Hemodynamic Regulation of
Metalloendopeptidases EC3.4.24.15 and
EC3.4.24.16; Expression and Function in
the Vascular Endothelium

A Dissertation Submitted for the degree of
Doctor of Philosophy

By

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Under the Supervision of Dr. Philip M. Cummins

June 2005

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Declaration

I hereby declare that this material which I now submit for the assessment on the program of study leading to the award of degree 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.

Signed

Date}

Student No. 77306223
Acknowledgments

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Abstract

Hemodynamic forces, namely shear stress and cyclic strain, have been well characterised as modulators of vascular endothelial function, and have been assigned an important role in the maintenance of vascular tone, haemostasis, and regulation of vascular growth and health. They exert their influence in part by effecting changes in the production and release of vasoactive compounds by the endothelium, and by effecting changes in the levels and activity of various enzymes. Thimet oligopeptidase (EC3.4.24.15, EP24.15) and neurolysin (EC3.4.24.16, EP24.15) are closely related zinc metalloendopeptidases that have been shown to be expressed and active in the vascular endothelium. Their substrates include the vasoactive peptides bradykinin and angiotensin I, which have been identified as important regulators of both blood pressure and angiogenic processes. Other related peptidases, namely endothelin converting enzyme (ECE) and angiotensin converting enzyme (ACE), have been shown to be regulated by hemodynamic forces in the vascular endothelium. As such EP24.15 and EP24.16 are likely candidates for regulation by hemodynamic forces.

In this regard we have investigated the effect of cyclic strain on the expression and activity of EP24.15 and EP24.16 in cultured bovine aortic endothelial cells (BAECs). We have shown that exposure to cyclic strain significantly increases the mRNA expression as well as both the cellular and secreted activity of both enzymes. We have demonstrated that up-regulation of both enzymes is dependent on Gi-protein mediated signalling, although with varying Giα/Gβγ subunit specificity for either enzyme. Using
immunocytochemistry, we have also demonstrated a strain-dependent increase in EP24.15 protein expression within the nucleus and cytoplasm in parallel with an increase in membrane associated EP 24.15.

The effects of strain on the ability of BAECs in culture to cleave both Ang I, and BK in an EC24.15/EC24.16 specific manner was also studied. We observed that exposure to cyclic strain induces a significant increase in the EP24.15 specific hydrolysis of both exogenously added BK and Ang I.

The potential of the observed effects of cyclic strain on EP24.15 to effect changes in endothelial cell function were also examined. Use of the dual EP24.15/EP24.16 inhibitor, cFP-AAF-pAB, and the EP24.15 specific antisense, FLIP, was seen to significantly attenuate cyclic strain-induced endothelial cell tubule formation and migration. We also found that the effects of FLIP transfection on cyclic strain-induced endothelial cell tubule formation could be largely reversed by addition of exogenous Ang-(1-7). Taken together these results suggest that strain-induced endothelial cell angiogenesis and migration putatively involves EP24.15 cleavage of vasoactive peptide substrates.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>ADAM</td>
<td>A Disintegrin and Metalloproteinase</td>
</tr>
<tr>
<td>ANG I</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>ANG II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANG 1-7</td>
<td>Angiotensin 1-7</td>
</tr>
<tr>
<td>AP 1</td>
<td>Activating Protein 1</td>
</tr>
<tr>
<td>AP 2</td>
<td>Activating Protein 2</td>
</tr>
<tr>
<td>BAEC</td>
<td>Bovine Aortic Endothelial Cell</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>BK 1-5</td>
<td>Bradykinin 1-5</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy Nucleic Acid</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECE</td>
<td>Endothelin Converting Enzyme</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetracetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Regulated Kinase</td>
</tr>
</tbody>
</table>
ET-1  Endothelin
FAK  Focal Adhesion Kinase
FBS  Fetal Bovine Serum
FGF  Fibroblast Growth Factor
FGFR  Fibroblast Growth Factor Receptor
GAPDH  Glyceraldehyde Phosphate Dehydrogenase
GFP  Green Fluorescent Protein
GPCR  G-protein Coupled Receptor
GnRH  Gonadotrophin Releasing Hormone
Grb-2  Growth Factor Binding Protein 2
HBSS  Hanks Buffered Saline Solution
HPF  High Powered Field
HPLC  High Performance Liquid Chromatography
ICAM-1  Intracellular Adhesion Molecule-1
iNOS  Inducible Nitric Oxide Synthase
JNK  c-Jun-Terminal Kinases
LB  Luria Bertrani
LDH  Lactate Dehydrogenase
MAPK  Mitogen Activated Protein Kinase
MAPKK  Mitogen Activated Protein Kinase Kinase
MAPKKK  Mitogen Activated Protein Kinase Kinase Kinase
MCP-1  Monocyte Chemoattractant Protein 1
MMP  Matrix Metalloproteinase
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NAPD(P)</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein Tyrosine Kinase</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>QFS</td>
<td>Quenched Fluorescent Substrate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
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</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>SAPK</td>
<td>Stress Activated Protein Kinase</td>
</tr>
<tr>
<td>Shc</td>
<td>Src Homology/Collagen</td>
</tr>
<tr>
<td>SiRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>SSRE</td>
<td>Shear Stress Response Element</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>THOP</td>
<td>Thimet Oligopeptidase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinases</td>
</tr>
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<td>TNF-α</td>
<td>Tumour Necrosis Factor α</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue Plasminogen Activator</td>
</tr>
<tr>
<td>u-PA</td>
<td>Urokinase Plasminogen Activator</td>
</tr>
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<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>Symbol</td>
<td>Unit</td>
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</tr>
<tr>
<td>Bp</td>
<td>Base Pairs</td>
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<tr>
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<td>Centimeters</td>
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<tr>
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<td>Grams</td>
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<td>Hours</td>
</tr>
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<td>Kilodaltons</td>
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<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Miligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Mililitre</td>
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<tr>
<td>mM</td>
<td>Milimolar</td>
</tr>
<tr>
<td>mm</td>
<td>Milimetre</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolution Per Minute</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>U</td>
<td>Enzyme Units</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
</tbody>
</table>

X
\( \mu m \)  Micrometre

\( \mu M \)  Micromolar

v/v  Volume per Volume

w/v  Weight per Volume
Publications


Cummins P.M., Cotter E.J., Cahill P.A. Hemodynamic regulation of metallopeptidases within the vasculature. Protein Pept Lett. 2004 Oct;11(5):433-42. Review


Posters


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The vascular endothelium, once simply considered a physiologically inert lining of the blood vessel, is both a complex multifunctional organ in its own right and an essential component of every other organ in the body. The endothelial monolayer forms a lining of the entire inner surface of the vascular system, and has been shown to act as a dynamic interface between the circulating blood and the underlying tissues. Its location, at the interface between the circulating blood and the vessel wall, allows it to monitor and respond to changes in blood flow and biochemical composition. It is for this reason that it plays an important role in the maintenance of vascular tone in response to changes in blood flow and pressure and in the progression of diseases such as hypertension and atherosclerosis. The endothelium also provides a selective permeable blood brain barrier and is involved in the regulation of hemostasis and the inflammatory response, while endothelial cell migration, proliferation and tube formation are essential for the growth of new vessels (Angiogenesis).

The endothelial cell can be activated by both physical and chemical stimuli; it can respond to changes in both shear stress and pulsatile cyclic strain, the hemodynamic forces associated with blood flow, as well as a variety of so-called vasoactive peptides (e.g. angiotensin, endothelin I), reactive oxygen species (ROS), and nitric oxide (NO). Activation of the endothelium can result in the expression of specific genes, cell migration and proliferation, and the release of various substances, which can in turn alter vascular function (Duffy et al., 2004).
It is the purpose of this part of the introduction to give an overview of the structure of the vascular endothelium, and the means by which it responds to changes in blood flow and composition.

1.1.1 Organisation and Structure

The vascular endothelium is composed of a monolayer of endothelial cells, which have a luminal surface constantly in contact with the circulating blood, an abluminal surface which interacts with the sub-endothelial matrix composed of pericytes and smooth muscle cells, and a lateral surface, adjacent to the surrounding cells which provides an area for cell-cell bridging.

The luminal surface provides an interface between the circulating blood and the vascular endothelial cell. The presence of multiple surface receptors and adhesion molecules allow the stimulation of second messenger molecules, leading to changes at the level of gene expression and cellular function. Vascular permeability, coagulation, and the inflammatory response are governed by stimulation of the luminal surface, as are changes in vascular endothelial cell fate decisions regulating these functions.

The abluminal surface rests upon the subendothelium, an extracellular matrix composed of proteins, including collagen type IV, elastin, laminin, fibronectin, thombospondetin, and von Willebrand factor (vWF), which are predominantly secreted by the abluminal surface of the endothelial cell (see Fig 1.1) (Duffy et al., 2004)
The lateral surfaces of the endothelial cell provide an area for cell-to-cell bridging, with intercellular clefts, approximately 6-7 nm wide, forming between adjacent cells (Guyton et al., 1996). Bridging the cells and thus joining adjacent endothelial cells together is a specialized complex consisting of gap junctions, demosomes, adherens junctions and the tight junctions. Adherens and tight junctions provide contact with the actin cytoskeleton, allowing changes in the shape and size of the cell to occur, and providing adhesive intercellular connections which strengthen the endothelial barrier and affect cell polarity (Dejana et al., 1995). They form a selectively permeable barrier, allowing the passage of certain solutes, with their selectivity also varying with

Figure 1.1 Diagram representing the structure of the vascular endothelium showing molecules involved in secretion, cell-cell interaction, and in the endothelial cell cytoskeleton.
pathological and physiological changes (Balda et al., 1998; Hunt et al., 2002). Their distribution varies along the vascular tree, showing a greater frequency in larger arteries, which require greater permeability control (Duffy et al., 2004). Gap and adherans junctions also provide contact points for cell-cell interaction both between adjacent endothelial cell and the pericytes and intimal smooth muscle cells of the subendothelial matrix (Baldra et al., 1998, Duffy et al., 2004).

The entire surface of the endothelial cells is coated with a large number of glycoproteins, who’s asymmetric, cell-surface specific, distribution accounts for the polarity exhibited by the endothelium. The luminal glycocalyx lining also contains a layer of immobile plasma and is thought to adsorb plasma proteins (Copley et al., 1983). The resulting combination of glycocalyx, plasma, and plasma proteins is known as the endothelial surface layer. This endothelial surface layer is thought to help regulate a number of physiological responses, such as shear stress mechanotransduction, oxygen diffusion across capillaries, capillary permeability, coagulation and interaction with immune cells (Davies et al., 1997; Secomb et al., 1999; Henry et al., 1997; Linjen et al., 1997; Silvestro et al., 1994).

Intercellular tethering forces of cell-cell and cell-matrix junctions exist in equilibrium with the intracellular contractile forces generated and maintained by the actin-myosin interactions. Constitutive tension exists within the cell with a basal actin-myosin interaction, which allows the cell to be poised to respond to changes in its biological environment. Stimulation of the endothelial cell due to changes in
haemodynamic forces, or disease, can lead to an alteration of cell shape due to a reorganization of contractile proteins. Changes in endothelial cell morphology are associated with a number of disease states such as atherosclerosis (see section 1.2.2.4) (Garcia et al., 1995).

The endothelial cell membrane also possesses a large number of flask shaped luminal invaginations, generally 50-100 nm in diameter, which are known as caveolae. Caveolae are involved in the endocytosis and transcytosis of macromolecules. Caveolae are suspected to be a site of plasminogen activation by urokinase and tissue plasminogen activator (uPA and tPA) (Anderson et al., 1993). They also have been implicated in the direct transduction of luminal forces to the cytoskeleton through actin-associated signaling molecules. In addition two key structural components of caveolae, calmodulin and caveolin, are known to regulate the activity of endothelial nitric oxide synthase (eNOS) (Duffy et al., 2004).

1.1.2 Functions of the Endothelium

The endothelial cells internal structure, location and intercellular arrangement allows it to respond to a wide variety of stimuli and allows it to fill a number of unique roles within the vasculature, namely the maintenance of a selectively permeable blood tissue barrier, regulation of hemostasis, regulation of inflammation and maintenance of vascular tone. It is intended to give a brief overview of each of the above-mentioned
functions with particular reference to role of hemodynamic forces in the maintenance of vascular tone in this section.

1.1.2.1 Selectively Permeable Barrier

With the larger vessels serving the role of major conduits, the majority of transfer of water, gases and solutes to and from the circulation occurs primarily across the capillaries and post-capillary venules. The vascular endothelium forms a semi-permeable barrier that selectively allows specific substances to move between the blood and the interstitium.

Low molecular weight hydrophilic and lipid soluble molecules such as CO$_2$ and O$_2$ move virtually unimpeded through the lateral clefts of the endothelium. The endothelium maintains its selective barrier to macromolecules through several distinct mechanisms (Garcia et al., 1995); firstly, the negatively charged glycocalyx provides an electrostatic repellant, preventing negatively charged macromolecules from crossing the endothelium; secondly, there is evidence to suggest serum albumin can cross the endothelium via a vesicular transport system; and thirdly, the tight junction and adherens junction proteins govern the size of the intercellular cleft and therefore solute permeability (Lui et al., 1997).

In addition some capillaries, such as the renal glomerular tufts, contain fenestrations up to 100 nm wide, which increase the passage of water across the
endothelium without affecting transendothelial transport of macromolecules. Also in some regions of the vasculature, such as the bone marrow, liver and spleen, the endothelial lining of the vessel is discontinuous allowing for the free passage of solutes (Wisse et al., 1983).

Barrier function is maintained by the regulated apposition of tight junction and adherans protein complexes between adjacent endothelial cells. The organization of these protein complexes is controlled by a number of physiological and pharmacological mediators. The proteins that form tight junctions linking adjacent cells include occludin, claudin family members, junctional adhesion molecules 1-3 (JAMs), cingulin, 7H6, spectrin and linker proteins such as the zonula occludins family members (ZO-1/2/3), the latter linking these proteins to each other and the actin cytoskeleton (Alexander et al., 2002; Furuse et al., 1993; Furuse et al., 1993; Gardner et al., 1996; Hirase et al., 1997). Adherans junctions contain trans-membrane cadherins and their cytoskeletal linkers, catenins (Daniel et al., 1997; Kaibuchi et al., 1999). Although spatially and biochemically distinct, functional interaction between adherans junctions and tight junctions has been demonstrated (Rubin et al., 1999).

The barrier function of the endothelium can vary in disease states, with increased micro-vascular permeability to macromolecules and water being the basis of edematous tissue injury and a hallmark of inflammation and atherosclerosis (Duffy et al., 2004). Hemodynamic forces can also lead to changes in vessel permeability (see section 1.2).
The majority of this increased transport appears to be paracellular, i.e. due to widening of the inter-endothelial junctions (Garcia et al., 1995). This widening can be either leukocyte independent or dependent. Leukocyte independent widening is thought to reflect contraction of the cytoskeleton and recoil of the cell-cell borders. Leukocyte dependent widening is associated with increased display of luminal selectins, which leads to greater recruitment of leukocytes on the luminal surface. It is suspected that leukocyte proteases such as elastase and metalloproteinases may break down junction elements, allowing leukocyte infiltration to the subendothelial matrix and damaging the overall endothelial barrier function (Alexander et al., 2002; Hunt et al., 2002).

