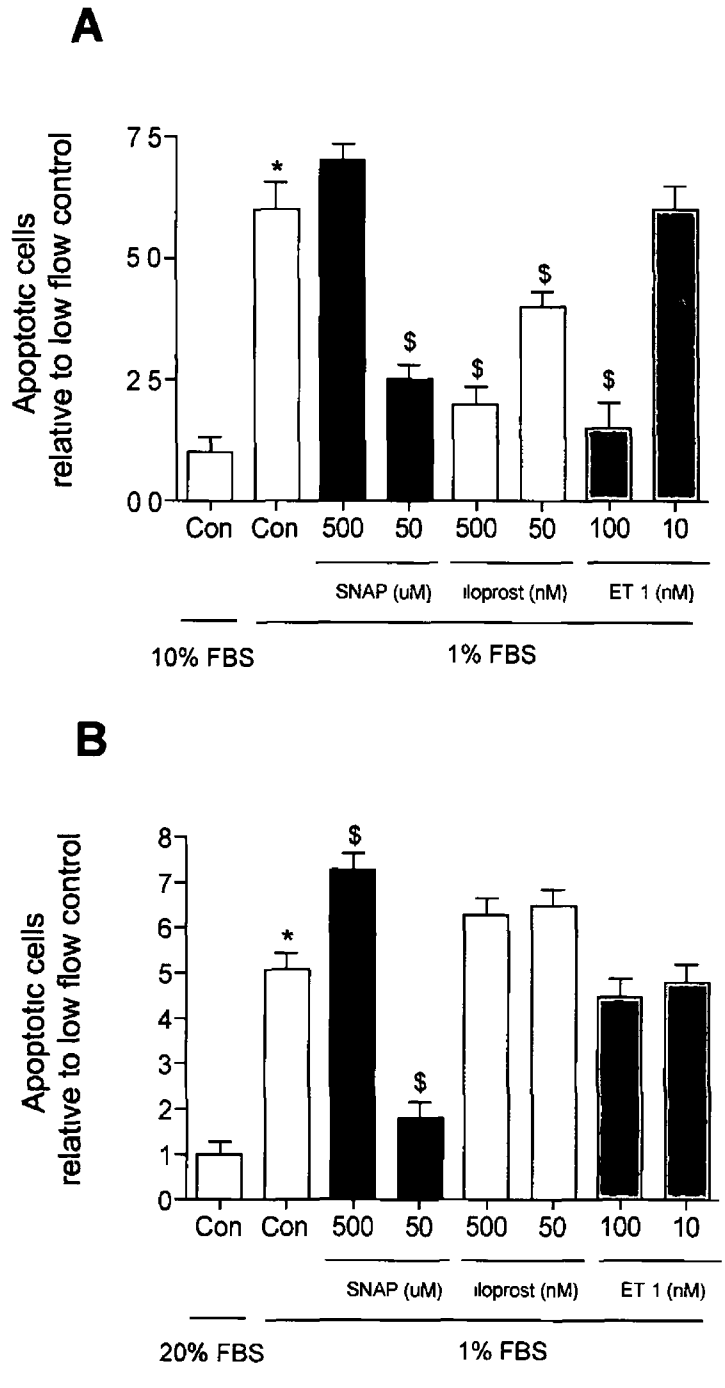


Figure 4 8 The effect of SNAP, Iloprost & ET-1 peptide on BREC and BRP apoptosis Serum starvation of BRECs or BRPs static mono-cultures and Annexin V FACs analysis of BRECs **(A)** and BRPs **(B)** apoptotic cells BRECs or BRPs exposed for 72 hours to nitric oxide generator SNAP or prostacyclin analogue, iloprost Histogram represents mean values ± SEM (n=6), *P<0 05 compared to low flow control, $P<0 05 compared to high flow control

4 3 Discussion

ECs in normal blood vessels produce NO, PGI_2 and ET-1 which contribute to maintainance of vessel homeostasis (Osanai et al , 2000) We have previously demonstrated the acute effect of pulsatile flow on ECs and BRPs in co-culture, with increased release of NO, PGI_2 and ET-1 in response to high pulsatile flow at 24 hours In this study, we examined the effect of sustained increases in pulsatile flow on BREC/BRP co-cultures after 72 hours Similar to the acute BREC response, a sustained increase in NO and PGI_2 was found, with increased BREC eNOS and COX-2 protein expression These results concur with numerous reports of increased NO upon exposure to increases in mechanical forces (Boo and Jo, 2003, Davis et al , 2001, Gallis et al , 1999, Hendrickson et al , 1999, Jin et al , 2003, Jin et al , 2005, Kuchan and Frangos, 1994, Malek et al , 1999a, Ziegler et al , 1998) Similarly, several investigators report increased EC PGI_2 synthesis or secretion in response to increased shear stress (Okahara et al , 1998, Inoue et al , 2002, Hendrickson et al , 1999, Meyer-Kirchrath et al , 2004, Osanai et al , 2000, Dancu et al , 2004) Previous reports suggest elevated PGI_2 production by shear stress is mediated by increased arachidonic acid release and a combination of increased expression of COXs and PGI_2 synthase (Okahara et al , 1998) We demonstrate an increase in BREC COX-2, but not COX-1 protein, whereas BRP COX-2 expression was unchanged Circulating PGI_2 in mono-cultured BRPs exposed to low or high pulsatile flow for 72 hours was negligible compared to co-culture experiments, demonstrating that the endothelial cell is the predominant source of PGI_2

Our previous analysis of ET-1 peptide in BREC/BRP co-cultures, demonstrated an increase in media perfusate ET-1 at high pulsatile flow after 24 hours (Chapter 3) Chronic exposure however, results in decreased circulating ET-1 peptide, as determined by enzyme-immunoassay Moreover, BREC ET-1 mRNA decreased at high compared to low pulsatile flow Several publications document a bi-phasic EC ET-1 response to either increased shear stress (Morawietz et al , 2000, Wang et al , 2002a, Malek et al , 1999b, Kuchan and Frangos, 1993) or cyclic stretch (Cattaruzza et al , 2000) ECs adapt to increased mechanical forces with

reorganisation of the cytosleton and these changes in shape and cytoskeletal organisation are integral to transducing shear stress into altered ET-1 gene expression (Malek et al , 1999b), and may account for the bi-phasic EC response to changes in flow

Binding of ET-1 to ET_A and ET_B receptors on VSMCs results in vasoconstriction, cell growth and cell adhesion Binding of the same peptide to ET_B receptors on endothelial cells causes vasodilation by stimulating the release of PGI_2 and NO, via increased protein kinase B/Akt and eNOS phosphorylation (Liu et al , 2003a) The *overall* vasoactive effect *in vivo* however, is a potent vasoconstriction Luscher *et al* demonstrated that endothelial denudation *in vivo* enhances ET-1 dependent contraction, confirming the vasodilatory effect of ET-1 (Luscher et al , 1990b) Interestingly, Lerman *et al* demonstrated the ability of NO and PGI_2 to limit ET-1 dependent effects *in vivo*, demonstrating cross-talk between these opposing vasoactive pathways (Lerman et al , 1992) With a doubling of baseline ET-1 plasma levels caused by exogenous ET-1 infusion in anesthetized pigs, there was a fourfold greater increase in systemic, pulmonary, coronary and renal vascular resistances after blockade of NO formation Inhibition of ET-1 peptide binding with PD142893 in our co-culture model did not significantly affect either NO or PGI_2 levels in media perfusate, however both L-NAME and indomethacin attenuated the flow induced decrease in ET-1, thus confirming the inhibitory effect of each dilator on ET-1 secretion

A plethora of clinical studies have correlated ET-1 plasma levels with systemic and peripheral vascular diseases ET-1 is activated in hypertension, atherosclerosis, restenosis, heart failure, idiopathic cardiomyopathy, and renal failure (Luscher and Barton, 2000) Tissue concentrations and receptor expression more reliably reflect the activation of the endothelin system however, as increased vascular ET-1 levels can occur in the absence of changes in plasma (Luscher and Barton, 2000) ET-1 peptide circulating in media perfusate decreased at high flow in our culture model, however ET_B receptor mRNA increased ~10-fold in BRECs exposed to high flow rates, resulting in two key outcomes BREC NO and PGI_2 would increase at high flow, and secondly, BREC ET_B receptors compete with pericyte ET_A and ET_B

receptors for ET-1 binding, thus reducing the vasoconstrictor capacity of ET-1 Morawietz *et al* (2000) similarly demonstrated a shear stress dose-dependent upregulation of HUVEC ET_B receptor The importance of endothelial ET_B is highlighted by the growing trend of pharmaceutical companies utilising ET_A specific antagonists, rather than non-selective ET-receptor antagonists in the treatment of hypertension (D'Orleans-Juste et al , 2002) Reports by Strachan *et al* (1999) demonstrated increased peripheral vascular resistance in normal subjects with systemic administration of specific ET_B receptor antagonist, suggesting that the overall balance of effects of endogenous ET-1 at the vascular ET_B receptor favors vasodilatation (Strachan et al , 1999) Additionally, infusion of ET-1 in humans reduces forearm blood flow in NTG patients or controls, presumably via SMC ET_A mediated vasoconstriction However, infusion of ET-1 and a selective ET_A inhibitor increased forearm blood flow in both groups, although the vasodilation was lower in NTG patients than in controls, suggesting an impaired EC ET_B mediated release of vasodilators (Henry *et al* – *IOVS* – in press)

BRP ET_B receptors also increased at high flow, however ET_A receptors are the predominant pericyte receptor subtype, and high flow reduced BRP ET_A expression These results mirror those published by Cattaruzza *et al* (2000), who demonstrated a ~10-fold increase in SMC ET_B and a ~2-fold decrease in ET_A, when exposed to cyclic stretch (Cattaruzza et al , 2000) Interestingly, in the same study, stretching increased SMC apoptosis and was mediated by ET_B, but not ET_A SMC receptor activation Several *in vivo* reports have also demonstrated a potential role of increased expression of ET_B VSMC receptors responsible for apoptosis in atherosclerosis (Kobayashi et al , 2000, Babaei et al , 2000), whereas Chakrabarti *et al* demonstrated increased ET_B in diabetic rat retinas (Chakrabarti et al , 1998) The present results demonstrate a ~2-fold increase in both BRP apoptosis and BRP ET_B receptor, however, inhibition of ET-1 binding with PD142893 only partially reversed increased BRP apoptosis at high flow Increased BRP ET_B at high flow may mediate increased apoptosis, however further experiments with selective ET-receptor inhibitors are required Finally, increased ET-1 in ocular and retinal tissue of diabetic rats has also been reported (Chakrabarti et al , 1998, Takagi et al ,

1996a)

Retinal blood flow is autoregulated, such that within certain limits retinal vessels either contract or relax in order to maintain a constant blood supply We have demonstrated an EC vasoactive response to altered biomechanical forces, which may represent the primary mechanism whereby EC can 'sense' variations in blood flow In addition to the EC vasoactive response to altered flow rates, however, local interaction among endothelial factors may also be an important variable For example, when NO formation is disrupted, there is often a compensatory upregulation of PGI_2 This compensation is thought to be a protective mechanism for maintaining endothelium-dependent vasodilation (Li et al , 2004b) However, various examples of cross-talk between endothelial dilators indicate that the nature and mechanisms involved may vary depending on the vessels and conditions studied As such, several apparent contradictions can be found in the literature A report by Osanai on PGI_2/NO cross-talk in HUVECs exposed to shear stress demonstrated a two-fold enhancement of flow-induced PGI_2 production upon inhibition of NO synthase (Osanai et al , 2000), while PGI_2 inhibition did not alter shear induced release of NO Similarly, our BREC/BRP co-culture results demonstrate a significant increase in PGI_2 at both low and high flow with L-NAME, compared to controls In contrast, with indomethacin treated co-cultures, NO increased at both low and high flow compared to controls Interestingly, an *in vivo* report of NO-induced retinal and choroidal vasorelaxation in the piglet, suggested NO relaxation is actually mediated by PGI_2, with NO donors leading to increased circulating PGI_2 (Hardy et al , 1998) In addition, Vassalle *et al* (2003) report increased PGI_2 in response to sodium nitroprusside, an NO-donor, though they also report a compensatory increase in NO with inhibition of COX (Vassalle et al , 2003) Conversely, in human microvascular endothelial cells and in umbilical vein endothelial cells, PGI_2 did not compensate for decreased NO following pharmacological inhibition in cell culture (Vassalle et al , 2003) Quinn *et al* however, report a compensatory PGI_2 vasodilation upon NO inhibition in the porcine ciliary artery (Quinn et al , 2003)

ET-1 inhibition did not alter NO or PGI_2 in our co-culture model, however inhibition of either NO or PGI_2 increased ET-1 at both low and high flow ET-1 induces pericyte proliferation in EC/pericyte co-culture (Yamagishi et al , 1993), and as such, NO or PGI_2 inhibition of ET-1 would presumably stabilise mature vessels by inhibiting pericyte proliferation Kuchan and Frangos (1993) first demonstrated flow-induced decrease in ET-1 was mediated by NO (Kuchan and Frangos, 1993) Also, endothelium-derived hyperpolarising factor (EDHF), which is speculated to be cytochrome P450, a product of arachidonic acid metabolism (Coats et al , 2001), has not been investigated in this study A recent report suggests that EDHF mediates vasodilation in retinal vessels, in particular when NO, PGI_2 or ET-1 levels are inhibited (Ding and Triggle, 2000)

In this study we have demonstrated that chronic exposure to high pulsatile flow for 3 days causes increased BRP apoptosis, whilst simultaneously conferring a protective effect on BRECs when compared to low flow Several investigators have previously demonstrated the anti-apoptotic effect of laminar shear stress and also cyclic strain on EC (Bartling et al , 2000, Kaiser et al , 1997, Urbich et al , 2000, Haga et al , 2003, Yoshizumi et al , 2003) In addition, Sakao *et al* (2005) demonstrated reduced EC apoptosis at high pulsatile flow, with the same pulsatile flow apparatus used in this study (Sakao et al , 2005) Moreover, several *in vivo* results demonstrate the pro-apoptotic effect of reduced blood flow, with increased EC apoptosis at vessel branch points or bifurcations (Bercelı et al , 1990, Garcia-Cardena et al , 2001, Hosoya et al , 2005) Considering less EC apoptosis at higher flow rates in our co-culture model, it is tempting to speculate that reduced retinal blood flow, as is reported in glaucoma, may result in increased EC apoptosis and thus facilitate regression of vessels Similarly, in DR where blood flow velocities are reduced during the latter stages of disease, increased EC apoptosis may facilitate increased transport across the blood retinal barrier and lead to basement membrane thickening and possibly pericyte cell death – a hallmark of DR

Whereas ECs are exposed to shear stress and cyclic strain, pericyte/VSMCs cells are predominantly exposed to cyclic strain (Redmond et al , 1995) Cyclic strain is an important determinant of VSMCs cell phenotype, however the role of cyclic strain in protecting against, or inducing apoptosis is less clear For example, exposure of VSMCs to varying magnitudes of cyclic strain at different points within the vessel wall (e g advential or medial layers) confers a specific SMC phenotype (Shi et al , 1996, Cappadona et al , 1999) Pericyte cells on retinal capillaries and in our co-culture model exist as a single layer of cells, and as such, the magnitude of cyclic strain is directly correlated with retinal blood flow High pulsatile flow resulted in increased pericyte apoptosis, similar to previous results from our laboratory with aortic SMCs exposed to pulsatile flow (Birney et al , 2004) This may be particularly relevant to the onset of DR where blood flow velocities initially increase, followed by increased pericyte 'drop-out' or apoptosis Studies within our laboratory are ongoing to determine the combined effect of hyperglycaemia and varying pulsatile flow rates

The role of the vasoactive mediators NO, PGI_2 and ET-1 in this divergent EC/pericyte apoptotic response to increased pulsatile flow was also examined To date, pericyte-endothelial cell interactions have largely documented the cross-talk regulating proliferation of each cell type Pericytes are known to inhibit endothelial cell proliferation by releasing cytokines such as TGF-β (Martin et al , 2000), whereas ultrastructural studies have shown that newly formed capillaries stop growing when pericytes migrate into the basement membrane (Crocker et al , 1970) Loss of capillaries in diabetic retinopathy is implicated in proliferation of endothelial cells in new vessel formation (Orlidge and D'Amore, 1987) EC/pericyte cross-talk involves a plethora of molecules, most of which are as yet undefined The most extensively studied to date is VEGF Pericyte secretion of VEGF induces NO production in EC (Breslin et al , 2003) and also protects EC from NO induced EC apoptosis (Hata et al , 2001) NO is a known anti-apoptotic stimulus in numerous cell types, however excessive local concentrations can lead to reactive oxygen species, apoptosis and also necrosis (Kim et al , 2001b) (Lau, 2003) The present results demonstrate a protective effect of high compared to low pulsatile flow

against EC apoptosis in both mono-cultured EC and also co-cultured EC/Pericytes Comparison of EC harvested from mono-culture and EC harvested from co-culture however, resulted in a several-fold increase in baseline apoptosis, demonstrating that the presence of pericytes has a protective effect on EC These results might explain the phenomenon of endothelial cell apoptosis occurring after pericyte apoptosis in DR, leading to formation of acellular capillaries (Witmer et al , 2003)

Our results demonstrate increased BRP apoptosis at high compared to low pulsatile flow We therefore analysed whether this increase may be due to NO, PGI_2 or ET-1 production upon exposure to flow Increased BRP apoptosis at high flow was not attributable to either NO or ET-1, however inhibition of PGI_2 synthesis with indomethacin attenuated the increased apoptosis at high compared to low pulsatile flow Interestingly, the PGI_2 analogue iloprost did not rescue BRP from serum starvation induced apoptosis in static cultures, though it did not increase apoptosis either Reports by Li *et al* demonstrated that iloprost induces apoptosis via a cAMP-mediated suppression of ERK activity in VSMCs in static cell cultures (Li et al , 2004a) The synthetic PPARγ ligand, rosiglitazone, induced prostaglandin release and apoptosis in rat aortic SMCs (Bishop-Bailey and Warner, 2003) Whereas, two recent reports also with rat aortic SMCs, demonstrated reactive oxygen species and reactive nitrogen species (Upmacis et al , 2004) induce apoptosis via a prostaglandin dependent mechanism Inhibition of NO at high flow did not alter BRP apoptosis, however at low flow BRP apoptosis significantly increased This is possibly due to nitric oxide protecting BRP against apoptosis at lower flow rates, or it may also be a result of PGI_2, which increased significantly in the presence of L-NAME at low flow

Though PGI_2 may have been responsible for increased BRP apoptosis at high flow rates, and possibly at low flow rates when NO is inhibited, inhibition of PGI_2 did not have a significant effect on the protective effect of increased pulsatile flow in BRECs Furthermore, addition of iloprost to serum-starvation induced apoptotic BRECs had a dose-dependent protective effect on BREC apoptosis Inhibition of both NO and ET-1 with L-NAME and PD142893 respectively, abolished the protective effect of increase pulsatile flow This is in agreement with reports that

ET-1 functions as an apoptosis survival factor for endothelial cells in an autocrine/paracrine manner via the ET_B receptor (Shichiri et al , 1997) Even though ET-1 decreased ~2-fold at high flow, ET-1 may have an important protective effect, since BRECs ET_B receptor mRNA increased ~10-fold at high flow Interestingly, ET-1 induces NO production via activation of the ET_B receptor and this may be the mechanism by which it protects endothelial cells exposed to pulsatile flow Furthermore, in static cultures, ET-1 also protected EC from serum-starvation induced apoptosis

Numerous publications on the effect of NO and EC apoptosis have demonstrated either a pro- or anti-apoptotic response Indeed, our static experiments with an NO donor (SNAP), demonstrate a dual role, depending on concentration NO protects EC from apoptosis at low concentrations, however excessive amounts can induce reactive oxygen species which can inhibit the protective effect of NO and also cause apoptosis Our co-culture model demonstrates that increased NO at high pulsatile flow protects these cells from apoptosis Reports by Hata *et al* indicate that VSMCs protect ECs from NO-induced apoptosis through inhibiting down-regulation of Bcl-2, possibly through VEGF (Hata et al , 2001), demonstrating the importance of EC-pericyte cross-talk in maintaining vessel homeostasis

These results help explain the damaging effects of endothelial dysfunction, which is frequently associated with DR (Deng et al , 1999, McVeigh et al , 1992, Calver et al , 1992), and in particular normal tension glaucoma (Buckley et al , 2002) (Henry et al , 1999) Endothelial dysfunction impairs the ability of ECs to respond to fluctuations in blood flow, reducing levels of NO or PGI_2 and also increasing ET-1, thus causing vasospasm The present results highlight the particular importance of NO signalling in the retinal vasculature At low flow, for example, reduced NO resulted in increased PGI_2 possibly leading to increased pericyte apoptosis Furthermore, reduced NO at low flow increased ET-1 levels which would compound the detrimental effects of low flow by constricting vessels At high flow we demonstrate that increased NO is required to protect EC against apoptosis, however

endothelial dysfunction may impair this protective mechanism and reverse the protective effect of increased flow on the endothelium

The crucial role of NO in maintaining vascular homeostasis is also apparent with the association of eNOS polymorphisms with disorders that have in common a dysfunctional endothelium coronary heart disease, ischemic stroke, hypertension and Fabry's disease(Wilcox et al , 1997, Heltianu et al , 2005, Abe et al , 2005, Howard et al , 2005) Similarly, systemic factors such as hypercholesterolemia and hyperglycaemia are thought to impair endothelial NO signalling via oxidative stress damage Oxidative stress manifests as an imbalance between the levels of NO and reactive oxygen species (ROS) such as the superoxide anion (SO) (Miller et al , 2005, Napoli et al , 2001) Studies on various animal models of diabetes have showed that administration of scavengers of ROS such a superoxide dismutase (SOD) and catalase (CAT) improved or normalised the agonist induced endothelium dependent dilation of arteries, suggesting that elevated levels of superoxide and hydrogen peroxide may inactivate NO after its release Clinical studies demonstrated decreased NO synthesis and increased ROS production in patients with essential hypertension, renovascular hypertension and malignant hypertension (Higashi et al , 2002, Lip et al , 2002) Indeed, recent reports targeting ECs with adenovirus expressing NOS prevents elevation of blood pressure in stroke-prone spontaneously hypertensive rats (Miller et al , 2005)

We believe this pulsatile flow study is relevant to blood flow abnormalities observed in DR and glaucoma In addition, these results may shed light on hypertensive retinopathy – a condition characterised by a spectrum of retinal vascular abnormalities in people with elevated blood pressure Our co-culture model exposes retinal cells to increases in pulse pressures from low to high flow 6 to 56 mm Hg in the extra-capillary space, and 11 to 70 mm Hg at the capillary inlet

Hypertension is a polygenic, multifactorial disorder, which induces structural and functional changes of arteries, forming thick, rigid vessels at a greater risk of atherosclerosis The underlying pathological defect in hypertension is due, at least in part, to the endothelial dysfunction, possibly due to pulse pressure induced increases in cyclic strain and shear stress in the vessel wall With hypertension, the retinal vasculature is initially protected from increases in systemic blood pressure by a local autoregulatory response, such that vasospasm occurs and the tone of retinal arterioles increases This stage is seen clinically as a generalized narrowing of the retinal arterioles Arteriolar narrowing may result from, but also lead to, hypertension A 'vicious cycle' may exist through which the microcirculation maintains or even amplifies an initial increase in blood pressure Thus, a rise in blood pressure may lead to increased resistance in the microcirculation, leading to further elevation of blood pressure (Porta et al , 2005)

The retinal circulation undergoes a series of pathophysiological changes in response to elevated blood pressure There is disruption of the blood–retina barrier, necrosis of the smooth muscles and endothelial cells, exudation of blood and lipids, and retinal ischemia (Wong and Mitchell, 2004) In addition, arteriovenous "nicking" is seen in chronic stages of hypertension This is caused by the enlargement of the retinal arteriolar wall, which compresses a retinal vein at their common adventitial sheath(Luo and Brown, 2004), increasing the risk of retinal venous occlusion During chronic stages of the pathology, large areas of the inner retina become infarcted as a result of occlusions of the precapillary arterioles Eventually their smooth muscle cells necrose This necrosis destroys the arterioles' ability to constrict, thereby leading to focal vasodilation and transmission of the high blood flow to the endothelium of smaller vessels Leakage of plasma proteins in the

posterior retina as hard exudates soon follows because the endothelial damage causes a breakdown of the blood retinal barrier (Walsh, 1982, Luo and Brown, 2004)

Elevated blood pressure is a definitive risk for poor microvascular and macrovascular outcomes of diabetes In Type 2 diabetes, blood pressure status was shown to be even more important than blood glucose status in predicting and preventing significant vision loss from diabetic retinopathy (UK Prospective Diabetes Study Group, 1998) Furthermore, when diabetes is associated with hypertension, retinopathy accelerates and is also more severe (Porta et al , 2001) Moreover, tight control of blood pressure can prevent deterioration of DR In the ARIC prospective cohort study, retinal arterioles were significantly narrower in persons who subsequently developed diabetes over the following 3 5 years compared with those who did not (Porta et al , 2005)

4.4 Conclusion

Primarily as a result of increased NO, chronic exposure to high pulsatile flow protects ECs from apoptosis On the other hand, high pulsatile flow induces pericyte cell apoptosis via increased prostaglandin products Moreover, ECs respond to fluctuations in pulsatile flow via altered NO/PGI_2/ET-1 signalling, demonstrating that EC and pericytes have an inherent capacity to autoregulate blood flow in response to perfusion pressure changes

Chapter 5

Hedgehog signalling is anti-apoptotic and also regulates Notch signalling in both BRECs and BRPs

5.1 Introduction

5.2 Results

5.3 Discussion

5.4 Conclusion

5 1 Introduction

Formation of the vascular system is one of the earliest and most important events during embryogenesis in mammals During the early stages of vascular development, the *de novo* formation of blood vessels occurs from mesodermally-derived endothelial cell precursors termed angioblasts Angioblasts differentiate and subsequently assemble into primitive blood vessels, termed the primary vascular plexus, in a process known as vasculogenesis Formation of the primordial vessels in the central retina is mediated by vasculogenesis, whereas angiogenesis is responsible for increasing vascular density and peripheral vascularization in the inner retina In contrast, the outer plexus and the radial peripapillary capillaries are formed by angiogenesis only These mechanisms of retinal vascularization appear similar to those of vascularization of the central nervous system during development (Hughes et al , 2000, Gariano, 2003) Identification of a wide range of molecules whose targeted disruption results in defective vascular development has begun to expose the extent of the complexity of this process Mice deficient in flk-1/KDR, flt-1, PDGF-B, PDGFRβ,TGFβ1, TGFβRII, endoglin, SHh, Notch, tissue factor, neuropilin-1 and -2, ephrinB2, EphB4, tie-1, tie-2, hypoxia-inducible factor, and angiopoietin-1 and -2, to name a few, exhibit defective vascular development The role that each of these plays in vessel formation, and whether their influence is direct or indirect, is not fully understood (D'Amore and Ng, 2002)

Several studies have reported the importance of the Notch signalling pathway in the development of many tissues and organs in the body (Artavams-Tsakonas et al , 1999, Miele and Osborne, 1999) Mutation of many components of the Notch signalling pathway in mice, for example, results in embryonic lethality due to defects in the formation of the vascular system Mice homozygous for null mutations in Jagged1 and Notch1 die *in utero* due to defects in vascular morphogenesis and angiogenic vascular remodelling respectively (Han et al , 2000, Singh et al , 2000) There is an increasing appreciation that pathways studied predominantly for their

role during development, rather than being silent in adult vasculature, are in fact active and largely responsible for pathologic and non-pathologic tissue remodelling

As with Notch, the importance of the Hedgehog signalling pathway within the vasculature is increasingly recognized The transmembrane Hedgehog receptor, Ptc1, is expressed throughout the vasculature in both juvenile and adult mice, suggesting that adult cardiovascular tissues may contain several cell populations responsive to Hedgehog signalling (Pola et al , 2001) The Hedgehog pathway also plays a pivotal role in development, regulating many of the same functions as the Notch signalling pathway, such as limb and neural tube development, highlighting the functional interactions between these pathways (Artavanis-Tsakonas et al , 1995, Artavanis-Tsakonas et al , 1999, Weed et al , 1997) Hedgehog signalling also appears to be important in proper development of the vascular system Similar to the Notch signalling pathway the correct level of Hedgehog signalling appears to be important, as both up- and down-regulation of hedgehog proteins results in vascular defects (Sullivan and Bicknell, 2003) Mutations of the Hedgehog signalling pathway, for example, result in lack of proper vascularization in the developing mouse lung (Pepicelli et al , 1998)

As with the Notch signalling pathway, the Hedgehog pathway is implicated in regulating cell fate decisions SHh regulates both proliferation and survival of oligodendrite precursors, and acts to promote proliferation and inhibit differentiation in both neuronal and non-neuronal cell types (Ho and Scott, 2002) Dysregulation of both the Hedgehog and Notch pathways lead to dysregulated cell growth and abnormal cellular accumulations, contributing to many types of cancers (Villavicencio et al , 2000, Miele and Osborne, 1999) Therefore, although it is likely, whether the Hedgehog signalling pathway regulates cell fate decisions through the Notch signalling pathway remains to be fully established

The aim of this study was to identify the presence of Notch & Hedgehog signalling pathway components in retinal endothelial & pericyte cells in static culture and also in human normal and glaucomatous eye sections In addition, the effect of exogenous Sonic Hedgehog addition on Notch signalling and apoptosis was also examined in retinal endothelial & pericyte cells

5 2 Results

5 2 1 Components of the Notch Signalling Pathway are present in BRECs

Notch1, -2 and -4 have been reported to be expressed in endothelial cells *in vivo*, and similar results have been reported in cultured endothelial cells (Liu et al , 2003b) Analysis of BRECs has revealed the presence of components of the Notch signalling pathway (Fig 5 1 & 5 2) The Notch receptors, Notch1 (Fig 5 1(a)) and Notch3 (Fig 5 1(c)) are present in BRECs as determined by immunocytochemistry The antibodies used in this study are directed against the active intracellular portion (IC) portion of the Notch receptors Therefore, these antibodies detect both the cleaved IC portion of the Notch receptor, in addition to the full-length receptor Routine detection of Notch1IC & Notch 3IC with antibodies from two different commercial sources, and competition with an appropriate blocking peptide confirmed the specificity of the antibody binding In these experiments, the nuclei were also stained with a fluorescent dye – DAPI (blue) (Fig 5 1 to 5 6, right panel) The presence of Notch1IC & Notch3IC protein was also confirmed by western blot analysis, and the presence of mRNA transcripts were verified through PCR analysis (western blot data – Chapter 6)

Immunocytochemical analysis revealed the sub-cellular localization of the components of the Notch signalling pathway Fig 5 1 (a) illustrates that Notch1IC is predominately located in the nuclei of BRECs, however, it is present both in the cell cytoplasm and on the plasma membrane Membrane-tethered Notch1IC appears to constitute a small proportion of the overall cellular distribution of Notch1IC Similarly, Notch3IC appears to be primarily located in the nuclei of BREC (Fig 5 1 (c)) Notch3IC is also present in the cytoplasm of the cell The presence of the Notch ligand, Jagged, has also clearly been shown in BRECs (Fig 5 2 (e)) Immunocytochemical analysis also revealed the presence of Notch target genes of the HRT and Hes families in BRECs Hes-1 (Fig 5 1 (e)), HRT-1 (Fig 5 1 (g)), HRT-2 (Fig 5 2 (a)) and HRT-3 (Fig 5 2 (c)) all exhibit a strong nuclear localization pattern within the cell, as is evident with Notch receptors and ligands

Both Hes-1 and HRT-2 are present within the cytoplasm, however, relative to the Hrt-1 and Hrt-3 staining pattern, they exhibit a more specific nuclear localization

In all cases the appropriate primary and secondary controls were performed in parallel with each experiment

5 2 2 Components of the Hedgehog Signalling Pathway are present in BRECs

Using BRECs in culture, we examined for the presence of Sonic Hedgehog (SHh), Indian Hedgehog (IHh) and the transmembrane Hedgehog receptor Patched (Ptc1) in microvascular endothelial cells *in vitro* Using antibodies targeted against Ptc1 (Fig 5 3 (a)), SHh (Fig 5 3 (c)) and IHh (Fig 5 3 (e)), each of these proteins stained positive in BRECs

Using PCR and Western blot analysis, the presence of SHh, IHh and Ptc1 mRNA and protein expression were further confirmed in BRECs (data not shown) Furthermore, the presence of mRNA transcripts for the Hedgehog signalling downstream targets, Gli2 and Smo, were confirmed by PCR analysis (data not shown)

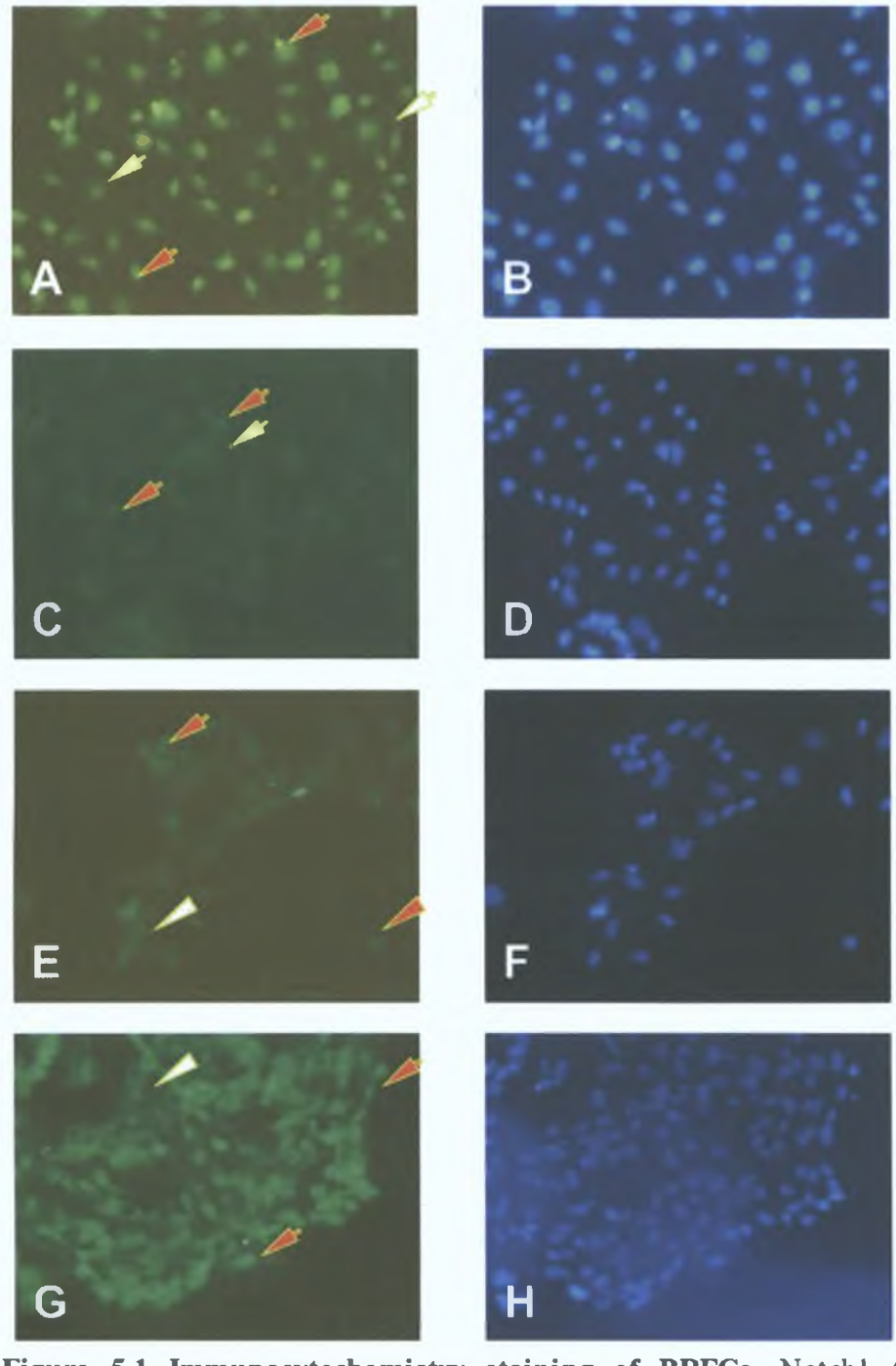


Figure 5.1 Immunocytochemistry staining of BRECs. Notch1 **(A)**, Notch3 **(C)**, Hes1 **(E)** and Hrt1 **(G)**, with corresponding Dapi nuclear staining **(B) (D) (F) (H).** Arrows indicate staining localised in nuclear region (red) and in cytoplasm (white) Magnification x20.