1.1.2.2 Hemostasis

Hemostasis is defined as the maintenance of a balance between pro- and anticoagulant forces in the circulation. A dynamic ongoing interaction exists between the endothelium, blood cells, plasma coagulation factors, fibrinolytic factors, and their inhibitors. In its normal state the endothelium favours anticoagulant mechanisms, which preserve blood fluidity. Firstly the endothelial cell monolayer provides a smooth covering which prevents the activation of the clotting cascade by the subendothelial matrix. In addition the negatively charged glycocalyx layer can repel coagulation factors and platelets in the circulation (Duffy et al., 2004).

Hemodynamic forces also play an important role in hemostasis. The maintenance of normal blood flow, through the maintenance of vascular tone, allows activated products to be carried away from the local area, eliminating an environment conducive to
thrombosis. Endothelium derived products such as, prostaglandins, NO, and tissue factor pathway inhibitor (TFPI) also favour anticoagulation (see section 1.1.2.4 and 1.2) (Bunting *et al.*, 1976; Marcum *et al.*, 1985).

The membrane protein thrombomodulin, constitutively expressed by the endothelial cells of most vascular beds, can dramatically inhibit the catalytic activity of thrombin, a pro-coagulant protease, by removing it from circulation and concentrating it at the endothelial cell surface where it is internalized and degraded (Heeb *et al.*, 1994). The anticoagulant activity of the endothelium includes fibrinolysis as well as inhibition of platelets and serine proteases. Inactive plasminogen can be converted to plasmin by the plasminogen activators, tPA and uPA. The activated plasmin then degrades fibrin into fibrin degradation products (Levine *et al.*, 1982). The formation of a fibrin clot is the end product of the thrombogenic pathway. Destruction of the endothelial lining allows direct contact between the circulating platelets and the subendothelial collagen. This leads to primary hemostasis, the stimulation of platelet adherence and aggregation. Activated serine proteases then initiate a cascade of reactions, which result in fibrin formation (secondary hemostasis). The resultant fibrin clot, in normal conditions, combines with transient vasoconstriction to reduce blood flow, allowing endothelial repair (Duffy *et al* 2004; Hunt *et al* 2002).

**1.1.2.3 Inflammation**

Injury to the vascular endothelium not only stimulates hemostatic events, but also leads to a localized inflammatory response in order to potentiate the healing process.
Chemotactic mediators, which are released from damaged tissue, white blood cells and pathogens initiate the response, which leads to an increase in the recruitment and adhesion of leukocytes to the endothelial surface. This leukocyte recruitment occurs via adhesion molecules expressed on both leukocyte and endothelium such as selectins located on the endothelium, integrins primarily located on endothelial cells, and immunoglobulins primarily located on endothelial cells (Duffy et al., 2004).

In the initial stages of adhesion leukocytes roll rapidly along the endothelial surface, interacting with the endothelial cells via P-selectin and E-selectin on endothelial cells and L-selectin on leukocytes (Geng et al., 1990; Bevilacqua et al., 1987). Leukocytes that are not swept away by the shear stress resulting from the blood flow become activated by signals either originating either from the luminal or extravascular space, or at the endothelial surface. Activation leads to an increased affinity for endothelial ligands of the immunoglobulin superfamily (IgSF), the vascular cell adhesion molecule (VCAM-1), and the intercellular adhesion molecules 1 and 2 (ICAM 1 & 2) (Pober et al., 1986). Interaction between the leukocyte and the IgSF leads to initiation of leukocyte spreading, followed by interaction between platelet-endothelial adhesion molecules located on both endothelial cells and leukocytes, leading to the trans-endothelial migration of the leukocyte to the sub-endothelial matrix.

1.1.2.4 Maintenance of Vascular Tone

Vascular tone is one of the main determinants of blood flow. The ability of the vessel to respond to changes in the microenvironment to maintain optimal flow is a result
of a finely tuned balance of vasodilator and vasoconstrictor stimuli. Systemic control of vascular tone is directed and maintained by the nervous system via the smooth muscle layer which makes up the bulk of the blood vessel wall, however the specific needs of individual vascular beds are directed by local mediators produced in the endothelium, for example prostacyclin (PGI₂), nitric oxide (NO), Endothelin 1 (ET-1), Bradykinin (BK), and Angiotensin I (Ang-I) (Duffy et al., 2004; Hughes, 2002).

Prostacyclin (PGI₂) is a member of the prostacyclin family of lipid mediators. PGI₂, as with other prostaglandins, is a 20-carbon unsaturated carboxylic acid with a cyclopentane ring. Within the vasculature it is produced in endothelial cells from arachnadonic acid by two constitutively expressed enzymes, prostacyclin synthase and cyclooxygenase-1 (COX-1) (Weksler et al., 1977). Circulating PGI₂ has an antithrombotic effect, via interaction with its IP receptor on platelets. Interaction with the IP receptor on smooth muscle cells leads to vasodilation. PGI₂ production increases in response to hypoxia, histamine, thrombin and hemodynamic forces (Baenziger et al., 1980; Duffy et al., 2004; Vane et al., 2003).

Nitric oxide is produced constitutively at a low level in the endothelium by calcium dependent endothelial nitric oxide synthase (eNOS), from molecular oxygen and L-arginine. Like PGI₂ it acts primarily as a vasodilator, causing local smooth muscle relaxation. An increase in NO production occurs in response to BK, thrombin, histamine, vasopressin, serotonin, and hemodynamic forces. An additional source of NO in the endothelium results from the actions of inducible nitric oxide synthase (iNOS), which is
expressed in response to inflammatory cytokines, and unlike eNOS, is non-calcium dependent (Duffy et al. 2004; Hughes 2002).

Endothelin, as its name suggests is predominantly found in the vascular endothelium, though it is also present in tissues such as the kidney. It is the most potent vasoconstrictor peptide known. The predominant isoform of endothelin in the vasculature is Endothelin-1. Its is produced as preproendothelin-1 (ppET-1), which is converted to Big-Endothelin 1, which is in turn converted to Endothelin-1 (ET-1) by the endothelin converting enzyme (ECE). Big-Endothelin 1 is approximately 100 times less potent a vasoconstrictor than ET-1, and its rapid extracellular conversion in conditions such as heart failure may provide biologically significant quantities of ET1. Two receptors of ET-1 exist within the vasculature; ET\textsubscript{B} is found predominantly on endothelial cells, while ET\textsubscript{A} predominates in vascular smooth muscle cells. Its is though the ET\textsubscript{A} receptor that ET-1 causes vasoconstriction, while its interaction with the ET\textsubscript{B} receptor leads to release of NO and PGI\textsubscript{2} and consequent vasodilation (Levin 1995). The expression of ppET-1 mRNA and release of ET-1 are stimulated by interleukin-1, Ang-II, hypoxia, and hemodynamic forces (Yoshiumi et al., 1989; Yoshizumi et al., 1990; Hughes 2002; Levin, 1995).

Angiotensin (Ang-II) is produced primarily from Ang-I (Ang-I) by the angiotensin converting enzyme (ACE). Although Ang-I has only slight vasoconstrictive properties, Ang-II is a potent vasoconstrictor, mediating the release of ET-1 though the AT1 receptor (Duffy et al., 2004). The inhibition Ang-II mediated vasoconstriction, either through blockade of the AT1 receptor or inhibition of ACE, has been exploited as a
successful clinic treatment for hypertension (Randall et al., 2003; Bakris et al., 2001). As will be discussed later Ang-II also plays an important, though not yet fully elucidated role in other important vascular endothelial processes, such as migration and angiogenesis. In addition angiotensin (1-7) (Ang-(1-7), the product of cleavage of Ang-I by metalloendopeptidases such as thimet oligopeptidase (EP24.15), neurolysin (EP24.16) and neutral endopeptidase (NEP). Numerous studies implicate Ang-(1-7) as acting in an opposing fashion to that of Ang-II, evoking vasodilation through release of NO. (Fetterik et al., 2000; Duffy et al., 2004; Turner et al., 2002; Ferrario et al., 1998). It has also been shown by Chappell and co-workers, that infusion of Ang-(1-7) considerably potentiates the antihypertensive effects of ACE inhibition and AT1 receptor blockade in the rat hindlimb (Chappell et al., 2000).

Bradykinin (BK) is a nine amino acid peptide which acts as a vasodilator by activating eNOS via the B2 receptor in endothelial cells. It can also cause contraction in smooth muscle cells, and is associated with the induction of pain and the recruitment of inflammatory cells. Like Ang-II it is believed to influence endothelial cell migration and angiogenesis. BK is cleaved to a number of inactive fragments by EP24.15, EP24.16, ACE, and NEP (Duffy et al., 2004; Meini et al., 2004; Venema et al., 2002).

Hemodynamic forces, namely shear stress and cyclic strain are, as has been mentioned above, important stimuli for the production of numerous vasoactive substances. It is intended, in the following section to give an overview of the
hemodynamic forces and the mechanisms by which endothelial cells transduce these stimuli.

1.2 Hemodynamic Forces and the Endothelium

Hemodynamic forces have been well established as important modulators of vascular tone and vascular remodeling. The interplay between hemodynamic forces and endothelial cell function also plays a critical role in a number of diseases, most importantly atherosclerosis.

Figure 1.2(a); Laminar Shear Stress occurs as a result of the frictional forces exerted on the vessel wall by blood flow. (b); Pulsatile cyclic strain is the deformation of the vessel wall in a direction perpendicular to blood flow which occurs due to the pulsatile nature of blood flow.

There are two major components of hemodynamic force; wall shear stress, resulting from the drag force exerted on the endothelium by the flowing blood, and cyclic mechanical strain or stretch, which occurs due to the pulsatile nature of the blood flow (see Fig 1.2 a, b). In healthy tissue, alterations in the magnitude of cyclic strain or shear stress invariably produce transformations in the vessel wall that accommodate the new
conditions and ultimately restore basal levels of blood pressure. (Lehoux et al., 1998; Topper et al.; 1999; Poston, 2002).

1.2.1 Cyclic Strain

1.2.1.1 Effects of Cyclic Strain on the Vessel Wall

The relationship between circumferential (cyclic) stress and the vessel wall has been extensively studied. It has long been known that blood pressure is the determining factor in vessel wall thickness; for example the comparison between the pulmonary artery and the aorta. Both have walls of a similar thickness in utero as they experience similar blood pressure. However after birth the aorta thickens with increasing blood pressure while the pulmonary artery undergoes atrophy due to a relative fall in blood pressure (Leung et al., 1977). It has also been observed that increases in blood pressure can lead to an increase in smooth muscle cell (SMC) size (hypertrophy) and increase in extracellular matrix (ECM) production, with decreases in blood pressure conversely leading to vessel atrophy (Bomberger et al., 1980). A normal level of mechanical straining of VSMCs may also be essential for the maintenance of their differentiated state; in vitro studies using cultured rabbit aortas showed that an abnormally low level of intraluminal pressure (10 mm Hg) over a period of 3-6 days led to a decrease in smooth muscle marker proteins h-caldesmon and filamin in the vessel wall. This was despite the presence of fetal calf serum, a noted mitogen. This decrease in marker proteins was reversed in aortic segments kept at physiological intraluminal pressure (80 mm Hg). In segments maintained at high
intraluminal pressures (150mm Hg) there was an overall increase in protein production (Lehoux et al., 1998).

Cyclic strain is a powerful stimulus and can regulate cell fate decisions both in endothelial and smooth muscle cells. 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).

1.2.1.2 Effects of Cyclic Strain on the Endothelium

The significant role of cyclic strain in determining SMC development and structure is coupled with a less obvious, but no less important, effect on the endothelium. Cyclic strain has been shown to induce the alignment of actin microfilaments in the direction of strain, and to modulate the sensitivity of endothelial cells towards shear stress (Zhao et al., 1995). Recent studies have also demonstrated that cyclic strain can induce an increase in the migratory and angiogenic capabilities of endothelial cells in vitro (Von Offenberg-Sweeney et al., 2005).

1.2.1.3 Cyclic Strain as a Modulator of Gene Expression

Mechanical strain is a potent stimulator of gene expression in both ECs and SMCs, as has been demonstrated. DNA microarray analysis with human vascular SMC
showed that COX-1, PAI-1 and tenascin were all un-regulated in response to cyclic strain while matrix metalloproteinase-1 (MMP-1) and thrombomodulin were down-regulated; suggesting a response of defense against excessive deformation (Feng et al., 1999).

Table 1.1 Genes regulated by cyclic strain within the vasculature: eNOS; endothelial Nitric Oxide Synthase, ppET-1; pre-pro-Endothelin-1, COX-1; Cyclic Oxygenase I, COX-II; Cyclic Oxygenase II, PDGF-BB; Platelet Derived Growth Factor-BB, MMP-1, -2, -9, and -14; Matrix Metallopeptidase-1, -2, -9, and -14, MCP-1; Monocyte Chemoattractant Protein, PAI-1; Plasminogen Activator Inhibitor-1, uPA; Urokinase Plasminogen Activator. Up-regulation is indicated by †, and down-regulation by ‡. References: 1; Feng et al., 1999. 2; Awoleski et al., 1995. 3; Cheng et al., 1996. 4; Cheng et al., 1996. 5; de Jonge et al., 2002. 5; Sumpio et al., 1998. 6; Wang et al., 2003. 7; Wung et al., 2001. 8; Wung et al., 2001. 9; Von Offenberg-Sweeney et al., 2004. 10; Von Offenberg-Sweeney et al., 2005.

<table>
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<tr>
<th>Vascular Tone</th>
<th>Growth/Remodling</th>
<th>Adhesion/Chemoattraction</th>
<th>Coagulation</th>
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<tr>
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<td>†MCP-1²</td>
<td>†PAI-1²</td>
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<td>†ppET-1⁵</td>
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<td>†MMP-2⁵</td>
<td>†Thrombomodulin⁷</td>
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<td>†COX-II⁶</td>
<td>†MMP-14⁶</td>
<td>†MMP-9⁶</td>
<td>†uPA¹⁰</td>
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<td>†COX-1¹</td>
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In endothelial cells cyclic strain has been demonstrated to up-regulate the expression of nitric oxide synthase (NOS), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-14 (MMP-14), monocyte chemotactic protein-1 (MCP-1), platelet derived growth factor-BB (PDGF-BB), endothelin-1 (ET-1), intracellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1), matrix metalloproteinase-9 (MMP-9) and urokinase plasminogen activator (uPA) (Awoleski et al., 1995; Cheng et al., 1996; Cheng et al., 1996; de Jonge et al., 2002; Sumpio et al., 1998; Wang et al., 2003; Wung et al., 2001; Von Offenberg-Sweeney et al., 2004; Von...
Offenberg-Sweeney et al., 2005). The release of NO from endothelial cells in response to changes in mechanical strain was one of the first responses identified (Leung et al., 1977; Galis et al., 1998). Other molecules secreted in response to cyclic strain include endopeptidases, Et-1, PAI-1, IL-8 and MCP-1 (Sumpio et al., 1998; Cheng et al., 1996; Okada et al., 1998). Intra-cellularly cyclic strain is responsible for the recruitment of a variety of signaling molecules including the MAPK kinase family. Sumpio and co-workers 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. 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; Li et al., 1999). As will be discussed further, this family of enzymes is involved in activating the transcription of a number of cyclic- and shear strain-induced genes. The various signal transduction mechanisms and downstream effects of these strain induced changes have not yet been fully elucidated but the ability of cyclic strain to influence changes in expression in the endothelium is believed to play a role in a number of pathologies including atherosclerosis, and hypertension.

1.2.1.4 Cyclic Strain and Vascular Health

The effects of cyclic strain on the vessel wall are believed to play a major role in the development of hypertension. The ability of increased intra-luminal pressure, and thus increased cyclic strain, to induce hypertrophy and hyperplasia (increase in cell numbers) in SMCs is believed to be a contributing factor in essential or primary
hypertension; an increase in blood pressure leads to an increase in the medial segment of the vessel, and a consequent reduction in the luminal radius. If one considers the equation $T = \frac{P r}{H}$, where $T$ is the tensile stress of the vessel, in order to maintain normal $T$ in cases of increasing $P$, it is necessary to increase the thickness of the vessel $H$, leading to a reduction in vessel radius, leading to a further increase in $P$ (Lehoux et al., 2003). Even small changes in luminal radius can greatly affect overall blood pressure as flowrate is proportional to vessel radius (Schachter, 2002).

The oxidative state of the blood vessel is a major contributor to vascular remodeling and the progression of disease. Under pathological conditions levels of reactive oxygen species (ROS) have been found to be increased (e.g. from the macrophage foam cells). Moreover the interaction of NO with these ROS such as superoxide may result in the formation peroxynitrite, a highly reactive intermediate found in vulnerable atherosclerotic plaques (Rajagopalan et al., 1996). Cyclic strain has been shown to increase the levels of ROS. ROS are believed to be an important mediator in modulating signaling pathways and gene expression by redox sensitive molecules such as PYK2 (Cheng et al., 2002). Mechanical stretch was found to increase expression of MMP-2 via a mechanism involving reactive oxygen species derived form NAD(P)H oxidase (Grote et al., 2003). Cyclic strain-induced increases in ROS have been linked to the regulation of a number of vasoactive compounds, signaling molecules and transcription factors (Awoleski et al., 1995; Chien et al., 1998; Cheng et al., 1998; Cheng et al., 1996; Wang et al., 2001, Wung et al., 2003).
Cyclic strain has also 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; Vahile et al., 1996; Banai et al., 1994). These and other studies clearly demonstrate the importance of cyclic strain in coordinating and regulating cell function by mediating changes in gene transcription, signaling pathways activation and the release of vasoactive compounds.

1.2.1.5 Modeling of Cyclic Strain In Vitro

Circumferential stress on ECs has been investigated in vitro by applying equibiaxial cyclic stretch to endothelial cells cultured on an elastic membrane mounted in a stretch device, such as that shown in Fig 1.3. In this approach endothelial cells are grown on flexible membranes, which can be precisely deformed by a microprocessor-controlled vacuum, providing equibiaxial tension. This allows the cells to be subjected to defined levels of cyclic strain, in a variety of wave patterns, including those which directly model cardiac output (Banes et al., 1985).

Figure 1.3 In Vitro Cyclic Strain Device; The Flexercell™ Tension Plus™ FX-4000™ system (Flexcell International Corp. - Hillsborough, NC)
1.2.2 Shear Stress

1.2.2.1 Shear Stress and the Endothelium

Shear stress is primarily sensed by the endothelium. The endothelial cell, located at the interface between the blood flow and the vessel wall, acts as an effective biological mechanotransductor which senses the physical stimuli of the shear forces associated with blood flow and converts these to intracellular biochemical signals (Poston et al., 2002).

The nature of the shear stress experienced by endothelial cells is a function of the blood flow patterns throughout the vasculature generated by the cardiac cycle. The typical level of shear stress experienced by the endothelium is in the range 0-50 dynes/cm². In roughly linear areas of the vasculature, blood flows in a smooth pulsatile pattern, subjecting the endothelium to laminar shear stress with fluctuations in magnitude that yields a net positive shear stress. Cells exposed to positive shear stress undergo reorientation and elongation in a direction parallel to the direction of blood flow, leading to a streamlining effect, which decreases the effective resistance and shear stress (Flaherty et al., 1972; Barbee et al., 1995). In areas of the vasculature where the laminar blood-flow pattern is disrupted, such as at branch points and bifurcations, the flow can become reversed and oscillatory resulting in low mean shear stress. In these conditions the endothelial cells do not reorient, and may be exposed to high shear gradients, as there is an absence of the membrane streamlining evident in areas of positive mean shear stress (Davies et al., 1986).
1.2.2.2 Shear Stress as a Modulator of Vascular Tone

It has long been observed that in healthy vessels increases in shear stress is an important regulator of vascular tone; it was first observed in 1933 by Schetzenmayr that the femoral artery in the dog hind limb dilates in response to the stimulus of increased blood flow (Schetzenmayr, 1933). Relaxation due to flow is widely considered to result from the release of NO and to a lesser extent PGI₂, although NO independence has occasionally been reported (Poston, 2002). The involvement of PGI₂ and NO has been demonstrated by the flow-induced synthesis of 6-ketoprostagladin-\(f_{10}\), a stable metabolite of PGI₂ (Heckler et al., 1993), and the fact that inhibitors of NO synthase blunted flow induced vasodilation (Cooke et al., 1990). Shear stress is the most potent physiological stimulus for NO production in endothelial cells. Acute changes in NO production are due to posttranslational activation of eNOS, while chronic alterations in eNOS expression are due to changes in gene expression (Traub et al., 1998). Shear also effects the levels of vasoconstrictors such as ET-1; It has been demonstrated that low levels of shear stress (4-5 dyn/cm²) transiently up-regulates ET-1 mRNA expression, with a peak at 2 h and a return to baseline by 4h (Yoshizumi et al., 1989). In contrast higher levels, (15-25 dyn/cm²), were shown to induce a mild increase (within first hour), followed by a five-fold sustained decrease in ET-1 mRNA in a force-dependent manner in both bovine aortic (Malek et al., 1993) and human umbilical vein endothelium (Sharefkin et al., 1991). It has been suggested that this up-regulation of ET-1 at low shear levels may be in order to increase blood pressure via vasoconstriction in hypotensive environments. The resulting increase in pressure and flow causes a down regulation of ET-1, removing its potent vaso-
constrictive effect. The expression of other shear stress activated genes is also known to be a function of shear magnitude; for example t-PA expression is only increased above 5 dynes/cm² (Diamond et al., 1990).

1.2.2.3 Shear Stress Modulates Gene Expression

As alluded to previously, shear stress may alter the transcription rate of a specific subset of genes. One of the best characterised responses is the regulation of endothelial nitric oxide synthase (eNOS). Shear stress is the most potent physiological stimulus for NO production. Although rapid increases in NO production are due to post-translational modification of eNOS, exposure to chronic increases in shear stress leads changes in eNOS gene expression (Traub et al., 1998). A selection of genes known to be important for a variety of vascular functions and known to be regulated by shear stress are shown in Table 1.2.

A motif present in the promoters of many shear regulated genes is the so-called shear stress response element (SSRE), which has the sequence GAGACC. This was first identified in 1993 by Resnick and co-workers in the platelet derived growth factor-B (PDGF-B) promotor (Resnick et al., 1993). Some genes have an apparently functionless GAGACC sequence, such as MCP-1, while others are shear regulated in the apparent absence of this sequence, such as PDGF-A (Reisnick et al., 1995). Alternative SSRE sequences have also been identified, such as the MCP-1 gene which has a shear stress element with the sequence TGACTCC which is a divergent TRE (phorbol ester tissue 12-
O-tetradecanoylphorbol 13 acetate responsive element), the site that binds AP-1 (Chien et al., 1998).

Table 1.2 Genes Regulated by Shear Stress in the Endothelium CNP; C-type naturic peptide. bFGF; basic fibroblast growth factor. HB-EGF; heparin binding epidermal growth factor. VCAM-1; vascular cell adhesion molecule 1. TF; tissue factor. T-Pa; tissue plasminogen activator. SOD; superoxide dismutase. (Adapted from Chien et al., 1998). † indicates up-regulation, while ‡ indicates down-regulation.
References: 1; Kuchan et al., 1994. 2; Yoshizumi et al., 1989. 3; Masatugu et al., 2003. 4; Frangos et al., 1985. 5; Okahara et al., 1995. 6; Chun et al., 1997. 7; Reider et al., 1997. 8, 9; Hsieh et al., 1991. 10; Malek et al., 1993. 11; Ohno et al., 1995. 12; Morita et al., 1993. 13; Nagel et al., 1994. 14; Tsuboi et al., 1995. 15; Shyy et al., 1994. 16; Lin et al., 1997. 17; Diamond et al., 1990. 18; Malek et al., 1994. 19, 20; Topper et al., 1996. 21; Inoue et al., 1996. 22; Hsieh et al., 1993. (adapted from Chien et al., 1998).

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<td>†bFGF†</td>
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<td>†Thrombomodulin†</td>
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<td>†Prostacyclin‡</td>
<td>†TGF-β1†</td>
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<td>†COX-II†</td>
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<td>†CNP§</td>
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<td>‡Adrenomedulin§</td>
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1.2.2.4 Shear Stress and Vascular Health

There is considerable evidence to suggest that laminar shear stress exerts an atheroprotective effect on the vessel wall; steady laminar shear stress promotes release of factors from endothelial cells that inhibit coagulation (†PGI₂, NO), migration of leukocytes, and smooth muscle cell proliferation (†NO), while simultaneously promoting
endothelial cell survival. On the contrary, in areas of low shear stress and flow reversal, such as at bifurcations and branch points in the vessel, the profile of secreted factors and expressed surface molecules shifts towards one that favors increased coagulation (\( \downarrow \) NO), leukocyte adhesion (\( \uparrow \) MCP-1, \( \uparrow \) VCAM-1), endothelial cell apoptosis and smooth muscle cell proliferation (\( \uparrow \) Ang-II, \( \uparrow \) PDGF, \( \uparrow \) ET-I) (Frangos et al., 1985; Morgi et al., 1995; Kaiser et al., 1997; Traub et al., 1998).

![Figure 1.4 Diagram of the formation of an atherosclerotic plaque; showing accumulation of lipids in foam cells, monocyte infiltration and smooth muscle cell migration (www.peprotech.com)](image)

The interplay between hemodynamic forces and the endothelium plays a major role in a number of vascular diseases, such as atherosclerosis. Atherosclerosis is a progressive disease characterised by the accumulation of lipids and fibrous elements in large arteries. Early lesions consist of subendothelial accumulations of cholesterol-laden macrophages called foam cells. These fatty streaks develop over time into plaques comprising of a lipid rich necrotic core and typically have a fibrous cap consisting of SMCs and ECM (see Fig 1.4). Plaques become increasingly complex and can grow
sufficiently large enough to occlude blood flow. One of the more common clinical complications is thrombus formation as a result of the rupture of a plaque and consequent vessel occlusion. (Traub et al., 1998).

There is a strong correlation between areas of low mean shear stress and oscillatory flow and endothelial dysfunction leading to the formation of atherosclerotic plaques (Traub et al., 1998; Topper et al., 1999). Manifestations of dysfunctional endothelium can be readily observed in certain areas of the arterial tree such as branch points, which experience low mean shear stress and flow reversal. These sites exhibit factors that favor the development of atherosclerotic plaques, such as increased uptake of lipoproteins, the expression of leukocyte adhesion molecules, and the secretion of growth factors which cause the proliferation of local macrophages and smooth muscle cells (Ku et al., 1985; Ross et al., 1990). Smooth muscle cells synthesize a connective matrix composed of elastic fibers, proteins, collagen, and proteoglycans, and the accumulation of lipids and free and esterified cholesterol follows, leading to the formation of a plaque (Ross et al., 1990, Hunt et al. 2002).

Even allowing for the presence of pro-atherogenic risk factors such as diabetes and hyperlipidemia, the earliest lesions of atherosclerosis show a striking predilection for the sites associated with major arterial branch points and curvatures (see Fig 1.5) (Cornhill et al., 1977, Topper et al., 1999).
Figure 1.5 Non-random localization of early atherosclerotic lesions in a Watanabe heritable hyperlipidemic (YVHHL) rabbit. This strain of rabbit has a natural mutation in the gene encoding its low-density lipoprotein receptor and, as a result, develops severe hypercholesterolemia and spontaneous atherosclerosis. The aorta has been stained with Oil-Red-O, which shows lipid-rich atherosclerotic lesions in the arterial wall. Despite the systemic nature of the hyperlipidemia, the lesions in this animal are largely confined to areas around curvatures and branch points, suggesting that patterns of blood flow are important in localizing this disease. The upper arrow indicates the arch of the thoracic aorta, which has been opened up to display the intimal lining *en face*. The lower arrow indicates the localized nature of the atherosclerotic lesions adjacent to the paired ostia of the intercostal arteries originating from the descending thoracic aorta (Topper et al., 1999).

1.3 Means by which Endothelial Cells Transduce Hemodynamic Forces
Both cyclic strain and shear stress can induce changes in the endothelium both at an autocrine and paracrine level, allowing the vessel to respond and adapt to changes in the hemodynamic environment with the goal of maintaining steady state homeostatic conditions.

The various mechanisms by which endothelial cells sense and transduce the frictional and stretch forces resulting from the shear and cyclic stresses associated with blood flow, respectively, have yet to be fully elucidated. For obvious reasons the putative sensory structures involved must be in a position to directly respond to changes in the blood flow, either by physical displacement (conformational change), or indirectly by mass transfer gradients (which change ligand-receptor interactions). Such membrane-associated receptors include ion channels, integrins, caveolae, tyrosine kinases and G-protein coupled receptors (GPCR) (Papadaki et al. 1997). Another possible mechanism by which shear stress may be transduced is via interactions of activated receptors with cytoskeletal elements, for example at focal adhesion sites and cell-cell junctions (Poston, 2002).

1.3.1 Ion Channels as Mechanosensors

Ion Channels were first suggested as potential mechanosensors based on the observation that shear stress evokes an immediate increase in current though endothelial cell potassium channels (Nakache et al. 1988, Cooke et al., 1991). It has been demonstrated that exposure of endothelial cells to a physiological level of shear stress for 10 sec activated a K⁺ channel, which was not rapidly desensitized, and the magnitude and
duration of which was shear stress dependent (Alevriadou et al., 1993, Oleson et al., 1988). The immediacy of this response, one of the fastest recorded responses to shear stress, led many to suggest that K⁺ channels may be effective mechanosensors (Poston, 2002). Studies which demonstrated that the shear stress induced increase of transforming growth factor β1 (TGF-β1) could be attenuated by blockage of K⁺ channels lent weight to the theory that K⁺ channels play a role in flow stimulated gene expression (Ohno et al., 1995). This shear stress sensitive K⁺ channel seems to be endothelial cell specific, as it was not found in vascular cell smooth muscle cells or atrial myocytes (Papadaki et al., 1997).

Sodium channels have also been implicated in shear-mediated responses. For example Traub et al. (1999) have suggested a tonic inhibition of flow-mediated MAP kinase (ERK) stimulation by Na⁺ channels, since shear-mediated ERK1/2 stimulation was enhanced by addition of the Na⁺ channel inhibitor, tetrodoxin. The hyperpolarisation associated with increased K⁺ channel opening is believed to account for the transient rise in cell calcium reported in response to shear (Schwartz et al., 1992; Shen et al., 1992; Corson et al., 1996). This increase in endothelial cell calcium was originally considered to be the reason for the increased NO release associated with increasing flow, as eNOS is strongly activated by Ca²⁺ (Luckhoff et al., 1990). However it has since been shown that NO-mediated relaxation in endothelial cells is largely calcium independent (Ayajiki et al., 1996; Poston, 2002).
1.3.2 Integrins as Mechanosensors

Integrins are basal membrane associated glycoproteins composed of $\alpha$ and $\beta$ subunits; of which some 18 $\alpha$ and 8 $\beta$ subunits have been identified, arranged in 24 possible combinations. Each subunit has a large extracellular domain, a transmembrane spanning region and a short cytoplasmic domain (Shyy, et al., 2002). The cytoplasmic domains of both the $\alpha$ and $\beta$ subunits interact with signaling molecules, and cytoskeletal and extracellular components such as fibronectin, vitronectin and collagen (Lehoux et al., 1998).