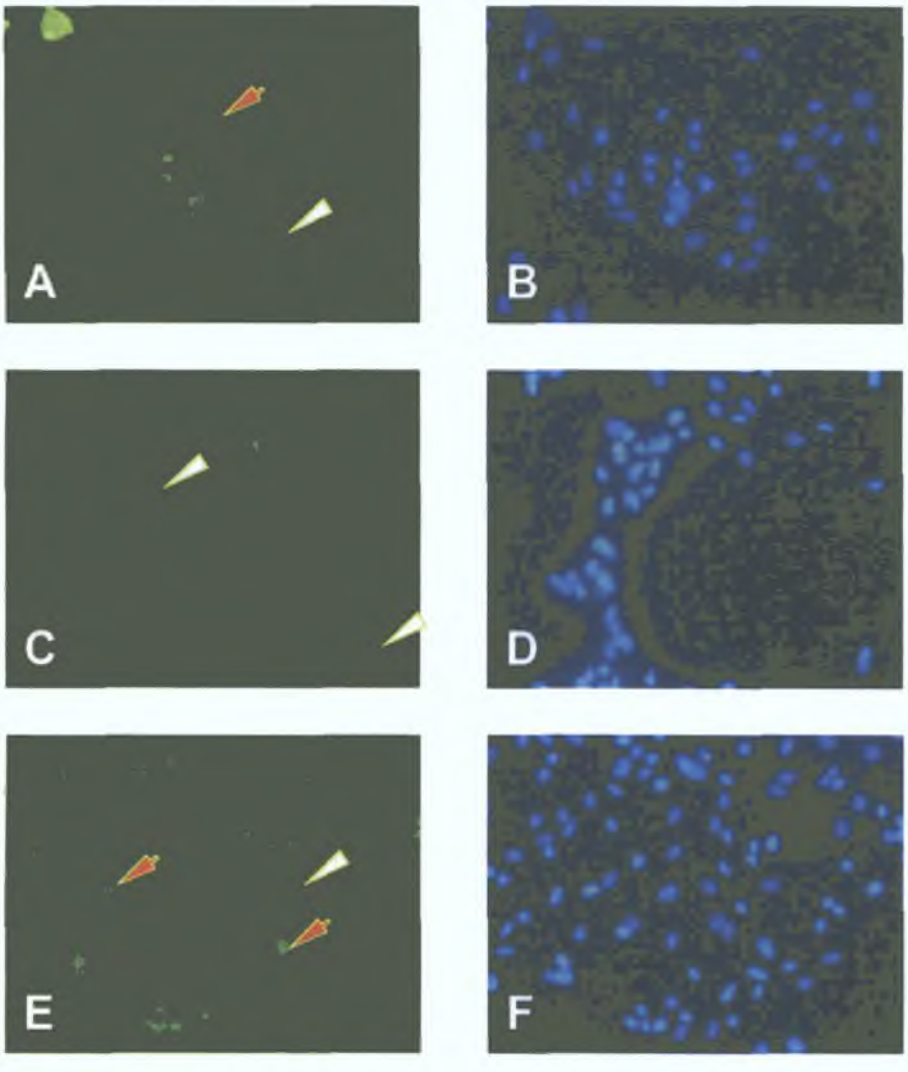


Figure 5.2 Immunocytochemistry staining of BRECs. Hrt2 **(A)**, Hrt3 **(C)** and Jagged **(E)**, with corresponding Dapi nuclear staining **(B) (D) (F).** Arrows indicate staining localised in nuclear region (red) and in cytoplasm (white) Magnification x20.

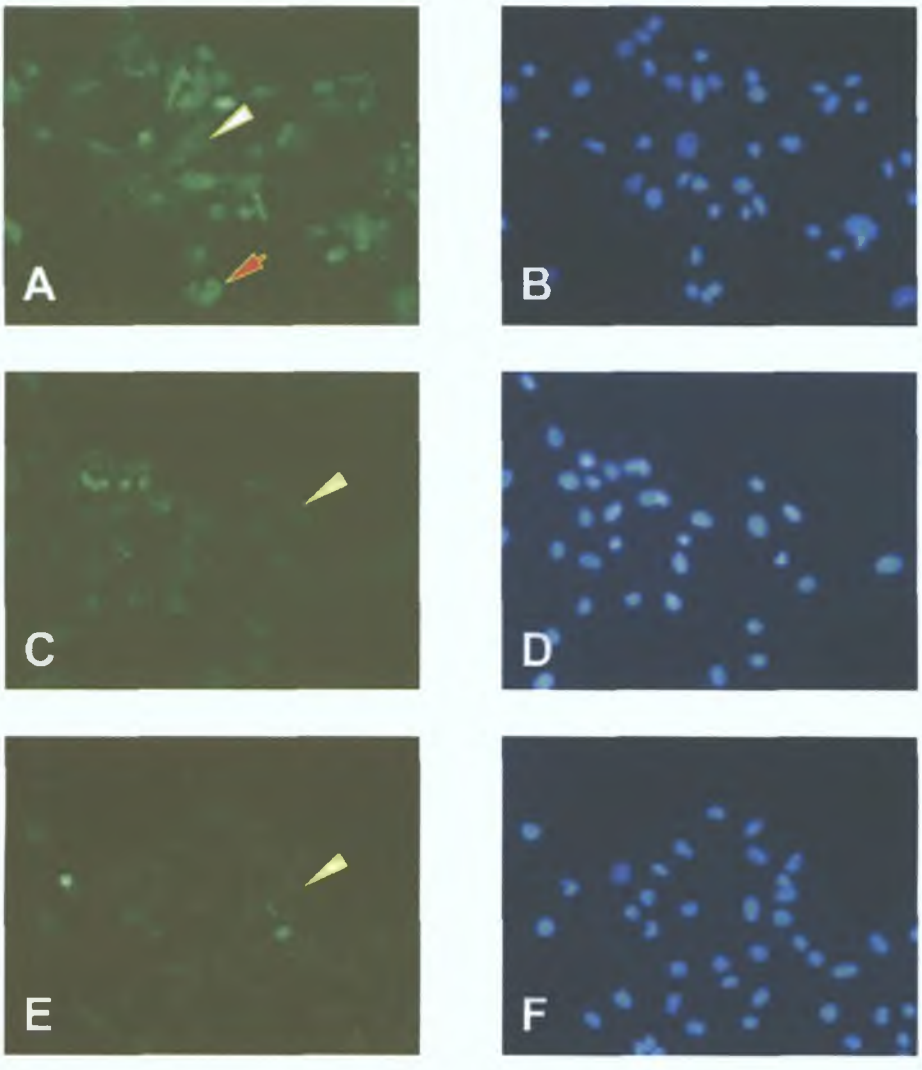


Figure 5.3 Immunocytochemistry staining of BRECs. Ptc1 **(A)**, Indian **(C)** and Sonic **(E)**, with corresponding Dapi nuclear staining **(B) (D) (F).** Arrows indicate staining localised in nuclear region (red) and in cytoplasm (white) Magnification x20.

5 2 3 Components of the Notch Signalling Pathway are present in Bovine Retinal Pericytes (BRPs)

Immunocytochemistry staining of BRPs with monoclonal antibodies has revealed the presence of components of the Notch signalling pathway in retinal pericytes (Fig 5 4 – 5 5) Fig 5 4 (c) illustrates that Notch3IC is predominately located in the nuclei of BRPs, however, it is also present both in the cell cytoplasm and on the plasma membrane Nuclear Notch3IC does not appear to be present in the nucleoli of the cell, and the distribution of Notch3IC in the cytoplasm appears to be clustered around the nucleus Membrane-tethered Notch3IC appears to constitute a small proportion of the overall cellular distribution of Notch3IC Similarly, Notch1IC appears to be primarily located in the nuclei of BRPs (Fig 5 4 (a)), however in this case, a discrete localization of the receptor within the nucleolus is also evident The presence of both Notch1IC & Notch3IC protein was further confirmed by western blot analysis, and the presence of mRNA was verified through PCR analysis (data not shown)

Additionally, homogenous staining of the Notch target genes Hes-1 (Fig 5 4 (c)), HRT-2 (Fig 5 4 (a)), HRT-3 (Fig 5 4 (c)) and the Notch ligand Jagged (Fig 5 4 (e)) was also established Analysis of HRT-1 staining (Fig 5 4 (g) revealed a specific nuclear sub-cellular localization In all cases the appropriate primary and secondary controls were performed in parallel with each experiment

5 2 4 Components of the Hedgehog Signalling Pathway are present in BRPs

Immunocytochemical analysis of SHh, IHh and Ptc1 receptor proteins in BRP cells indicated the presence of these proteins in retinal pericytes Rather than being freely secreted proteins, mature Hedgehog are tethered to the plasma membrane through a covalently bonded cholesterol moiety (Lee et al , 1992) Expression of the Hedgehog morphogens SHh and IHh was uniformly distributed throughout the cell Their presence in this mural cell type is in agreement with previous immunohistological observations of SHh smooth muscle localisation in rat microvascular tissue sections (Podlasek et al , 2003)

Patched associates with caveolin-1 at caveolae, which are small, flask-shaped membrane invaginations enriched in cholesterol and sphingolipids Staining of BRPs for Ptc1 immunofluorescense was positive (Fig 5 6 (a)), indicating retinal pericytes are a target of Hedgehog morphogens The staining pattern of Ptc1 had some clusters of fluorescense at the membrane which suggests that Ptc1 may function by trafficking Smoothened at caveolae Ingram *et al* previously suggested that Patched mediates the trafficking of Smoothened through a membrane compartment, either by promoting its trafficking to the plasma membrane or by promoting its degradation via the lysosome (Ingham et al , 2000)

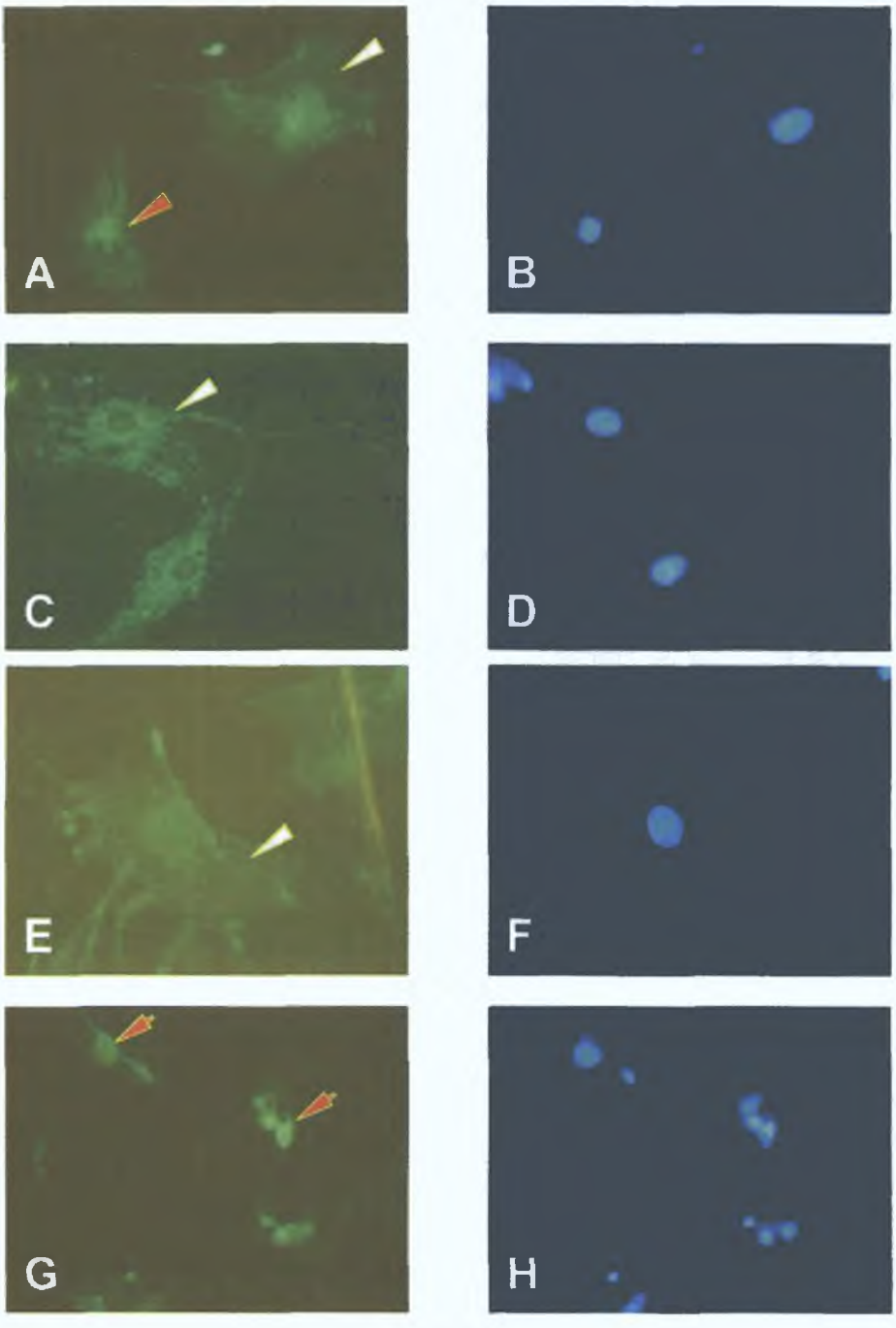


Figure 5.4 Immunocytochemistry staining of Pericytes. Notch1 **(A)**, Notch3 **(C)**, Hes1 **(E)** and Hrt1 **(G)**, with corresponding Dapi nuclear staining **(B) (D) (F) (H).** Arrows indicate staining localised in nuclear region (red) and in cytoplasm (white) Magnification x20.

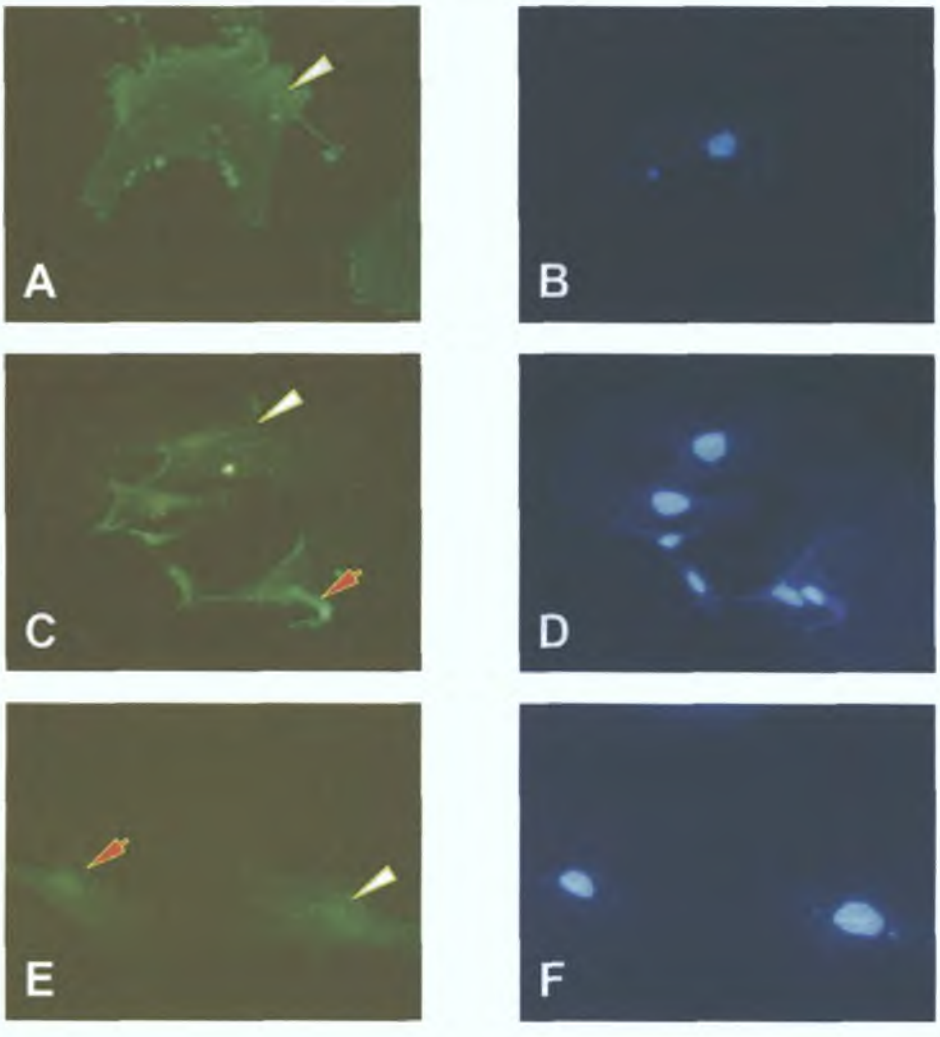


Figure 5.5 Immunocytochemistry staining of Pericytes. Hrt2 **(A)**, Hrt3 **(C)** and Jagged **(E)**, with corresponding Dapi nuclear staining **(B) (D) (F).** Arrows indicate staining localised in nuclear region (red) and in cytoplasm (white) Magnification x20.

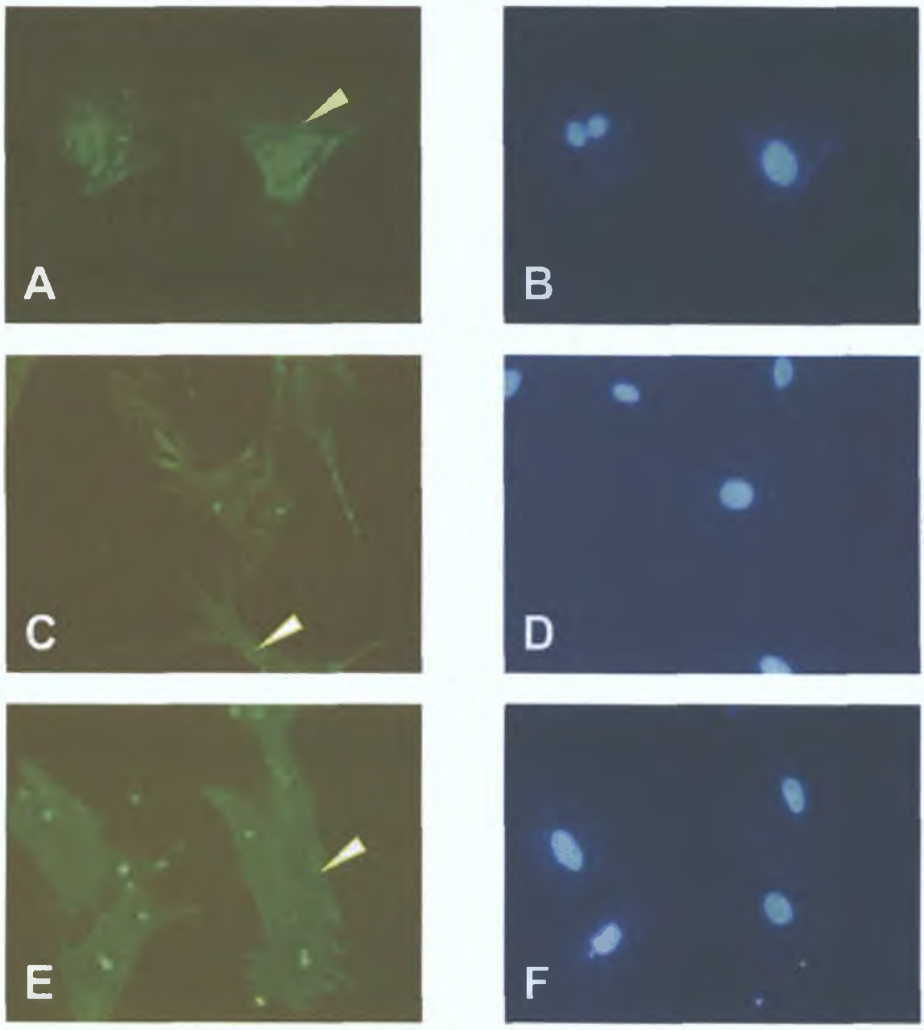


Figure 5.6 Immunocytochemistry staining of Pericytes. Ptc1 (A), Indian **(C)** and Sonic **(E)**, with corresponding Dapi nuclear staining **(B) (D) (F).** Arrows indicate staining localised in nuclear region (red) and in cytoplasm (white) Magnification x20.

5 2 5 Pathway Components of Notch & Hedgehog Signalling are present in the human retina – comparison of normal & glaucomatous eyes

Immunohistochemical staining for Notch & Hedgehog Signalling Pathways components was performed on formalin fixed, paraffin wax embedded sections of 12 human eyes, of which, 6 were from glaucoma patients It is noteworthy that these glaucomatous eyes are from patients with raised intra-ocular pressure Concurrent control experiments with omission of the primary antibody excluded the possibility of non-specific immunolabelling by the secondary antibody and allowed the effectiveness of endogenous peroxidase quenching to be assessed

Three different regions in the optic nerve head (ONH) were identified (1) the Pre-Laminar Region (PLR), localized at the level of the choroid and retina, which includes the superficial nerve fiber layer and is characterized by minimal connective tissue and the absence of clearly myelinated axons, (2) the Lamina Cribosa (LC), localized at the level of the sclera and characterized by large connective tissue septa, and (3) the retro-laminar region (RLR), localized outside the eye and characterized by myelinated axons and delicate connective tissue septa These ONH regions, along with the retina were examined for immunoperoxidase vessel staining

There was no detectable Notch3IC, Hrt-1, Hes-1, Hes-5, Sonic or Patched immunoreactivity in the retinas of either normals or glaucomas This is not to conclude that these proteins are absent however, as the epitope used to generate the commercial monoclonal antibodies may have simply been masked in these tissue samples

The majority of PLR microvessels at the ONH were positive for Notch1IC (Fig 5 7 (b)) Similarly, immunostaining of these vessels for Notch target genes, Hrt-1, Hrt-2 and Hrt3 (Fig 5 7, b to f) were positive Some smaller vessels which are found in the retina (Fig 5 7 (a) & (f)) and the RLR (Fig 5 7 (d)), were also positive for Notch1IC and its transcription factor targets (Hrt-1, Hrt-2 and Hrt3) Subjective analysis of Notch1IC staining suggests an increase in Notch1IC in glaucomatous patients, though staining is also evident in normals It is difficult to ascertain for

certain if Notch1IC is increased in glaucoma patients however, with the small number of eyes used in this study

In contrast, the PLR microvessels of the ONH were clearly negative for the Hedgehog morphogen Indian However, large numbers of vessels within the retina (Fig 5 8 (A) (B) & (C)) of normal eyes examined, were positively stained for IHh (n=6) Interestingly, microvessels in all of the glaucomatous eyes, though present, were negative for IHh (n=6) The specific vessels positively staining for IHh are the interconnecting vessels between the two layers of vasculature within the retina – the superficial and the deep or inner vascular plexus Furthermore, no immunoreactivity was observed for IHh in RLR vessels in either normals or glaucomas

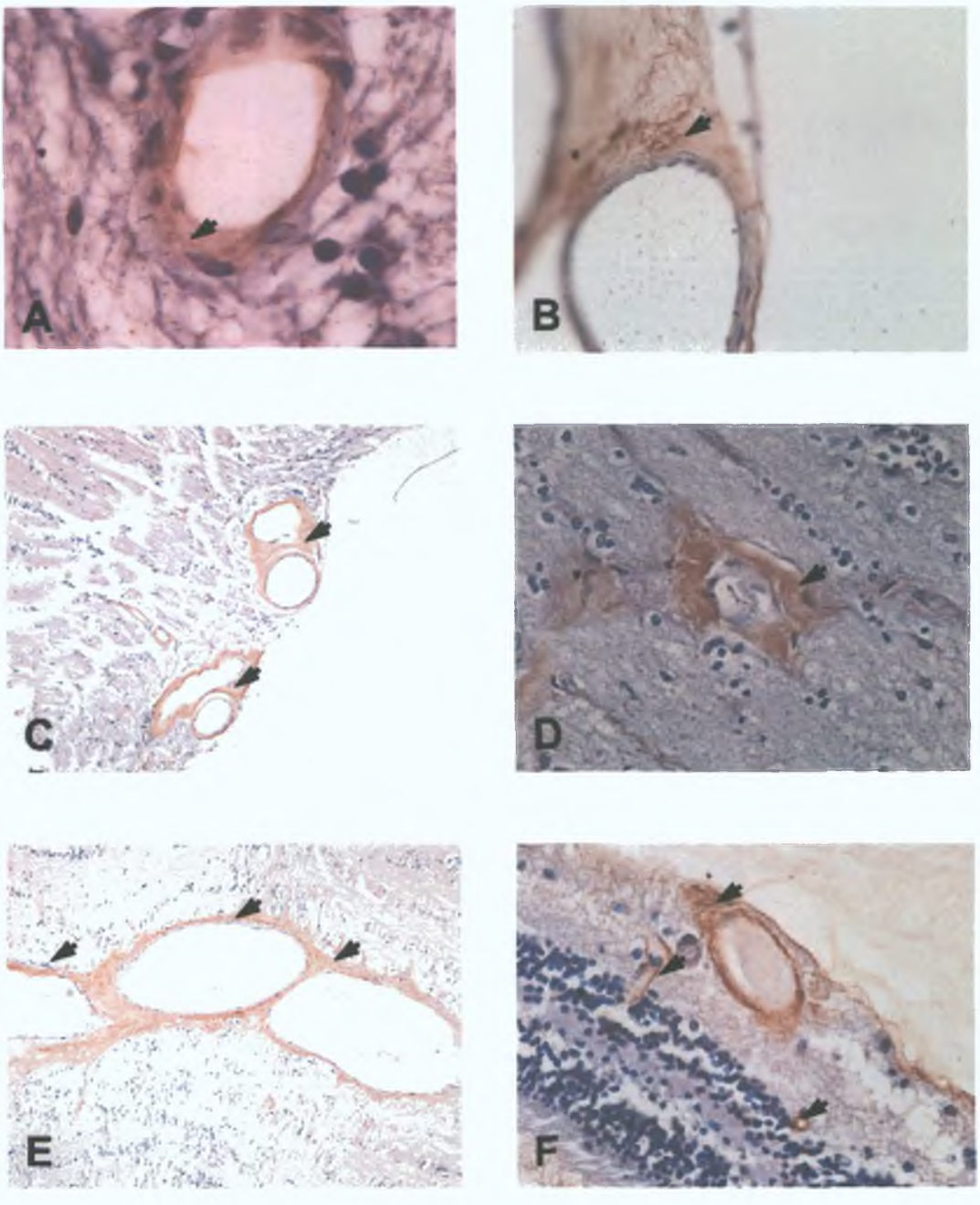


Figure 5.7 Immunohistological diaminobenzidine (DAB) staining of human retina/Optic Nerve Head. Notch1 **(A)**, Hrt1 **(B)**, Hrt2 **(C)** & **(D)** and Hrt3 **(E)** & **(F)** (arrows = DAB)

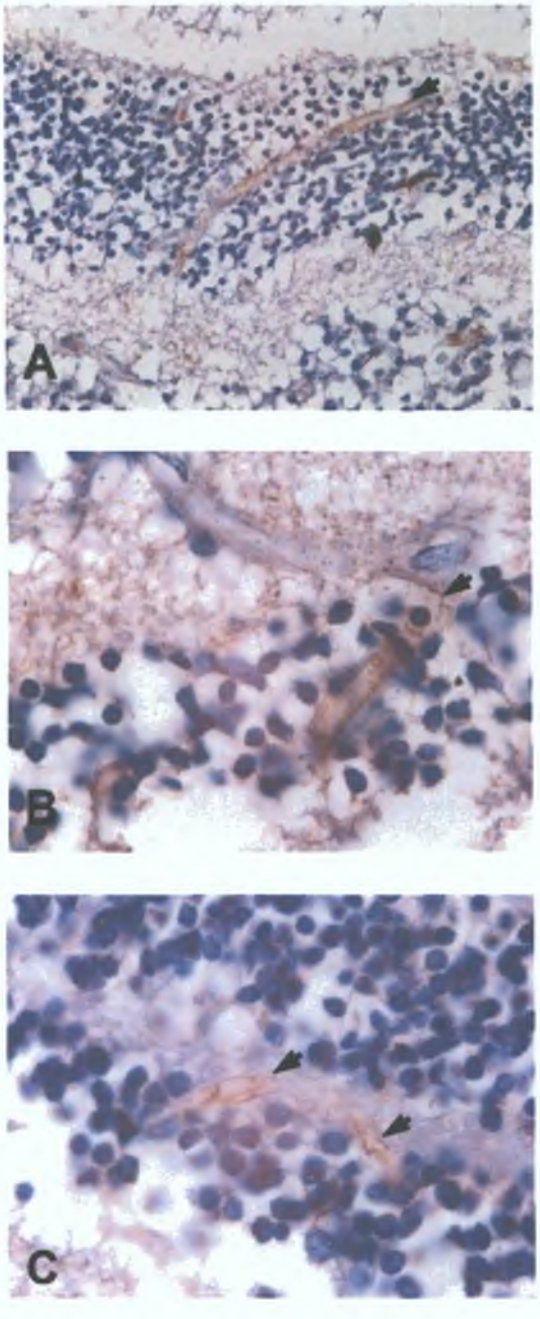


Figure 5.8 Immunohistological DAB staining of human retina.
Indian **(A) (B)** & **(C)** (arrows = DAB)

5 2 6 Exogenous addition of Sonic Hedgehog Protects against serum-starvation induced apoptosis & activates Notch Signalling in BRECs & BRPs

Vascular cell fate decisions are hallmarks of the vascular cell response to injury and play a crucial role in the pathogenesis of vascular disease Notch receptor-ligand interactions and the Hedgehog (Hh) signalling pathway have been strongly implicated in vascular morphogenesis and remodelling of the embryonic vasculature, with Hh activation upstream of Notch signalling during development This study has successfully detected components of the Notch & Hh signalling pathway in cycling BRECs and BRPs We therefore tested the hypothesis that the Hh and Notch pathways interact to promote changes in vascular cell fate in BRECs & BRPs *in vitro*

The interaction of Hh proteins with their specific receptor patched-1 (Ptc1) inactivates the repression of the transmembrane protein smoothened (Smo), leading to activation of the transcription factor Gli, the principal mediator of the Hh signalling pathway Gli induces expression of downstream target genes of the Hh pathway, including Ptc1 and Gli itself Thus, Ptc1 and Gli are both components and transcriptional targets of the Hh signalling pathway Activation with SHh recombinant protein (3μg/ml) resulted in a significant fold increase in Ptc1 target gene mRNA expression in both BRECs and BRPs (4 50 ± 0 18 and 1 70 ± 0 09 fold, n=3, respectively) concomitant with a significant fold increase in gli2 mRNA levels (1 38 ± 0 05 and 2 45 ± 0 19 fold, respectively) as determined by real-time PCR analysis (Fig 5 9 (a) & (b)) Smo mRNA also increased in BRECs and BRPs treated with exogenous SHh (1 53 ± 0 07 and 1 47 ± 0 05 fold, n=3, respectively) As expected, treatment of both BRECs and BRPs with SHh in the presence of the specific SHh inhibitor cyclopamine (40μM) abrogated the effect of SHh induction of Ptc1, Gli2 and Smo in each cell type (Fig 5 9) Cyclopamine is a naturally occurring steroidal alkaloid which specifically inhibits the Hh pathway by interacting with the Hh signalling protein Smoothened Cylopamine was dissolved in DMF at 40μM and as such, all controls contained the equivalent volume of DMF

To investigate whether the induction of the Hedgehog signalling pathway resulted in an increase in transcription of Notch pathway components, we performed real-time PCR analysis of BREC & BRP mRNA samples following activation with SHh SHh stimulated the expression of Notch1 and Notch3 in BRECs (4 26 ± 0 25 and 2 32 ± 0 11 fold, n=3, respectively) (Fig 5 10 (a)) and Notch1, but not Notch3 in BRPs (1 50 ± 0 06 and 0 93 ± 0 09 fold, n=3, respectively) (Fig 5 10 (b)) Abrogation of this response with the addition of cyclopamine, demonstrates SHh peptide acts by de-repressing the Ptc1-Smo complex (Fig 5 10)

Activation of Hedgehog signalling with recombinant SHh peptide had an overall anti-apoptotic effect on both BRECs and BRPs BREC anti-apoptotic transcripts *BCl-2* and *BCl-xl* both increased in the presence of SHh (1 53 ± 0 06 and 1 54 ± 0 06 fold, n=3, respectively), while the pro-apoptotic marker *Bax* decreased (0 48 ± 0 04 fold, n=3) (Fig 5 11) In BRPs, exogenous SHh increased *BCl-2* but not *BCl-xl* (1 54 ± 0 10 and 1 03 ± 0 05 fold, n=3, respectively), while *Bax* decreased (0 68 ± 0 09 fold, n=3) (Fig 5 11) The overall anti-apoptotic effect in both BRECs and BRPs due to Hedgehog activation was reversible in the presence of cyclopamine (Fig 5 11)

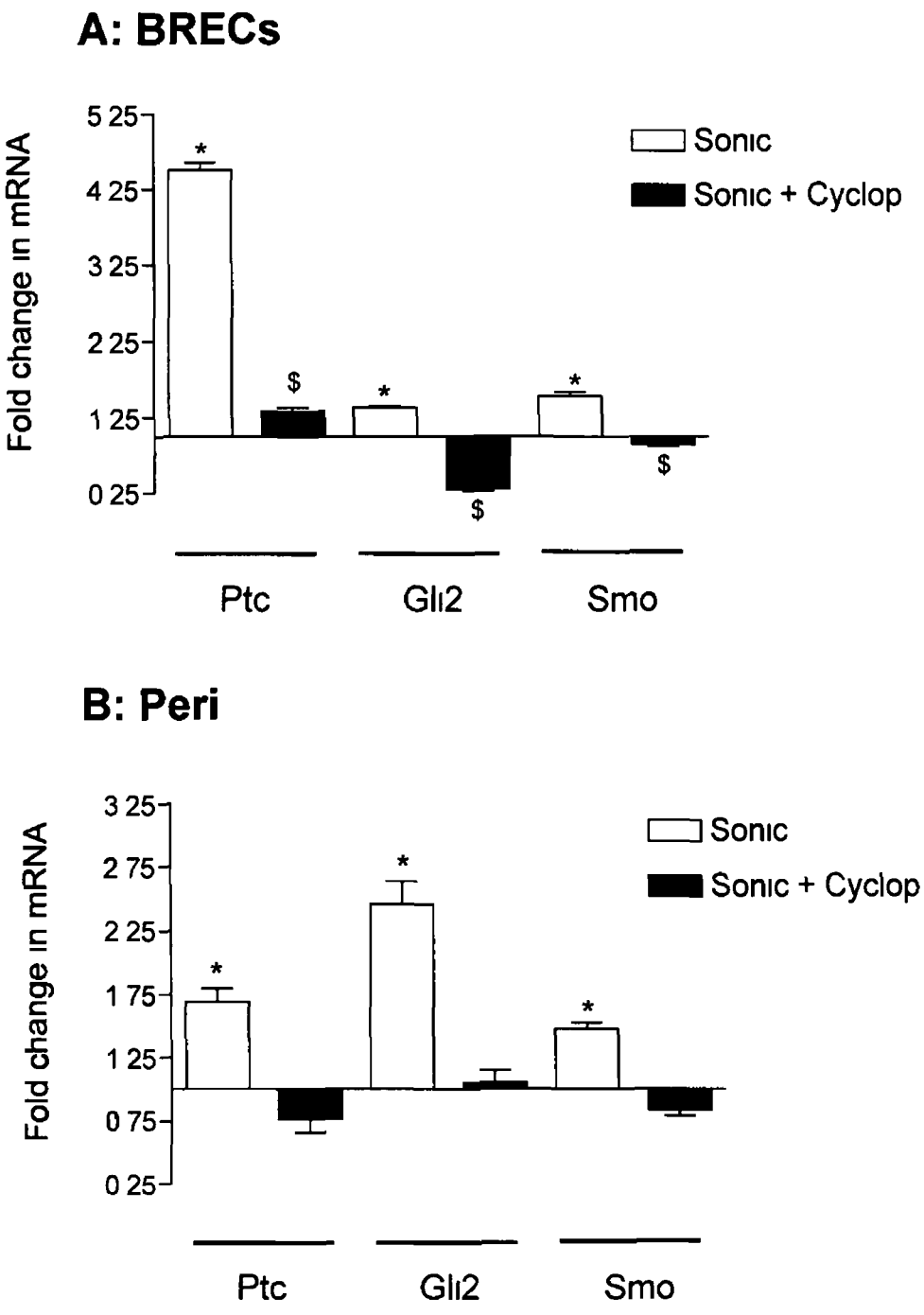


Figure 5 9 Realtime PCR analysis of *Ptc1*, *Gli2* and *Smo* mRNA. BRECs (A) or BRPs (B) were exposed to recombinant SHh (3μg/ml) or SHh (3μg/ml) + Cyclopamine (an inhibitor of Shh) (40μM), in static culture Histogram represents mean values ± SEM (n=3), *P<0 05 compared to low flow control (normalised to 1) $ P<0 05 compared to Sonic treatment

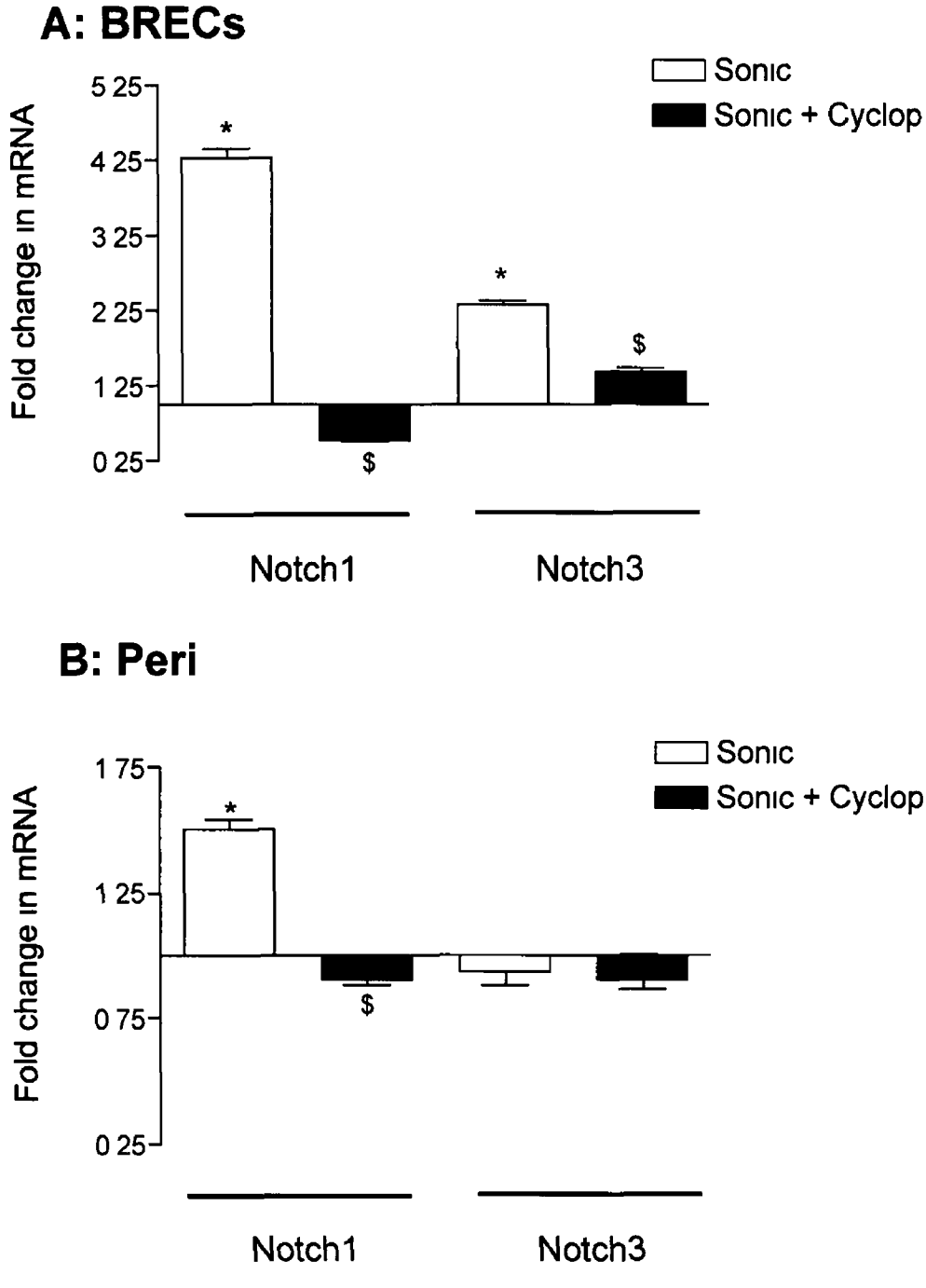


Figure 5 10 Realtime PCR analysis of *Notch1* and *Notch3* mRNA. BRECs **(A)** or BRPs (B) were exposed to recombinant SHh (3µg/ml) or SHh (3µg/ml) + Cyclopamine (an inhibitor of Shh) (40µM), in static culture Histogram represents mean values ± SEM (n=3), *P<0 05 compared to low flow control (normalised to 1) $ P<0 05 compared to Sonic treatment

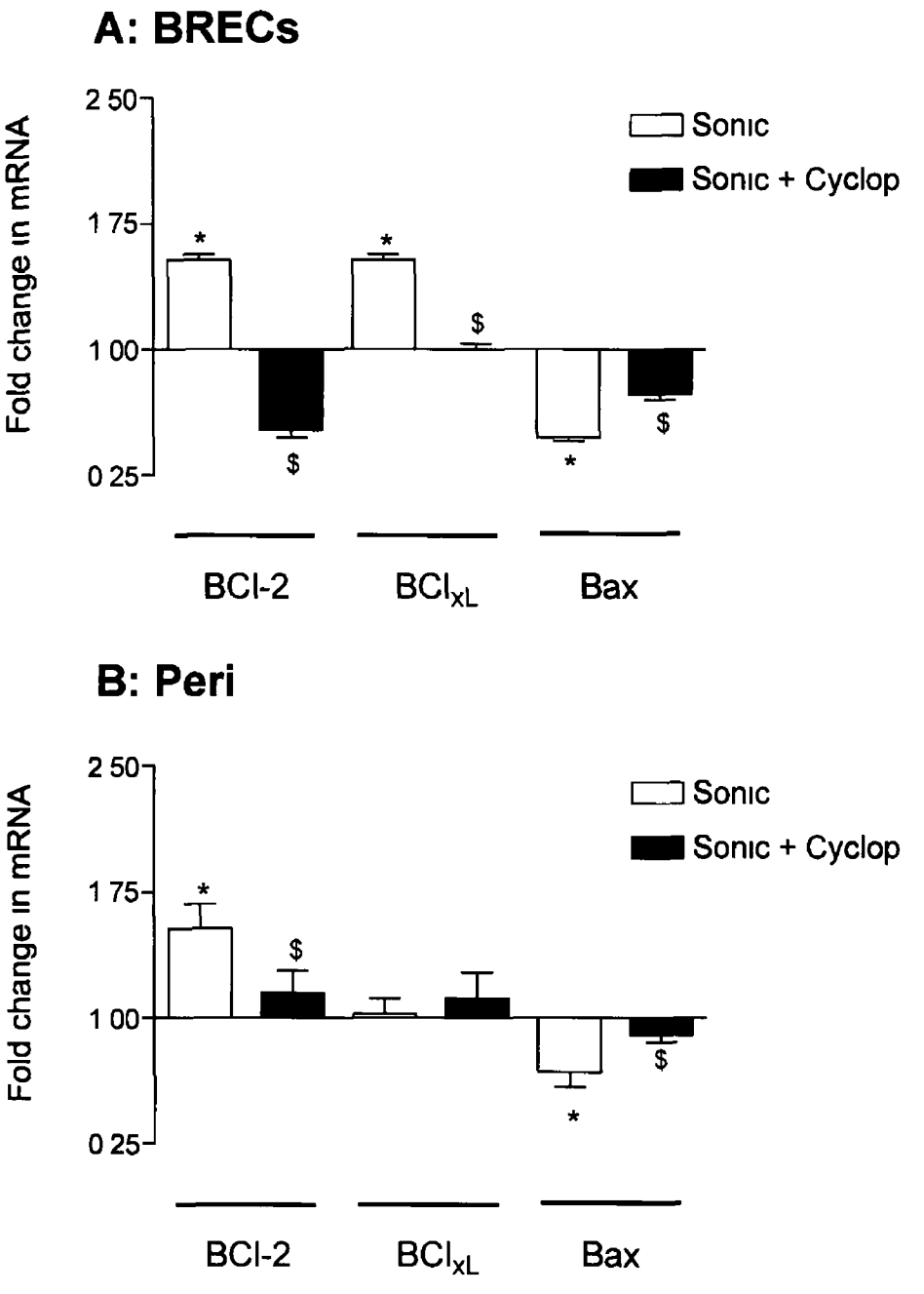


Figure 5 11 Realtime PCR analysis of *bcl-2, bcl-xl* and *bax* mRNA. BRECs (A) or BRPs (B) were exposed to recombinant SHh (3μg/ml) or SHh (3μg/ml) + Cyclopamine (an inhibitor of Shh) (40μM), in static culture Histogram represents mean values ± SEM (n=3), *P<0 05 compared to low flow control (normalised to 1) $^{\$}$ P<0 05 compared to Sonic treatment

5 2 7 Real-time PCR analysis of Notch & Hedgehog Signalling components in retinal tissue isolated from normal & glaucomatous human eyes

In earlier immunocytochemistry analysis of Notch and Hedgehog signalling components, we confirmed the presence of Notch1, Notch3, Hes1, Hrt1, Hrt2, Hrt3, Jagged, Ptc1, SHh and IHh in BRECs (Fig 5 1 to 5 3) and BRPs (Fig 5 4 to 5 6) Furthermore, we confirmed the presence of Notch1, Hrt1, Hrt2, Hrt3, Ptc1 and IHh in vessels of normal and/or glaucomatous human eyes by immunohistochemistry (Fig 5 7 & 5 8) We therefore sought to confirm by real-time PCR analysis if SHh is present in the human eye, since there was none detectable by immunohistochemistry Furthermore, a comparative analysis of mRNA for Notch1, Notch3, IHh & SHh in normal versus glaucomatous eyes was performed

Four normal and four glaucomatous RNA preparations of retinal tissue were analysed There was no significant difference between normal and glaucoma eye mRNA levels of Notch1 (Fig 5 12 (a)), Notch3 (Fig 5 12 (b)), SHh (Fig 5 13 (a)) or IHh (Fig 5 13 (b)) Previous immunohistochemical analysis of the same eyes demonstrated presence of IHh in normal, but not in glaucoma eyes Though IHh is present in glaucomatous eyes, the real-time data suggest increased mRNA in normal eyes compared to glaucoma eyes Due to the small sample size, these results are not significant, however they are suggestive of a role of IHh in normal microvessels in the retina, which are impaired in glaucomatous retinas Impairment of these vessels may occur secondary to loss of neurons or possibly due to increased IOP

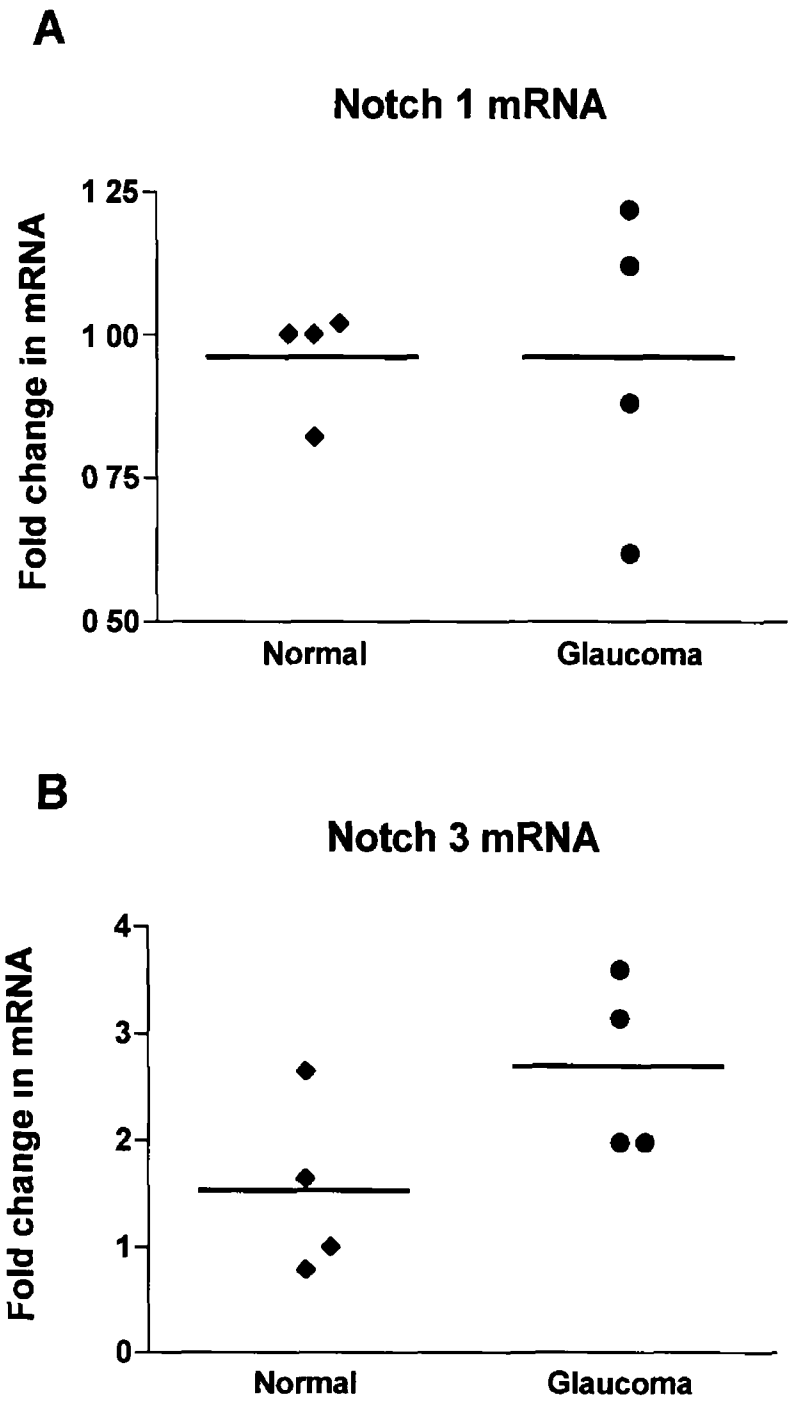


Figure 5 12 Realtime PCR analysis of *Notch1* (A) and *Notch3* (B) mRNA levels in human normal and glaucomatous retinas (n=4)

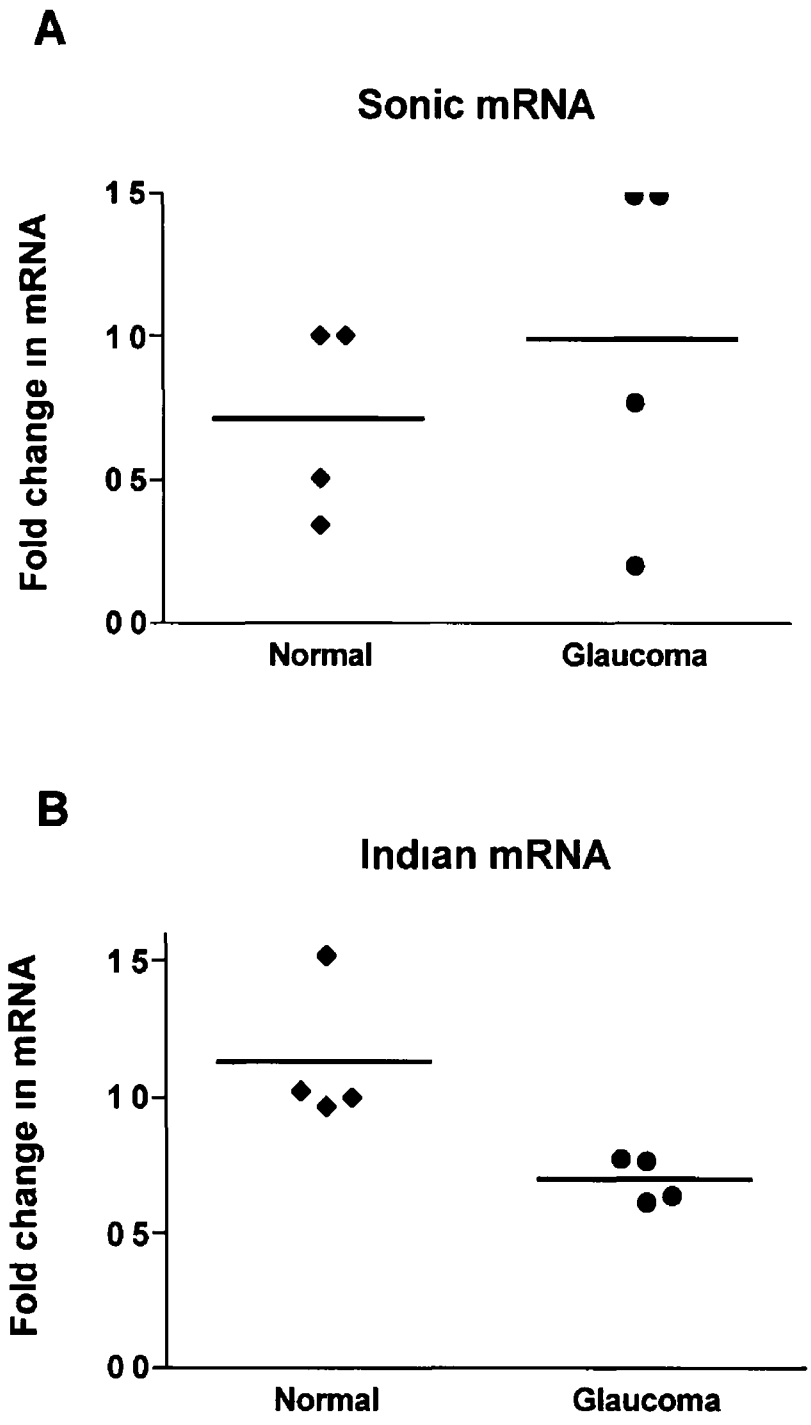


Figure 5 13 Realtime PCR analysis of *Sonic* (A) and *Indian* (B) gene expression in human normal and glaucomatous retinas (n=4)

5.3 Discussion

A number of different receptor-ligand interactions have been implicated in the development and maintenance of the vasculature including: the vascular endothelial growth factor (VEGF) family, angiopoietins and their cognate receptor-Tie2, the fibroblast growth factor (FGF) family, the platelet derived growth factor (PDGF) family, ephrins and Eph receptors, and various other cytokines and chemokines (Karsan and Harlan, 1996). Further to this plethora of signalling molecules and pathways, the Notch and Hedgehog family of receptors and ligands also play a significant and non-redundant role in development and maintenance of the vasculature.

Several studies point to a role for Notch and its ligands in influencing vascular development. For example, Notch signalling is required for arterial-venous differentiation in zebrafish (Lawson et al., 2001). Mutant mice that are null for Notch1 show defects in the vasculature, and the severity of these vascular defects is enhanced in mice that are null for both Notch4 and Notch1, although Notch4 single knockouts are viable and healthy (Krebs et al., 2000). A homozygous Notch2 hypomorphic allele in mice disrupts development of vasculature of the glomerulus, heart, and eye (McCright et al., 2001). Mice that are rendered null for the Notch ligand, Jagged1, exhibit defects in vascular remodelling (Xue et al., 1999). In addition, mice with mutations in both Delta-like1 and Notch2 show embryonic haemorrhage, possibly resulting from poor development of vascular structures (Kojika and Griffin, 2001). Mutations in Notch target genes also highlight the importance of the Notch signalling pathway in vascular development. Zebrafish embryos harbouring a mutation in Gridlock, a HRT-2 orthologue, show impairment of vascular formation in the form of aortic coarctation (Weinstein et al., 1995). In contrast, HRT-2 null mice do not present with aortic coarctation, suggesting a level of redundancy between mammalian Notch target genes, however, they present with massive post-natal cardiac hypertrophy, and a resulting high rate of lethality in the first 10 days of life (Gessler et al., 2002). Interestingly, constitutive activation of Notch4 specifically in EC, also causes defects in vascular remodelling (Leong et al.,

2002) (Uyttendaele et al , 2001) The similar vascular phenotypes that are induced by either constitutive activation or constitutive loss of Notch suggest a requirement for specific and finely tuned activation of Notch within the context of the developing vasculature

The human disorders CADASIL and AGS provide evidence of the importance of the Notch signalling pathway in the adult vasculature These disorders involve dysregulation of components of the Notch signalling pathway resulting in cardiovascular abnormalities CADASIL, which is caused by mutations in Notch3, is characterized by degeneration of SMC, primarily in cerebral arteries and arterioles, but also in systemic arteries and some veins CADASIL patients present with an abnormal accumulation of Notch3 in VSMC, impaired Jagged1/Notch3 binding and defective CBF-1-dependent Notch3 signalling (Brulin et al , 2002, Prakash et al , 2002, Joutel et al , 2004, Prakash et al , 2002, Brulin et al , 2002) AGS also highlights the importance of the Notch signalling pathway, and in particular Jagged1, in the development and maintenance of the cardiovascular system AGS patients typically present with congenital heart defects, heart murmurs and/or peripheral pulmonary stenosis (Joutel and Tournier-Lasserve, 1998, Loomes et al , 1999)

Several studies have revealed the presence of Notch1, Notch2 and Notch 3 in VSMC, both *in vivo* and *in vitro* (Leimeister et al , 2000, Lindner et al , 2001, Campos et al , 2002, Kitamoto et al , 2005) However, Notch3 expression is previously reported as SMC specific (Joutel et al , 2000) Joutel *et al* indicated that Notch3 function is required in adult mice for the structural and functional integrity of arteries, particularly smaller-diameter arteries (Joutel et al , 1996) To date, vascular expression of Notch4 has not been documented in VSMC, which is reported to be endothelial cell specific (Uyttendaele et al , 1996) In addition to Notch4, Notch1 and Notch2 have been reported to be expressed in endothelial cells in vivo (Lindner et al , 2001, Nijjar et al , 2001) Therefore, whilst significant advances have been made in recent years in detailing the regulation, expression and role of the Notch signalling pathway in the adult vasculature, the pathway remains poorly characterized

This study documents the presence of many components of the Notch signalling pathway in retinal endothelial and pericyte cells, both *in vivo* and *in vitro* and is largely in agreement with several recent studies of the macrovascular VSMC (Campos et al , 2002, Iso et al , 2002) Campos *et al* , for example, documented the presence of Jagged1, Notch3, HRT-1, HRT-2 and HRT-3 protein and mRNA expression in Rat VSMC *in vitro* We demonstrate the presence of HRT-1, HRT-2, HRT-3, Hes-1, Notch1 and Notch3 in BRECs, in BRPs, and also in the adult vasculature of the human retina and optic nerve head The Notch receptor antibodies used in this study are directed against the IC portion of the Notch receptor and, as such, detect both the IC and full-length forms of the Notch receptor Several studies have revealed the predominance of the IC portion of the Notch receptor in vascular cells (Campos et al , 2002, Wang et al , 2002c, Wang et al , 2002b) and in other cell types (Fehon et al , 1991, Aster et al , 1994) This study also investigated the sub-cellular localization of the components of the Notch signalling pathway using immunocytochemical analysis The components of the Notch signalling pathway appear to localize predominantly in the nucleus, which is in agreement with several reports (Fortini et al , 1993, Kopan et al , 1996, Lieber et al , 1993)

Since Hh signalling plays a key role in pattern formation, differentiation, and proliferation in the early mouse embryo, the vascular system was not initially identified as a target of Hh action when knockout mutants of individual Hh genes were examined Nonetheless, a role for Hh signalling in blood vessel formation in the embryo is supported by a number of observations, including the hypervascularization of neurectoderm in response to overexpression of SHh (Saika et al , 2004), and the decreased vascularization of lung tissue in SHh-deficient mice (Pepicelli et al , 1998) Support for Hh's role in vascular development came from the observation that zebrafish carrying mutations in components of the Hh signalling pathway have a defect in circulation and vascularization (Lawson et al , 2002, Brown et al , 2000) During normal development, two trunk axial vessels form—the aorta and the posterior cardinal vein The aorta develops immediately adjacent to a midline source of SHh The loss of SHh leads to a single large vessel that expresses

only venous and no arterial markers (Lawson et al , 2002) Hh does not act directly on the axial vessels, but through a mesodermal intermediate

The consensus is that the signals encoded by the three Hh genes (Sonic, Indian and Dessert) all activate the same downstream signalling cascade, and that the presence of genes controlled by separate regulatory elements facilitates the expression of the signal at multiple sites and times during embryogenesis Hh signalling is used throughout embryogenesis in many differentiating tissues to establish cell fate, promote cell proliferation, and mediate programmed cell death Although the expression of DHh and IHh are more restricted, recent analysis has revealed previously unobserved overlapping domains of expression for SHh and IHh in the early embryo—for example, in the node and somites (Zhang et al , 2001) A role for IHh in early embryogenesis is supported by the observation that double IHh/SHh knockouts have an earlier, more severely altered phenotype than do SHh mutants, and resemble Smo mutations, which have no Hh downstream signalling (Zhang *et al* 2001) Hh ligand binding relieves Ptc1-mediated suppression of Smoothened (Smo) Without the Hh ligand, kinases glycogen synthase kinase 3, casein kinase 1, and protein kinase A mediate phosphorylation of Gli and its processing to a repressor form With Hh binding to Ptc1, the full-length Gli promotes transcription of downstream targets, including Ptc1 Interestingly, one of the genes expressed downstream of Hh signalling encodes the Hh receptor Ptc1, thereby making Ptc1 expression an indicator of Hh responsiveness In this study, we have demonstrated the presence of Ptc1 in both BRECs and BRPs in cell culture Furthermore, both Hedgehogs, Indian and Sonic, were also present in BRECs and BRPs, suggesting a possible autocrine/paracrine signalling mechanism

Similar to Notch signalling, Hh is now appreciated as an important molecular pathway involved in maintenance of the adult vasculature For example, several reports indicate the adult vascular system in the mouse can respond to Hh Ptc1-LacZ expression, an indicator of Hh response in Ptc1-LacZ heterozygous mice, is measurable in both endothelial cells and adventitial fibroblasts that surround the vessels, with the fibroblasts capable of robust Ptc1 upregulation in response to administered Hh (Pola et al , 2001) In a hind limb ischemia model, SHh treatment

promotes an increase in capillary density and blood flow (Pola et al , 2001) Furthermore, Ptc1 is upregulated in the interstitial mesenchymal cells and an SHh-blocking antibody inhibits angiogenesis (Pola et al , 2003) SHh addition also promotes neovascularization and the formation of large, well-branched vessels in a corneal angiogenesis assay (Pola et al , 2001) The data documenting upregulation of Ptc1 expression in adventitial fibroblasts suggest that Hh acts via a support cell intermediate, rather than directly on endothelial cells to promote adult vessel remodelling

Activation of the Hh pathway has previously been associated with several tumours For example, mutations in the Ptc1 gene are associated with basal cell carcinomas and with medulloblastomas (Bale, 2002), and the Gli genes are named for their role in glioma formation (Matise and Joyner, 1999) Defects in Hh signalling cascade components are also associated with holoprosencephaly and congenital malformations (Kim et al , 2001a) It has now been established that in many digestive tract tumours, the Hh system is activated, with increased expression of SHh and IHh This occurs in oesophageal, stomach, pancreatic and biliary tumours, but not in colonic SHh expression seems particularly important in pancreatic tumourigenesis (Thayer et al , 2003)

Interestingly, our immunohistolgical analysis of adult human eyes had no obvious SHh or Ptc1 staining, either in the retinal or optic nerve head vessels However, IHh was visible in capillaries of the retina of normal eyes, but not in glaucomatous eyes No IHh staining was visible in larger vessels of normal or glaucomatous eyes in the lamina cribosa, pre- or retro-laminar regions These eyes from patients with glaucoma, have raised intra ocular pressure as the principal cause of the pathology In these high tension glaucoma patients, it is likely that the vessels which stain positive for IHh would be exposed to considerable increases in pressure Furthermore, similar to NTG, HTG patients have reduced ocular blood flow (Findl et al , 2000, Fuchsjager-Mayrl et al , 2004, Garhofer et al , 2004) and prolonged arterio-venous passage times (Duijm et al , 1999, Arend et al , 2004), indicating vascular blood flow abnormalities are at least a secondary effect of HTG Our realtime PCR analysis of IHh also suggests mRNA levels in patients compared to

normals are lowered, however a larger patient/control size is required to confirm the significance of these results