Integrin signaling is a crucial component in development, maintenance and function of the vascular system. Binding of an extracellular ligand (such as fibronectin or collagen) to the integrin results in localized clustering of integrins in the basal plasma membrane. Integrins have the unique characteristic that they can signal through the cell membrane in either direction, essentially forming a bridge between the ECM and the cytoskeleton. Integrins can also act like a traditional receptor in binding an agonist and activating an intracellular response. The cytoplasmic tail of integrins, though devoid of intrinsic enzyme activity, transduces signals via adaptor proteins such as CAS, She, and paxicillan connecting it with cytoplasmic kinases, transmembrane growth factors and the cytoskeleton (Giancotti et al., 1999; Shyy et al., 2002).

A role for integrins as mechanosensors is supported by the observation that inhibition of integrin binding to ECM proteins using the RGD peptide inhibitor, prevented the shear stress induced dilation of coronary arterioles exposed to intraluminal flow. Similar results were observed with a $\beta$-integrin neutralizing antibody (Muller et al.,
1997). Other studies have demonstrated that pretreatment of a confluent endothelial cell monolayer with a αvβ3 neutralizing antibody blocked shear stress activation of ERK, c-jun N-terminal kinase, and the IκB complex (Li, et al., 1997; Bhullar et al., 1998).

Integrin-mediated mechanotransduction is believed to include multiple kinases (FAK, c-Src, and Fyn), adaptor molecules (p130Cas and Shc), guanine nucleotide exchange factors (GEFs) (C3G and SOS) and small GTPases (e.g. rap1 and Ras) in activating MAPKs such as ERK. Under static conditions the integrin is in an inactive conformation. Shear stress activates integrins by switching them into an active conformation, which increases their affinity and avidity for the cognate ECM proteins.

Figure 1.6 Representation of Integrin mediated mechanotransduction; Green circles represent adaptor proteins, red ovals kinases, white circles GEFs, and white squares GTPases. The black oblong boxes represent the portions of the integrin molecule, while phosphorylation is indicated by a red asterix (adapted from Shyy et al., 2002).
Through specific interaction of the α and β subunits of the activated integrins, the FAK/c-Src pathways and the Cav-1/Fyn pathways are activated to elicit cascades of phosphorylation on various downstream effectors and their assembly through SH2 and SH3 interactions, converging at the level of the Raf-MEK-ERK signaling pathway, in endothelial cells (see Fig.1.6) (Shyy et al., 2002; Poston et al., 2002; Lehoux et al., 1998).

1.3.3 Caveolae as Mechanosensors

Another possible candidate for the transduction of hemodynamic forces into biochemical signals are caveolae. Caveolae are specialized domains of the plasma membrane that are rich in cholesterol. Their high cholesterol content makes them more rigid than other portions of the cell membrane. Caveolae are abundant in endothelial cells and have been implicated in transcytosis, ion movement across the membrane, and signal transduction (Schnitzer et al., 1995). The principle component of caveolae is a 21- to 24-kD integral membrane protein called caveolin. Caveolin functions as a scaffold for the recruitment and sequestration of signaling molecules. Among signaling molecules known to associate with caveolae are G proteins, c-Src-family tyrosine kinases, ras, PKC, eNOS, shc, Grb2 SOS, Raf-1, and ERK 1/2 (Garcia-Cardena et al., 1996; Couet et al., 1997). Based on their biophysical characteristics and interactions with signaling molecules, caveolae represent an attractive site for mechanotransduction in the endothelium (Traub et al., 1998).
1.3.4 Heterotrimeric G-proteins as Mechanosensors

G-protein signaling represents a highly sophisticated molecular system with the ability to receive, integrate, and process information from extracellular stimuli. G-protein coupled receptors (GPCRs) constitute a large family of single polypeptide chain receptors, with seven trans membrane α-helices composed primarily of hydrophobic residues looping between the extracellular and intracellular phases. GPCRs couple to heterotrimeric G-proteins, which consist of α, β, and smaller γ subunits. Most α and all γ subunits contain sites for reversible acylations which target G-proteins to the membrane compartment which is the principle site of G-protein signaling (Sugden *et al.*, 1997).

In the inactive G-protein heterotrimer, α subunits exist as GDP ligated forms (*Fig 1.7*). The activation of G-proteins occurs as follows; binding of an extracellular agonist to a GPCR leads to a conformational change in the receptor and its associated G protein. This decreases the affinity of the α subunit for GDP, and GTP replaces GDP. The GPCR disassociates from the heterotrimer, which in turn disassociates into an α and βγ subunits (the latter of which always remains tightly associated. Both α and βγ dimers then activate their different effector molecules (see section 1.3.4.1 and 1.3.4.2). The innate GTPase activity of the α subunit returns it to a GDP ligated (*Fig 1.7*) form and αGDP reassociates with both βγ dimers and the subsequently with the GPCR (Sudgen *et al.*, 1997; Seifert *et al.*, 2003).

More than twenty G-protein α subunits have been described which have been loosely divided into four families based on structural and functional homologies; αi/o, αs,
The majority of GPCRs are capable of activating more than one G-protein sub-type, which leads to initiation of various intracellular signaling cascades. There are some characteristic patterns of G-protein activation by specific receptors, with the cellular or physiological effect of a receptor being dependent on which G-protein sub-type it is coupled to.

**Figure 1.6 The G-protein binding cycle.** G protein-linked receptors contain seven transmembrane segments that form a ligand-binding site on the outside of the cell and a G protein-binding site on the inside. (1) When the ligand binds, (2) the receptor activates a G protein by causing the Ga subunit to release GDP and acquire GTP. (3) The G(alpha) and G(beta/gamma) subunits then separate and (4) initiate signal transduction events. (5) The GTP-G(alpha) subunit eventually hydrolyzes its bound GTP, converting the subunit back to its inactive GDP-G(alpha) form. (6) The inactive GDP-G(alpha) subunit then recombines with G(betagamma) to form the inactive G heterotrimer. (© 1999 by Addison Wesley Longman)
1.3.4.1 The Gα Subunits

1. Gαs-; there are two members of this family, αs and αolf, as well as four known splice variants. Upon activation, this group stimulates adenylyl cyclase to increase levels of intracellular cAMP and to activate calcium channels. αs is ubiquitously expressed while αolf is restricted to neuronal cells, specifically olfactory sensory neurons.

2. Giα/β: This family consists of α1i, α2i, α13, αo1, α02, αand, α4-cone, αgust and αz, all of which are highly homologous and have the ability to inhibit adenyl cyclase, in addition to activation of potassium channels. The high degree of homology between sub-types may suggest partially redundant functions. Expression of the various αi subtypes may be diverse depending on the tissue examined; for example αi2 is predominant in the mammalian heart. A defining characteristic of this family is their sensitivity to petussis toxin (PTX), which catalyzes the adenosine diphosphate (ADP)-ribosylation of αi and αo subunits at a cysteine residue near the C-terminus resulting in the uncoupling of G-protein and receptor. An exception to this rule is αz which is pertussis toxin-insensitive.

3. Giaq: This family stimulates phospholipase C in a pertussis toxin-insensitive manner. αq and α11 are ubiquitously expressed and receptors activating αq family members do not discriminated between αq and α11. α15/16 are only expressed in hematopoietic cells and α14 is restricted to the kidneys, lungs and testes.
4. G\textsubscript{i12}; $\alpha_{12}$ and $\alpha_{13}$ constitute the members of this family and appear to be widely expressed. The function of these proteins is somewhat unclear. One recently discovered function is the interaction of these proteins with cadherins causes the release of transcriptional activator $\beta$-catenin (Megis et al., 2001, Seifert et al., 2003).

1.3.4.2 The G$\beta\gamma$ Subunit

This complex is assembled from a repertoire of five $\beta$ subunits and twelve $\gamma$ subunits. The sequence similarity is higher between $\beta$ subunits (79-90% homology) than $\gamma$ subunits and it is not yet clear how many combinations will actually form stable dimers (Clapham et al., 1993). $\beta\gamma$ sensitive effectors include adenyl cyclase, phospholipase C, phospholipase A, potassium channels, a calcium pump, and phosphoinositide 3 kinase (PI-3 kinase) (Tang et al., 1991; Exton et al., 1996; Yamada et al., 1989; Lotersztajn et al., 1992). With a few exceptions there appears to be no major differences between different $\beta\gamma$ combinations in their ability to activate effector enzymes.

1.3.4.3 G-Proteins as Mechanosensors

A role for G-protein involvement in mechanotransduction was first suggested following the observation that intracellular messengers associated with G-protein pathways were also activated in response to flow. These included inositol triphosphate (IP\textsubscript{3}), diacylglycerol (DAG), protein kinase C (PKC) and a rise in cell calcium. Involvement of the $G_{ia}$ subunits was suggested by the pertussis toxin-sensitivity of shear-induced NO release in some experiments (Poston 2002; Ohno et al., 1993). Later studies by Gudi and
co-workers demonstrated the rapid activation of $G_{i_0q_{a11}}$ and $G_{i_3o_{a2}}$ proteins by shear, with activation occurring as little as one second after onset of flow (Gudi et al., 1996). This group also demonstrated that treatment of endothelial cells with antisense $Ga_q$ oligonucleotides inhibited shear stress-induced ras-GTPase activity, while scrambled oligonucleotide treatment had no effect. Further studies on G-proteins reconstituted on liposomes in the absence of protein receptors showed an increase in activity in response to shear stress (Gudi et al., 1997; Frangos et al., 1997). Jo and co-workers also demonstrated that that the treatment of endothelial cells with pertussis toxin prevented stress mediated activation of ERK1/2 (Jo et al., 1997), indicating a role for $Gi_{a1}$ subunit in mechanotransduction.

Both the $G\alpha$ and $G\beta\gamma$ subunits of the G-protein complex can initiate signaling by divergent pathways in response to shear. Jo and co-workers demonstrated that expression of the carboxy terminus of $\beta$-adrenergic receptor kinase ($\beta$ARK-ct), which acts as a $G\beta\gamma$ scavenger inhibits the shear stress activation of HA-JNK, whereas blockade of $Gi_{a2}$ with the dominant negative mutant $\alpha_{i2}$ (G203T) or with antisense $Go_{i2}$ prevented shear dependent activation of HA-ERK (Jo et al., 1997).

It is unclear whether G-protein activation is secondary to the deformation of the cytoskeleton or to activation of hitherto unidentified luminal membrane receptors. It has been proposed that G-protein activation could be achieved through shear-mediated alteration in membrane fluidity (Haidecker et al., 2000).
1.3.5 Tyrosine Kinases as Mechanosensors

Tyrosine kinases (PTKs) play an important role in the signaling pathway which leads to the activation of MAPKs, as indicated by the finding that genistein, a PTK inhibitor, can attenuate the shear stress activation of ERK and JNK (Li et al., 1997; Jo et al., 1997; Takahasi et al., 1996). PTKs are also critical in the shear stress-regulation of endothelial cell shape and stress fibres (Chien et al., 1998), as well as in the early phase of flow-dependent NO production (Corson et al., 1996). Reports also indicate that the activities of PTKs in cardiac myocytes, platelets, and ECs are increased by mechanical stimuli such as cyclic stretch and shear stress (Sadoshima et al., 1997; Ishida et al., 1996). It has been shown that shear stress induced a rapid and transient tyrosine phosphorylation of FLK-1 and its concomitant association with the adaptor protein Shc in endothelial cells (Labrador et al., 2003; Ravichandran et al., 2001). The adaptor protein Shc is implicated in signaling via many different types of receptors, such as growth factor receptors, G-protein coupled receptors, hormone receptors, integrands and tyrosine kinases. (Lopez-Ilasaca et al., 1998).

PTKs can be divided into two major categories; receptor and non-receptor tyrosine kinases.
1.3.5.1 Receptor PTKs

These PTKs distinguished as a sub-class from other enzyme linked membrane receptors by the presence of an intrinsic tyrosine kinase activity within the receptor molecule itself. Receptors of this class generally respond to circulating hormonal stimuli which trigger the activation of intracellular signaling pathways leading to cell shift in metabolism proliferation and/or differentiation. Members of this family include the insulin-like growth factor receptor (ILGFR), the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR). Many of these receptors exist as monomers in the cell membrane (e.g. EGFR) (see Fig 1.8). Agonist binding to the extracellular portion leads to the dimerisation of two receptors, and a subsequent conformational change, which causes the autophosphorylation of the cytoplasmic domain, creating binding sites for intracellular adaptor molecules such as Grb2 and Sos, and leading to the subsequent activation of RAS. (Adapted from Sudgen et al., 1997).
Sos and Grb2. These adaptor molecules subsequently bring other signaling molecules, such as Ras, into close proximity to the receptors intercellular domain, leading to activation of a signaling cascade (Stone et al., 1998; Sugden et al., 1997).

1.3.5.2 Non-receptor PTKs

The so-called non-receptor PTKs include molecules such as FAK and c-Src, these represent cellular enzymes that have intrinsic kinase activities but do not possess extracellular domains. FAK and c-Src, for example are present in focal adhesion sites and are tyrosine phosphorylated in response to cell adhesion and the stimulation of a number of growth factors (Schaller et al., 1994). Focal adhesion associated kinases such as FAK and c-Src are rapidly activated in ECs by shear stress (Li et al., 1997).

1.3.6 Intracellular Signaling Pathways; The Role of Mitogen Activated Protein Kinases and Small GTPases in the Transduction of Shear Stress Responses.

The proposed mechanosensors discussed previously fulfill the first step in mechanotransduction; namely the transmission of an extracellular physical response to an intracellular biochemical signal. This intracellular signal needs to recruit and activate further effector molecules in order to induce changes at the level of transcription.

The family of kinases termed mitogen activated protein kinases (MAP kinases) are potential candidates as second messengers in mediating some of the effects of shear
stress on endothelial cells. MAP kinases are ubiquitously expressed serine/threonine protein kinases that are activated in response to a variety of extracellular stimuli involved in cell growth, transformation and differentiation. The extracellular signal related kinases (ERK1/2), and the stress activated c-Jun NH(2)-terminal kinase (JNK) are members of the MAP kinase family and have many potential substrates, including other protein kinases (p90rsk, AP-1, Raf-1, MEK), transcription factors (c-myc, c-jun, c-fos, p62TCF), enzymes (cPLA₂), and cell surface proteins (EGF receptor) and thus have many effects on cellular physiology and gene expression (Traub et al., 1998; Berk et al., 1995; Go et al., 1999; Poston et al., 2002).