SHh, Notch1 and Notch3 are all present in human eyes, as determined by RT-PCR, however no obvious differences are apparent between normals and glaucomas RNA samples from these eyes were generated from the retinal tissue and the prelaminar region (PLR) of the optic nerve head (ONH), which is involved in several eye diseases In particular, a variety of optic neuropathies are located at the PLR and circulatory disturbances in this area are thought to be one of the causes of glaucoma The reasons for the specific vulnerability of this region to pathologic conditions are as yet unclear

How does the Hh signal interact with the other signalling pathways demonstrated to play a role in vascular development? Loss-of-function mutation of a number of genes implicated in angiogenesis results in embryonic vascular remodelling defects For example, targeted mutation of the Ang1 or Tie2 genes results in severe yolk sac angiogenesis defects by midgestation, reminiscent of the Smo homozygous mutant phenotype (Thurston, 2003, Zhang et al , 2001) This observation suggests that this angiogenesis growth factor and its receptor may act in the same pathway as Hh This hypothesis is further supported by the observation that SHh treatment upregulates the angiopoietins in adventitial fibroblast cells, placing Hh upstream of these vascular-specific growth factors (Pola et al , 2001) Addition of SHh also promotes expression of VEGFs in fibroblasts up to 72 hours In contrast, the same group report an absence of Ptc1 upregulation by SHh in endothelial cells of corneal neovessels *in vivo*, which was mirrored *in vitro* by the inability of human umbilical vein endothelial cells or microvascular endothelial cells to respond to SHh by Ptc1 upregulation, proliferation, migration or serum-free survival (Pola et al , 2001) Olsen *et al* (2004) propose the inability of many cell types to respond to exogenous Hh addition expressing the necessary targets – Ptc1, Gli and Smo – is due to large intracellular levels of Hedgehog-interacting protein (HIP), a Hh pathway antagonist HIP is abundantly expressed in endothelial, but low or undetectable in many other cell types Pola *et al* do not specify from which microvascular bed they

derive their ECs, however our retinal microvascular ECs do repond to exogenous SHh addition by upregulating not only Ptc1 and Gli2, known downstream targets of the Hh cascade, but also Smo Our ECs were stimulated in media containing reduced serum, thereby possibly sensitizing our cells Furthermore, we demonstrated a similar induction of Hh components in retinal microvascular pericytes upon addition of SHh peptide Analysis of HIP in BRECs compared to macrovascular endothelial cells would be an interesting future study

Notch signalling has also been implicated in blood vessel differentiation and arrest at the capillary plexus stage is observed in embryos deficient in Notch1 (Krebs et al , 2000) Notch4 is also implicated in vessel remodelling (Krebs et al , 2000) (Uyttendaele et al , 2001) Recent chimera studies (Damert et al , 2002) have demonstrated a role for VEGF produced by the visceral endoderm in yolk sac angiogenesis VEGF has also recently been shown to induce Notch1 and Delta-like4 (Dll4) expression in arterial endothelial cells via the PI3K/Akt pathway (Liu et al , 2003b) Dll4, the likely vascular Notch receptor, is expressed in arteries but not veins, implying a role for this cascade in establishing vessel identity, a key step during vascular development (Shutter et al , 2000) Dll4 expression is also known to play a role in retinal capillary development possibly in conjunction with PDGF signalling (Claxton and Fruttiger, 2004) Taken together, these data are consistent with a regulatory cascade for vascular remodelling that begins with Hh promoting Ang1, Ang2, and VEGF expression, which in turn promotes Notch expression and signalling Strong support for this hierarchy comes from work performed in zebrafish, where exogenous VEGF can restore normal arteriogenesis in the absence of SHh, but not in the absence of Notch function, and addition of Notch can compensate for the loss of VEGF activity (Lawson et al , 2002) SHh from the notochord promotes VEGF expression by the adjacent somite, which promotes Notch activity and expression of the artery-specific ephrin B2 in the dorsal aorta Absence of this pathway permits expression of venous markers and differentiation of the posterior cardinal vein Our studies demonstrate increased Notch1 expression in both BRECs and BRPs upon addition of SHh, while Notch3 is increased in BRECs but not in BRPs. In most cell types Notch1IC expression results in increased CBF-1-

dependent activity, however, the role of Notch3IC appears to be cell-type specific In 1999, Beatus *et al*, suggested that Notch3IC acts as an antagonist of the CBF-1-dependent Notch signalling pathway, as Notch3IC expression inhibited the Notch1IC CBF-1-dependent increase in Hes-1 activity in a human chorion carcinoma cell line In addition, Wang *et al*, have shown that co-expression of Notch1 and Notch3IC in VSMC results in a potentiation of the Notch1IC response (Wang et al, 2002c) This contradicts the finding in embryonic VSMC, that the activation of Notch3IC on the CBF-1 promoter is considerably more potent than that of Notch1IC (Campos et al, 2002) The reason for this discrepancy is not clear, but perhaps points to a more important role for Notch3 signalling developmentally, or indeed, a more significant role for Notch1 in adult VSMC A Notch3 inhibition of Notch1 in VSMCs may explain a functional reason for SHh activation of Notch1, but not Notch3 in our pericytes Future studies are required to ascertain if this is the case

The process of apoptosis is tightly regulated through a number of gene products that promote or inhibit cell death Apoptosis constitutes a systematic means of cell suicide within an organism during normal morphogenesis, tissue remodelling and in response to pathogenic infections or other irreparable cell damages The most extensively studied gene products, and perhaps the most important, are the Bcl-2 family (Gupta, 2003) The net influence of the Bcl-2 family on apoptosis appears to be the ratio between the pro- and anti-apoptotic molecules in a cell at any given time A number of signalling pathways such as the MAPK and NFkB pathways can influence the relative concentration of Bcl-2 family proteins in mammalian cells (Gupta, 2003) This study investigates whether the Hedgehog signalling pathway should be added to this list

Activation of Hedgehog signalling resulted in increased anti-apoptotic *BCl-2* and *BCl-xl* in BRECs, and a decrease in pro-apoptotic *Bax* Similarly, in BRPs, *BCl-2* was also increased, concomitant with decreased *Bax* expression No change in BRP *BCl-xl* expression levels was found, however Therefore addition of SHh had an overall anti-apoptotic effect on both BRECs and BRPs Discrepancies in

downstream target activation, such as *BCl-xl* in this case, may be due to differential intermediate Notch signalling in the signalling cascade It is conceivable, and indeed likely, that the Notch signalling pathway could be part of a complex multi-signalling network that exerts an influence over EC/pericyte cell fate decisions, including apoptosis This, however, remains largely undefined and requires further study Notch1 has been shown to physically interact with the NFkB signalling pathway in T cells (Guan et al , 1996) exhibiting an IkB-like activity, physically interacting with the p50 NFkB subunit, sequestering it in the cytoplasm, thus inhibiting NFkB activity It is currently not documented whether Notch1IC, exerts the same effect on NFkB activity in vascular cells

Evidence from a number of sources suggests that the Hh-VEGF-Notch cascade may not be the only means whereby Hh promotes cell fate decisions For example, zebrafish embryos lacking SHh have a more severely abnormal vascular phenotype, failing to form two distinct trunk axial vessels, than those that lack VEGF or Notch, which have two vessels with the aorta transformed from artery to vein This discrepancy suggests that Hh also promotes vascular development via other signalling cascades (Lawson et al , 2002) The simple pathway of Hh promoting VEGF production by an adjacent cell type is further complicated by the observation that both IHh and VEGF are produced by the visceral endoderm layer, and this source of VEGF is required for proper angiogenesis (Damert et al , 2002) These data suggest that Hh and VEGF may act in concert rather than in tandem to promote vascular remodelling This model is supported by an investigation of the role played by retinoic acid (RA) in supporting yolk sac capillary plexus remodelling (Bohnsack and Hirschi, 2004) In this study, RA acts by promoting visceral endoderm survival and therefore the expression of the visceral endoderm products VEGF-A, basic fibroblast growth factor, and IHh Rescue of the vascular remodelling phenotype observed in retinaldehyde dehydrogenase-2-deficient embryos, unable to synthesize RA, could only be achieved upon addition of all three endoderm-specific factors, addition of any one alone was not sufficient (Bohnsack and Hirschi, 2004)

This angiogenic cascade involving Hh, VEGF, and Notch involves communication between three different cell types the Hh producing cell, the Hh responding cell, and the target cells However, our evidence also suggests that Hh may act directly on endothelial cells as has previously been demonstrated In an assay using either murine brain capillary endothelial cells or human umbilical vein endothelial cells, SHh promoted endothelial network and lumen formation in the absence of support cells (Kanda et al , 2003) Furthermore, a vascular network-like structure formed in response to SHh treatment of the bEnd3 endothelial cell line (Vokes et al , 2004) This morphologic transition suggests that Hh may also play a direct role in tubulogenesis In both of these *in vitro* experiments, the induction of capillary morphogenesis occurred in the apparent absence of VEGF, suggesting that the direct action of Hh on endothelial cells is independent of VEGF Taken together, the evidence suggests that Hh acts both via the VEGF–Notch cascade, and via an alternate pathway

5 4 Conclusion

This study demonstrates the presence of many components of the Notch signalling pathway in microvascular endothelial & pericyte cells and also in the retinal & optic nerve head microvasculature We have demonstrated activation of the hedgehog pathway in each cell type, resulting in an overall anti-apoptotic cellular response This effect is possibly mediated by activation of Notch1 in pericytes and Notch1 and/or Notch3 in endothelial cells

Chapter 6

The effects of Pulsatile Flow on Hedgehog & Notch Signalling Control of Microvascular retinal endothelial and pericyte cell fate

6.1 Introduction

6.2 Results

6.3 Discussion

6.4 Conclusion

6 1 Introduction

An important aspect of this study was to investigate the effect of pulsatile flow on Notch and Hedgehog signalling in co-cultured BRECs & BRPs In addition, the role of these pathways in determining BREC & BRP cell fate decisions, namely apoptosis and proliferation were examined

Cells of the vasculature are exposed to two principal haemodynamic forces, shear stress and cyclic strain generated as a result of blood flow Shear stress is described as the dragging frictional force created due to blood flow, and primarily affects EC under normal conditions Additionally, vessels are exposed to an oscillating transmural pressure due to the pulsatile nature of blood flow, resulting in the "stretching" of vascular cells in multiple planes Both pericytes and endothelial cells absorb this pressure-induced cyclic strain Several retinal pathologies such as high tension glaucoma, normal tension glaucoma & diabetic retinopathy report fluctuations in blood flow, thus altering mechanical forces within the vessel wall Therefore, this study focuses on the effect of alterations in pulsatile flow on EC and pericyte apoptotic and proliferative profiles As retinal EC & pericytes reside in a mechanically active environment and are subjected to variable mechanical loads, the response of these cells to deformation may represent an important defence mechanism against fluctuating mechanical load

Apoptosis is an essential physiological process, important in both vascular development and maintenance of homeostasis within the adult vasculature Dysregulation of apoptosis, however, is a common response to vascular injury, contributing to the progression and ultimate clinical outcome of vascular disease states, such as hypertension, transplant arteriopathy, diabetic retinopathy, glaucoma and atherosclerosis Despite its clinical importance however, regulation of apoptosis within the retinal vasculature is poorly understood As such, these experiments examined the role of both Notch and Hedgehog signalling and apoptosis in retinal cells

The Notch signalling pathway has previously been implicated in several aspects of vascular development, including arterio-venous differentiation, angiogenic processes, and in the regulation of developmental VSMC fate decisions (Artavanis-Tsakonas et al , 1999, Gridley, 2001, Iso et al , 2003a) Moreover, mutations in Notch receptors result in embryonic lethality due to defects in both vasculogenesis and angiogenesis (Xue et al , 1999, Singh et al , 2000) In addition, the fact that dysregulation of the Notch signalling pathway is involved with human pathologies involving cardiovascular abnormalities, such as CADASIL and AGS, highlights the importance of the Notch signalling pathway in the adult vasculature Numerous studies have implicated the Notch signalling pathway in the regulation of cell fate decisions, including apoptosis, in many cell types (Artavams-Tsakonas et al , 1995, Artavams-Tsakonas et al , 1999, Greenwald, 1998) Whilst the presence and function of the Notch signalling pathway was not established in adult VSMC or ECs until recently, several lines of evidence indicated that components of this pathway could be present, and possibly regulate apoptosis in both VSMC and ECs Results from our laboratory demonstrated cyclic strain decreased VSMC Notch, which resulted in increased VSMC apoptosis, thus demonstrating the anti-apoptotic role of Notch signalling in VSMCs (Morrow et al , 2005) Furthermore, results also demonstrated Notch modulation of VSMC migration and growth rate (Sweeney et al , 2004) To date, there are no documented reports of the effect of mechanical forces on EC Notch *in vitro*

Our results with microvascular retinal endothelial and pericytes have clearly established Hedgehog upstream of Notch in a signalling cascade (Chapter 5) Furthermore, we also demonstrated the anti-apoptotic effect of Hedgehog/Notch signalling in serum deprivation-induced apoptosis in both BRECs and BRPs As such, we examined the effect of pulsatile flow on Hedgehog signalling in BREC/BRP cultures and investigated the interaction between Hedgehog and Notch signalling using specific inhibitors of each We hypothesized that Hedgehog/Notch signalling effect changes in apoptosis due to altered pulsatile flow

Therefore, the principal aims of this study were to establish the presence and activity of Hedgehog and Notch signalling components in co-cultured retinal EC/pericyte cells exposed to pulsatile flow Furthermore, the role of Notch/Hedgehog signalling in regulating EC/Pericyte cell apoptosis exposed to pulsatile flow was also examined

6 2 Results

6.2 1 Pulsatile flow regulates Pericyte Proliferation and Apoptosis

Proliferation of BRPs when co-cultured with BRECs *in vitro*, was determined using carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) dye flow cytometry cell tracer assay CFDA-SE dye is a succinimidyl ester (SE) fluorescent dye The fluorescent SE covalently couples to both intracellular and cell-surface proteins by reaction with lysine side chains and other available amine groups When cells divide, the SE labeling is distributed equally between the daughter cells, which are therefore half as fluorescent as the parents As a result, each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity that is readily followed by flow cytometry Using this technique we examined the proliferative profile of BREC & BRP after co-culture for 3 and 18 days, at both low and high flow Our results demonstrate high flow (25 mls/min) inhibits BRP proliferation after 3 (Fig 6 1 (a)) and 18 days (Fig 6 1 (b)), when compared to low flow (0 3 mls/min)

Western blot analysis of proliferating cell nuclear antigen (pCNA) was also performed pCNA is a 36 kDa molecular weight protein also known as cyclin (Sherr, 1995) The protein has also been identified as the polymerase-associated protein and is synthesized in early G1 and S phases of the cell cycle (Sherr, 1995) The anti-proliferative effect of increased pulsatile flow on BRPs was confirmed with reduced pCNA protein levels at high flow (Fig 6 2) as determined by western blot analysis

BRPs exposed to high pulsatile flow for 3 days with BRECs resulted in a significant increase in annexin V positive (green/apoptotic) cells with respect to low flow control (1 48 ± 0 068 fold, n=6) (Fig 6 3) The effect of cyclic strain on the *bcl-2* family of apoptotic genes was also investigated High pulsatile flow increased BRP mRNA of the pro-apoptotic *bax* gene by 1 45 ± 0 04 fold (n=3) over low flow (Fig 6 5) Additionally, the anti-apoptotic *bcl-2* gene decreased in BRP following increased pulsatile flow (0 49 ± 0 05 fold, n=3) (Fig 6 5) The role of Bcl-2 related proteins mediating increased BRP apoptosis at high flow was confirmed via Western blot analysis BRP Bax protein increased 1 40 ± 0 05 fold (n=3) (Fig 6 4 (b)),

whereas BCl-2 decreased 0 36 ± 0 04 fold (n=3) in BRPs exposed to high relative to low flow (Fig 6 4 (a)) In contrast, high pulsatile flow resulted in no significant change of anti-apoptotic *bcl-x_L* mRNA (n=3) (Fig 6 5) The progression of apoptosis is determined by the relative concentration of both pro- and anti-apoptotic mediators within a cell The pattern of *bcl-2* family gene expression is in agreement with our flow cytometry findings that increased pulsatile flow induces apoptosis in BRPs

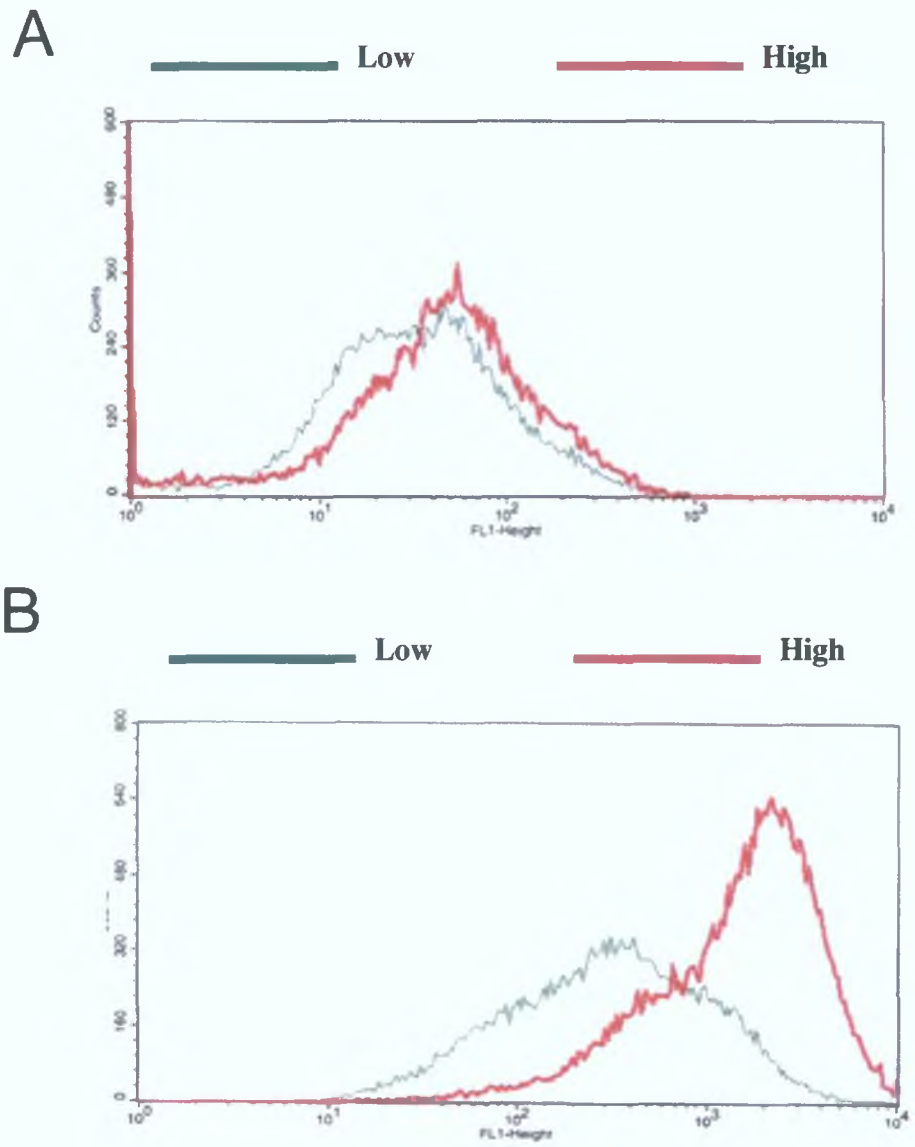


Figure 6.1 The effect of Pulsatile Flow on Pericyte Proliferation. Pericytes were stained with CFDA dye pre co-culture with BRECs and exposed to low **(green)** or high **(red)** pulsatile flow for 3 **(A)** (n=3) or 18 **(B)** days (n=1)

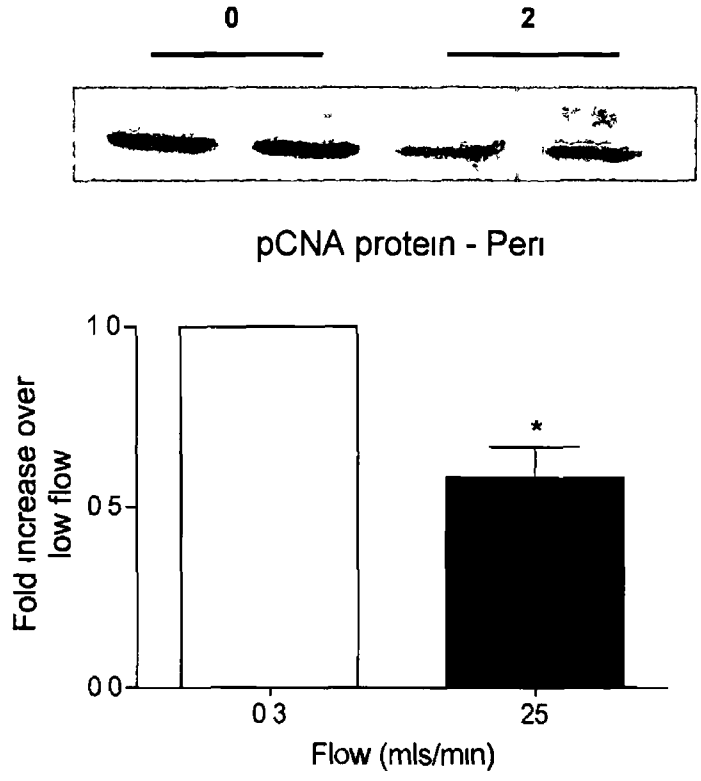


Figure 6 2 The effects of Pulsatile Flow on BRP pCNA protein levels Western Blot Analysis of pericyte protein lysates for pCNA protein Pericytes were exposed to low or high pulsatile flow in co-culture with BRECs for 3 days Histogram represents mean values ± SEM (n=3)., *P<0 05 compared to low flow control

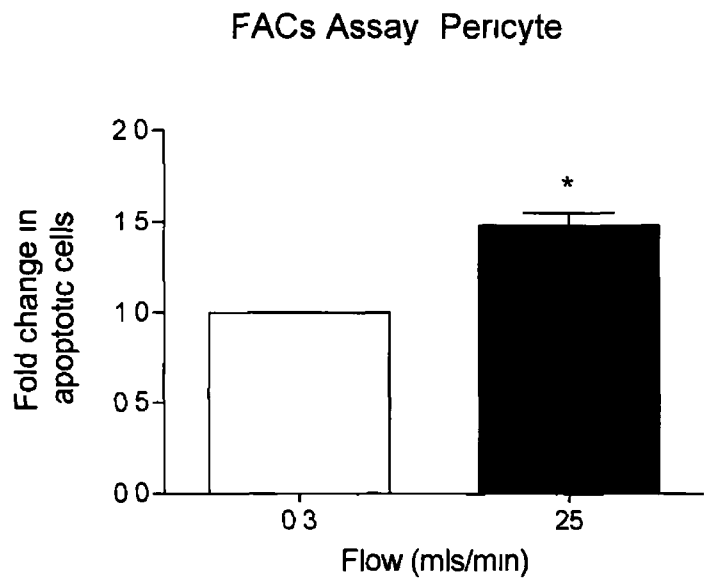


Figure 6 3 The effects of Pulsatile Flow on BRP apoptosis. Annexin V FACs Pericyte Apoptosis Assay Pericytes were exposed to low or high pulsatile flow in co-culture with BRECs for 3 days Histogram represents mean values ± SEM (n=4)., *P<0 05 compared to low flow control

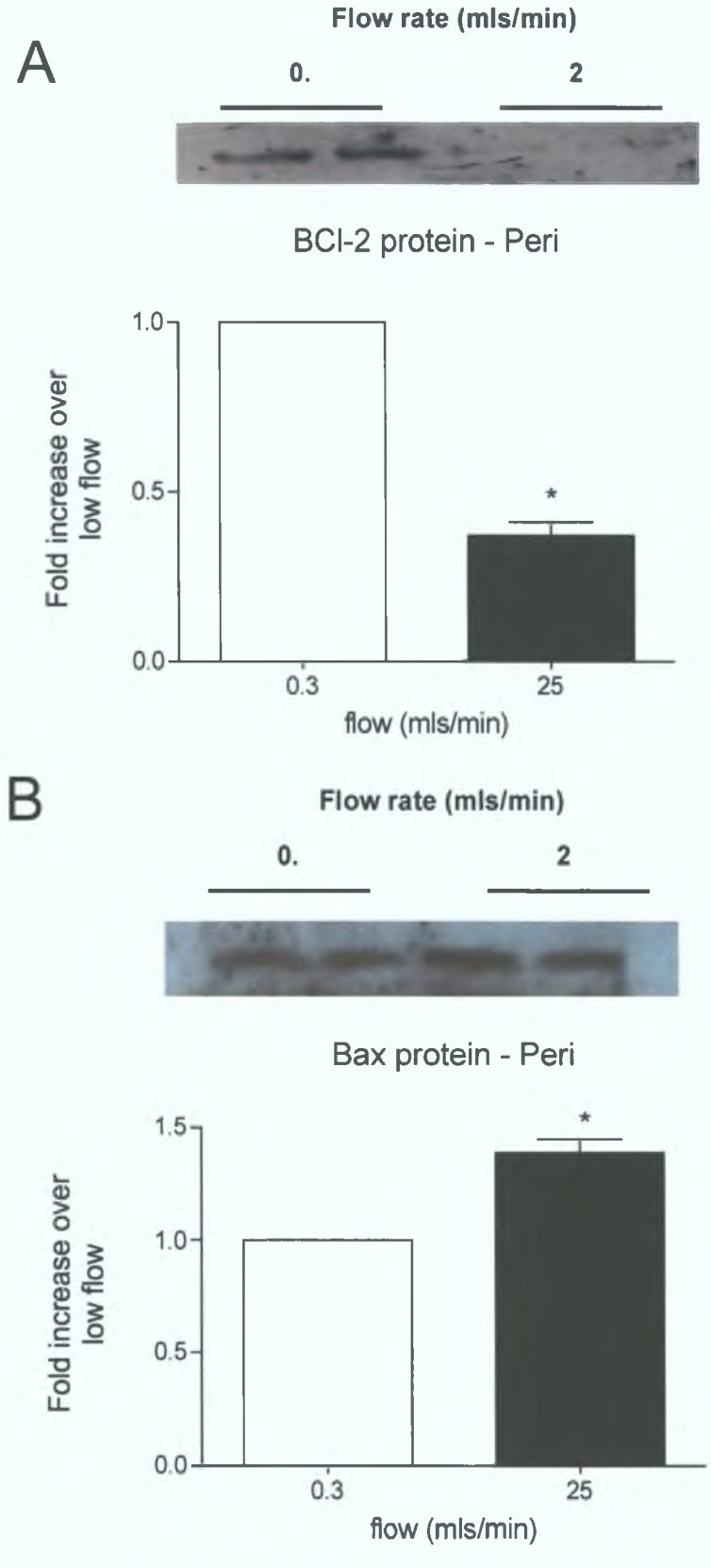


Figure 6.4 The effect of Pulsatile Flow on BRP Bcl-2 and Bax protein levels. Western Blot Analysis of pericyte protein lysates for BCl-2 (**A**) and Bax protein (**B**). Pericytes were exposed to low or high pulsatile flow with BRECs for 3 days. Histogram represents mean values ± SEM (n=3), *P<0.05 compared to low flow control

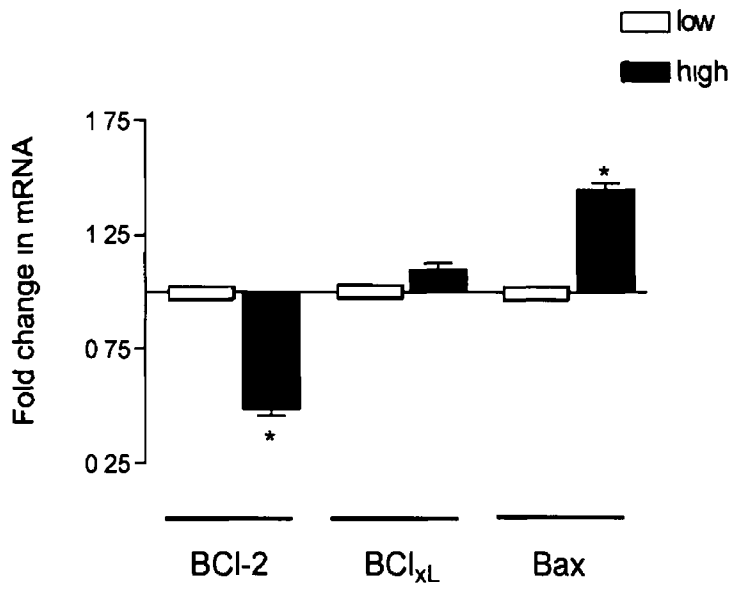


Figure 6 5 The effect of Pulsatile Flow on BRP *bcl-2, bcl-xl* and *bax* mRNA levels Realtime PCR analysis of Pericyte *bcl-2, bcl-xl* and *bax* mRNA levels after 3 day co-culture with BRECs at low or high pulsatile flow Histogram represents mean values ± SEM (n=3), *P<0 05 compared to low flow control

6 2 2 Pulsatile flow regulates Pericyte Hedgehog & Notch Signalling pathway components

The effect of pulsatile flow on components of the Notch and Hedgehog signalling pathway was determined in this study by Western blot and quantitative real time PCR analysis BRPs/BRECs co-cultures were exposed to low or high pulsatile flow for 3 days, separated and analysed

When compared to low flow, high pulsatile flow reduced BRP Indian, Sonic, Ptc1, Notch1IC and Notch3IC protein levels (0 44 ± 0 07, 0 70 ± 0 07, 0 77 ± 0 03, 0 58 ± 0 08, 0 84 ± 0 03 fold, respectively) (n=3) (Fig 6 7 (a) & (b), 6 8 (a) & (b), 6 9 (a), respectively) The effect of pulsatile flow on the components of the Notch/Hedgehog signalling pathways was further examined using quantitative real time PCR analysis Increased pulsatile flow caused significant decreases in *indian, sonic, Ptc1, Gli2, notch1* and *notch3* mRNA levels (0 49 ± 0 16, 0 38 ± 0 09, 0 41 ± 0 08, 0 58 ± 0 02, 0 47 ± 0 09 fold, n=3 respectively) with respect to low flow (Fig 6 6) Interestingly, *Smo* mRNA increased in BRPs exposed to increases in pulsatile flow (1 65 ± 0 06 fold, n=3) (Fig 6 6)

In order to determine if these changes in BRP Notch1IC protein were due to signalling between BRECs and BRPs, or due to increased mechanical forces at high flow, we exposed BRPs to low or high flow in the absence of BRECs for 3 days Mono-cultured BRPs Notch1IC protein decreased 0 61 ± 0 07 fold (n=3) (Fig 6 9 (b)) at high relative to low pulsatile flow, similar to results of BRPs harvested from co-culture

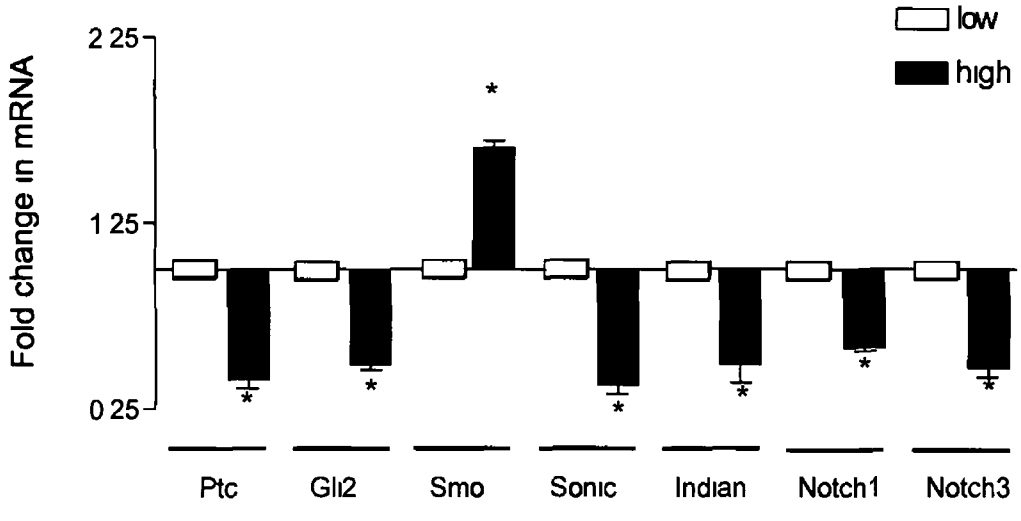


Figure 6.6 The effect of Pulsatile Flow on BRP *Ptc1, Gli2, Smo, Sonic, Indian, Notch1* and *Notch3* mRNA levels Realtime PCR analysis of Pericyte *Ptc1, Gli2, Smo, Sonic, Indian, Notch1* and *Notch3* mRNA levels after 3 day co-culture with BRECs at low or high pulsatile flow Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control