The pathway for ERK1/2 activation in response to growth factors has been well characterized. The MAP and ERK kinase (MEK-1) is a dual specificity kinase that phosphorylates ERK1/2 on T-E-Y. MEK-1 itself is regulated by a MAP kinase kinase kinase (MAPKKK), Raf-1. Raf-1 is mediated by translocation to the membrane and association with the small GTP binding proteins Rho, Rap1 and ras. The GTPase activity of Rap1 and ras is regulated by a complex which involves the so called adaptor proteins Grb2 and Sos which are recruited and activated by tyrosine kinase receptors (Pelech et al., 1992; Shyy et al., 2002).

It has been demonstrated that ERK1/2 is activated by shear stress in endothelial cells in a time- and force-dependent manner (Tseng et al., 1995). Shear stress stimulation of ERK1/2 was unaffected by treatment with the Ca²⁺ chelator BAPTA-AM, indicating that the response was Ca²⁺-independent. These findings, combined with observations that
eNOS contains multiple sites for phosphorylation by a number of kinases, suggested that this pathway was a likely candidate in the stimulation of sustained NO production in response to shear stress (Berk et al., 1995). More recent work by Davis and co-workers has indicated, however that the immediate activation of eNOS in response to shear involves the c-Src, with the increase in eNOS mRNA expression observed in response to prolonged shear stress exposure was shown to involve the c-Src activation of Raf, MEK1/2, and ERK1/2 (Davis et al., 2001; Davis et al., 2003). ERK1/2 has also been shown to mediate the cyclic strain dependent up-regulation of MMP-2 in the endothelium. Von-Offenberg Sweeney and co-workers demonstrated that use of PD98059 to selectively inhibit ERK1/2 significantly attenuated the effects of cyclic strain on MMP-2 (Von-Offenberg Sweeney et al., 2004).

Another member of the MAPK family, which has been shown to be regulated by shear stress, is the stress-activated protein kinase JNK (Jo et al., 1997). Studies have demonstrated that shear stress inhibits tumor necrosis factor-stimulated JNK activity in endothelial cells (Surapisitchat et al., 1998), a finding consistent with the findings that shear stress has an anti apoptotic effect on the endothelium.

Cyclic strain also activates Erk1/2 and JNK in cultured SMC are transiently activated in arterial wall by acute hypertension (Xu et al., 1996). Kito and co-workers found that cyclic strain activated Erk1/2, JNK and p38 in pulmonary ECs with subsequent AP-1/TRE activation and was found to modulate the Egr-1 transcription factor (Kito et al., 2000).
1.3.7 Downstream Effectors of ERK1/2 Activity; the Protein Kinase C (PKC) Family

PKCs are well-characterized serine/threonine kinases that are activated by a variety of stimuli. The dominant isoforms in endothelial cells are believed to be PKCα, PKCe and PKCζ (Traub et al., 1998).

The requirement of PKC for ERK1/2 activation has been demonstrated by the fact that pretreatment of endothelial cells with staurosporine, a PKC inhibitor, attenuated ERK1/2 activation by shear stress (Tseng et al., 1995). In further studies using antisense oligonucleotides to inhibit expression of various PKC isoforms, PKCe was identified as the isoform responsible for the activation of ERK1/2 in response to shear stress (Traub et al., 1997).

Studies by Cai and co-workers, demonstrated the mechanism by which PKC mediates ERK1/2 activation. These investigations demonstrated that activation of Raf-1, a key mediator of ERK 1/2 activation is dependent on several criteria; 1) recruitment of Raf-1 to the plasma membrane; 2) activation of Raf-1 by the GTP-bound form of ras; 3) tyrosine phosphorylation of Raf-1 by c-Src or c-Src family kinase; and 4) phosphorylation of Raf-1 on serine and threonine residues. Both PKCα and PKCe were directly responsible for the phosphorylation of serine and threonine residues on Raf-1 in response to various stimuli. This redundancy in PKC signaling allows for signaling regardless of a rise in intracellular Ca²⁺, as PKCe is Ca²⁺-independent (Cai et al., 1997).
1.3.8 Transcription factors

Transcription factors or “third messengers” are proteins typically located in the cytosol or in the plasma membrane. These readily available elements are activated post translationally by second messengers and can rapidly regulate the expression of selective immediate early genes (IEG). Shortly after the cell is stimulated IEG are transcribed and code for immediate early proteins, which play a regulatory role in the transcription of other late response proteins (Davies et al., 1995; Papadaki et al., 1997). Endothelial IEGs transiently up-regulated by shear stress are c-myc, c-fos, and c-jun (Hsieh et al., 1993). c-fos, and c-jun encode proteins that belong to nuclear activator protein 1 (AP-1), while c-myc protein can associate with other proteins to form other transcription factors. Two transcription factor families present in endothelial cells are stimulated by a number of defined stimuli which includes shear stress. These are the AP-1 and Rel-related nuclear factor kappa B (NFκB) (Papadaki et al., 1997).

1.3.8.1 AP-1

The AP-1 complex is formed from the dimerisation of Fos/Jun families of proteins through so-called leucine zipper (Nollert et al., 1992). AP-1 can bind to cis acting elements in the promoter region of genes, such as the TRE [TGACTCA], and the cAMP responsive element or CRE [TGACGTCA]. The c-fos/c-jun dimer shows a higher binding affinity for the TRE than c-jun/c-jun, while c-fos/c-fos is unable to bind properly (Papadaki et al., 1997).
It has been demonstrated that c-jun/c-jun homodimer is the activator of the AP-1/TRE in endothelial cells exposed to arterial levels of shear stress, with latent c-jun being activated through the ras-MEKK-JNK pathway and translocating to the nucleus where c-jun/c-jun homodimers form and activate the TRE (Li et al., 1996). Although a number of genes possess the TRE/CRE motif in their promoter region, only one, MCP-1, has a well-studied response to shear stress (Chien et al., 1998; Poston, 2002).

1.3.8.2 NFκB

NFκB is a pluripotent transcription factor, activated by a number of stimuli, which is found in the cytosol of inactivated cells as a heterodimer of a p50 and a p65 protein, bound to an inhibitory cytoplasmic IkB protein (Lan et al., 1994). Upon activation the p50/p60 protein complex rapidly dissociates from IkB, allowing transport of free NFκB to the nucleus where it can bind to specific NFκB consensus sites [GGAAGATCCT]. NFκB is also known to bind to the SSRE [GAGACC], and has been shown to be involved in the shear stress-dependent up-regulation of a number of genes including PDGF-B (Resnick et al., 1993) and eNOS (Davis et al., 2003). It is generally considered that the activation of NFκB occurs via phosphorylation by protein kinases, including PKC, c-Src, and MEK1/2 (Papadaki et al., 1997; Davis et al., 2003).

1.3.9 Hemodynamic Forces and mRNA Stability

Evidence suggests that mechanical forces may alter protein levels, not at the level of transcription, but by altering the mRNA stability of certain genes. It has been proposed
that a shear-induced loss in mRNA stability may be the process by which shear down-regulates expression of certain proteins (Papadaki et al., 1997). A suggested mechanism for this is via the presence of AUUUA repeats in the untranslated regions of the gene, which are known to cause mRNA instability in certain genes. For example VCAM-1, which is down-regulated by shear stress, contains some six such repeats in the 3' untranslated region. In comparison, genes up-regulated by shear, such as tPA and PDGF-B have only one such sequence, while the constitutively expressed glyceraldehyde phosphate dehydrogenase (GAPDH) gene has none. However these motifs may have other functions, i.e. involvement in the transport of mRNA to the cytoplasm, and VCAM-1, or indeed any other gene down-regulated by shear stress, might be regulated at the transcriptional level (Patrick et al., 1995, Poston 2002).

1.4 Thermolysin Like Zinc Metallopeptidases

Metal dependent proteases constitute one of the four major types of protease, the others being aspartic, serine and cysteine proteases. This classification is based on the catalytic mechanism and the critical active site amino acid residues involved. In the case of metallopeptidases, an activated water molecule complexed to the divalent cation, usually Zn$^{2+}$, serves as the nucleophile, attacking the carbonyl group of the targeted peptide bond. Rawlings and Barrett proposed a classification system based not only on the catalytic mechanism, but also the evolutionary links between enzymes (Rawlings and Barrett, 1993). Most mammalian metallopeptidases fall into one of four clans or sub clans.
By far the largest group, Clan MA, is distinguished by the active site HEXXH motif, where the two histidine residues, together with a third distant residue, bind the zinc atom. This clan is divided into two major sub-clans: MA(E), the ‘gluzincins’, where the third ligand is a glutamate, and MA(M), ‘metzincins’, where a third histidine or an aspartate residue serves this purpose. This latter group is typified by the matrix metalloproteases and their relatives, including the ADAMs (a disintegrin and metalloprotease). The gluzincins are further divided into families based on homology. The families most relevant to mammalian signaling systems are M1 (aminopeptidases), M2 (angiotensin converting enzyme family), M3 (EP24.15/thimet oligopeptidase and EP24.16/neurolysin), and M13 (neutral endopeptidase family, which also includes endothelin converting enzyme). The metallocarboxypeptidases belong to the clan MC, while a smaller group of peptidases, in which the active site motif is reversed (HXXEH; the so-called ‘inverzincins’) and typified by insulin-degrading enzymes (insulysin), belong to the M16 family within the ME (Rawlings and Barrett, 1993; Lew, 2004).

Given the multifunctional roles of ACE, ECE, NEP, Neurolysin and THOP, the possibility that the expression and function of these metallopeptidases might be sensitive to hemodynamic stimuli has been considered. Inspection of the promoter regions of the genes coding for THOP, ACE, ECE-1 and NEP all display the shear stress response element SSRE [GAGACC], a motif, which as discussed previously frequently occurs in the promoters of mechanosensitive genes (McCool et al., 1998; McCool et al., 2000; Li et al., 1995; Shinoki et al., 1998).
1.4.1 Endothelin Converting Enzyme1 (ECE-1)

Endothelin converting enzyme (ECE-1, EC3.4.24.71) is involved in the production of one of the most potent vasoconstrictors known, endothelin-1, from its parent peptide big ET-1. The physiological significance of ET-1 within the vasculature has been discussed previously (see section 1.1.2.4). ECE-1 is expressed primarily in the endothelium (Levin, 1995). ECE-1 is known to exist in various isoforms; namely (ECE-1a, ECE-1b and ECE-1c in humans). A closely related peptidase, ECE-2 has also been identified, however it is thought that unlike ECE-1 which operates at the cell membrane, ECE-2 is likely to operate intracellularly (Lew, 2004).

The ability of hemodynamic forces to modulate the ECE and ET-1 has been demonstrated previously. Yoshizumi and co-workers found that low levels of shear stress (4-5 dyn/cm²) transiently up-regulates ET-1 mRNA expression, with a peak at 2 h and a return to baseline by 4 h (Yoshizumi et al., 1989), in contrast higher levels, (15-25 dyn/cm²), were shown to induce a mild increase (within first hour), followed by a five-fold sustained decrease in ET-1 mRNA in a force dependent manner in both bovine aortic (Malek et al., 1993) and human umbilical vein endothelial cells (Sharefkin et al., 1991). This suppression of ET-1 mRNA has been shown to be attenuated or abolished by inhibition of tyrosine kinase, disruption of cytoskeletal components (F-actin), chelation of intracellular calcium, and inhibition of stretch activated ion channels (Malek et al., 1999). A reduction in the production of ET-1 in response to high shear stress is an unsuprising result, as a reduction in the levels of ET-1 would aid vasodilation and thus lead to a
reduction in blood pressure. Cyclic or pulsatile strain has also been shown to elevate levels of ET-1 mRNA expression and secretion. (Chenga et al., 1998). This up-regulation in response to strain involved reactive oxygen species, as pre-treatment with catalase was shown to abolish the strain induced increase in transcription and secretion. Further studies indicated the involvement of a Ras/Raf/extracellular Signal-regulated pathway (Chenga et al. 2001).

Hemodynamic forces have also been shown to regulate levels of ECE-1. Masatsugu and co-workers demonstrated a significant and force dependent decrease in expression of both ECE-1α and ECE-1β mRNA in response to shear stress in both human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) cultured in a parallel plate flow chamber. This occurred with a similar decrease in levels of ET-1 mRNA. These responses to shear appeared to occur through oxidative stress, as treatment with an antioxidant attenuated the response to shear (Masatsugu et al., 2003). Similar studies by Morawietz and co-workers showed a 63.8% and 37.8% decrease in levels of ECE-1α and ECE-1β mRNA in HUVECs in response to chronic laminar shear stress. A decrease in ET-1 expression and release was also demonstrated and shown to occur in a protein kinase C insensitive and eNOS sensitive manner. In contrast an eNOS/PKC dependent and tyrosine kinase independent increase in levels of the ET<sub>B</sub> receptor in response to chronic laminar shear stress was also observed in this study (Morawietz et al., 2000).
The down-regulation of both ET-1 expression and release and of ECE expression in response to high levels of laminar shear stress, would putatively reduce the amount of ET-1 available within the vasculature. \textit{In Vivo}, in response to high blood flow rates and consequent high blood pressure, the reduction of ET-1 levels may aid the relaxation of the vessel wall, with a concomitant reduction in blood pressure.

1.4.2 Angiotensin Converting Enzyme (ACE)

ACE (EC.3.4.15.1) is a well characterised membrane bound metallopeptidase known to play a crucial role in cardiovascular homeostasis. ACE is involved primarily in the production of the potent vasoconstrictor Ang-II from Ang-I, but is also involved in the conversion of Ang(1-9) to Ang-(1-7) and the further degredation of Ang-(1-7) to inactive peptide fragments. ACE is also involed in the inactivation of the vasodilator BK (BK) by converting it firstly to BK(1-7) and then to BK(1-5) (Duncan et al., 1999; Guy et al., 2004). ACE is not only interesting because of its physiological and pathological characteristics, but also because it is the only known metallopeptidase with two active sites, undoubtedly the result of gene duplication (Lew, 2004). The two display distinct substrate and inhibitor affinities, a phenomenon recently exploited in the design of site specific inhibitors which limit the formation of the vasoconstricor Ang-II while preventing the degradation of the vasodilator BK (Georgadis et al., 2003). A testes specific single site isoform of ACE also has been identified, while the closely related ACE2 is believed to function as a carboxypeptidase rather than a dipeptidyl peptidase with Ang-II as its substrate (Vickers et al., 2000).
Using both *in vitro* and *in vivo* shearing paradigms, Rieder and co-workers have investigated the mechanosensitive properties of ACE. Employing a cone-plate viscometer to expose bovine pulmonary artery endothelial cells (BPAECS) to chronic laminar shear stress (20 dynes/cm², 18h), ACE activity was reduced by 49.5% and ACE mRNA expression by 82% (Rieder *et al.*, 1997). The same group modelled *in vivo* shear stress elevations by placing a stainless steel clip over a 12mm region of the rat abdominal aorta. This restriction in vessel diameter increased blood flow velocity and caused a reduction in vascular ACE activity by 40%. A significant shear-dependent reduction in ACE promoter activity was also observed in the same study (Rieder *et al.*, 1997). The signalling pathways mediating these events remain to be elucidated; however NO may play a significant role. It is well known that shear stress increases the production of NO in vascular endothelial cells. In this regard Takemoto and co-workers have demonstrated that eNOS inhibition results in an up-regulation of ACE expression in various vascular and myocardial tissues (Takemoto *et al.*, 1997), whilst Ackermann and co-workers demonstrated that NO and NO-releasing compounds inhibit ACE activity in a concentration-dependent and competitive manner (Ackermann, *et al.* 1998). In a later study, Gosgnach and co-workers employed a parallel plate flow chamber to expose cultured rat vascular smooth muscle cells to chronic shear stress (10 dynes/cm², 24h) in an effort to model SMC exposure to shear in the event of endothelial denudation (Gosgnach *et al.*, 2000). They present data demonstrating shear-dependent induction of ACE activity and mRNA expression in SMCs, which was mediated in part by basic fibroblast growth factor (bFGF), a finding which concurs with earlier studies which demonstrate the over-expression of ACE in the proliferating neointima following balloon injury (Rakugi, *et al.*, 1998).
Gosgnach also reports a shear-dependent induction (2.14 fold) of ACE activity in rat aortic endothelial cells in the same study, which appears to contrast with the earlier findings of Rieder (Reider et al., 1997). This may reflect differences in the cultured endothelial cell types used and/or the shearing paradigms employed. The relationship between cyclic strain and ACE in some vascular cell types has also been investigated, albeit less directly. Studies have demonstrated that strain-dependent SMC collagen production and SMC proliferation is mediated in part via elevated Ang-II production by ACE (Li et al., 1997; Li et al., 1998), whilst strain induced activation of p42 and p44 MAPK in vascular SMCs has also been reported to be Ang-II-and ACE-dependent (Hosokawa et al., 2002).

1.4.3 Neutral Endopeptidase (NEP)

Neutral Endopetidase (neprilysin, enkephalinase, EC.3.4.24.11), was first isolated from renal epithelial cell brush borders, and is primarily membrane bound. It catalyses the degradation of a number of vasodilator peptides arterial natriuretic peptide (ANP), brain natriuretic peptide, C-type natriuretic peptide, substance P and BK, as well as degrading vasoactive petides such as ET-1 and Ang-II. In addition to degrading vasoactive peptides to inactive fragments (eg, BK to BK-(1-7)), NEP also catalyses the production of Ang-(1-7), from Ang-I, and the cleavage of big ET-1 to form ET-1 (Ferro et al., 1998). Recently an NEP homologue, NEP-2, was identified by three separate groups. Termed either neprilysin-2 (NEP-2) (Ouimet et al., 2000), secreted endopeptidase (SEP) (Ikeda et al., 1999), or nephilysin like-1 (NL-1) (Ghadder et al., 2000), NEP-2 has a
similar substrate specificity to NEP-1. However the specific expression pattern of NEP2, being absent in the periphery (other than the testis) and restricted to brain regions low in NEP, as well as the existence of a secreted isoform, suggests a distinct function yet to be identified (Lew 2004).

To our knowledge there have as yet been no studies that indicate a role for hemodynamic forces in the regulation of NEP expression or activity in vascular cell types. However given its role in the regulation of vascular tone and blood presure, it is highly likely that like both ACE and ECE1, it is subject to mechanoregulation by hemodynamic forces.

1.4.4 The soluble Metalloendopeptidases; Thimet Oligopeptidase and Neurolysin

The closely related enzymes thimet oligopeptidase (THOP, EC3.4.24.15, EP24.15) and neurolysin (EC3.4.24.16, EP24.16) both belong to the clan MA series of peptidases, and share similar structural features and substrates with the previously discussed ACE, ECE and NEP. Unlike the latter three enzymes they exhibit no distinct transmembrane domain. However, it has been demonstrated in numerous tissue types that these enzymes can be constitutively secreted and membrane associated (for review see Shimpton et al., 2002). They were first purified from the soluble cytosolic fractions of the bovine pituitary (THOP, Horsthemke et al., 1980) and rat synaptic membranes (Neurolysin, Checler et al., 1986). They have a broad tissue distribution, having been identified in neuronal, vascular, pituitary and testicular tissue, and are believed to play a role in sexual maturation (THOP), pain perception and opioid processing (THOP),
neurotensin inactivation (THOP/Neurolysin), blood pressure regulation and renal homeostasis (THOP/neurolysin) (Shimpton et al., 2002).

1.4.4.1 Biochemical properties; Thimet Oligopeptidase

THOP was first isolated from the soluble fractions of bovine pituitary and rat brain (Horsthemke et al., 1980; Orlowski et al., 1983) and shown to hydrolyse a number of biologically relevant peptides with a general specificity for peptide bonds on carboxyl side of basic residues (See Fig 1.11).

Isolated brain THOP was shown to be optimally active at a neutral pH, be inhibited by metal ion chelators and reactivated by low concentrations of Zn$^{2+}$ (Orlowski et al., 1983). Later atomic absorption studies indicated the presence of a tightly bound zinc atom at the catalytic core (Tisljar, et al., 1989). This zinc atom is co-ordinated by the side chains of the active site histidines (H473, H477), and a glutamate residue located 25 residues carboxy to the HEXXH motif (E502). A second glutamate residing within the active site (E474) also participates in the coordination of the zinc via an activated water molecule (Cummins et al., 1999). The recently published crystal structure of human THOP showed that the active site is partially blocked by a large shielding element, which prevented the hydrolysis of large, folded substrates but allowing the small largely unstructured oligopeptides favoured by THOP to bind in an extended conformation along the long narrow channel leading to its active site (Ray et al., 2004) (see Fig 1.9, 1.11).

THOP also displays thiol activation at low levels of treatment with dithioltheitol (0.5 M), while higher concentrations (5 M) lead to inhibition. This inhibition has been suggested
Figure 1.9 Overview of the thimet oligopeptidase structure and active site density. (a) ribbon diagram of thimet oligopeptidase. Individual α helices and β sheets are numbered, and the regions structurally similar to other zinc metallopeptidases is in gold. The active site zinc is shown as a blue sphere. (figure created using RIBBONS). (b) Active site of thimet oligopeptidase. Key residues of the HEXXH motif are marked along with the third zinc ligand from the protein Glu-502. The active site zinc and catalytic water molecule are shown as blue and red spheres respectively (figure created using Swiss-PDB viewer). (Adapted from Ray et al., 2004)

to be as a result of the disruption of disulphide bridges in the enzyme and the thiophilicity of the catalytic zinc ion (Barret et al., 1990). The activation of THOP by thiols has been shown to involve the conversion of an inactive multimer, where the access of substrates to the active site is blocked, to an active monomer via disruption of intermolecular disulphide bridges (Shimpton et al., 1997). This may allow for the regulation of the activity of THOP in vivo by the alteration of inter- and intra-cellular redox potential.
The purification to homogeneity of THOP from rat testis in 1989 (Orlowski et al., 1989), and its subsequent cloning from the same source (Pierotti et al., 1990), allowed the DNA and protein sequence of the enzyme to be studied in detail. It was revealed to be a protein of 78 kDa, with an amino acid sequence comprising 645 amino acids, to be the product of a single gene and to lack a secretory signal sequence or a membrane spanning domain. In addition putative sites for N glycosylation and phosphorylation were identified. Although there is no evidence that THOP undergoes glycosylation, Tullai et al. have demonstrated that THOP can be phosphorylated by protein kinase A at Ser^{644}, and that this modifies it’s kinetic parameters for the cleavage of certain subjects including gonadotrophin-releasing hormone (GnRH) (Tullai, et al., 2000). In addition phosphorylated forms of THOP were detected in rat PC12 cells and mouse AtT-20 cells, both basally and in response to the forskolin stimulation of protein kinase A. Furthermore, cleavage of a specific fluorescent substrate by THOP was shown to be decreased after treatment by cAMP analogs (Ferro et al., 1995).

1.4.4.2 Biochemical Properties; Neurolysin

Neurolysin (EC3.4.24.16, EP24.15) was originally purified and characterised from rat synaptic membranes (Orwolksi et al., 1986) on the basis of its ability to cleave neurotensin (NT) at Pro^{10}-Tyr^{11}, allowing its separation from THOP, which cleaves neurotensin (NT) exclusively at Arg^{8}-Arg^{9} (Checler et al., 1986). The cleavage of NT represents one of the few differences in substrate specificity between the two enzymes, with most other natural substrate cleaved at identical sites by either enzyme (see Fig.1.11). Studies involving highly purified or recombinant enzymes and synthetic
substrate/inhibitor libraries have revealed only subtle differences in the specificites of the two enzymes (Rioli et al., 1998).

Some distinction between THOP and neurolysin can be made using the inhibitors (N-[(R,S)-carboxyl-3-phenylpropyl]-Ala-Ala-Tyr-\(p\)-aminobenzoate (cFP-AAY-pAB), which preferentially inhibits THOP (\(K_{i}\) [THOP] = 19nM, \(K_{i}\) [Neurolysin] = 700nM) and Pro-Ile which inhibits neurolysin (\(K_{i}\) [THOP] = 5mM, \(K_{i}\) [Neurolysin] = 90\(\mu\)M) (Dauch et al., 1991). However Pro-Ile is not a very potent inhibitor and cFP-AAY-pAB can also strongly inhibit neurolysin. Highly potent and specific phosphinic inhibitors, Pro-Phe\(\Psi\) (PO\(_2\)CH\(_2\))Leu-Pro-NH\(_2\) (PF\(^{\alpha\epsilon}\)LP-NH\(_2\) (\(K_{i}\) [THOP] = 66.5\(\mu\)M, \(K_{i}\) [Neurolysin] = 12nM) and Pro-Phe\(\Psi\) (PO\(_2\)CH\(_2\))-\((LD)\)Arg-Arg-Phe(Z-FPCA RF (\(K_{i}\) [THOP] = 66.5\(\mu\)M, \(K_{i}\) [Neurolysin] = 12nM) have been developed by and Jiracek and co-workers, which allow the assessment of the distinct functional role of both THOP and neurolysin (Jiracek et al., 1995; Jiracek et al., 1996).

One major difference between the enzymes is the activation of THOP by low levels of reducing agents, which as previously mentioned occurs due to the disassociation of the multimeric form of the enzyme, while neurolysin is strictly monomeric (Shimpton et al., 1997).

Cloning of rat brain neurolysin revealed a 704 amino acid protein that is approximately 60% homologous to THOP (Dauch et al., 1991). It was later shown that relatively few of the difference between neurolysin and THOP relate to the substrate
binding site (Ray et al., 2004). Like the crystal structure of THOP, the crystal structure of neurolysin displays a narrow peptide binding channel.

Modeling of the binding of neurolysin to neurotensin suggests that the N terminus of the peptide slides into the channel, aligning the cleavage site (Pro<sup>10</sup>-Tyr<sup>11</sup>) with the catalytic zinc ion (see Fig 1.10). The presence of flexible loop elements lining the channel were suggested to be responsible for allowing cleavage at multiple sites (Brown et al., 2001). This hypothesis was strengthened by the discovery that one of the differences in between the binding sites of THOP and neurolysin involve a conformational difference in a loop segment between residues 599-611 (Ray et al., 2004).

1.4.4.3 Substrate Specificities

With the exception of the cleavage of neurotensin discussed above, both THOP and neurolysin display a similar specificity for a range of natural substrates. Both enzymes have been shown to cleave peptides between 6-17 residues long, and to preferentially cleave at the carboxyl side of hydrophobic residues. They also display an additional preference for an aromatic residue at the P<sub>2</sub> and P<sub>3</sub>' position, and to favour cleavage with the P<sub>1</sub> position occupied by Phe, Ala, or Arg (Shimpton et al., 2002).
Figure 1.10 Model of NT bound to the deduced structure of neurolysin. A molecular surface representation of neurolysin sectioned to show the large cavity at the bottom of the active site channel is shown with the 13-residue substrate NT, with the N-terminus of the peptide at the top (Brown et al., 2001).

Both enzymes hydrolyse BK at the Phe\(^5\)-Ser\(^6\) bond, GnRH at the Try\(^5\)-Gly\(^6\) bond, the resulting N-terminal fragment at the His\(^2\)-Trp\(^3\) bond, dynorphin A\(_{1-8}\) at the Leu\(^5\)-Arg\(^6\) bond, Ang-I at the Pro\(^7\)-Phe\(^8\) bond and somatosatin at both the Phe\(^6\)-Phe\(^7\) and the Th\(^{10}\)-Phe\(^{11}\) bond. By contrast neurolysin cleaves NT at Pro\(^{10}\)-Tyr\(^{11}\), unlike THOP, which cleaves NT exclusively at Arg\(^8\)-Arg\(^9\) (Shimpton et al., 2002). A number of studies have demonstrated that both THOP and neurolysin display differing affinities for a range of natural substrates. Barrelli and co-workers showed that rat kidney neurolysin displayed a greater affinity for NT and Ang-I than BK (IC\(_{50}\)[NT] = 2.1\(\mu\)M, IC\(_{50}\)[Ang-I] = 4.1\(\mu\)M.
Figure 1.11 Cleavage sites of natural substrates of THOP and Neurolysin. Sites of cleavage for both enzymes are shown by arrows, while the different cleavage site in neurotensin are clearly marked (adapted from Shimpton et al., 2002.)

IC$_{50}$[BK] = 13.2μM (Barrelli, et al., 1993). Similar studies with rat testes THOP demonstrated that the enzyme had a greater affinity for BK and NT than Dynorphin A$_{1-8}$ (Km[BK] = 4.9μM, Km[NT] = 2.8μM, and Km[Dynorphin A$_{1-8}$] = 4.9μM) (Lew et al., 1995). Interestingly this study showed that the substrate specificity of rat testes THOP differed significantly from that of THOP expressed in the brain, not least of all in its inability to efficiently cleave GnRH. This suggested that tissue specific forms of THOP, with differing substrate specificities may exist. Comparative studies with recombinant rat THOP and porcine neurolysin have also shown that although the rate of hydrolysis of BK was roughly similar for both enzymes, neurolysin displays a greater affinity for
neurotensin and Ang-I than THOP, while GnRH and dynorphin A are cleaved at a greater rate by THOP relative to neurolysin (see Table 1.3) (Rioli et al. 1998).

Table 1.3 Relative rates of hydrolysis on naturally occurring peptides of recombinant EP24.15 and EP24.16; 100% of hydrolysis represents the EP24.15 cleavage of 16.6 nmol BK in 10 min at 37°C, in 50 mM Tris-HCl, pH 7.5. (adapted from Rioli et al., 1998).

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>% Hydrolysis EP24.15</th>
<th>% Hydrolysis EP24.16</th>
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<tr>
<td>BK</td>
<td>100</td>
<td>108</td>
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<tr>
<td>NEUROTENSIN</td>
<td>80</td>
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<td>METORPHINAMIDE</td>
<td>130</td>
<td>85</td>
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<tr>
<td>GnRH</td>
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<td>DYNORPHIN</td>
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<td>ANG-I</td>
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1.4.4.4 Subcellular Localisation

The subcellular localisation of both THOP and neurolysin, as identified immunocytochemically, displays variation with cell and tissue type. The initial immunocytochemical localisation of THOP in brain with a polyclonal antibody against the rat testis enzyme showed that the enzyme was primarily localised to the nucleus (Healy et al., 1992). This finding was surprising given that THOP had been associated with the soluble cytosolic cellular fraction (Orlowski et al., 1983). More recently, using both THOP and neurolysin antibodies it was identified that rat brain THOP is primarily nuclear, while rat brain neurolysin was primarily extranuclear, being found particularly in the cell processes (Massarelli et al., 1999).
Other studies with the mouse AtT-20 cell lines have shown that immunoreactive THOP stains in a punctate fashion throughout the cytoplasm and in the cell processes, with occasional intense nuclear staining. This contrasted with the staining for neurolysin, which was confined to the perinuclear area (Garrido et al., 1999). Co-localisation of both THOP and neurolysin with the trans golgi marker syntaxin-6, and with a secretory granule associated protein was also demonstrated in mouse AtT-20 cells by Garrido et al., suggesting that a portion of these peptidases are putatively secreted. A similar conclusion was reached by Ferro and co-workers who found that 15% of total THOP activity and immunoreactivity in AtT-20 cells sedimented in the denser fractions of a Ficoll gradient. These fractions over-lapped with those containing β-endorphin immunoreactivity, again suggesting localisation within the secretory pathway. This was surprising, considering that neither THOP or neurolysin contain a secretory signal sequence. Later studies demonstrated that THOP was both secreted and membrane associated in AtT-20 cells (Ferro et al., 1999; Crack et al., 1999). More recently it has been demonstrated by Jeske and co-workers that THOP is associated with lipid rafts in both mouse AtT-20 cells and mouse neuronal GT1-7 cells, and that in GT1-7 cells it is constitutively released from the exofacial leaflet of lipid rafts, indicating a putative mechanism by which it may be constitutively secreted (Jeske et al., 2003; Jeske et al., 2004).