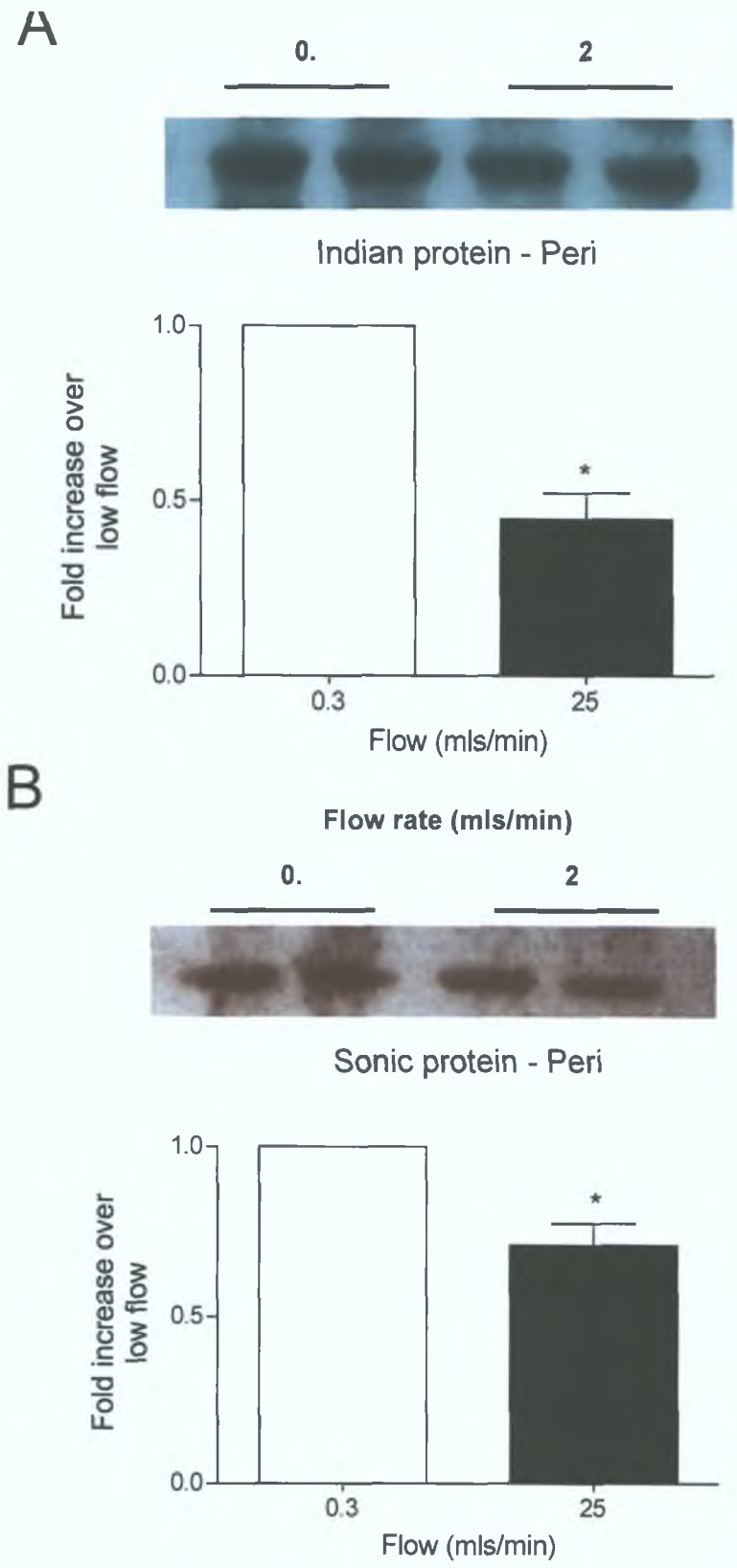


Figure 6.7 The effects of Pulsatile Flow on BRP Indian & Sonic Hedgehog protein levels. Western Blot Analysis of pericyte protein lysates for Indian (**A**) and Sonic protein (**B**). Pericytes were exposed to low or high pulsatile flow with BRECs for 3 days. Histogram represents mean values ± SEM, (n=3) *P<0.05 compared to low flow control

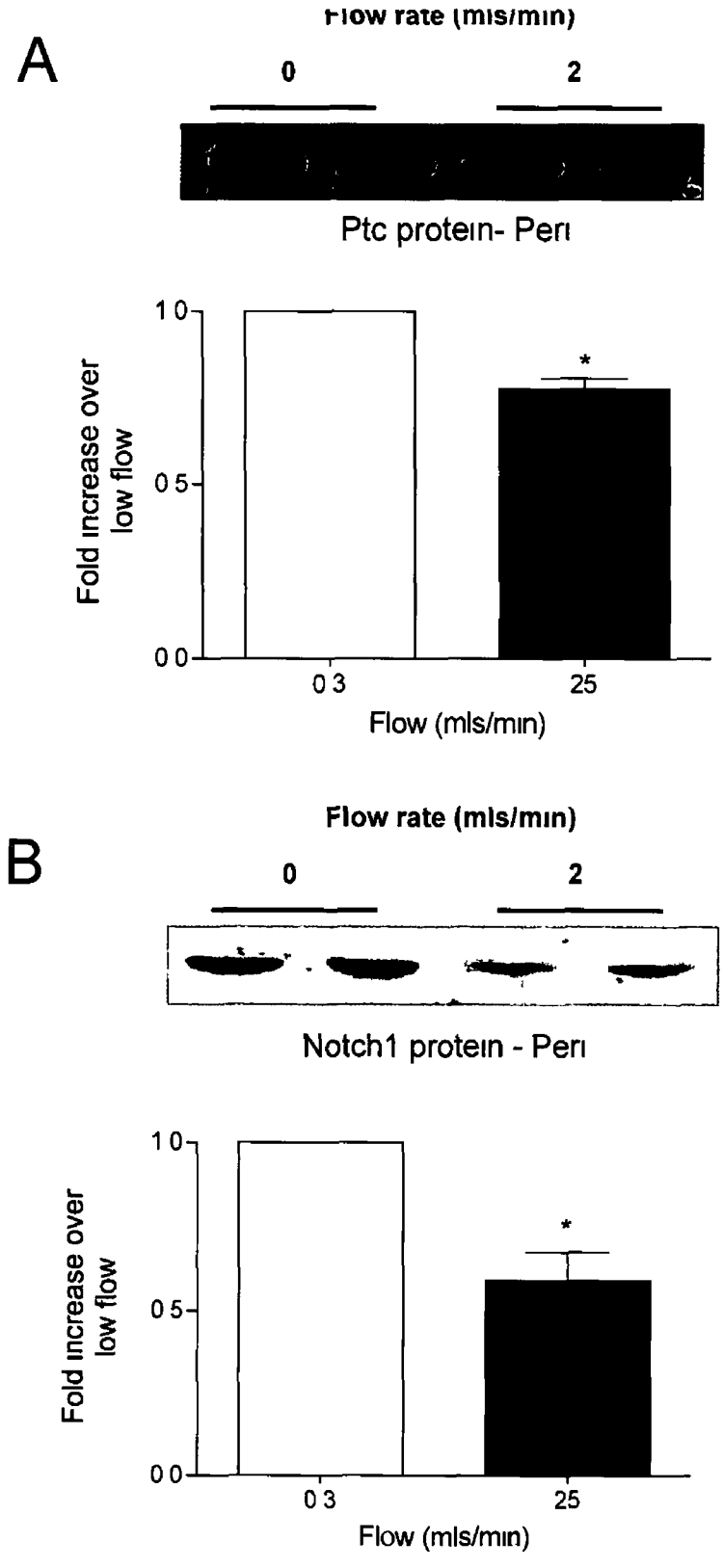


Figure 6 8 The effects of Pulsatile Flow on BRP Ptc1 & Notch1IC protein levels Western Blot Analysis of pericyte protein lysates for Ptc1 (**A**) and Notch1 protein (**B**) Pericytes were exposed to low or high pulsatile flow with BRECs for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control

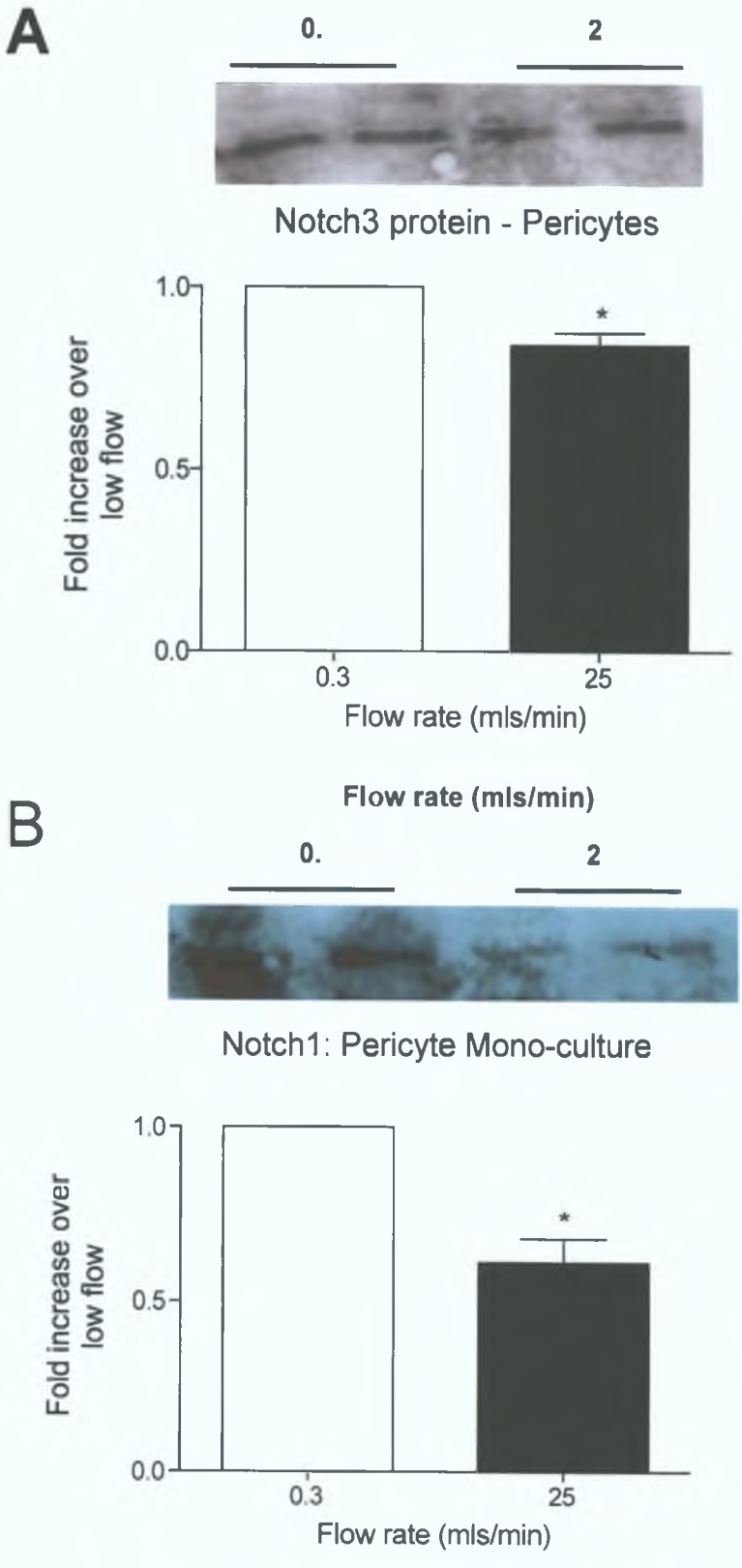


Figure 6.9 The effects of Pulsatile Flow on BRP Notch3 & Notch1IC (mono-culture) protein levels. Western Blot Analysis of pericyte protein lysates for Notch3 (**A**) and Notch1 protein (**B**). Pericytes were exposed to low or high pulsatile flow for 3 days with BRECs (**A**) and without BRECs (**B**). Histogram represents mean values ± SEM, (n=3) *P<0.05 compared to low flow control

6 2.3 Pulsatile flow regulates BREC Proliferation and Apoptosis

In contrast to the anti-proliferative effect of increased pulsatile flow on BRPs, BREC proliferation was unchanged after 3 days (n=3) and 18 days (n=3) exposure to low or high pulsatile flow, as determined by CFDA-SE cell tracer assay (Fig 6 10) Moreover, no changes in BREC pCNA levels were found by Western blot analysis (n=3) (Fig 6 11)

In contrast to increased BRP apoptosis at high pulsatile flow, increased pulsatile flow had an anti-apoptotic effect on BRECs (0 43 ± 0 06 fold, n=3), as determined by flow cytometry Annexin V assay (Fig 6 12) Similar to BRPs, BREC apoptotic profile is altered via changes in BCl-2 protein family members High pulsatile flow increased anti-apoptotic BCl-2 protein (2 05 ± 0 27 fold, n=3), whereas pro-apoptotic protein Bax decreased compared to low flow (0 58 ± 0 06 fold, n=3) (Fig 6 13 (a) (b), respectively) The modulation of each of these family members by pulsatile flow was validated with real-time PCR analysis *Bcl-2* mRNA levels increased (2 73 ± 0 38 fold, n=3), concomitant with decreased *Bax* mRNA (0 45 ± 0 06 fold, n=3) at high pulsatile flow (Fig 6 14) Furthermore, anti-apoptotic *Bcl-xl* mRNA levels also increased (1 86 ± 0 11 fold, n=3) in BRECs exposed to high pulsatile flow (Fig 6 14)

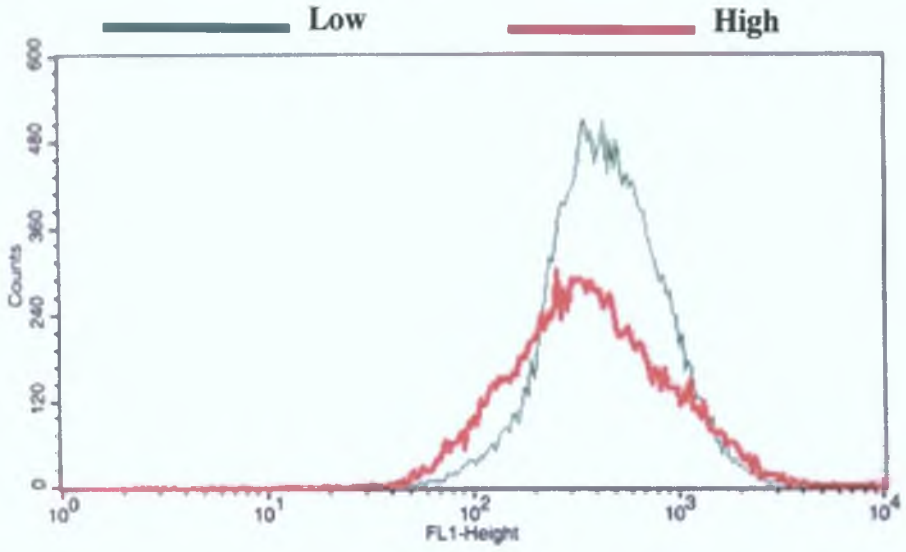


Figure 6.10 The effect of Pulsatile Flow on BREC Proliferation. BRECs were stained with CFDA dye pre co-culture with BRECs, exposed to low (**green**) or high (**red**) pulsatile flow for 18 days (n=1)

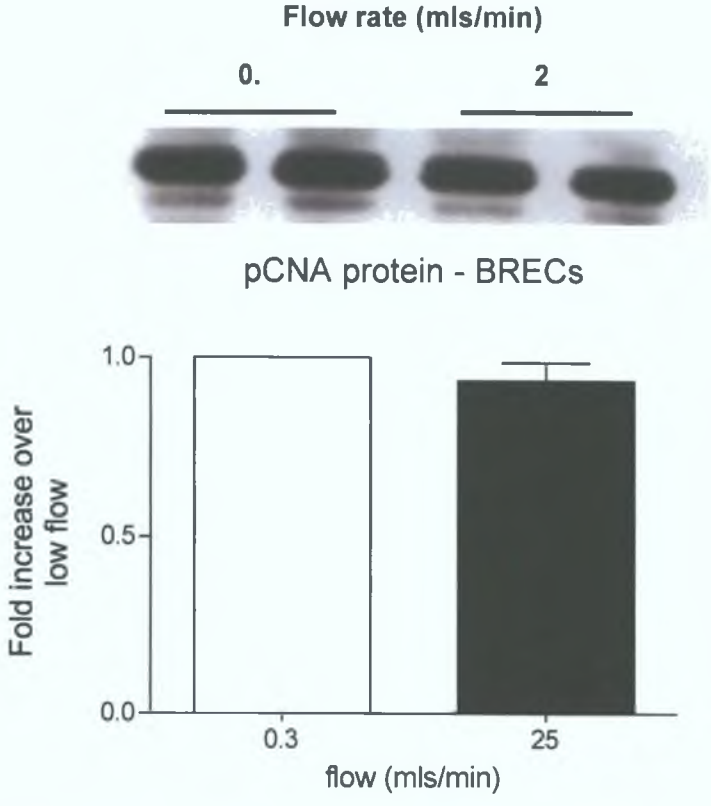


Figure 6.11 The effect of Pulsatile Flow on BREC pCNA protein levels. Western Blot Analysis of BRECs protein lysates for pCNA protein. BRECs were exposed to low or high pulsatile flow with BRECs for 3 days. Histogram represents mean values ± SEM, (n=3)*P<0.05 compared to low flow control

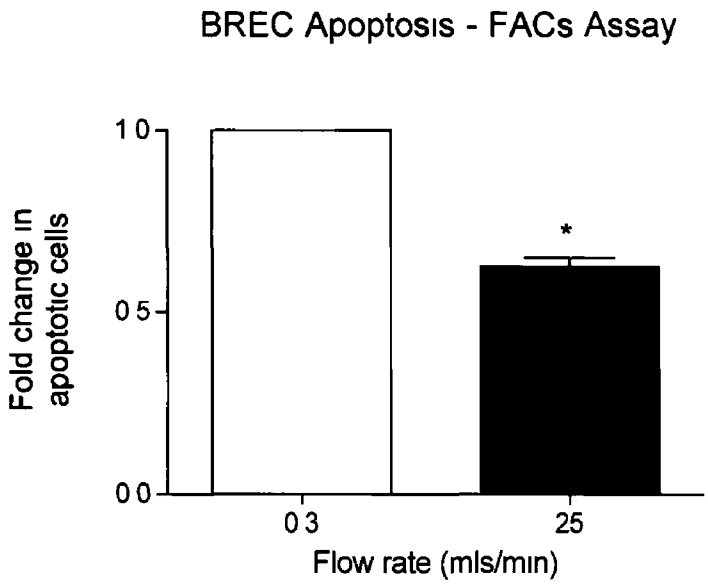


Figure 6 12 The effects of Pulsatile Flow on BREC apoptosis. Annexin V FACs BREC Apoptosis Assay BRECs were exposed to low or high pulsatile flow in co-culture with BRPs for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control

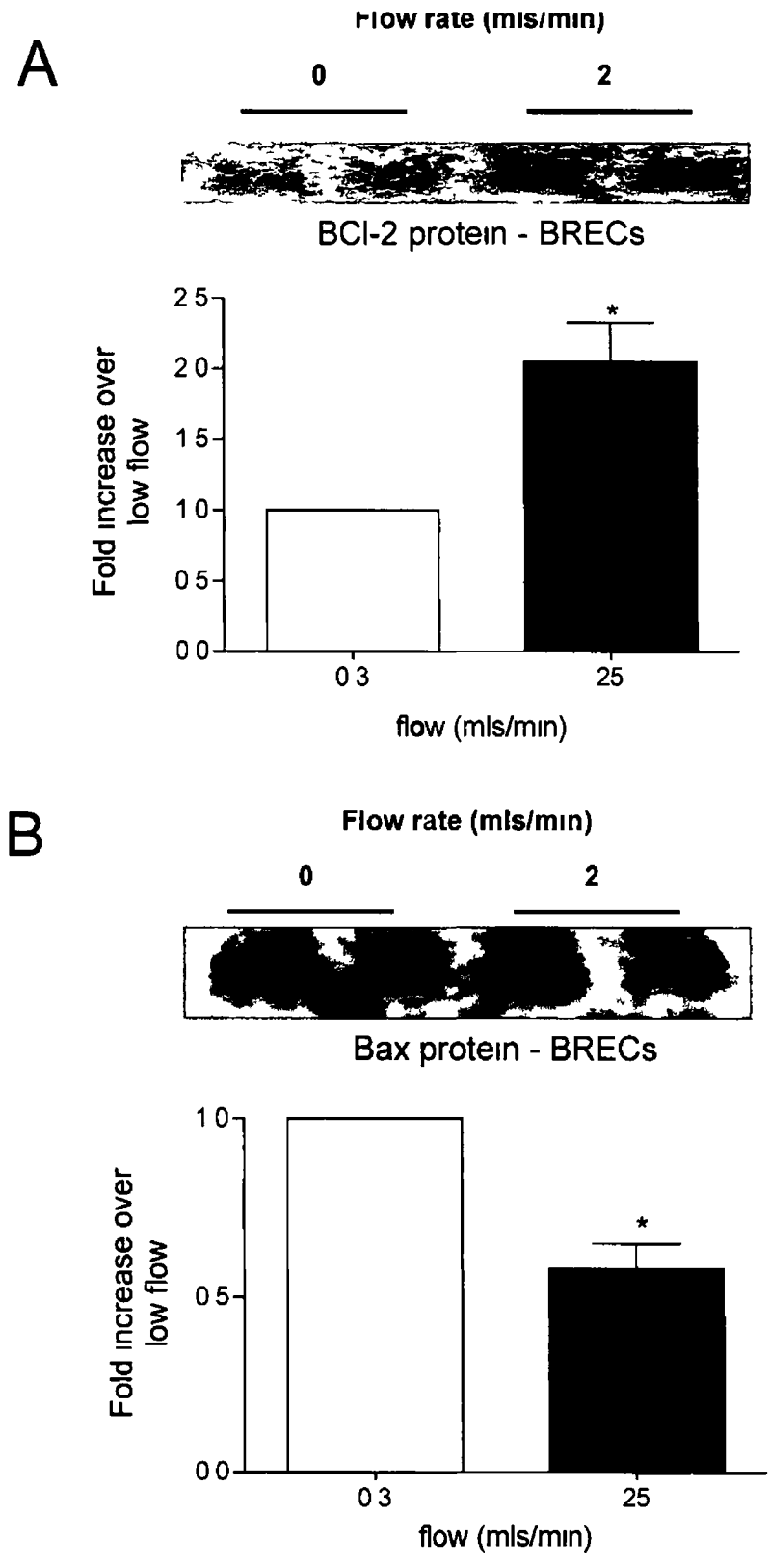


Figure 6 13 The effects of Pulsatile Flow on BREC Bcl-2 and Bax protein levels. Western Blot Analysis of BREC protein lysates for BCl-2 (**A**) and Bax protein (**B**) BRECs were exposed to low or high pulsatile flow with Pericytes for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control

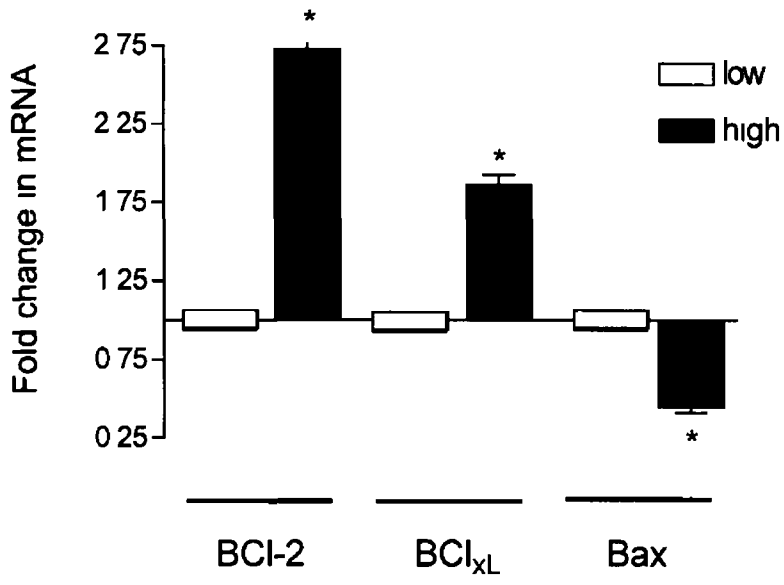


Figure 6.14 The effect of Pulsatile Flow on BREC *bcl-2, bcl-xl* and *bax* mRNA levels Realtime PCR analysis of BREC *bcl-2, bcl-xl* and *bax* mRNA levels after 3 day co-culture with Pericytes at low or high pulsatile flow Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control

6 2 4 Pulsatile flow regulates BREC Hedgehog & Notch Signalling pathway components

The effect of low and high pulsatile flow on Notch and Hedgehog signalling in BRECs was also investigated, after co-culture with BRPs for 3 days In contrast to the effect of increased pulsatile flow on BRP Notch and Hedgehog components, protein levels of Indian, Sonic, Notch1IC and Notch3IC all increased in BRECs exposed to high compared to low flow (2 83 ± 0 34, 1 56 ± 0 11, 2 26 ± 0 19, 2 30 ± 0,20 fold, respectively, n=3) (Fig 6 15 (a)(b), 6 16 (a)(b), respectively) Furthermore, increased pulsatile flow significantly increased *indian, sonic, Ptc1, Gli2, Smo, notch1* and *notch3* mRNA levels (13 46 ± 4 2, 9 27 ± 2 55, 2 38 ± 0 52, 8 98 ± 1 03, 1 49 ± 0 06, 2 99 ± 0 63, 11 41 ± 2 83 fold, respectively, n=3) (Fig 6 17) Therefore, to summarise, high pulsatile flow resulted in increased Notch and Hedgehog signalling components in BRECs, whilst the opposite occurred in BRPs The only exception is Smo, which increased in both BRECs and BRPs at high pulsatile flow

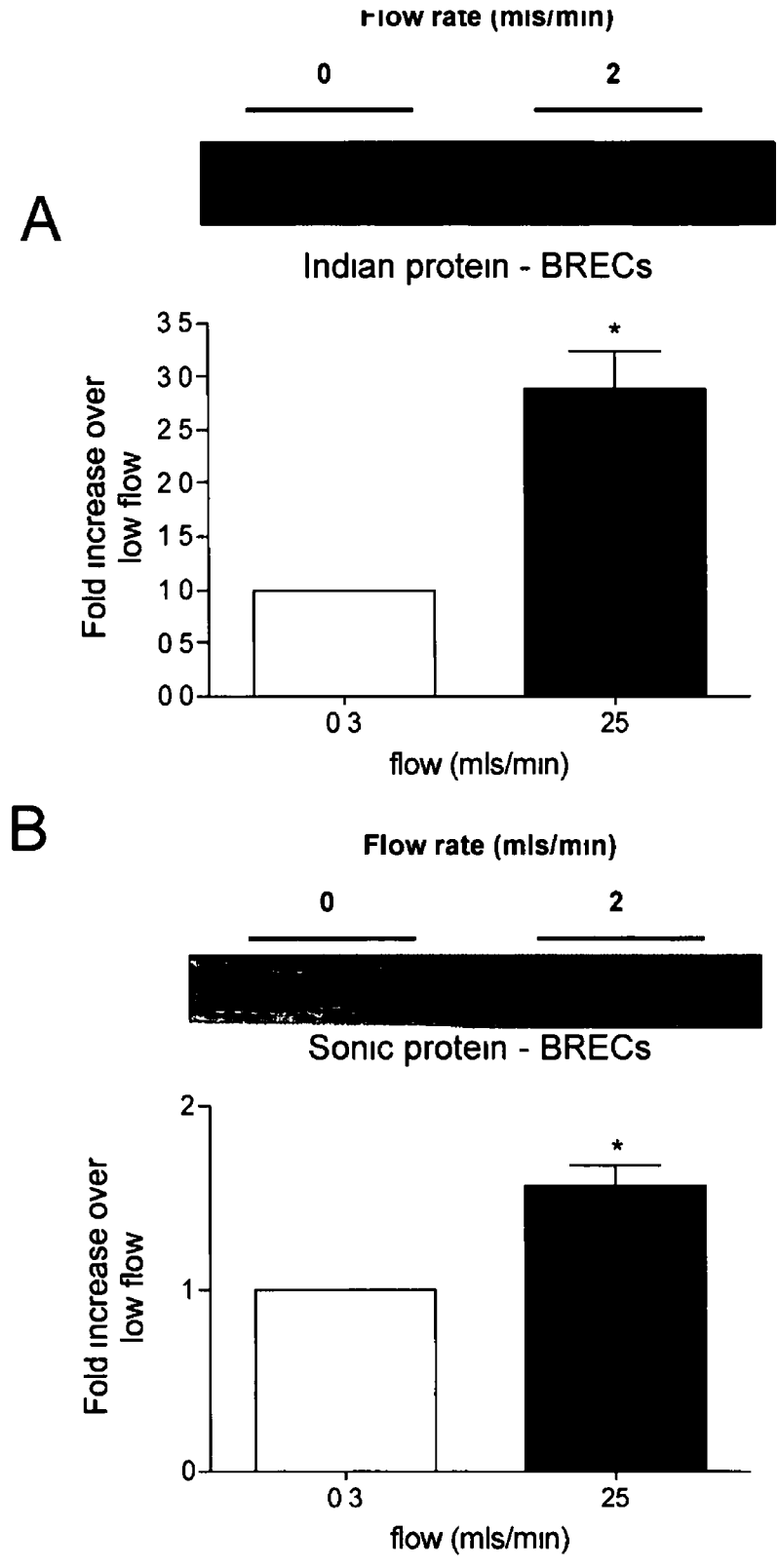


Figure 6 15 The effects of Pulsatile Flow on BREC Indian & Sonic Hedgehog protein levels. Western Blot Analysis of BREC protein lysates for Indian (**A**) and Sonic protein (**B**) BRECs were exposed to low or high pulsatile flow with Pericytes for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control

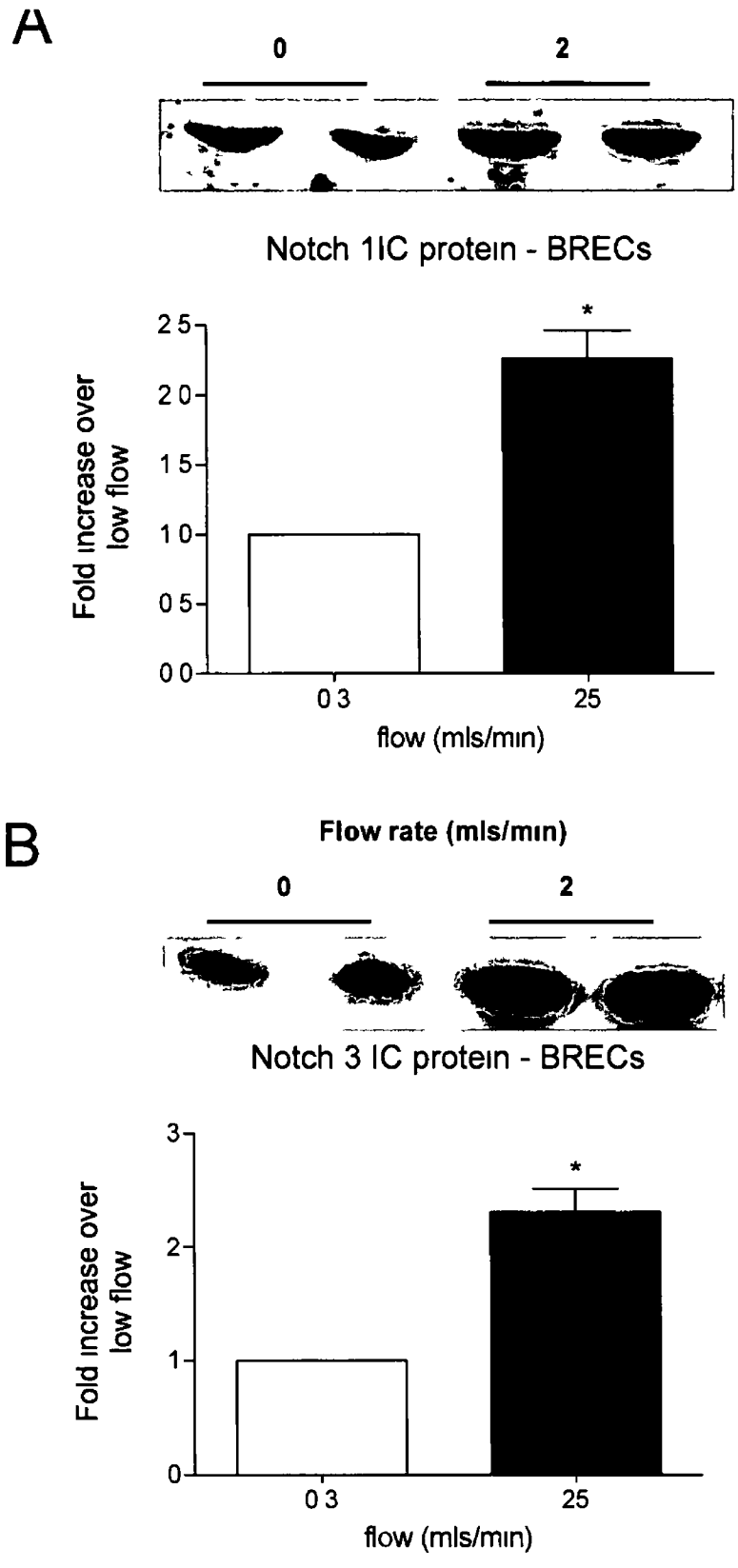


Figure 6 16 The effects of Pulsatile Flow on BREC Notch1 & Notch3 protein levels Western Blot Analysis of BREC protein lysates for Notch1(**A**) and Notch3 protein (**B**) BRECs were exposed to low or high pulsatile flow with Pericytes for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control