The secretion of neurolysin activity has also been observed in astrocytes, but not neurons, in culture. However, its release is insensitive to a range of agents that affect classical secretion. In addition, its release was demonstrated to be temperature-dependent. Despite lacking a secretory signal sequence it seems that neurolysin is
secreted via a non classical mechanism (Rubartelli et al., 1990). Membrane association of the enzyme was also observed in the rat brain (Woulfe et al., 1992).

In addition to cytosolic and plasma membrane localisation neurolysin has been shown to exist in the mitochondrial compartment (Tisljar et al., 1990; Serizawa et al., 1995). The fact that neurolysin is the product of a single gene has raised the question as to how it can be located in more than one compartment. The presence of a putative mitochondrial target sequence at the N terminus of the preceursor to neurolysin (mitochondrial oligo peptidase) was noted by Serizawa and co-workers. More recently, Kato and co-workers have demonstrated the existence of alternative initiation site for transcription of neurolysin. The longer transcript contains a cleavable mitochondrial target sequence, which directs the enzyme to the mitochondria, whereas the shorter form lacking such a signal remains in the cytosol (Kato et al., 1997).

1.4.4.5 Involvement of THOP and Neurolysin in the Vasculature

The ability of both THOP and neurotensin to cleave the vasoactive peptides Ang-I and BK suggests that the enzymes may play a role in the maintainence of vascular tone and other vascular functions.

In 1991, Genden and Molineaux showed that the infusion of the inhibitor eFP-AAF-pAB produced an immediate drop in mean arterial pressure in normotensive rats, and that this decrease could be blocked by the administration of B₂ BK receptor
antagonists (Genden et al., 1991). Based on these results the authors suggested that THOP, and to a lesser extent neurolysin played a role in the maintainence of blood pressure by the inactivation of the vasodilator BK. However the discovery of the instability of cFP-AAF-pAB \textit{in vivo} and the fact that it could be degraded into cFP-AA, a potent ACE inhibitor, led to the suggestion that the results observed by Genden and Molineaux may occur as a result of ACE inhibition (Telford et al., 1995). However later studies with the equipotent but more stable inhibitor (N-\{1(R,S)-carboxyl-3-phenylpropyl\}-Ala-Aib-Tyr-p-aminobenzoate (termed JA-2) demonstrated a potentiation of BK induced hypotension in rabbits, without affecting the hypertensive effects of Ang-II, thereby strengthening the hypothesis that THOP and neurolysin are involved in the metabolism of circulating vasoactive peptides and blood pressure maintainence (Smith, et al., 2000). More recent \textit{in vitro} studies by Norman and co-workers demonstrated THOP and neurolysin specific BK and NT cleavage of that in both the endothelial hybrid cell line EA.hy926 and in ovine aortic endothelial cells. Interestingly, it was found that neurolysin was responsible for a greater proportion of the cleavage of both substrates observed by both membrane and cytosolic fractions than THOP, indicating that in their model neurolysin may be the more relevant in the maintainence of blood pressure (Norman et al., 2003).

To our knowledge there as yet have been no other studies which demonstrate the regulation of THOP and neurolysin by hemodynamic forces. However, given their putative role in the maintainence of blood pressure, and the identification fo the SSRE
sequence in the EP24.15 promotor, both THOP and Neurolysin are candidates for regulation by hemodynamic forces (McCool et al., 1998; McCool et al., 2000).

1.5 Physiological Phenomena Associated with Hemodynamic Forces.

Alterations in physiological hemodynamic forces have long been known to have the potential to influence vascular morphology at both the cellular and whole vessel level. This obviously has implication for both healthy tissue and disease states.

For example, the reorientation of endothelial cells in the direction of flow, as observed in regions of laminar flow and normal mean shear stress, seems to be directly correlated with vascular health. In regions of disturbed oscillatory flow and low mean shear stress where the cells are non aligned, there is a greater occurrence of atherosclerotic lesions, increased vascular permeability to leukocyte adhesion and infiltration, and potentially a greater likelihood of an apoptotic profile amongst the endothelial cells in that region (see section 1.2.2.4). In addition, in regions of low mean shear stress there is endothelial production of substances such as ET-1, PDGF and Ang-II, which favour SMC proliferation, a phenomenon which has implications for atherosclerotic plaque formation.

1.5.1 Vascular Remodeling; Restenosis and Neointimal Formation

Atherosclerosis is a progressive disease characterised by the accumulation of lipids and fibrous elements in large arteries. Early lesions consist of subendothelial accumulations of cholesterol-laden macrophages called foam cells. These fatty streaks
develop over time into plaques comprising of a lipid rich necrotic core and typically have a fibrous cap consisting of SMCs and ECM. Plaques become increasingly complex and can grow sufficiently large enough to occlude blood flow. One of the more common clinical complications is thrombus formation as a result of the rupture of a plaque, thrombus formation and consequent vessel occlusion (Traub \textit{et al.}, 1998).

A common treatment for vessel occlusion in humans is balloon Angioplasty. In this treatment a balloon catheter is introduced surgically to the affected vessel and inflated \textit{in situ}, with the resulting destruction of the occlusion. In some cases a stent (a metal or plastic support) may also be introduced to prevent vessel collapse. However in the long term this procedure may often fail due to restenosis (see \textbf{Fig 1.12}), the reduction in vessel lumen diameter due to smooth muscle cell growth and resulting intimal thickening (termed neointimal formation) (Poston, 2002).

It has been proposed that both endothelial damage and the disruption of flow patterns, with resultant reduction in mean shear stress post-angioplasty, may be responsible for this phenomenon. It has been demonstrated in a rat model of carotid artery angioplasty, in which vascular damage was induced by balloon catheter injury, that increasing the blood flow (and thusly the shear stress) was found to reduce early neointimal formation (Kohler and Jaiwien, 1992). An important role for endothelium derived factors produced by endothelial cells in response to shear stress was also indicated by a series of studies which showed that polyurethane vascular grafts seeded with endothelial cells and exposed to high shear rates before implantation in rats showed a marked reduction of neointimal thickness when compared to grafts not exposed to shear
(Dardik et al., 1999). Further studies have demonstrated the importance of factors produced by the endothelium in conditions of disrupted flow and low mean shear stress in neointimal formation; it was demonstrated that inhibition of the PDGFβ receptor reduced neointimal thickness in baboons implanted with polytetrafluoroethylene grafts (Davies et al., 2000). PDGF, as discussed previously, is produced by the endothelium in conditions of low mean shear stress and has a pro-proliferative effect on smooth muscle cells.

Figure 1.12 Diagram showing restenosis and neointimal formation as a result of balloon Angioplasty; Following treatment for vessel occlusion with balloon angioplasty, damage to the endothelium may result in SMC proliferation leading to reduction in lumen diameter. (www.heartcenteronline.com)
1.5.2 Angiogenesis; New Vessel Formation

Angiogenesis is the formation of new capillaries from the pre-existing vasculature by controlled migration and proliferation of endothelial cells and it plays a fundamental role in the growth survival and function of normal and pathological tissues. The process of angiogenesis requires the loosening of intercellular junctions and degradation of the pericellular matrix by endothelial cells, migration of the endothelial cells towards the angiogenic stimulus, sprout formation, formation of a lumen and the joining of sprouts to form a new capillary bed (see Fig 1.13)/(Quarmby et al., 2002).

Figure 1.13 Diagram showing the stages of Angiogenesis; After the loosening of intercellular junctions and degradation of the pericellular matrix, ECs migration through the vessel wall in the direction of an angiogenic stimulus (e.g. VEGF), forming endothelial sprouts, which eventually grow together to form a lumen. Subsequent SMC and pericyte recruitment leads to the development of a new vessel (Quarmby et al., 2002).
In some conditions angiogenesis may be beneficial; for example, in tissue repair after reperfusion of ischemic tissue or cardiac failure. However there are numerous clinical instances where angiogenesis is maladaptive, such as cancer and intraplaque formation (Carmeliet et al., 2000).

A clear association between blood flow/shear stress and angiogenesis is supported in the literature. High blood flow occurs concomitantly with capillary growth in physiological conditions such as exercise or exposure to altitude. Experimental subjects subjected to physical training have been shown to have so 20% more capillaries in their musculature than untrained controls (Breen et al., 1996). Angiogenesis is also a feature of tumor development, and the restriction of tumor Angiogenesis has been used successfully to regulate tumor growth (Quarmby et al., 2002). It has been demonstrated that physiological shear stress enhances the enhances wound closure in cultured human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECS) via the action of endothelial cells spreading and migration (Albuquerque, et al., 2000).

The two most important direct-acting positive regulators of angiogenesis are thought to be vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). It has been demonstrated in vitro that VEGF and FGF positively regulate many endothelial cellular functions relevant to angiogenesis, including proliferation, migration, extracellular proteolytic activity, and tube formation (Dvorak et al., 1995).
Evidence that cyclic strain is a stimulus for angiogenesis comes from both *in vitro* and *in vivo* studies (Rivilis *et al.*, 2002; Zheng *et al.*, 1999; Vailhe *et al.*, 1996; Banai *et al.*, 1994). Conditioned media from cyclically strained myocytes *in vitro* has been found to stimulate tube formation in coronary endothelial cells, a feature of ECs demonstrating an angiogenic profile. *In vivo* studies have demonstrated that short-term exercise, leading to increase levels of mechanical load on the vessel wall resulted in increased angiogenesis in skeletal muscle of Sprague-Dawley rats. Angiogenesis associated with cyclic strain are believed to involve a number of contributors such as MMP-2, VEGF, FGF, and TGF-β and may be associated with a number of conditions in which blood flow is affected including ischemia and bradycardia (Bani *et al.*, 1994; Ausprunk *et al.*, 1977; Pepper *et al.*, 1997; Egginton *et al.*, 2001).

Both NO and prostaglandins are also positive regulators of angiogenesis, though their mode of action is believed to be intermediary and indirect. It has been demonstrated that inhibition of these so-called intermediary cytokines block cell growth and migration *in vitro* (Quarmby *et al.*, 2002). As both production of NO and prostaglandins is up-regulated by increased shear and cyclic strain, it is though likely that they contribute to the increase in angiogenic potential observed in endothelial cells exposed to shear stress. NO production in particular is an important effector mechanism in angiogenesis and may contribute to VEGF-related pathways; it has been demonstrated that the angiogenic response to VEGF was impared in mice deficient in the gene coding for eNOS (Murohara *et al.*, 1998; Silva-Azevedo *et al.*, 2002).
In addition to the above mentioned cytokines it has become evident in recent years that vasoactive peptides, in particular BK, Ang-II and Ang-(1-7), play an important role in angiogenic processes in both healthy and diseased vasculature.

Recent in vivo studies have clearly demonstrated the important pro-angiogenic role of BK, in a rat model (Hu et al., 1993), and in several tumor models (Wu et al., 1998; Ishihara et al., 2002). This BK-induced angiogenesis seems to be mediated largely via the B2 receptor, and involves up-regulation of VEGF production (Ishihara et al., 2002). As BK also stimulates the up-regulation of eNOS via the B2 receptor (Duffy et al., 2004), it is likely that NO production is involved in the phenomenon. Inhibition of BK induced angiogenesis has recently been highlighted as a potential treatment for certain cancers; Stewart and co-workers have demonstrated that BK antagonists inhibit lung and prostate tumor formation in Nude mice models (Stewart et al., 2005).

In contrast to the clear pro-angiogenic properties of BK, the roles played by Ang-II and Ang-(1-7) in angiogenesis is still unclear, with many studies generating contradictory findings. Studies have demonstrated that Ang-II stimulates both endothelial and smooth muscle cell proliferation in vitro (Stoll et al., 1995), increases vessel density in rat cremaster muscle (Munzemaier et al., 1996), in the chorioallanic membrane of the chick embryo (Le Noble et al., 1993), and in the rat ischemic hindlimb (Radi et al., 2002). Inhibitors of angiotensin converting enzyme (ACE) have been shown to block neovascularisation in the rat cornea (Volpert et al., 1996) and micro vascular growth in normotensive rats (Wang et al., 1990). However ACE inhibition was also shown to
increase vessel density in rat ischemic hindlimb (Silvestre et al., 2001). Recent in vivo studies by Desideri and co-workers have also demonstrated that the migratory capacity of HUVECs could be inhibited by treatment with Ang-II (Desideri et al., 2003).

It has been suggested that Ang-(1-7) plays an opposing role to Ang-II in angiogenesis; Machado and co-workers have demonstrated that while Ang-II stimulated angiogenesis in a murine sponge model, treatment with Ang-(1-7) diminishes angiogenesis (Machado et al., 1999). Other studies have demonstrated that Ang-(1-7) treatment causes the release of NO from the endothelium, a substance which is known to promote angiogenesis (Fetterik et al., 2000).

Most actions of Ang-II in the vasculature occur via the AT1 rather than the AT2 receptor. The AT1 receptor has been implicated in both pro- and anti-angiogenic responses to Ang-II treatment. Numerous studies have demonstrated the involvement of VEGF and requirement of eNOS in these events (Tamarat et al. 2002; Otani et al., 1998). Otani and co-workers have demonstrated that Ang-II potentiated the angiogenic response to VEGF in microcapillary endothelial cells, while Tamarat et al. demonstrated that the proAngiogenic effect of Ang-II in a mouse hindlimb Ischemia model was linked to increase VEGF production and blocked in mice lacking the gene for eNOS (Tamarat et al. 2002; Otani et al., 1998).

Although a universal mechanism for the effects of BK, Ang-II and Ang-(1-7) on angiogenesis has yet to be elucidated, it is clear that they play an important role. In
common with other vasoactive peptides their production and release can be mediated in response to hemodynamic forces.

1.6 Summary

Hemodynamic forces, namely shear stress and cyclic strain, have been well characterised as modulators of vascular endothelial function, and have been assigned an important role in the maintainance of vascular tone, hemostasis, and regulation of vascular growth and health. They exert their influence in part by effecting changes in the production and release of vasoactive compounds by the endothelium, and by effecting changes in the levels and activity of various enzymes.

THOP (EP24.15) and neurolysin (EP24.16) are closely related zinc metalloendopeptidases that have been shown to be expressed and active in the vascular endothelium. Their substrates include the vasoactive peptides BK and Ang-I, which have been identified as important regulators of both blood pressure and angiogenic processes. In addition other closely related metallopeptidases, namely ECE and ACE, have been shown to be regulated by hemodynamic forces in the vascular endothelium.