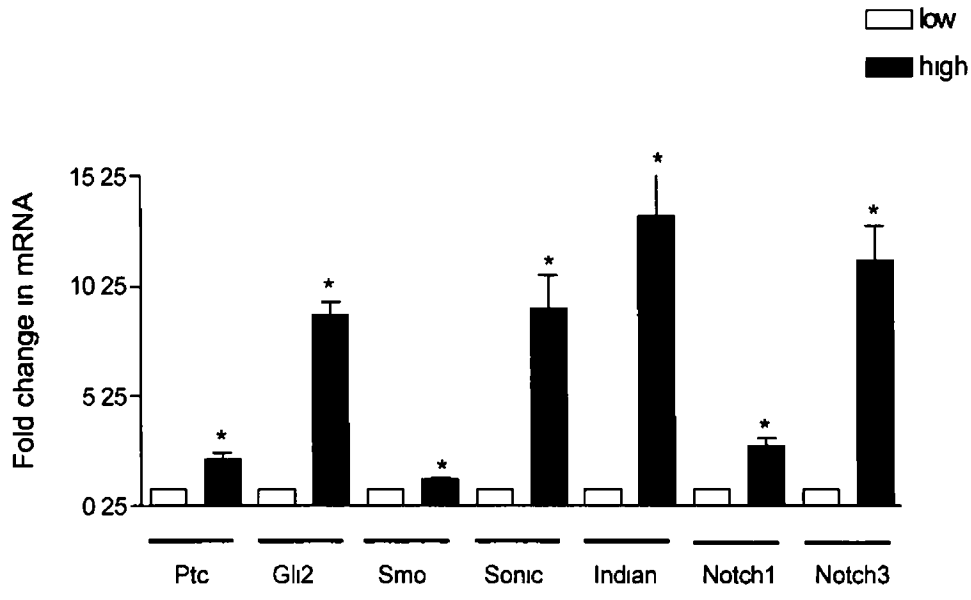


Figure 6 17 The effect of Pulsatile Flow on BREC *Ptc1, Gli2, Smo, Sonic, Indian, Notch1* and *Notch3* mRNA levels Realtime PCR analysis of BREC *Ptc, Gli2, Smo, Sonic, Indian, Notch1* and *Notch3* gene expression after 3 day co-culture with Pericytes at low or high pulsatile flow Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control

6.2 5 Inhibition of Hedgehog Signalling in mono-cultured BRECs exposed to low or high pulsatile flow

Cyclopamine is a naturally occurring steroidal alkaloid which specifically inhibits the Hh pathway by interacting with the Hh signalling protein Smoothened Inhibition of Hedgehog signalling in static BREC cultures (low serum) with cyclopamine (40μM) induced apoptosis via reduced *Bcl-2* and *Bcl-xl* and increased *Bax* gene expression (Section 5 2 6) Furthermore, exposure of BRECs to increased pulsatile flow in co-culture with BRPs, decreased apoptosis via increased BREC *Bcl-2 & Bcl-xl,* and decreased *Bax* mRNA levels (Section 6 2 3) We therefore examined whether inhibition of Hedgehog signalling with cyclopamine would abrogate the anti-apoptotic effect of high pulsatile flow in BRECs In order to elucidate the effect of specific Hh inhibition on BRECs exposed to pulsatile flow, these experiments were performed on mono-cultured BRECs

Similar to the anti-apoptotic effect of increased pulsatile flow on BRECs co-cultured with BRPs, high pulsatile flow conferred a protective effect on mono-cultured BRECs when compared to low flow (0 43 ± 0 06, Fig 6 18) Inhibition of Hh signalling with cyclopamine however, increased apoptosis in both low and high flow BRECs, when compared to low flow control (1 88 ± 0 08 fold, 1 24 ± 0 11 fold, respectively, n=3) Furthermore, cyclopamine abrogated high flow-induced increases in *Bcl-2* mRNA levels (2 06 ± 0 09 fold, n=3) (Fig 6 19), at both low and high pulsatile flow (0 25 ± 0 03 fold, 0 78 ± 0 07 fold, repectively, n=3) Similarly, cyclopamine addition inhibited flow induced increases in Bcl_{XL} mRNA (1 35 ± 0 03 fold, n=3) (Fig 6 20) Furthermore, high pulsatile flow reduced expression of pro-apoptotic *Bax* mRNA in control samples (0 39 ± 0 06 fold, n=3), which was reversed upon addition of cyclopamine (1 30 ± 0 12 fold, n=3) (Fig 6 21)

Previously, in chapter 5 we demonstrated activation of Hh signalling components with exogenous addition of recombinant SHh peptide in static serum-starved BRECs Similarly, high pulsatile flow increased mRNA levels of Hh related genes *Ptc1, Gli2* and *smo* (2 38 ± 0 52, 11 68 ± 1 23, 1 65 ± 0 08 fold, n=3, respectively) (Fig 6 22 – 6 24) Hh inhibition with cyclopamine abrogated any

increases due to high pulsatile flow in each of these 3 genes (0 62 ± 0 09, 1 00 ± 0 09, 0 70 ± 0 07, n=3, respectively) (Fig 6 22 – 6 24), thereby demonstrating increased Hh signalling due to high pulsatile flow increases BREC *Ptc1*, *Gli2* and *Smo* mRNA levels

Exposure of BRECs to recombinant SHh in static culture increased Notch1 and Notch3 mRNA levels (Fig 5 10) As such, we examined BREC Notch1 and Notch3 mRNA levels in the pulsatile flow system upon inhibition of Hh signalling with cyclopamine to determine if increased Hh at high flow mediates flow induced increases in Notch1 or Notch3 mRNA levels Addition of cyclopamine abolished flow-induced increases in *Notch1*, but not *Notch3* mRNA (1 03 ± 0 06, 12 00 ± 1 83 fold, n=3, respectively) (Fig 6 25 – 6 26) These results demonstrate increased Hh at high pulsatile flow modulates Notch1 mRNA levels

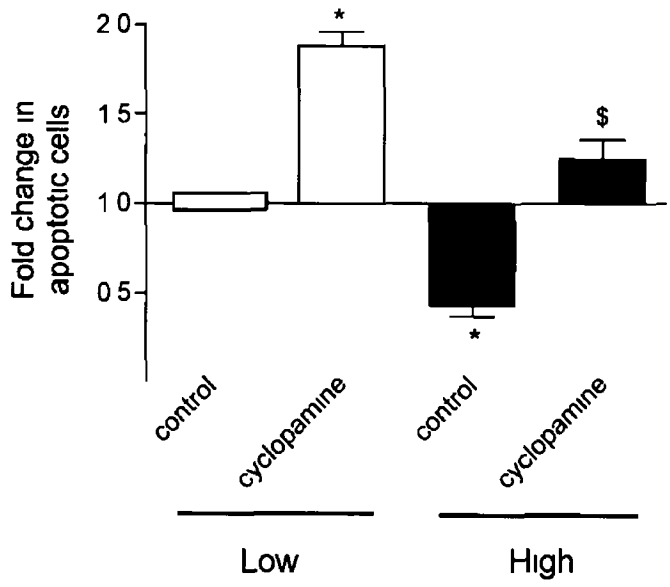


Figure 6.18 The effect of Cyclopamine on BREC apoptosis exposed to Pulsatile Flow. Annexin V FACs Apoptosis Assay Mono-cultured BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

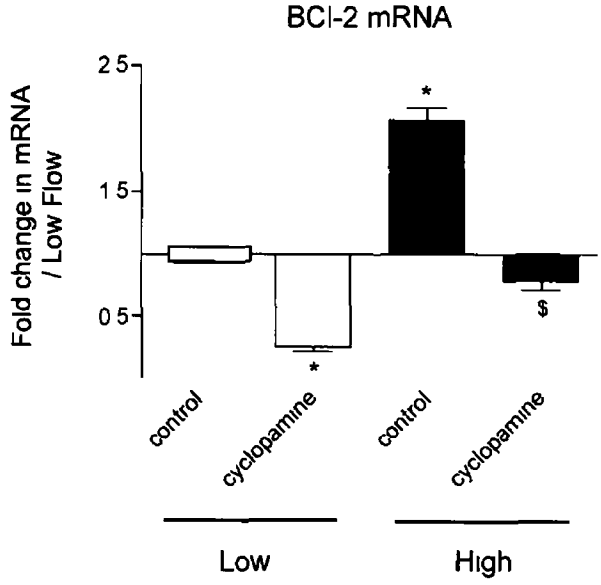


Figure 6 19 The effect of Cyclopamine on BREC *Bcl-2* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *bcl-2* gene expression BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

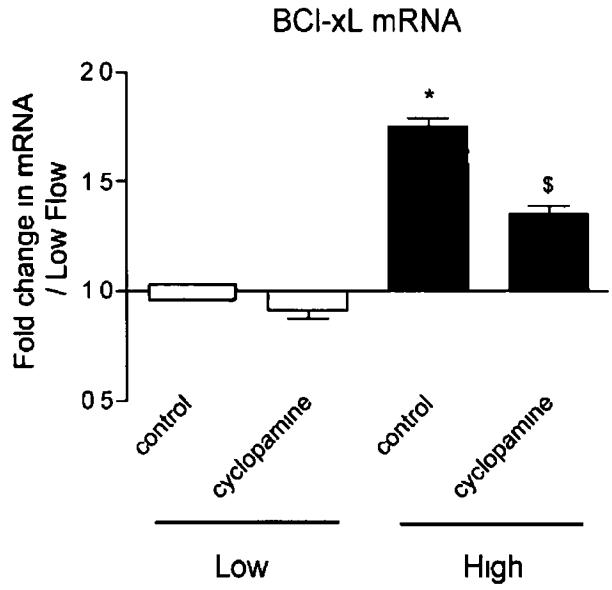


Figure 6 20 The effect of Cyclopamine on BREC *Bcl-X$_L$* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *bcl-xl* gene expression BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

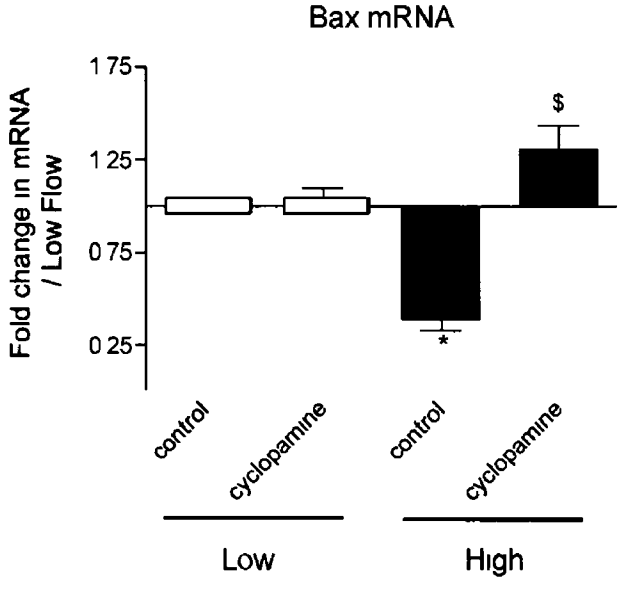


Figure 6.21 The effect of Cyclopamine on BREC *Bax* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *bax* gene expression BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

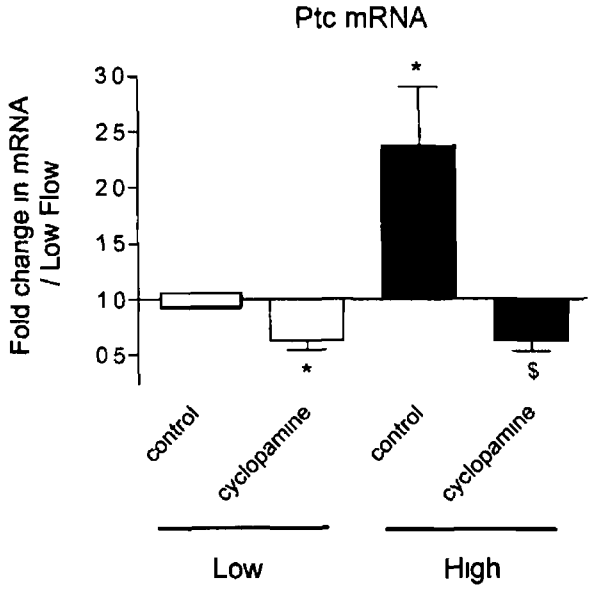


Figure 6 22 The effect of Cyclopamine on BREC *Ptc1* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Ptc* gene expression BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

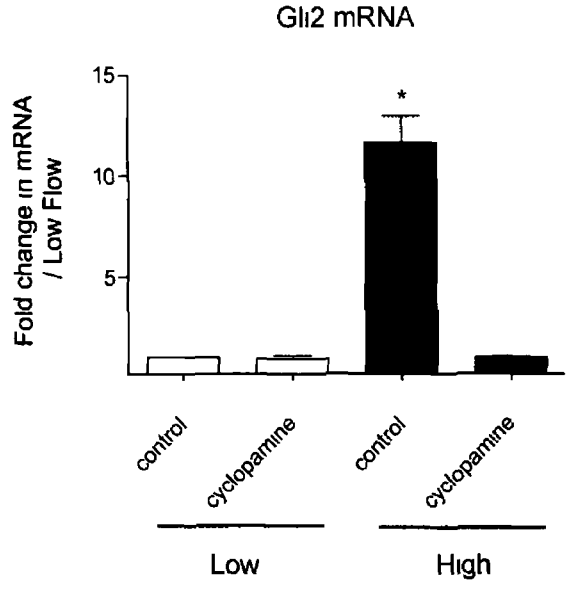


Figure 6 23 The effect of Cyclopamine on BREC *Gli2* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Gli2* gene expression BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

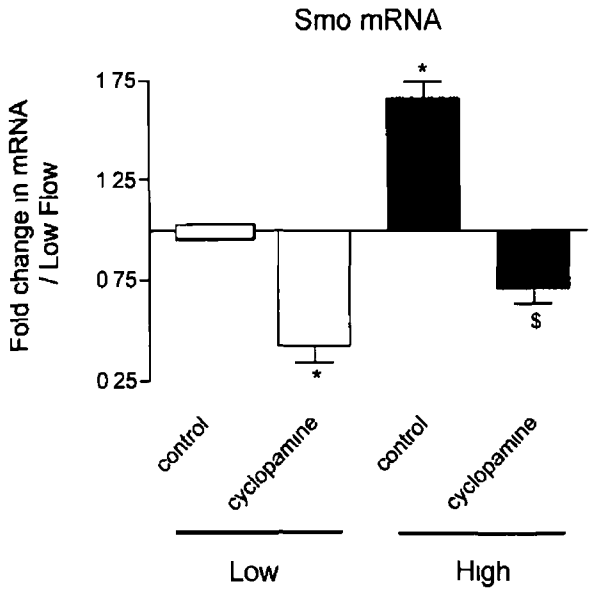


Figure 6.24 The effect of Cyclopamine on BREC *Smo* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Smo* gene expression BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

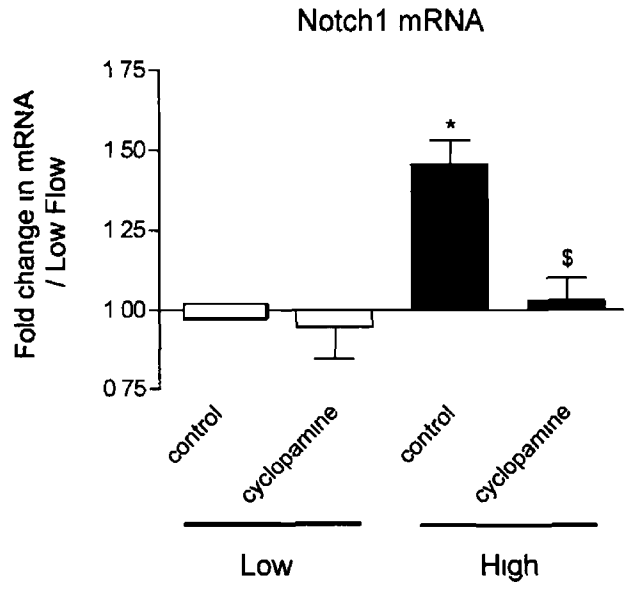


Figure 6 25 The effect of Cyclopamine on BREC *Notch1* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Notch1* gene expression BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

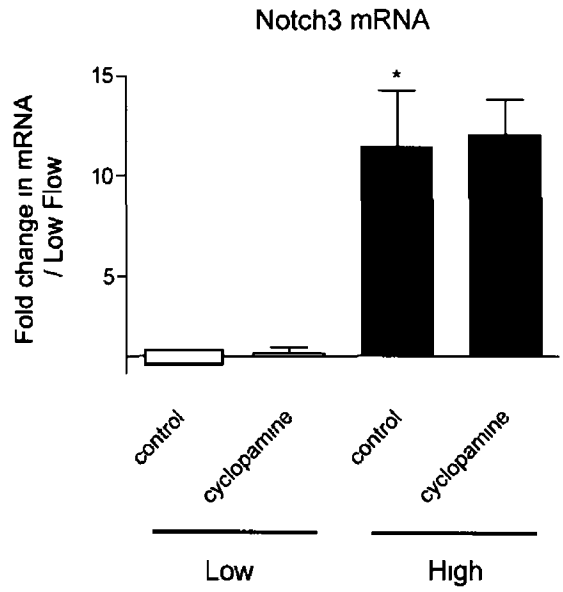


Figure 6 26 The effect of Cyclopamine on BREC *Notch3* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Notch3* gene expression BRECs were exposed to low or high pulsatile flow for 3 days with DMF (control) or Hedgehog inhibitor - Cyclopamine Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control $^{\$}$P<0 05 compared to high flow control

6.2.6 Inhibition of Notch Signalling in mono-cultured BRECs exposed to low or high pulsatile flow

To determine the role of the Notch signalling pathway in modulating BREC apoptosis exposed to pulsatile flow *in vitro,* we inhibited Notch signalling in our flow experiments. This was achieved through transfection of BRECs with an inhibitor of the Notch signalling pathway, RPMS-1. RPMS-I has previously been shown to prevent NotchIC-mediated activation at promoters with CBF-1 binding sites by stabilizing the CBF-1/HDAC co-repressor complex. This prevents effective displacement of the co-repressor complex by NotchIC (Smith et al., 2000a). Cells were co-transfected with the β-gal plasmid to allow for normalisation of transfection efficiency, while control cells were transfected with pCMV (mock) - the vector system of RPMS-1. The number of apoptotic cells upon inhibition of NotchIC with RPMS-1 significantly increased with respect to mock transfected control, at both low and high pulsatile flow (1.58 ± 0.13, 2.08 ± 0.09 fold, n=3, respectively) (Fig. 6.27). These results suggest an important role for NotchIC in BRECs exposed to pulsatile flow.

The downstream effect of the inhibition of Notch signalling on the *bcl-2* family of apoptotic genes was also investigated. No change in *Bcl-2* expression was found with RPMS-1 treated BRECs compared to mock transfected control (2.08 ± 0.16, 1.99 ± 0.16 fold, n=3, respectively) (Fig. 6.28). RPMS-1 transfection abrogated flow-induced decreases in Bax mRNA, with increased *bax* mRNA at both low and high flow (2.86 ± 0.06 fold, 3.29 ± 0.22 fold, respectively, n=3), compared to low flow control (Fig. 6.30). High flow increased BREC BCl_{XL} mRNA in control cells (1.75 ± 0.12 fold, n=3) (Fig. 6.29), however RPMS-1 treatment reduced this flow induced increase in BCl_{XL} mRNA (1.41 ± 0.09 fold, n=3) (Fig. 6.29).

Our experiments have demonstrated Hh signalling modulates the Notch signalling pathway in BRECs static and pulsatile flow studies. To determine if Notch can modulate Hh signalling, we analysed *Sonic, Indian, Gli2, smo* and *Ptc1* gene expression in RPMS-1 transfected BRECs exposed to pulsatile flow. As expected, high flow induced gene expression of each of these Hh signalling

components in control experiments (2 73 ± 0 11, 3 33 ± 0 14, 5 55 ± 0 54, 1 40 ± 0 05, 4 64 ± 0 20 fold, n=3, respectively) Inhibition of NotchIC with RPMS-1 however, had no significant effect on gene expression of any of these Hh genes (Fig 6 31 – 6 35)

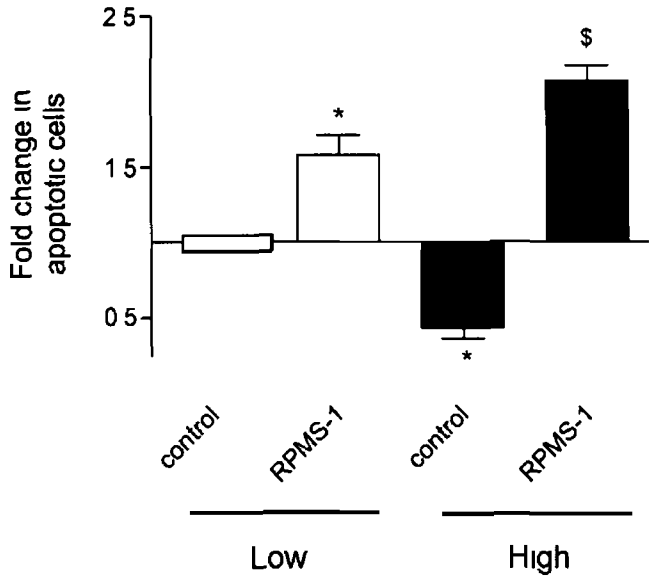


Figure 6 27 The effect of RPMS-1 on BREC Apoptosis exposed to Pulsatile Flow. Annexin V FACs Apoptosis Assay Mono-cultured BRECs transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $P<0 05 compared to high flow control

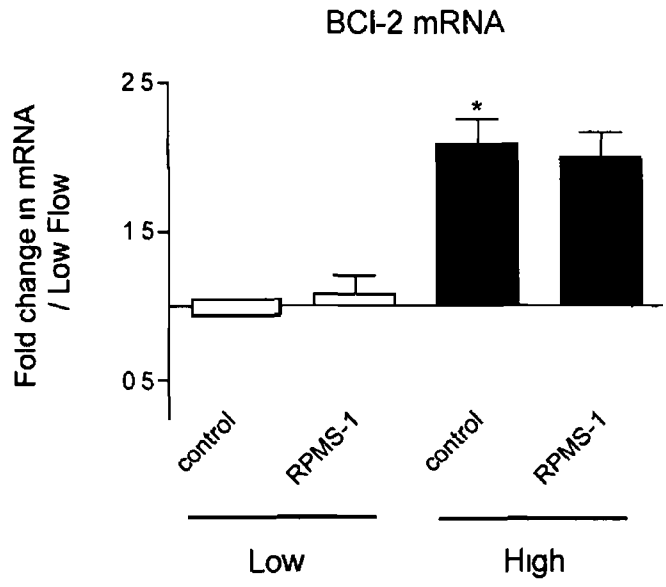


Figure 6 28 The effect of RPMS-1 on BREC *Bcl-2* mRNA levels exposed to Pulsatile Flow. Realtime PCR analysis of mono-cultured BREC *bcl-2* gene expression BRECs were transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $P<0 05 compared to high flow control

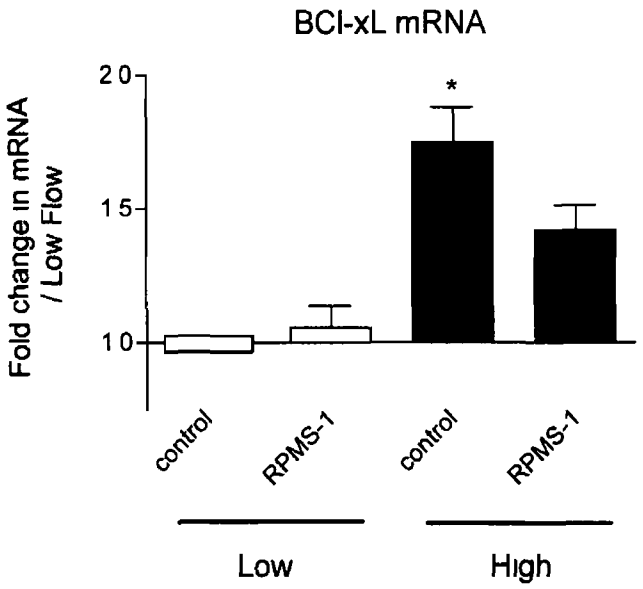


Figure 6.29 The effect of RPMS-1 on BREC *Bcl-X$_L$* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *bcl-xl* gene expression BRECs were transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

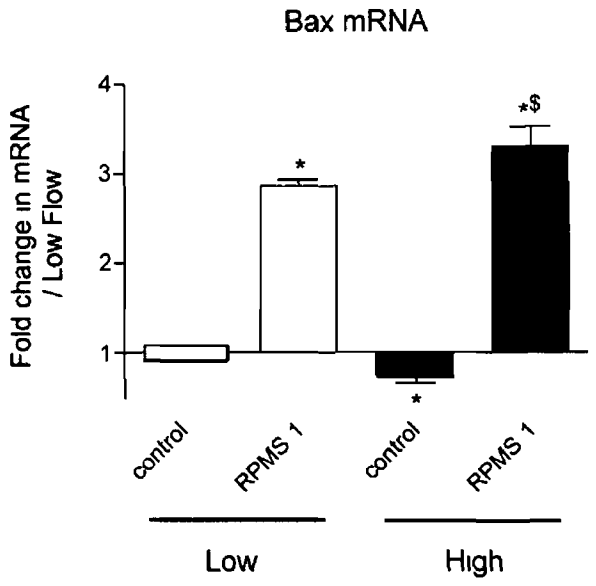


Figure 6 30 The effect of RPMS-1 on BREC *Bax* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *bax* gene expression BRECs were transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

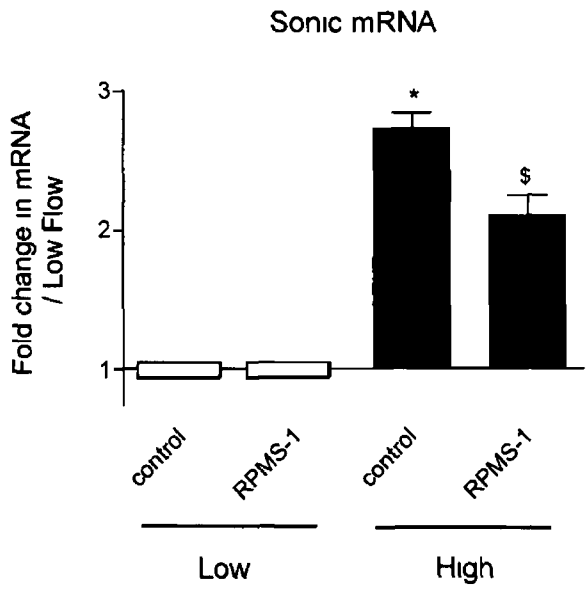


Figure 6 31 The effect of RPMS-1 on BREC *Sonic* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Sonic* gene expression BRECs were transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $P<0 05 compared to high flow control

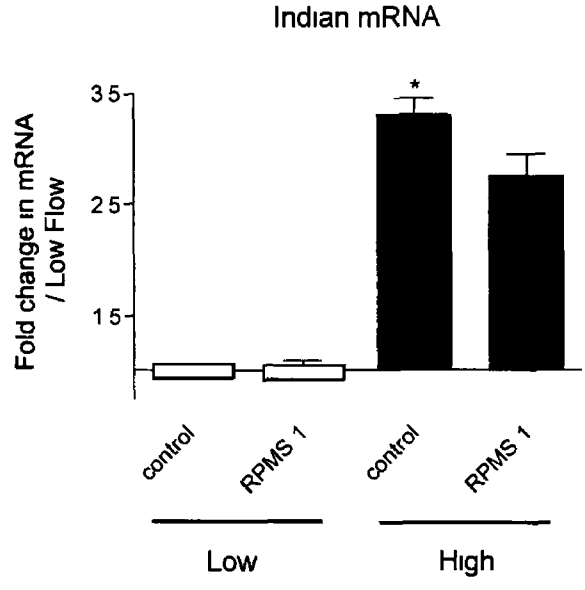


Figure 6 32 The effect of RPMS-1 on BREC *Indian* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Indian* gene expression BRECs were transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $P<0 05 compared to high flow control

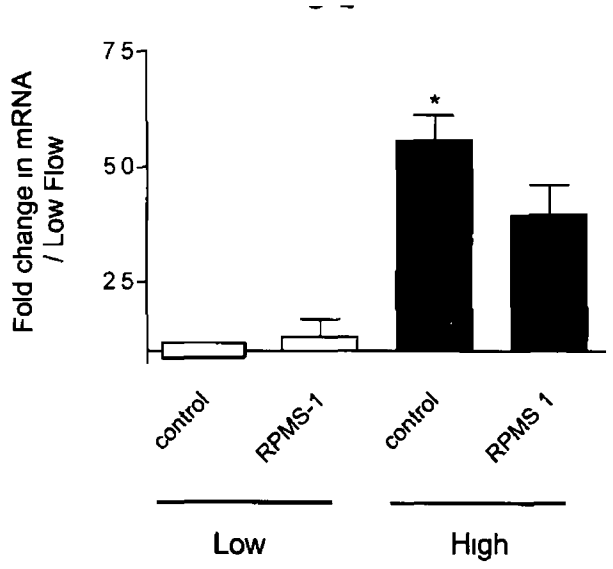


Figure 6.33 The effect of RPMS-1 on BREC *Gli2* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Gli2* gene expression BRECs were transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

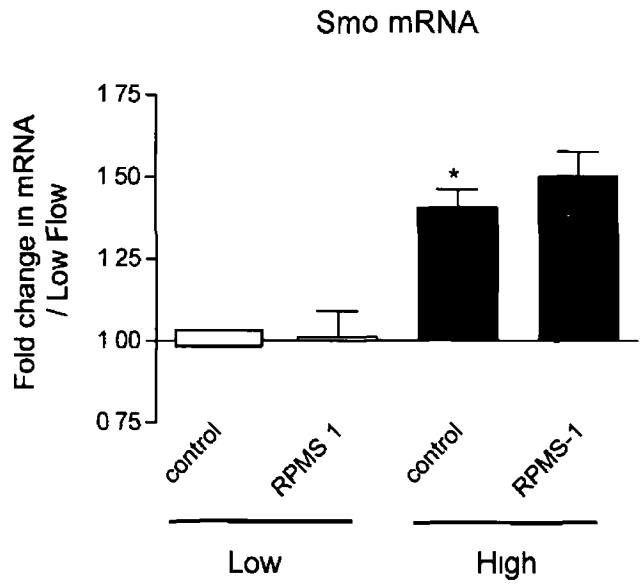


Figure 6 34 The effect of RPMS-1 on BREC *Smo* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Smo* gene expression BRECs were transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

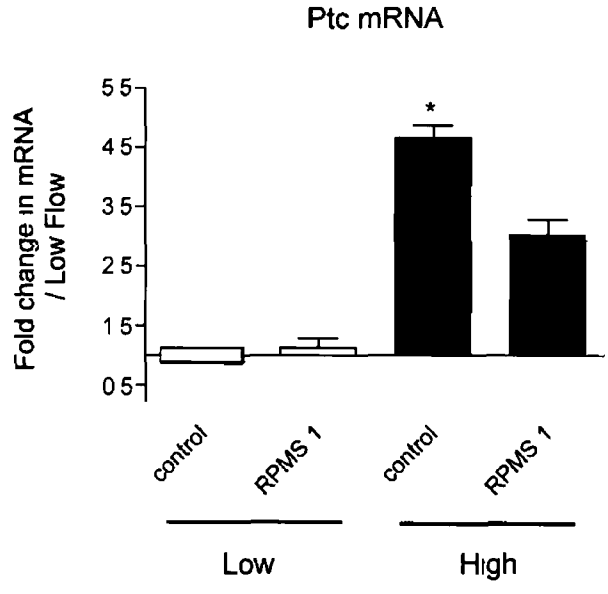


Figure 6.35 The effect of RPMS-1 on BREC *Ptc1* mRNA levels exposed to Pulsatile Flow Realtime PCR analysis of mono-cultured BREC *Ptc* gene expression BRECs were transfected with pCMV (control vector) or RPMS-1 (Notch inhibitor) and exposed to low or high pulsatile flow for 3 days Histogram represents mean values ± SEM, (n=3) *P<0 05 compared to low flow control $^{\$}$P<0 05 compared to high flow control

6.3 Discussion

Local retinal blood flow haemodynamics greatly influence remodelling of developing and mature vessels in both normal and pathologic settings Remodelling of the retinal vasculature involves restructuring of extra-cellular matrix through synthesis, degradation, and reorganisation, and it also involves regulation of cell populations through control of cell fate decisions, namely apoptosis, proliferation, migration and differentiation The mechanisms that effect these aspects of restructuring of the vessel wall are now being elucidated and we have clearly demonstrated a role of Notch & Hedgehog in mediating these processes

This study demonstrates that increased pulsatile flow induces apoptosis and inhibits proliferation in BRP This concurs with macrovascular studies from our laboratory, and others, which report increased VSMC apoptosis both *in vivo* and *in vitro* due to increased mechanical forces (Mayr et al , 2002, Mayr et al , 2000, Vouyouka et al , 2004, Birney et al , 2004, Hirsch et al , 1998) In addition, the degree of pulsatile flow induced BRP apoptosis observed in this study is comparable to those reported in macrovascular *in vitro* studies, which describe a 2- to 4 fold increase in VSMC apoptosis due to increased cyclic strain (Mayr et al , 2002, Wernig et al , 2003)

This study has clearly established that increased pulsatile flow induces apoptosis in BRPs concomitant with decreased Notch1 and Notch3 mRNA and protein The Notch receptor is activated when engagement by one of its ligands triggers a series of cleavages that releases the notch intracellular domain Processing of Notch receptors requires the activity of two proteases, namely tumor necrosis factor α-converting enzyme (TACE) and presenilin/γ-secretase (Baron et al , 2002) TACE cleaves the Notch receptor between the extracellular and transmembrane domains, whereas further cleavage takes place within the transmembrane domain by γ-secretase, which is dependent on the presence of presenilins These cleavage events release the intracellular domain of Notch, thereby allowing translocation to the nucleus (Sainson et al , 2005) In addition, recent evidence implicates glycogen

synthase kinase-3 (GSK-3) in post-translational modification of NotchIC GSK-3 is a serine/threonine kinase which has been implicated in the regulation of several metabolic enzymes and transcription factors in response to extracellular signals Recently, reports demonstrate GSK-3β phosphorylates NotchIC, thus protecting NotchIC from proteasome-mediated degradation (Foltz et al , 2002)

Functional coupling between the Notch signalling pathway and BRP apoptosis was difficult to establish since pericytes proved extremely difficult to transfect Our static BRP experiments however, suggest Notch may modulate BRP apoptosis For example, addition of recombinant SHh to BRPs in static cultures inhibited serum starvation-induced apoptosis, concomitant with increased *Notch1* and *Notch3* mRNA Moreover, studies within our laboratory have confirmed the potent anti-apoptotic effect of both Notch1 and Notch3 in VSMCs and overexpression of either Notch1IC or Notch3IC can reverse cyclic strain induced VSMC apoptosis (Sweeney et al , 2004) These findings concur with *in vivo* findings by Wang *et al* , (2002a), who report a decrease in Notch receptor and Notch target gene expression following balloon injury, with the most dramatic decrease evident for the Notch3 receptor and the HRT-1 target gene

In addition, exposure of BRPs to exogenous SHh in static cultures increased Notch1 and Notch3 and reduced Bax mRNA, suggesting Bax may be a downstream target of Notch signalling in BRPs The importance of the Bcl-2 family in the regulation of apoptosis in a variety of cells is well established, with changes in Bcl-2 family expression often observed during the pathogenesis of vascular disease (Bai et al , 1999, Cook et al , 1999) Therefore, this study investigated the effect of pulsatile flow on the pro-apoptotic gene *bax*, and the anti-apoptotic genes *bcl-2* and *bcl-*x_L This study shows that high pulsatile flow alters the ratio of *bcl-2* family genes in favour of apoptosis in BRP, with an increase in Bax mRNA and protein, and a concomitant decrease in Bcl-2 mRNA and protein No significant change in anti-apoptotic *bcl-xl* mRNA levels was observed Furthermore, work with VSMCs in our laboratory demonstrated that the pro-apoptotic *bax* gene, and the anti-apoptotic *bcl-*x_L gene are both regulated by the Notch signalling pathway in VSMC in response to cyclic strain (Morrow et al , 2005) Down-regulation of the Notch signalling

pathway in response to increased cyclic strain results in a decrease in *bcl-x_L* expression in VSMCs Bcl-x_L acts to protect against apoptosis in part by binding to the pro-apoptotic protein Bax, thus inhibiting the ability of Bax to form homodimers This inhibits the ability of Bax to increase mitochondrial membrane potential and cause activation of the caspase cascade Our pulsatile flow studies concur with these macrovascular cyclic strain experiments

The regulation of both *bax* and *bcl-2* by pulsatile flow is somewhat unsurprising as both have been implicated in neonatal vascular remodelling and the pathogenesis of vascular disease (Pollman et al , 1999, Gibbons and Pollman, 2000, Kim et al , 2000) *Bax* expression is up-regulated in the rat heart following coronary occlusion (Liu et al , 1998) and its over-expression in the ventricles of spontaneously hypertensive rats is said to contribute to myocyte apoptosis (Fortuno et al , 1998) In addition, an increased level of apoptosis and *Bax* expression was observed in human umbilical vessel SMCs during delivery, which coincided with increased mechanical forces in the vessel during delivery (Kim et al , 2000) Similarly, Bax-associated apoptosis was also observed at other sites undergoing dramatic haemodynamic changes during the perinatal period, such as the ductus arteriosus and the branching point of large arteries (Kim et al , 2000) These observations indicate that Bax may play a key role in both neonatal and pathological vascular remodelling, and that the stimulus for *Bax* up-regulation may be exposure to increased haemodynamic forces

Several investigators have proposed that the stimulus for increased *bax* gene expression due to cyclic strain is likely due to increased p53 activity as *bax* is a direct transcriptional target for p53 Two recent studies have shown increases in both p53 activity and Bax expression due to cyclic strain (Mayr et al , 2002, Wernig et al , 2003) In contradiction of these reports, we observed a decrease in p53 mRNA levels in BRPs exposed to increased flow (data not shown) Several distinct experimental differences may account for this Firstly, the fact that BRPs were analysed in co-culture with BRECs is a key difference, since BRECs can modulate BRPs cell fate decision, as determined by our co-culture studies (Chapter 4) For example nitric oxide, prostacyclin and endothelin-1 to name a few, are each known to modulate VSMC fate decisions Kibbe *et al* report the *absence* of p53 renders VSMC more

susceptible to NO-induced apoptosis than p53 competent cells, suggesting an anti-apoptotic effect of p53 due to differential expression and activation of the MAPK pathways in response to NO (Kibbe et al , 2002) Therefore, reduced BRP p53 at high pulsatile flow may in fact be a pro-apoptotic stimulus Moreover, recent studies with VSMCs exposed to pulsatile flow in our laboratory demonstrated an increase in VSMC apoptosis at high flow, concomitant with no change in VSMC p53 (Birney et al , 2004) Furthermore, Chae *et al* demonstrated increased Bax and decreased Bcl-2 in VSMCs exposed to exogenous NO donor, suggesting NO from the underlying BRECs may also alter the Bax/Bcl2 ratio in BRPs (Chae et al , 2004)

This study has provided the basis for further analysis within our laboratory of the mechanism of Notch signalling pathway regulation of *bax* and *bcl-x_L* in VSMCs Using siRNA knockdown technology preliminary results indicate that the down-regulation of *hrt-1*, *hrt-2* and *hrt-3* results in increased *bax* expression, with the most significant effect being mediated by *hrt-3* (Dr Catherine Sweeney – personal communication) In contrast, down-regulation of *hes-1* results in decreased *bax* expression In addition, targeted inhibition of *hrt-1*, *hrt-2* and *hrt-3* results in a decrease in *bcl-x_L* expression in VSMC, whereas *hes-1* down-regulation results in a slight increase in bcl-x_L protein expression (D Morrow – personal communication)

Several investigators have described the role of Hh in vasculogenic and angiogenic vessel formation In particular, EC sprouting, tube formation and migration by Hh is well described, however Hh signalling in SMC/pericytes is less well described We demonstrated the potent anti-apoptotic effect of exogenous addition of recombinant SHh to BRP in static cultures (Chapter 5), via increased *BCl-2* and decreased *Bax* mRNA Furthermore, SHh also increased BRP *Notch1* and *Notch3* mRNA levels In haemodynamic experiments, BRPs exposed to high pulsatile flow resulted in decreased Indian and Sonic Hh protein and mRNA levels, concomitant with increased apoptosis and NotchIC Thus, it is tempting to speculate that increased BRP apoptosis at high flow is due to less NotchIC as a result of decreased Hh Indeed, recent reports demonstrate SHh activates GSK-β, a molecule known to inhibit intracellular proteolytic degradation of NotchIC Future

experiments should determine if Hh modulates Notch post-translational modification via GSK-3, or membrane cleavage by any one of several enzymes such as γ-secretase or presenilin

Chronic hypoxia is, at least in the retina, sufficient to induce the expression of angiogenic growth factors, resulting in the characteristic retinal neovascularization associated with proliferative diabetic retinopathy (PDR) The observation that retinal neovascularization occurs adjacent to the nonperfused area supports the hypothesis that angiogenic factors are released from hypoxic tissue New vessel formation in PDR thus requires VEGF-mediated recruitment of ECs, however pericyte recruitment is also required to form a mature and stable vessel Recruitment of pericytes is largely regulated by the PDGR family of proteins and receptors (Hammes et al , 2002) Therefore, in addition to the effect of pulsatile flow on BRP apoptosis, we also examined the proliferative profile of BRPs The effect of flow is a known modulator of EC/pericyte cell fate decisions during vasculogenesis and may play an important role in angiogenic sprouting of new vessels in PDR High pulsatile flow decreased BRP proliferation in co-culture with BRECs, as determined by pCNA protein expression and a cell tracer assay This suggests flow is an anti-proliferative force for pericytes and may contribute to maturation of new vessels Additionally, experimental observations suggest that pericyte proliferation may also be a clinically significant factor in hypertension, a risk factor in several retinopathies (Herman et al , 1987, Herman and Jacobson, 1988) Vouyouka *et al* (2004) also demonstrated high pressure inhibits VSMC proliferation It is tempting to speculate that inhibition of BRP proliferation at high flow is due to reduced BRP Notch1IC and/or Notch3IC at high flow Wang *et al* demonstrated HRT1, a downstream target of Notch, promotes VSMC growth by inhibiting the expression of a principle cell cycle inhibitor, p21 WAF1/CIP1, and attenuates both growth factor deprivation and Fas ligand (FasL)-induced cell death by inducing the expression/activity of Akt, a well-established anti-apoptotic mediator (Wang et al , 2003b, Wang et al , 2002c) Further experiments are required to elucidate any potential modulation of BRP proliferation by NotchIC

In contrast to high flow induced BRP apoptosis, high flow conferred a protective effect on BREC apoptosis Accordingly, a plethora of studies on EC responsiveness to shear stress demonstrate a protective effect of increased shear stress (Bartling et al , 2000, Kaiser et al , 1997, Caplan and Schwartz, 1973, Haga et al , 2003) Moreover, at certain positions along the vasculature, such as bifurcations in the vessel or points of extreme curvature, where flow patterns are disturbed, preferential formation of atherosclerotic lesions occur at these points (Caplan and Schwartz, 1973) These regions are characterized by an enhanced turnover of endothelial cells, which could be mediated by apoptosis activation

Exposure of BRECs to increased pulsatile flow resulted in increased Notch1 and Notch3 mRNA, whereas Western blot analysis demonstrated an increase of intracellular cleavage components of both Notch1 and Notch3 receptors As such, we hypothesized that increased Notch at high flow mediated the protective effects of high pulsatile flow

The function of the Notch signalling pathway has often times been demonstrated as being cell type specific (Yang *et al* , 2004) For example, activated Notch4 is able to inhibit endothelial apoptosis in response to the inflammatory mediator, lipopolysaccharide, through inhibition of a mitochondrial-directed death pathway, through a CSL-dependent and independent pathway mechanism (MacKenzie *et al* 2004a) Moreover, Notch1 increases *Bcl-2* expression in a thymic lymphoma cell line (AKR1010) but not in a T-cell hybridroma line (2B4 11), however Notch1 confers resistance to glucocorticoid-induced apoptosis in both cell types Interestingly, a number of recent papers suggest that Notch promotes *Bcl-2* expression, and that this is achieved in a CBF-1-independent manner Notch1 and Notch4 upregulate *Bcl-2* expression in T-cells and EC respectively (MacKenzie et al , 2004b, Jang et al , 2004) and inhibit induction of apoptosis in the respective cells Our studies demonstrate high flow confers a protective anti-apoptotic effect on ECs compared to low flow, via increased Bcl_2 and Bcl_{XL}, and decreased Bax, concomitant with increased NotchIC

Using the same CELLMAX™ pulsatile flow apparatus, Sakao *et al* (2005) also demonstrated increased *Bcl-xl* expression at high flow in human pulmonary microvascular ECs (Sakao et al , 2005) Similarly, exposure of human umbilical vein ECs to increases in laminar shear stress resulted in increased *Bcl-xl* expression (Bartling et al , 2000) Our studies reveal RPMS-1 inhibition of CBF-1 dependent Notch binding reversed the protective effect of high flow RPMS-1 inhibits NotchIC activation of CBF-1 through interference with the SKIP CBF-1 repressor complex Our findings demonstrate high flow increases BREC Bcl_{XL} and decreases BREC Bax mRNA via a CBF-1 dependent mechanism Recent reports however, demonstrate Notch can upregulate Bcl-2 via a CBF-1 *independent* mechanism, though this mechanism is unclear at present (MacKenzie *et al* 2004a) RPMS-1 did not inhibit high flow-induced increases in BREC Bcl-2 mRNA Accordingly, high flow-induced increases in NotchIC may increase Bcl-2 mRNA via a CBF-1 independent mechanism Further investigations are required to confirm these intial findings This dual anti-apoptotic mechanism makes the activation of Notch a particularly potent inhibitor of the intrinsic apoptotic pathway

This is the first account of EC Notch1 or Notch3 modulation by mechanical forces Furthermore, this is the first account of Notch3 in *any* EC type Several reports suggest Notch3 is specific to mural cells, however there may be many reasons we have localised Notch3 to BRECs Firstly, this is the first *in vitro* account of Notch in any vascular EC type exposed to pulsatile flow Exposure of BRECs to pulsatile flow enables culture of these cells in supplemental media, even after a confluent monolayer has formed These cells maintain a healthy phenotype, and do not overgrow, as would be the case in static cell culture, since pulsatile flow presumably confers an anti-proliferative effect on the endothelial monolayer at *both* low and high pulsatile flow Secondly, our model enabled co-culture of ECs with pericytes, the presense of which can modulate the EC proteome through many secretory molecules, most of which are as yet undefined For example, we demonstrate Notch3 activation in static BRECs with exogenous SHh addition In our co-culture model, BRPs may be a source of Hh Several investigators have demonstrated that SHh is tethered to the cell membrane, however reports also

demonstrate diffusion of Hh through tissues. Our report of Notch activation upon exposure to increased pulsatile flow, is in agreement with reports demonstrating increased EC Notch ligand (Jagged) and downstream target (Hes-1) in human umbilical vein endothelial cells (HUVECs) exposed to increases in shear stress (Wasserman et al., 2002). Notch signalling is activated via cell-to-cell contact, such that Notch receptors bind ligands on an adjacent cell. Using immunocytochemistry, we have demonstrated the Notch ligand Jagged is present in BRECs (Figure 5.2).

Our studies did not evaluate the effect of pulsatile flow on Notch4 expression in ECs, though Notch4 is widely expressed in ECs. Recent unpublished observations in our laboratory however, suggested Notch4 is not regulated by cyclic strain in aortic ECs (Dr. von Offenberg Sweeney – personal communication). This is an area which will require further work to determine the role, if any, of Notch4 in modulating retinal EC responses to mechanical forces *in vitro*.

Using bioinformatics, we examined whether any Notch target gene promoter sites are present on the *bax, bcl-2* or *bcl-x_L* genes. Nakagawa *et al.*, (2000) have determined that the HRT family of Notch target genes preferentially bind to an E box motif, CACGTG, but also bind to other E box motifs (CAACTG, CACCTG, CACTTG, and CATCTG) to regulate transcription of other genes. Therefore, through sequence alignment of cloned *bax, bcl-2* and *bcl-x_L* promoters (Grillot et al., 1997, Igata et al., 1999), we have determined that the Notch family HRT target genes can bind to, and therefore possibly directly regulate, both the *bax* and *bcl-x_L* genes (MultiAlin software). The HRT family can bind to the CACGTG or CATCTG sequences of *bax*, and the CACCTG or CACTTG sequences of *bcl-x_L*, however, this would have to be further confirmed with mutational analysis. Therefore, it is likely that the HRT family of Notch target genes act as effectors of the Notch signalling pathway, at least in part, by repressing *bax* expression and promoting *bcl-x_L* expression, which has been confirmed in additional experiments within our laboratory.

Similar to BREC modulation of BRP cell fate decisions, BRPs have a modulatory effect on BRECs. For example, Hata *et al* (2001) demonstrate that VSMCs protect ECs from NO-induced apoptosis by releasing VEGF. In addition,

they demonstrate VSMCs may maintain the levels of anti-apoptotic protein Bcl-2 in adjacent ECs, demonstrating VSMCs play important roles in the regulation of EC survival Furthermore, Gerber *et al* reported that binding of VEGF to the VEGF receptor-2 (VEGFR-2) on ECs, enhanced EC survival through the phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway (Gerber et al , 1998) Since growth factor activation of the PI3K/Akt induces the phosphorylation of the Bcl-2 family member BAD thereby suppressing apoptosis and promoting cell survival, VEGF could directly inactivate the cell-intrinsic death machinery BAD by its phosphorylation in ECs Future studies within our laboratory will attempt to elucidate the role of VEGF signalling and apoptosis in both ECs and pericytes

The effects of Notch activation on proliferation can be stimulatory or inhibitory depending on the cell type, and the mechanisms mediating cell cycle inhibition can be cell-type specific (Noseda et al , 2005) Proliferation of endothelial cells is inhibited by a constitutively active NotchIC or activation of the Notch pathway by Jagged1 (Noseda *et al* 2004, Liu *et al* 2003) Notch activation inhibits proliferation of endothelial cells in a cell autonomous manner by inhibiting phosphorylation of the retinoblastoma protein (Noseda *et al* 2004b) During endothelial cell cycle entry, activated Notch inhibits mitogen induced upregulation of p21Cip1 and delays phosphorylation of retinoblastoma suppressor protein, Rb, by cyclin D-cdk4 complexes Notch-dependent downregulation of p21Cip1 inhibits nuclear localization of cyclin D and cdk4, and targeted downregulation of p21Cip1 hinders nuclear translocation of cyclin D-cdk4, reducing S-phase entry in endothelial cells Furthermore, recent evidence demonstrates that Notch-mediated cell cycle arrest is associated with downregulation of minichromosome maintenance (MCM) proteins in ECs and human fibroblasts (Noseda et al , 2005) MCM proteins form a complex with helicase and participate in the formation of prereplicative complexes that allow chromatin licensing to ensure that DNA replication initiates at specific sites Thus, MCM proteins are essential for DNA replication and cell cycle progression Indeed, downregulation of MCM proteins is also observed on activation of CBF1 and is mediated by inhibition of Rb phosphorylation (Noseda et al , 2005)

Our results however demonstrate no change in BREC proliferation from low to high pulsatile flow even though both Notch1 and Notch3 are increased upon exposure to high pulsatile flow BRECs within our 3-D model are fully confluent for several days prior to increases in flow rate and thus presumably quiescent at this point (t=0) Interestingly, it has previously been reported, when endothelial cells reach confluence, the Notch pathway is activated and p21Cip1 is downregulated, suggesting that Notch activation contributes to contact inhibition of endothelial cells, in part through downregulation of p21Cip1 (Noseda *et al* 2004)

Similarly, various studies raise the notion that Notch activation may be required for the establishment of a mature, quiescent endothelial phenotype, in part by downregulating VEGFR-2 (Liu *et al* 2003, Taylor *et al* 2002) Furthermore, the prominent vascular defects observed in Notch and Notch ligand-deficient mice suggest that inappropriate apoptosis and/or proliferation may play a role in the observed phenotypes (Krebs *et al* 2000, McCright *et al* 2001, Xue *et al* 1999) Interestingly, the lack of Notch1 and (or) Notch4 does not prevent the differentiation of mesodermal precursors into endothelial cells in mice (Krebs *et al* 2000) The primary vascular plexus is laid down, but remodelling of this initial endothelial network does not take place (Krebs *et al* 2000) Thus, it is possible that Notch activation is required to maintain endothelial viability only in reorganizing or mature vasculature Notch may thus affect the non-proliferative state of an endothelial monolayer *in vivo* and also in our 3-D co-culture model

It has previously been shown that SHh indirectly induces angiogenesis by upregulating expression of VEGF (Pola et al , 2001) As described previously, VEGF can activate Notch in some cell types, as such, we examined Hh signalling components in BRECs exposed to pulsatile flow These results demonstrate high pulsatile flow increases BREC IHh, SHh, Ptc1, Smo and the downstream Hh transcriptional target – Gli2

Our static BREC experiments (chapter 5) demonstrate Hh modulation of Notch signalling Further evidence of the signalling cascade of Hedgehog – Notch in BRECs was confirmed in this pulsatile flow study Addition of the specific Hh

inhibitor cyclopamine abrogated flow-induced increases in *Notch1* mRNA Recent studies in our laboratory examined CBF-1-dependent Notch signaling pathway activation by luciferase reporter constructs These studies reveal that stimulation of BRECs with VEGF transactivates a CBF-1-dependent promoter (Dr Catherine Sweeney – personal communication) While recent reports by Liu *et al* (2003) demonstrate that VEGF upregulates Notch1 and Dll4 gene expression via the PI3K/AKT pathway in human arterial endothelial cells Further evidence of SHh activation of Notch via VEGF are apparent in studies by Kanda et al (2002), in which they demonstrate SHh activation of PI3K/AKT in microvascular ECs

This is the first demonstration of pulsatile flow regulation of Hedgehog in ECs, and also the first *in vitro* demonstration of Notch signalling in retinal endothelial or pericyte cells Interestingly, Wu *et al* previously demonstrated induction of IHh by cyclic strain and determined IHh functions as an autocrine signal to transduce and amplify mechanical stimulation of chondrocyte proliferation (Wu et al , 2001) Similarly, mechanical loading in a repeated manner in a mouse *in vivo* model, triggers the expression of IHh which in turn increases the number of replicating mesenchymal cells as well as the amount of the cartilage formed (Ng et al , 2006) Taken together these events increase condylar growth This lends weight to the link between Hh and Notch signalling, since notch regulation of cellular proliferation is well characterised Using zebrafish as a model system, Lawson *et al* (2001) demonstrated that the SHh signalling pathway has a role in artery formation Furthermore, the authors show that SHh is upstream of VEGF, and is necessary for Notch1 expression in arteries (Lawson et al , 2002)

Bone morphogenetic proteins (BMPs) are another family of secreted proteins that regulate cell fate decisions, such as growth and differentiation Interestingly, Wu *et al* also demonstrate that BMPs are a downstream target of IHh, and DPP, the equivalent of BMP in *Drosophila* is induced by hedgehog (2001) Several reports also demonstrate a link between BMPs and Notch signalling Thus, Hedgehog-VEGF-Notch signalling may be a link in a complex signalling mechanism involving several molecules and/or pathways regulating cell fate decisions For example,

Lawson *et al* demonstrated that the Notch signalling pathway lies upstream of Eph-B4/ephrin-B2 in arterial/venous specification In zebrafish, Notch is expressed solely in arteries, and lack of Notch signalling results in the loss of ephrin-B2 expression in the arterial tree (Lawson et al , 2002)

Our results demonstrating Hh modulation by changes in pulsatile flow in both ECs and pericytes may also be significant to the pathogenesis of diabetic retinopathy There is strong evidence that retinal blood flow is raised in the early stages of diabetes even before the onset of diabetic retinopathy and also considerable variation in the distribution of blood flow The early stages of retinopathy are characterised by an increase in retinal blood flow with a progressive decrease during advanced stages of disease The reason for this increase in retinal perfusion is most probably coupled to the cellular and intracellular alterations induced by glucose and to endothelial dysfunction Vascular endothelial growth factor (VEGF) is universally accepted as a primary factor in the regulation of vessel patency in vascular networks throughout the body, including the retina There is considerable evidence that the VEGF system in disturbed early in diabetes and interacts with other pathways and vasoactive factors to stimulate breakdown of the blood retinal barrier (BRB) and eventually promote angiogenesis, the hallmark feature of proliferative diabetic retinopathy (PDR) Furthermore, intravitreal injections of VEGF in animal studies mimic retinal blood flow alterations associated with the pathology Abberant regulation of Hedgehog signalling due to altered retinal blood flow may be responsible for increased VEGF in DR Work by Pola *et al* (2001) demonstrated SHh stimulates fibroblasts *in vitro* to produce a combination of potent angiogenic factors, including the 3 major isoforms of VEGF, Ang-1, and Ang-2 Therefore, SHh seems to act as an indirect angiogenic agent and may trigger neovascularization through SHh/Ptc1 signalling specifically in mesenchymal cells

The human disorders CADASIL and AGS provide additional evidence of the importance of the Notch signalling pathway in the adult vasculature These disorders involve dysregulation of components of the Notch signalling pathway, and resulting cardiovascular abnormalities CADASIL, which is caused by mutations in Notch3, is characterized by degeneration of SMC, primarily in cerebral arteries, but also in

systemic arteries and some veins CADASIL patients present with an abnormal accumulation of Notch3 in VSMC, impaired Jagged 1/Notch3 binding and defective CBF-1-dependent Notch3 signalling (Prakash et al , 2002, Brulin et al , 2002, Joutel et al , 2004) AGS also highlights the importance of the Notch signalling pathway, and in particular Jagged 1, in the development and maintenance of the cardiovascular system AGS patients typically present with congenital heart defects, heart murmurs and/or peripheral pulmonary stenosis (Joutel and Tournier-Lasserve, 1998, Loomes et al , 1999)

CADASIL patients have reduced retinal mean and peak systolic capillary flow This agrees with the previously reported reduction of capillary flow on the optic nerve head rim measured in CADASIL patients as well as with the irregular choroidal filling in retinal fluorescein angiography CADASIL might also be a risk factor for nonarteritic anterior ischemic optic neuropathy as recently reported (Rufa et al , 2004a), suggesting pathology of arteries supplying the optic nerve head Each of these suggest a strong regulatory role of Notch3 signalling in blood supply to the retina *in vivo* (Harju et al , 2004)

6 4 Conclusion

High pulsatile flow provides an anti-proliferative and pro-apoptotic stimulus to BRPs in co-culture with BRECs This may be due to reduced Hedgehog activation of Notch signalling at this flow rate In contrast, BRECs exhibit reduced apoptosis in a high flow environment, whereas proliferation is unchanged Hedgehog activates Notch signalling at high flow which protects ECs from flow-induced apoptosis by downregulating Bax protein & gene expression, and possibly also modulating Bc2 and Bcl-xl

Chapter 7

Summary

The current study describes the use of a novel *in vitro* transcapillary co-culture system as a basis for modelling the effects of haemodynamic forces on microvascular retinal endothelial and pericyte cells Blood vessels are continually exposed to haemodynamic forces generated in the vasculature in the form of fluid shear and circumferential stresses Changes in these conditions result in alterations in the signal transduction pathways and release of vasoactive substances from vascular endothelial and mural cells that are ultimately responsible for regulating vascular tone Changes in haemodynamics can result in endothelial dysfunction, a process that has been described in macrovascular disease states such as atherosclerosis and thrombosis (Drexler and Hornig, 1999) and is also of particular interest in the ocular microvasculature in conditions such as diabetic retinopathy (DR) (Endemann and Schiffrin, 2004) and normal tension glaucoma (NTG) (Henry et al , 1999), where there is growing evidence of endothelial dysfunction as a major risk factor

The work described in this thesis can be broadly classified in two parts Chapters 3 and 4 describe the acute and chronic effect of pulsatile flow on the release of vasoactive substances NO, PGI_2 and ET-1, and the resulting effect on EC and pericyte cell apoptosis The latter part, described in chapters 5 and 6, characterise Notch and Hedgehog signalling in human eyes, in static cell culture, and also co-cultures of retinal ECs and pericytes exposed to pulsatile flow Interaction between the Notch/Hedgehog pathways and their role in modulating apoptosis was also examined The following is a brief overview of the main findings of this research project and suggestions for future experiments and potential avenues of research For an in-depth analysis, please refer to the discussion sections of chapters 3 to 6

The retinal vasculature does not have sympathetic autonomic innervation, rather, it maintains optimal nutrition and oxygenation of the retina through vascular autoregulation (Jean-Louis et al , 2005) Autoregulation assures sufficient blood supply over a wide range of ocular perfusion pressures and regulates blood flow, depending on functional activity and the resultant metabolic demands (Garhofer et al , 2005) This autoregulatory capacity is evident with a recently described flicker stimulation technique (Garhofer et al , 2002) Retinal blood flow increases after flickering light provocation, indicating the ability of the retina to adapt its blood flow to changing energy demands caused by increased neural activity (Garhofer et al , 2005) Similarly, provocation through breathing pure oxygen has also been used to demonstrate retinal vascular autoregulation, seen as a vasoconstriction of retinal vessels or a decrease in retinal blood flow (Jean-Louis et al , 2005) As described previously, endothelial dysfunction disrupts autoregulation of retinal blood flow The term 'endothelial dysfunction' indicates a generalised alteration in EC phenotype and function characterised by an abnormal vasodilator response, such as decreased NO or prostacyclin release, or increased production of vasoconstrictors such as ET-1 (Ibrahim et al , 2005) The mechanism of these EC changes are not fully understood, but impaired NO bioactivity is a principal feature of this abnormality An impaired retinal blood flow autoregulation in both DR and glaucoma patients has been demonstrated with both flicker stimulation and pure oxygen breathing experiments (Garhofer et al , 2002, Riva et al , 2004, Patel et al , 1994, Rassam et al , 1995, Grunwald et al , 1984)

As mentioned previously, blood flow alterations are apparent in both DR and glaucoma, and may also be a causative factor of impaired retinal blood flow autoregulation Therefore, we examined the effect of altered pulsatile flow on the principal mediators of retinal vascular autoregulation NO, PGI_2 and ET-1 Analysis of conditioned media from these experiments revealed the vasodilators NO and PGI_2 were both significantly up-regulated following exposure to high pulsatile flow for 24 and 72 hours Moreover, the observed increase was associated with increases in protein expression and steady-state mRNA levels On the other hand, the potent vasoconstrictor ET-1 increased after 24 hour exposure to high flow, however chronic

exposure for 72 hours resulted in decreased ET-1 peptide and BREC *ET-1* mRNA levels. This is a possible mechanism that microvessels use to maintain blood flow at a constant rate, such that, acute increases in blood flow stimulate ET-1 release, returning blood flow to 'normal' levels. Chronic increases in blood flow inhibit ET-1 release however, as ET-1 is also a potent pericyte mitogen. Malek *et al.* (1999(b)) report a similar bi-phasic ET-1 release with aortic ECs exposed to increases in shear stress. In addition, Malek *et al* demonstrate this bi-phasic response is due to reoorganisation of the EC microtubule network, which proved crucial to transduction of shear stress into altered ET-1 gene expression.

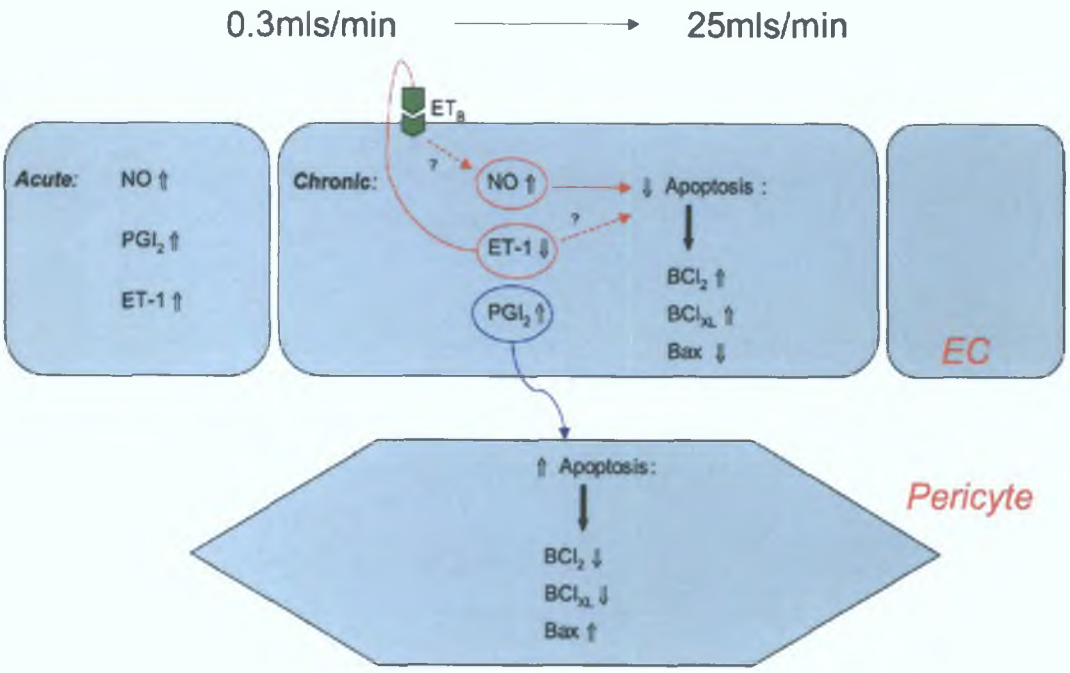


Figure 7.1 Schematic summary diagram of effects of high compared to low pulsatile flow on BRECs/BRPs co-culture. Exposure to increased flow for 24 hours induces NO, PGI_2 & ET-1 release. After 72 hours, NO & PGI2 increase, whereas ET-1 peptide release decreases. NO & ET-1 protect ECs from apoptosis at high flow. Apoptosis of BRPs increases at high flow due to PGI_2 release from BRECs.

Our pulsatile flow studies suggest blood flow inhibits ET-1 release, at least in part by NO, as we found a significant increase in ET-1 release in the presence of the NO inhibitor, L-NAME This may be particularly relevant in NTG NTG patients typically have an impaired EC NO response as a result of endothelial dysfunction Taken together, these results suggest reduced local levels of NO would increase local ET-1 peptide, and possibly explain why these patients have microvascular vasospasms At present, it is unclear which is the primary and which is the secondary cause of the vasculopathy in NTG reduced blood flow or reduced NO

The crosstalk between NO and ET-1 is a fundamental mechanism governing the expression and activity of ET-1 across species (Lavallee et al , 2001) In addition to this interaction, we demonstrated a similar ability of PGI_2 to inhibit ET-1 release, as indomethacin treatment resulted in increased ET-1 at both low and high flow compared to controls Moreover, crosstalk between these vasoactive mediators is further complicated by reports that ET-1 induces COX-2 expression in rat ECs and also in VSMCs (Chen et al , 2003), suggesting another autoregulatory mechanism Lastly, to elucidate crosstalk of PGI_2 and NO production, we investigated the effects of the inhibition of NO synthesis on the shear-induced PGI_2 production The results demonstrated that inhibition of NO synthase with L-NAME enhanced flow-induced production of PGI_2, indicating that endogenous NO functions as an inhibitor of PGI_2 production in an autocrine or paracrine fashion Similar studies with HUVECs exposed to shear stress corroborate these findings (Osanai et al , 2000)

Haemodynamic forces generated by the flow of blood are crucial in maintaining homeostasis within the blood vessel wall Abnormal blood flow however alters the mechanical forces in the vessel wall and can lead to vascular remodelling, a process which underlies the pathogenesis of cardiovascular diseases such as atherosclerosis and restenosis (Schwartz et al , 1995, Libby, 2003), and the microvascular complications DR and glaucoma In DR for example, hyperglycaemia induces EC metabolic changes, increased retinal blood flow, basement membrane thickening, pericyte dropout, and leaky vessels which are prone to rupture (Cai and Boulton, 2002, Frank, 2004, Hammes et al , 2002) During the later proliferative phase of DR, blood flow typically decreases and new vessels proliferate

In glaucoma, vessels typically regress, possibly due to loss of neurons, or possibly as a result of blood flow perturbations We hypothesize that fluctuations in mechanical forces within the vessel wall due to altered blood flow in DR and NTG will result in altered local vasoactive metabolites and possibly regulate EC and pericyte apoptosis As such, we examined the role of NO, PGI_2 and ET-1 on EC/pericyte cell apoptosis in co-culture In control cells, increased pulsatile flow resulted in reduced EC apoptosis, whereas pericyte apoptosis increased Using specific inhibitors of each vasoactive pathway, we have shown that the protective effect of increased flow on ECs was mediated by NO and ET-1 L-NAME inhibited the flow-induced increase in NO release into circulating media perfusate and also increased BREC apoptosis To our initial surprise, inhibition of ET-1 also abrogated the flow-induced anti-apoptotic BREC response These findings were intriguing, as high flow *inhibited* ET-1 release in control experiments The protective effect of ET-1 at high flow may be attributable to increased expression of the EC ET-1 receptor – ET_B Interestingly, activation of ET_B receptor on ECs typically results in increased NO Henry *et al* have reported an impaired EC ET_B receptor mediated peripheral vasodilatation in NTG patients *in vivo* using a selective ET_A antagonist (Henry *et al* – *IOVS* – in press, 2005) In the presence of ET_A receptor antagonism, endogenous ET-1 interacts solely with the ET_B receptor subtype, which is expressed by both endothelial and mural cells This produces vasodilatation resulting from reduced ET_A-receptor-mediated vasoconstriction and/or unopposed ET_B-receptor-mediated

release of NO from the endothelium Therefore, it is probable that the protective effect of high flow on EC apoptosis may be largely due to NO release This would explain why endothelial dysfunction is predominantly characterised by reduced EC secretion of NO, particularly in response to flow Furthermore, the disadvantage of two pro-apoptotic stimuli on ECs in NTG is apparent – NTG is associated with reduced blood flow velocities and also reduced bioavailability of NO

In contrast to the protective effect of increased flow on EC apoptosis, BRP apoptosis increased at high flow rates These results are consistent with reports from our laboratory of VSMCs exposed to pulsatile flow in the same pulsatile flow apparatus (Birney et al , 2004) Our studies demonstrated a cyclooxygenase product, possibly PGI_2, was primarily responsible for this increase in BRP apoptosis, as indomethacin treatment reversed the flow-induced BRP apoptosis Several other laboratories have also reported a similar effect on VSMC apoptosis (Bishop-Bailey and Warner, 2003, Upmacis et al , 2004, Pilane and LaBelle, 2004) Furthermore, we have shown exogenous addition of iloprost, a prostacyclin analogue, rescued BRECs but not BRPs from serum-starvation induced apoptosis We also observed a partial inhibition of BRP apoptosis at high flow with inhibition of ET-1 binding Similar to the protective effect of ET-1 on ECs at high flow, the increase in BRP apoptosis due to ET-1, though less peptide is circulating in media perfusate at high flow may be due to increased BRP ET_B receptor expression Moreover, studies by Cattaruzza *et al* (2000) have previously demonstrated that increased VSMC apoptosis due to cyclic stretch is mediated by increased VSMC ET_B receptor expression Future studies with specific ET_A or ET_B receptor inhibitors are required to elucidate the signalling mechanism involved

The second half of this thesis describes the role of Notch and Hedgehog signalling in retinal ECs and pericytes The principal findings of this aspect of the study relate to the cellular mechanisms by which these two signalling pathways interact, and alter cell fate Unlike the vasoactive mediators NO, PGI_2 and ET-1, Notch/Hedgehog are relatively recently described in vascular cell types Moreover, data of Notch/Hedgehog in microvascular cell types is limited and regulation of Notch/Hedgehog by mechanical forces has not yet been described in retinal vascular cells *in vitro* As such, our initial experiments sought to simply determine the presence or absence of Notch/Hedgehog signalling components in BRECs and BRPs, before proceeding to pulsatile flow experiments

This study comprehensively documents the presence of many components of Notch/Hedgehog signalling in both BRECs and BRPs, most of which had not yet been described in these cell types The Hedgehog signalling family was first identified in *Drosophila*, and was so named as haploinsufficiencies of this family of genes produces a phenotype resembling a hedgehog (Nusslein-Volhard and Wieschaus, 1980) The Hedgehog signalling pathway shows a high degree of evolutionary conservation from *Drosophila* to humans Similarly, the Notch signalling pathway is a highly conserved method of cell-cell communication that also controls cell fate decisions in many cell types, a function that is also conserved from *Drosophila* to humans (Miele and Osborne, 1999) As such, we postulated that Notch and Hedgehog signalling might also regulate cell fate decisions in BRECs and BRPs

This study has clearly shown that exogenous addition of recombinant Sonic Hedgehog exerts an anti-apoptotic effect in serum-starved BRECs and BRPs In an attempt to elucidate the mechanism through which the Sonic Hedgehog signalling pathway exerts its anti-apoptotic effect, we examined a number of known regulators of apoptosis from the Bcl-2 family of proteins This study provides previously unreported evidence that the Hedgehog signalling pathway regulates mRNA levels of the pro-apoptotic *bax*, and anti-apoptotic *bcl-2* and *bcl-xL* in BRECs and BRPs Furthermore, exogenous addition of Sonic Hedgehog increased BREC *Notch1* and *Notch3*, and BRP *Notch1* mRNA levels, suggesting SHh may mediate its anti-

apoptotic effect through regulation of Notch These results corroborate other studies from our laboratory, which document the anti-apoptotic effect of Hedgehog activation in VSMC, via increases in NotchIC (Morrow *et al* – in press) Additionally, Notch has recently been shown to prevent apoptosis in both ECs (MacKenzie et al , 2004b) and VSMCs (Wang et al , 2002c) Furthermore, in human diabetic retinas, pericyte cell apoptosis is an early pathological feature Increases in pro-apoptotic bax are known to occur in pericytes of DR patients (Podesta et al , 2000) Therefore, demonstration of SHh reducing bax mRNA, possibly *via* Notch in BRPs, suggests a potential role of Notch/Hh signalling in DR

These results are particularly interesting with respect to pulsatile flow regulation of BREC/BRP apoptosis As described previously, BRP apoptosis increased under high pulsatile flow conditions, concomitant with decreased Notch1IC and Notch3IC protein, and decreased *Notch1* and *Notch3* mRNA levels At present, we can only speculate that reduced NotchIC in BRPs exposed to high flow triggers increased BRP apoptosis However, studies of VSMCs exposed to cyclic strain in our laboratory substantiate this hypothesis Sweeney *et al* (2004) recently demonstrated that overexpression of NotchIC reversed cyclic strain-induced VSMC apoptosis Furthermore, we demonstrate that increased BRP apoptosis is mediated by increased bax and decreased bcl-2 mRNA levels, whereas Sweeney *et al* (2004) similarly demonstrated that Notch modulates VSMC apoptosis via bax and bcl-2

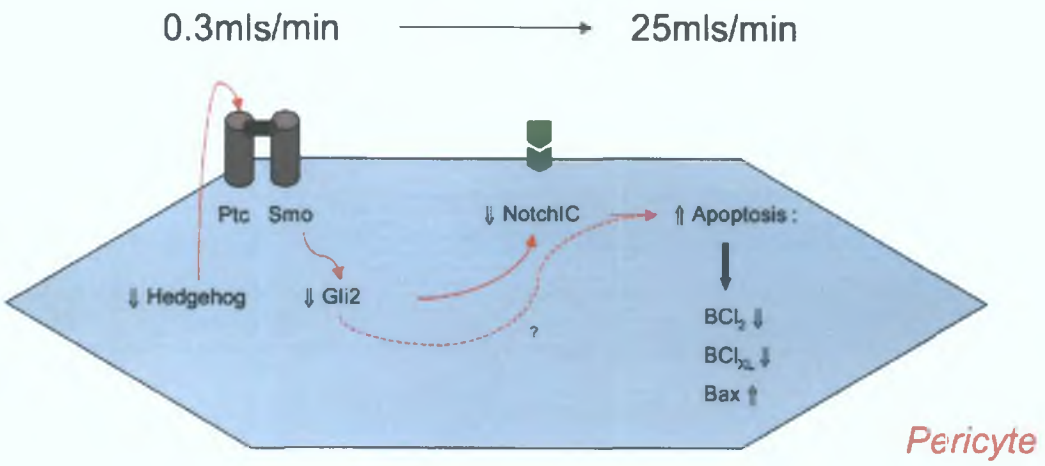


Figure 7.2 Schematic diagram of effects of pulsatile flow on BRPs Hedgehog/Notch Signalling in co-culture with BRECs. Exposure to increased flow for 72 hours decreased SHh, Ihh, Gli2, Notch1IC and Notch3IC. Apoptosis of BRPs increased at high compared to low flow, via decreased BCl_2 & Bcl_{XL}, and increased Bax.

Our static experiments demonstrate that Sonic Hedgehog activates Notch signalling in BRPs, therefore we also examined the regulation of Hh signalling components in BRPs exposed to pulsatile flow. The flow-induced regulation of BRP IHh and SHh mirrored pulsatile-flow regulation of Notch, leading us to postulate that Hh modulates Notch in BRPs. SHh can activate GSK-3β, a serine/threonine kinase which has been implicated in the regulation of several metabolic enzymes and transcription factors in response to extracellular signals. Indeed, GSK3-β is known to modulate Notch signaling through phosphorylation of NotchIC, such that inhibition of GSK3-β shortened the half-life of Notch1IC. Early studies of GSK-3β in mammalian systems focused on its pivotal role in glycogen metabolism and insulin-mediated signalling, suggesting a pivotal role of Notch/Hedgehog in DR. At present, both SHh and IHh are thought to activate the same signalling pathway via binding to the Ptc receptor, thus causing de-repression of the Ptc-Smo membrane complex. It is unclear if these two Hh peptides display the same binding affinity for the Ptc receptor however, and more than one Ptc receptor has now been described (Nieuwenhuis and Hui, 2005). Therefore, the significance of increased Ihh *and* SHh is unclear at present.

High flow reduces BREC apoptosis, concomitant with increased Notch1IC and Notch3IC protein, and increased *Notch1* and *Notch3* mRNA. Our findings demonstrate that inhibition of endogenous NotchIC CBF-1 dependent signalling reversed the protective effects of increased flow. Moreover, we also demonstrate that SHh and IHh protein and mRNA increased at high flow. Therefore, in conjunction with our static results, these results led us to hypothesize that Hh activation at high flow in BRECs is protecting against apoptosis via increased NotchIC. Indeed, Hh inhibition with cyclopamine abrogated flow-induced increases in Notch mRNA. Furthermore, either Hh inhibition, or Notch inhibition, each attenuated the flow-induced decrease in BREC apoptosis bax mRNA levels. Lastly, inhibition of Notch did not alter any Hh signalling components, suggesting Notch is downstream of Hh in a signalling cascade of Hh – Notch – Bcl-2 genes.

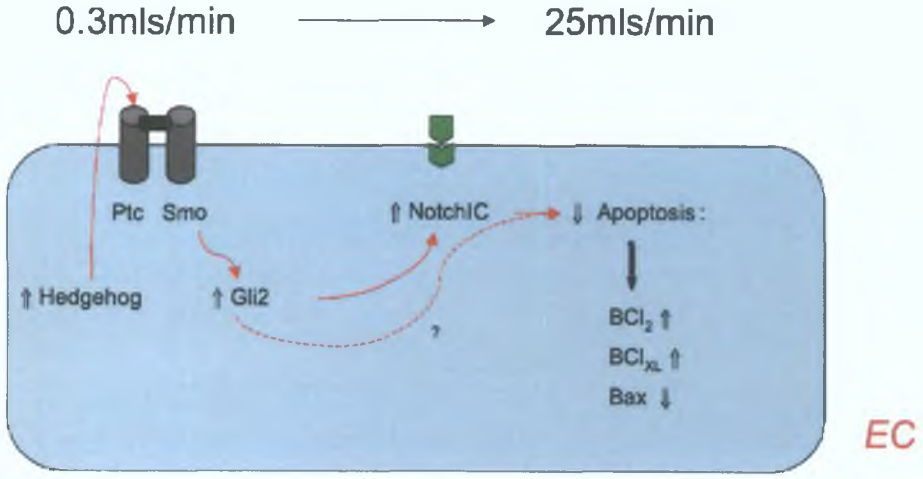


Figure 7.3 Schematic diagram of effects of pulsatile flow on BRECs Hedgehog/Notch Signalling. Exposure to increased flow for 72 hours increased SHh, Ihh, Gli2, Notch1IC and Notch3IC. Apoptosis of BRECs decreased at high compared to low flow via increased BCl_2 & Bcl_{XL} and decreased Bax. Inhibition of Hh with cyclopamine, or inhibition of NotchIC CBF-1 dependent gene transactivation, reverses the protective effect of high flow.

To date, there is no documentation of Notch3 in ECs in any vascular EC type, while most authors describe Notch3 as 'VSMC-specific' (Nijjar et al , 2001, Lindner et al , 2001, Wang et al , 2002b) Our results reveal this is not the case, with immunocytochemistry, Western blotting and realtime PCR each confirming the presence of Notch3 in BRECs Further evidence of EC Notch3 signalling is suggested in CADASIL patients – a disease caused by mutations in the Notch3 gene Haritoglou *et al* (2004 (a)) analysed the ocular vasculature of CADASIL patients and found ECs were detached with intracytoplasmatic vacuoles and mitochondrial changes In addition, findings in muscle and skin biopsies of CADASIL patients have suggested arterial ECs are also affected ECs appear to shrink, detach from the basal lamina and the tight and gap junctions are also disrupted (Prakash et al , 2002) Furthermore, results from co-workers with bovine aortic ECs, demonstrate the presence of Notch3IC and cyclic strain mediated increase in Notch3IC *in vitro* (Dr Von Offenberg Sweeney - personal communication) At present however, the distinctive functionality of the four Notch receptors in either ECs or pericytes remains to be elucidated

These results may be of particular relevance to the angiogenic process, which is principally regulated by VEGF Angiogenesis in the retina is a critical process in vascular development as well as in recovery from injury It may also have pathological, sight-threatening consequences when it produces neovascularization of the vitreous (e g , ROP, diabetes, retinal vein occlusion) or subretinal space (e g , age-related macular degeneration, ocular histoplasmosis) (Garner and Kissun, 1980, Garner, 1986) Elucidating the molecular mediators of angiogenesis is therefore of great clinical importance

VEGF has been linked to increased angiogenesis, proliferation, and migration of both EC and SMC Brown *et al* , (2000) demonstrated that cyclic strain-induced angiogenesis was associated with increases in VEGF (Brown and Hudlicka, 2003) The logical progression of this project would examine the interaction between Hh, VEGF and Notch, as Hh is reportedly upstream, and Notch downstream of VEGF in a signalling cascade in some cell types Indeed, recent studies in our

laboratory demonstrated that VEGF is the intermediate signalling molecule which enables SHh to activate VSMC Notch *in vitro* (D Morrow – personal communication) In addition, VEGF induces transactivation of the Notch promoter in static BREC cultures (Dr C Sweeney – personal communication)

The VEGF family comprises six known ligand and three known receptor sub-types The ligands include VEGF, placenta growth factor, and VEGFB, VEGFE, whilst the VEGF-receptor (VEGFR) sub-family includes VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1) and VEGFR3 (Flt-4) (Zachary, 2005) The most prominent ligand of the VEGF family is VEGF itself (VEGF-A), which binds to VEGFR1 and VEGFR2 (Veikkola and Alitalo, 1999) VEGFR1 is the predominant receptor subtype on retinal pericytes (Takagi et al , 1996b) and VSMCs *in vitro* (Parenti et al , 2002) *In vivo* studies of adult human and monkey tissues also demonstrate that VEGFR1 is the predominant pericyte receptor subtype (Witmer et al , 2002) The majority of *in vitro* studies on VEGFR1 signalling in ECs have found weak or no signalling, however Kanno *et al* (2000) demonstrated VEGF mediated induction of migration was *via* VEGFR1 signalling (Kanno et al , 2000) Furthermore, recent *in vivo* studies have demonstrated that VEGFR1 inhibition is capable of inhibiting tumour and retinal angiogenesis (Luttun et al , 2002)

Several groups have shown VEGF is an intermediate molecule in a signalling cascade of Hedgehog – VEGF – Notch (Lawson et al , 2002, Liu et al , 2003b) Currently it is unclear if VEGF activates Notch via VEGFR1 or VEGFR2, or independently of these receptors It is also possible that VEGF regulates enzymes responsible for Notch cleavage e g presenilin or γ-secretase VEGF upregulates both Notch1 and Delta-like 4 in arterial ECs (Lawson et al , 2002, Liu et al , 2003b) ECs undergo apoptosis when they are deprived of VEGF or are exposed to VEGF receptor antagonists (Sakao et al , 2005) Consistent with our findings of SHh inhibiting EC apoptosis by upregulating Bcl-2 and downregulating Bax, VEGF also prevents EC apoptosis via activation of bcl-2 family genes (Liu et al , 2000) Furthermore, utilising the same pulsatile flow apparatus, Sakao *et al* (2005) recently demonstrated that high flow is an anti-apoptotic stimulus for ECs, however, inhibition of VEGFR1 and VEGFR2 increased apoptosis at high flow These

findings mirror our RPMS-1 results Thus, it is tempting to surmise that VEGF, acting on VEGFR1 or VEGFR2 on ECs mediates increased intracellular BREC NotchIC In addition, gene knockout studies in mice with dispruted VEGFR1 expression die embryonically due to vascular defects with features highly reminiscent of Notch signalling pathway disruption (Xue et al , 1999, Smith et al , 2000)

The phenotypic consequences of Notch1 signalling vary widely depending on cell type and cellular context For example, Notch1 signalling results in growth inhibition and apoptosis induction of human hepatoma cells (Qi et al , 2003), whereas in lymphoma or myeloma cells activated Notch1 induces proliferation and inhibits apoptosis (Jundt et al , 2002, Jundt et al , 2004) Notch1 is also anti-proliferative in SiHa and Caski cells (Jesudasan et al , 1995) and increased Notch1 signalling triggers cell cycle withdrawal and differentiation in primary mouse keratinocytes (Rangarajan et al , 2001) Furthermore, the induction of gene expression by Notch activation is likely to involve cross-talk with other pathways implicated in growth/differentiation Our results suggest Notch signalling may activate different proliferation pathways in BRECs and BRPs Several investigators have demonstrated that Notch signalling in ECs has a potent anti-proliferative effect At high flow, BREC proliferation was unchanged compared to low flow, concomitant with increased Notch These results suggest sufficient NotchIC is present at both low and high pulsatile flow to maintain a quiescent monolayer Sakao *et al* (2005) demonstrated that ECs become hyperproliferative in the presence of VEGFR inhibitor, again suggesting a potential signalling between VEGFR and Notch and also EC proliferative control

During development, VEGF acts on EC VEGFR2 to induce EC proliferation (Sainson et al , 2005) In the quiescent vasculature of the adult however, it is estimated that only 0 01% of cells are actively proliferating (Sainson et al , 2005) Quiescent endothelial cells are normally anchored by their abluminal surface to a collagen-rich matrix via Notch-β1integrin binding (Leong et al , 2002) Our pronectin-coated culture capillaries may facilitate EC anchorage in such a manner

Pronectin is a synthetic protein polymer which incorporates multiple copies of the RGD cell attachment ligand of human fibronectin Recent results demonstrate that Notch prevents EC proliferation via increased β1integrin mediated adhesion, via downregulation of p21Cip1 (reducing S-phase entry), and also by downregulating EC VEGFR2 (Sainson et al , 2005, Taylor et al , 2002) Henderson *et al* have shown that EC over-expression of HRT-1, a transcriptional target of Notch, downregulates VEGFR2 mRNA expression levels, and inhibits VEGF-meditated cell fate decisions *in vitro* (Henderson et al , 2001) Similarly, zebrafish notch5 has been shown to repress VEGFR3 expression in venous EC in vivo (Lawson et al , 2001) These results demonstrate that Notch plays a critical role in controlling EC proliferation and may also stabilise the vasculature by inhibiting vascular permeability (Taylor et al , 2002)

During angiogenesis, elongation of the new sprout depends on the proliferation of ECs It has been suggested that Notch activation is absent in vessels at the early stages of angiogenesis when ECs are proliferating and reactivated when ECs stop proliferating facilitating vessel stabilisation A recent publication by Claxton *et al* (2004), demonstrates an important regulatory role of Delta-like 4 (Dll4), a Notch ligand, in retinal angiogenesis Dll4 mRNA is strongly expressed in ECs at the very tips of growing vessels, however these ECs had no apparent Notch staining The authors demonstrated that astrocytes at the leading edge of the growing vessels were positive for Notch and thus the likely target of EC Dll4 Studies using an *in vitro* endothelial-sprouting assay, demonstrated that expression of constitutively-active Notch4 in human dermal microvascular endothelial cells inhibited endothelial sprouting (MacKenzie et al , 2004a) Moreover, Notch4 inhibited VEGF-induced angiogenesis in the chick chorioallantoic membrane *in vivo* and inhibited EC proliferation and migration through collagen but not fibrinogen (MacKenzie et al , 2004a) Furthermore, activation of β1-integrins is sufficient to inhibit VEGF-induced endothelial sprouting *in vitro* and angiogenesis *in vivo* suggesting that constitutive Notch4 activation in ECs inhibits angiogenesis, in part, by promoting β1-integrin-mediated adhesion to the underlying matrix (Leong et al , 2002)

In contrast, studies demonstrated that Notch1-expressing endothelial cell cultures formed cord-like structures on Matrigel in contrast to cells expressing a dominant-negative form of Notch1, emphasizing the relevance of the Notch1 pathway in vessel assembly (Soares et al , 2004) In EC that sprout from the surface of beads embedded in fibrin gels following stimulation with fibroblast-derived factors, Notch1, Notch4, and Dll4, as well as the downstream Notch effectors are robustly expressed (Nakatsu et al , 2003) reinforcing the pivotal role Notch plays in guiding EC during angiogenic sprouting Similar studies have shown that constitutive activation of Notch signalling stabilizes network formation of ECs on Matrigel and enhances formation of vessel-like structures in a three-dimensional angiogenesis model, whereas blocking Notch signalling can partially inhibit network formation (Liu et al , 2003b) Thus, results are inconclusive regarding the exact role of Notch signalling in vessel formation and stabilisation, and require further work to determine which transcription factors are activated by each NotchIC, in response to specific stimuli

NotchIC decreased in BRPs exposed to high pulsatile flow, concomitant with decreased proliferation, suggesting BRP NotchIC induces a pro-proliferative response Recent reports from our laboratory demonstrate that overexpression of either Notch1IC or Notch3IC resulted in enhanced VSMC growth, and inhibition of VSMC apoptosis and transmigration (Sweeney et al , 2004) Interestingly, pericytes at the tip of growing vessels in the retinal vasculature also express Notch3 (Claxton and Fruttiger, 2004), suggesting Notch3 may induce pericyte proliferation required during new vessel formation Previous studies have reported that Notch3 receptor signalling in VSMCs can promote proliferation by inhibiting the expression of p27KIP1, a critical cell cycle inhibitor, and can promote survival through induction of c-FLIP, a well-established anti-apoptotic mediator (Campos et al , 2002) In addition, Notch3 receptor expression is increased in neointimal formation in the rat balloon injury model, implying a role of Notch3 in VSMC proliferation (Campos et al , 2002, Wang et al , 2002b) Future studies are required to elucidate the proliferative signalling pathway in BRPs exposed to flow.

Exposure of BREC/BRP co-cultures to pulsatile flow led us to postulate why BREC increase NotchIC in response to increases in flow, yet NotchIC in BRPs decreases One possible reason for the differential response to flow could be due to EC growth in monolayers, whereas BRP do not form a monolayer EC monolayer growth would permit Jagged ligand binding to Notch receptors on adjacent cells, however this would not occur in BRPs Interestingly, antisense oligonucleotides directed against Jagged1 enhance FGF-2-induced endothelial tube formation in a collagen gel assay (Zimrin et al , 1996), suggesting Jagged-Notch binding is responsible for inhibiting EC proliferation Future studies should address whether increased BREC NotchIC at high flow is due to increased Jagged expression Additionally, analysis of Delta ligand expression may potentially explain NotchIC increases at high flow in ECs only, as Delta ligand expression is EC-specific

Retinal angiogensis is largely regulated by hypoxic stimuli, which presumably destabilise a 'normal' quiescent vessel The principle hypoxia mediators are hypoxia-inducible factors (HIF-1), which bind hypoxia response elements (HREs) in promoter sequences In normoxia and hyperoxia, hydroxylation of HIF-1 residues enables its capture by a ubiquitin ligase complex which directs it to the proteasome for destruction Under hypoxic conditions, HIF-1α is not hydroxylated, escapes ubiquitination, accumulates, and directs pro-angiogenic gene expression (Gutteridge, 2000) The promoter regions of several genes known to regulate angiogenesis have HRE sites, such as VEGF and VEGFR1 VEGFR2, which does not have any HRE sites, is also upregulated by angiogensis, presumably directly by VEGF In DR, VEGFA acting as an EC survival factor may be increased in preclinical DR as a mechanism to maintain the integrity of the vascular bed Intracellular 'pseudohypoxia', due to increased glucose and advanced glycation endproducts (AGEs) increases VEGF expression *in vitro* (Lu et al , 1998) In the later stages of the pathology, high VEGFA production in ischaemic areas where VEGFR2 is upregulated, then leads to well-known signs of DR, *i e* vascular leakage and neovascularisation

Whilst this study has provided evidence of the expression and activity of the Notch and Hedgehog signalling in BRECs/BRPs, and has contributed to the understanding of the regulation of BRECs/BRPs apoptosis, it also poses a considerable number of questions Specifically, the role of VEGF, hypoxia and glucose on Notch/Hedgehog signalling in EC/pericyte co-cultures remain unclear Analysis of VEGF receptor levels in each cell type may provide some clues to angiogenic mechanisms in a flow environment Several complications arise however Hedgehog activates VEGF in several cell types, yet Hedgehog and VEGF may not solely signal in a cell-autonomous manner, as such, co-culture experiments may involve BRECs/BRPs 'cross-talk' via VEGF and/or Hedgehog Moreover, VEGF can also signal independent of VEGFR1 and VEGFR2 In addition, Nagase *et al* demonstrate that SHh is responsible for angiogenesis in the neural tube via AngII and independently of VEGF (Nagase et al , 2005) Indeed, preliminary results in our laboratory demonstrate AngII can transactivate BREC Notch gene expression (Dr C Sweeney – personal communication) Therefore, the potential of SHh induction of Notch, independent of VEGF, needs to be examined

Evidence now suggests that retinal endothelial and pericyte cells also interact with another cell type during new vessel formation Primordial vessels are preceded by a plexus of glial cells, namely astrocytes Sinor *et al* (1998) demonstrated hypoxia upregulates VEGF expression and release in astrocytes (Sinor et al , 1998), however the role of astrocytes in modulating the angiogenic sprout has not yet been defined BREC, BRPs, and astrocyte exposed to pulsatile flow in a commercially available tri-culture CELLMAX™ flow system, may provide some clues to interactions between these cells and how the angiogenic vessels form

Another potential area of research might examine utilisation of novel sources of ECs in new vessel formation, such as recruitment of circulating stem cells or redeployment of mural cells from regressing vessels Indeed Claxton *et al* demonstrated two distinct populations of ECs were present in the developing vasculature, only half of which were Dll4 positive (Claxton and Fruttiger, 2004) Recent studies by Lee *et al* (2005) found circulating endothelial precursor cells were elevated in non-proliferative & proliferative Diabetic Retinopathy groups

compared to controls Similarly vasculogenesis-related progenitor cells are increased in coronary heart diseases (Shintani et al , 2001) Additionally, circulating stem cells systematically administered to adult mice participate in experimental retinal neovascularisation (Grant et al , 2002, Grant et al , 2003) Taken together, these results suggest ischaemia may somehow mobilise a systemic factor to signal to bone marrow cells for recruitment of progenitor cells

Other angiogenic pathways currently generating a lot of interest, are those involving Id (inhibitor of differentiation/DNA synthesis) proteins They are members of the basic HLH family of transcription factors, however they lack a DNA binding domain (Kadesch, 1993) By binding to basic HLH transcription factors, Id proteins regulate gene expression and regulate cell growth and differentiation in embryonic and adult tissues (Ouyang et al , 2002, Kreider et al , 1992) Notch target genes are basic HLH transcription factors and thus are potentially modulated by Id proteins Elevated Id-1 expression either at transcriptional or translational levels has been reported in over 20 types of human cancer including prostate, breast, cervical, colon, liver cancers (Lee et al , 2005, Ling et al , 2003) Furthermore, ectopic expression of Id-1 is able to promote cancer cell proliferation and inhibit apoptosis Recent reports demonstrated upregulation of Id-2 expression in response to hypoxia, with two functional HIF-1 binding sites identified in the Id-2 promoter region (Lofstedt et al , 2004) Furthermore, using microarray technology, our laboratory demonstrated that retinal SMCs increase Id-2 in response to hypoxia (Dr R Kane – personal communication) and Lyden *et al* (1999) indicated that normal Id expression in ECs is required to support tumour angiogenesis Id knockout animals lacked the ability to branch and sprout new tumour vessels (Lyden et al , 1999) Notch signalling in osteoblast cells signal bone-morphogenetic proteins (BMPs) - known activators of Id-1 promoter activity (Nobta et al , 2005) As such, these studies demonstrate a potential interaction between the Notch and Id signalling pathways

A new family of regulators of angiogensis has recently been identified Sprouty Sprouty inhibits signalling mediated by the FGF receptor and the epidermal growth factor (EGF) receptor in *Drosophila* (Casci et al , 1999, Kramer et al , 1999, Reich et al , 1999) and four mammalian genes (Sprouty1–4) have been identified (Tefft et al , 1999) The precise molecular mechanism by which the FGF receptor signal is blocked remains controversial however Recently Kwabi-Addo *et al* have established that decreased *Sprouty1* expression may play a role in prostate cancer Sprouty1 protein is downregulated in approximately 40% of prostate cancers (Kwabi-Addo et al , 2004) Direct evidence for a role for Sprouty in angiogenesis comes from a study in which the mouse Sprouty4 was overexpressed in the developing endothelium of a mouse embryo (Lee et al , 2001) It was found that embryos expressing Sprouty4 had decreased sprouting of smaller vessels from the larger ones In addition, when HUVECs were transfected *in vitro* with Sprouty4, there was a decrease in cell migration and cell cycle arrest at the G1/S phase with no apoptosis (Lee et al , 2001) Sprouty has also been shown to interact with Frizzled (Strutt and Strutt, 2003), a receptor of the Wnt family of molecules Furthermore, Frizzled has recently been shown to inhibit Notch signalling (Strutt, 2002), suggesting Sprouty and Notch pathways communicate Additionally, suggestions of Hedgehog – Sprouty interactions were demonstrated by co-localisation studies (Warburton et al , 2001)

Though the work in this thesis examined two seemingly disparate cell signalling mechanisms – Vasoactive regulation by flow and Notch/Hedgehog signalling – it is unlikely these pathways signal independently of one another in either BRECs or BRPs For example, recent evidence demonstrated that NO induces Notch1 expression in mouse cholangiocytes concomitant with decreased apoptosis (Ishimura et al , 2005) In addition, VEGF, which is intrinsically linked to Notch signalling, stimulates release of NO and prostacyclin In ECs, NO has been shown to signal the actions of VEGF in increasing cell proliferation, migration, and permeability (Leibovich et al , 1994, Papapetropoulos et al , 1997, Ziche et al , 1997), suggesting a role in vascular growth and remodelling Interestingly, VEGF

activates EC via VEGFR2 on the cell surface, however VEGFR2 is also a key mechanotransducer that activates eNOS in response to blood flow (Jin et al , 2003) Additionally, both NO and VEGF increase EC GLUT1 (Sone et al , 2000), a membrane-bound glucose transporter, thus increasing glucose uptake and possibly EC damage in DR Furthermore, studies by Lakshminarayanan *et al* indicate that VEGF elevates hydraulic conductivity in BRECs through signalling mechanisms involving NO (Lakshminarayanan et al , 2000a)

There is also a distinct relationship between VEGF and the prostaglandin-cyclooxygenase system Recent evidence suggests cyclooxygenase-2 modulates angiogenesis by interacting with the VEGF system (He et al , 1999, Gliki et al , 2001) Furthermore, Lipocalin-type prostaglandin D synthase (L-PGDS), a gene involved in the prostanoid biosynthesis, is a target of Notch signalling in rat leptomeningeal cells (Fujimori et al , 2003), though involvement of VEGF in this process is not yet clear Moreover, prostaglandin-D2 synthase is present in the vitreous humor of DR patients (Yamane et al , 2003) Lastly, the stimulatory interaction between VEGF and ET-1 on each other's gene expression in vascular endothelial cells and smooth muscle cells has also been demonstrated (Matsuura et al , 1998) Co-culture of BAECs and VSMCs resulted in enhanced gene expression in these cells of ET-1 and VEGF, respectively This interaction may play an important role in cardiovascular disorders characterised by disruption of vascular cell proliferation, as both ET-1 and VEGF are potent mitogens

In conclusion, advancing our understanding of retinal blood flow autoregulation via changes in microvascular endothelial cell function and the subsequent interaction with the retinal pericyte will be of crucial importance to the understanding and origin of events within the eye that lead to ocular diseases such as glaucoma and retinopathies Moreover, better understanding of the molecular and functional changes occurring in response to alterations in Notch and Hedgehog signalling in response to flow may lead to strategies and potential targets to modulate blood flow in disease

Chapter 8

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