Based on these observations THOP and neurolysin are likely targets for regulation by hemodynamic forces within the vascular endothelium. It is the purpose of this thesis to examine the putative effects of cyclic strain on the expression and activity of THOP and neurolysin in cultured bovine aortic endothelial cells (BAECs). It is also intended to
examine the effects of cyclic strain on the THOP and neurolysin mediated cleavage of both synthetic and natural vasoactive peptide substrates, and the possible functional influence this may have on endothelial cells.

**N.B. THOP and neurolysin will be subsequently referred to as EP24.15 and EP24.16 respectively for the remainder of this thesis.**

### 1.7 Experimental Approach

The experimental approach applied to investigate this hypothesis was as follows (See Fig 1.14); a flexercell *in vitro* culture system which uses vacuum to mechanically strain or deform cells cultured on a flexible, matrix-bonded growth surface was employed for culturing vascular ECs under conditions of equibiaxial defined cyclic strain (Banes *et al.*, 1985). Post-strain levels of secreted and cellular activity were determined for both enzymes using a specific fluorescent substrate (QFS). The levels of mRNA for both enzymes were also determined using RT-PCR with gene specific primers, and the levels of both total and membrane associated EP24.15 were determined immunocytochemically using an EP24.15 anti-sera. Both pharmacological and molecular inhibitors of Giα and Gβγ-signaling were also employed to determine if this mechanotransduction pathway was involved in the transduction of strain-induced changes in enzyme expression and activity. The effects of strain on the endopeptidase specific extracellular cleavage of both synthetic and natural peptide substrates by BAECs in culture were examined. Both the
Cyclic Strain

Bovine Aortic Endothelial Cells

Cyclic strain induced changes in EP24.15/EP24.16 Expression, Activity, and Extracellular Membrane Association

Involvement of Gα/βγ signalling pathways


Effect on Endothelial Cell Fate Decisions such as BAEC Migration and Tubule Formation

Figure 1.14 Flow Chart Representing Experimental Approach.

main product of EP24.15/EP24.16 cleavage of Ang-I, Ang-(1-7), and BK, which is degraded by both enzymes, have been shown to influence endothelial cell fate decisions such as angiogenesis and migration (Machado et al., 2001; Ishihara et al., 2002). In order to investigate the possible functional consequences of strain-induced increases in EP24.15/EP24.16 mediated cleavage of Ang-I and BK in BAECs for endothelial cell fate
decisions, the effects of EP24.15/EP24.16 inhibition on strain-induced BAEC tubule formation and migration was examined.

1.8 Thesis Overview

The research presented in the following chapters examines the regulation of EP24.15 and EP24.16 expression, activity, and subcellular location in cultured BAECs by mechanical forces. It also details the effect of strain on the ability of cultured BAECs in culture to cleave synthetic and natural peptide substrates in an EP24.15/EP24.16 specific manner, and examines the functional impact of these events on the cell.

The results of this research had been divided into three main chapters;

Chapter 2; Materials and Methods

Chapter 3;
Examination of the effects of cyclic strain on EP24.15 and EP24.16 mRNA levels and both cellular and secreted enzymatic activity in BAECs.

Chapter 4;
The involvement of $G_\alpha$ and $G_\beta\gamma$ signaling pathways in the strain mediated regulation regulation of EP24.15 and EP24.16 activity by cyclic strain in BAECs.
Chapter 5;
Examination of the influence of cyclic strain on the cleavage of synthetic and natural peptide substrates of EP24.15/EP24.16 in BAECs.

Chapter 6;
Chapter 2; Materials and Methods
2.1 Materials

AGB Scientific (Dublin, Ireland)
Whatmann Chromatography paper

Amersham Pharmacia Biotech (Buckinghamshire, UK)
Anti-mouse 2° antibody, HP-conjugated
Anti-rabbit 2° antibody, HP-conjugated
ECL Hybond nitrocellulose membrane
ECL Hyperfilm
Rainbow molecular weight marker, broad range (6-175kDa)

Antibodies (custom)
Rabbit anti-EP24.15 fAB was the kind gift of Prof. T.J Wu, Dept. of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, MD, USA.

Bachem UK Ltd. (St. Helens, UK)
Pro-Ile peptide
7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys(2,4-dinotrophenyl)

Bio Sciences Ltd (Dun Laoghaire, Ireland)
DMEM
dNTP's
DEPC-treated water
Trizol® reagent

BioRad (CA, USA)
IScript cDNA synthesis kit

Calbiochem (San Diego, CA)
Pertussis toxin
NF023

Cayman Chemical Company (Michigan, USA)
Anti- rabbit eNOS polyclonal antibody

Chemicon (Temecula, CA)
Anti-MMP-2 antibody
Recombinant MMP-2/MMP-9 standard

Coriell Cell Repository (NJ, USA)
Bovine Aortic Endothelial Cells (BAECs)

Guthie cDNA Resource Centre (Sayre, PA)
Giα1-G202T contract
Gi\textsubscript{12}-G203T construct

Gi\textsubscript{3}-G202T construct

**Invitrogen (Groningen, The Netherlands)**

Lipofectamine reagent

Lipofectamine 2000 reagent

**Molecular Probes (Oregon USA)**

Anti-rabbit antibody, Alexafluor 488 labelled

DAPI fluorescent stain

**MWG Biotech (Milton Keynes, UK)**

THOP(EP24.15) primer set

Neurolysin(EP24.16) primer set

COXII primer set

β-actin primer set

GAPDH primer set

**Inhibitors**

CFP-AAF-pAB was the kind gift of Dr. M. Glucksman, Dept. of Biochemistry and Biophysics, Rosalind Franklin University of Medicine and Science, IL, USA

**PALL Corporation (Dun Laoghaire, Ireland)**

Biotrace nitrocellulose membrane
Pierce Chemicals (Cheshire, UK)

BCA protein assay kit
Supersignal West Pico chemiluminescent substrate

Plamids

β-ARK-ct and GFP were the generous gifts of Dr. John Cullen, Dept. of Surgery (University of Rochester Medical Centre, Rochester NY)

EP24.15-FLIP was the generous gift of Dr. M. Glucksman, Dept. of Biochemistry and Biophysics, Rosalind Franklin University of Medicine and Science, IL, USA

PromoCell (Heidelberg, Germany)

Human Aortic Endothelial Cells
Endothelial Cell Growth Media MV

Promega (Madison, WI)

Taq DNA Polymerase
MLV-RT
RNase H
Oligo dT$_{12-14}$

Sarstedt (Drinagh, Wexford, Ireland)

T25 tissue culture flasks
T75 tissue culture flasks
T175 tissue culture flasks
6-well tissue culture plates
24-well tissue culture plates
5.10 and 25ml serological pipettes
15 and 50ml conical centrifuge tubes

Sigma Chemical Company (Poole, Dorset, England)
β-galactosidase
2-mercaptoethanol
Acetic Acid
Acetonitrile(Chomosolv)
Acetone
Agarose
Ammonium Persulphate
Ang-I
Ang-Iotensins II
Ang-(1-7)
Bestatin
Bisacrylamide
Bovine Serum Albumin
BK
BK 1-5
Brightline Haemocytometer

Bromophenol Blue

Captopril

Chloroform

EDTA

EGTA

Ethidium Bromide

Fibronectin

Foetal Calf Serum

Bovine Gelatin

Glycerol

Glycine

Hanks Balanced Salt Solution

Hydrochloric Acid

Isopropanol

Methanol

Penicillin-Streptomycin (100x)

Phosphoramidon

Ponceau S

Potassium Chloride

Potassium Iodide

Potassium Phosphate (Dibasic)

Rat tail type I collagen
RPMI-1640
Sodium Chloride
Sodium Hydroxide
Sodium Orthovanadate
Sodium Phosphate
Sodium Pyrophosphate
SYBERgreen Jump Start Taq Ready Mix
SDS
TEMED
Tris Acetate
Tris Base
Tris Cl
Triton X-100
Trypsin-EDTA Solution (10x)
Tween 20

2.2 Cell Culture Methods

All cell culture techniques were carried out in a clean and sterile environment using a Bio air 2000 MAC laminar air flow cabinet. Cells were visualized using an Olympus CK30 phase contrast microscope.
2.2.1 Culture Of Bovine Aortic Endothelial Cells (BAECs)

BAECs were obtained from the Corriell Cell Repository, New Jersey, USA (CAT NO: AG08500). The cells are derived from a one-year-old male Hereford cow. The thoracic aorta was removed immediately post-mortem on 10/22/85. Cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 5%CO₂. Cells were cultured in T175cm², T75cm², T25cm² and 6 well plates. In the case of cyclic strain experiments cells were grown on Bioflex® series culture plates which have a flexible, pronectin-bonded growth surface. Cells between passage 8 – 17 were used in these experiments.

BAECs are a strongly adherent cell line, forming a confluent monolayer with a distinct cobblestone-like cell morphology. 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 thee 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 for 5-10 min or until all the cells were removed from the flask surface. Trypsin was inactivated by the addition of growth medium and the cells were removed from suspension by centrifugation at 3500rpm for 5 min. Cells were then resuspended in culture medium and typically passaged 1:5 into culture flasks, or cryogenically preserved.
2.2.2 Culture of Human Aortic Endothelial Cells (HAECs)

Human aortic endothelial cells (HAECs) were obtained from PromoCell, Heidleberg, Germany (Cat. No.: C-12271). The cells were derived from a 21 year old male Caucasian. Cells were maintained in endothelial cell growth medium MVDB131 supplemented with endothelial supplement mix (5% (v/v) fetal calf serum (FCS), 10ng/ml Epidermal Growth Factor, 1μg/ml Hydrocortison, 50 ng/ml Amphotericin B, 50ng/ml Gentamicin) A 1:5 sub-culture ratio was generally used. Cultured cells were maintained in a humidified 5% v/v CO₂ atmosphere at 37°C.

2.2.3 Cyclic Strain Studies

For cyclic strain studies, BAECs/HAECs were seeded into 6-well Bioflex® plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of approximately 6X10⁵ cells/well, allowed to adhere and grown to confluence over 24h. After 24 h, the media was removed and replaced with fresh media and the cells exposed to varying levels of cyclic strain. Bioflex™ plates contain a pronectin-coated silicon membrane bottom that enables precise deformation of cultured cells by microprocessor-controlled vacuum. When cells had reached approximately 100% confluence, a Flexercell™ Tension Plus™ FX-4000™ system (Flexcell International Corp. - Hillsborough, NC) was employed to apply a physiological level of cyclic strain to each plate (0-10% strain, 60 cycles/min, 0-24 h) providing equibiaxial tension using the Heartbeat™ Simulation protocol.
2.2.4 Cell Counts

Cells counts were performed using a Sigma brightline haemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viability. 20 μl of trypan blue was added to 100 μl of cell suspension, and the mixture was left to incubate for two min. 20 μl 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:

\[ \text{Average Cell No.} \times 1.2 \times 1 \times 10^4 \times (\text{area under cover slip mm}^3) = \text{Viable cells/ml} \]

2.2.5 Treatment With Pharmacological Inhibitors

For these experiments, BAECs were grown until approximately 70-80% confluent after which the growth media was removed and cells rinsed 3 times in HBSS. Inhibitors were diluted in RPMI-1640 supplemented with 5% FBS and antibiotics. For DMSO-soluble inhibitors, a suitable stock concentration was prepared so that the final concentration of DMSO in working solutions was less than 0.5%. Cells were exposed to inhibitors for 1 hour prior to exposure to cyclic strain and were present in the media during the course of the mechanical strain experiment. Following 24 h of 5% cyclic strain cells were harvested for total RNA/protein and conditioned media. Inhibitors used were pertussis toxin (100 ng/ml), NF023 (10 μM), captopril (10 μM), phosphoramidon (10
μM), bestatin (10 μM) and cFP-AAF-pAB (4.00 μmol/L). Concentrations used were taken from current literature or based on manufacturers recommendations.

2.2.6 Preparation of Whole Cell Lysates

Following trypsinisation as described in section 2.1.1, the cell pellet was washed in 1X PBS to remove any trace levels of FBS. The cell suspension was then centrifuged at 3500 rpm for 5 min. The PBS supernatant was removed and the cells were resuspended in 1X lysis buffer (20 mM Tris, 150 mM NaCl; ImM Na$_2$EDTA; 1 mM EGTA; 1% Triton X-100 (v/v); 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1mM sodium orthovanadate; 1μg/ml leupeptin). The resulting lysates were frozen and thawed three times followed by three cycles of ultrasonication for 5 sec on ice using a sonic disembrator (Vibra Cell, Sonics and materials Inc). In the case of samples to be assayed for cellular EP24.15/EP24.16 activity pellets were resuspended vigorously in PBS, as the presence of zinc chelators in lysis buffer affected enzyme activity. These samples were then frozen and thawed five times followed by three cycles of ultrasonication for 5 sec on ice using a sonic disembrator (Vibra Cell, Sonics and materials Inc). All samples were stored at 20°C for short-term storage or −80°C for long-term storage, until required for analysis.
2.2.7 Bicinchoninic Acid (BCA) Protein Microassay

In this assay Cu$^{2+}$ reacts with protein under alkaline conditions to produce Cu$^+$, which in turn reacts with BCA to produce a coloured product (Pierce., 1997). 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; 200 µl of this mixture is added to 10 µl of protein lysate or BSA standards (standard curve in the range 0-2 mg/ml). The plate is incubated at 37°C for 30 min and the absorbance read at 560 nm using a microtitre plate reader.

2.2.8 Lactate Dehydrogenase (LDH) Assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells. Frequently used as a marker for plasma membrane damage or “leakage”. In vitro release of LDH from cells subsequently provides an accurate measure of cell membrane integrity and cell viability (Racher et al., 1998). As such, this assay was used to assess the effects of mechanical strain on viability of BAECs. This assay is based upon the ability of LDH to catalyze the reaction:
Pyruvate + NADH + H(+) → Lactate(+) + NAD(+)

Briefly, 5 μl of cell lysate or 50 μl of conditioned media was added to 900 μl of 0.2M Tris-HCl pH7.3, 100 μl of 1.1mM β-NADH and 20 μl of 115 mM sodium pyruvate. The samples were mixed by inversion and the absorbance read at 340 nm every 20 sec for 5 min. LDH activity can be derived from the rate of changes in optical absorbance (ΔOD/sec/μg cellular protein/ml) (Cao et al., 1993).

2.3 RNA Preparation Methods

2.3.1 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 based on the method of Chomczynski and Sacchi (Chomczynski et al., 1987). Trizol reagent maintains the integrity of the RNA while disrupting the cells and dissolving the cell components.

After washing briefly with HANKS, cells were lysed directly in culture plates by the addition of 1ml of Trizol per 10 cm². A volume less than this can result in contamination of the RNA with DNA. To ensure complete homogenization, cells were lysed by titrating through a pipette a number of times. The samples were then incubated for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per ml of Trizol reagent used and was then
mixed vigorously for 15 sec before being incubated for 5 min at room temperature. Samples were then centrifuged at 12,000 g for 15 min 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 0.5 ml of isopropanol per 1ml of Trizol used. Samples were incubated for 15 min at room temperature and then centrifuged at 12,000 g for 10 min 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 1 ml of 75% ethanol per ml of Trizol used followed by centrifugation at 7,500 g for 5 min at 4°C. The resultant pellet was air-dried for 5-10 min before being resuspended in DEPC-treated water. The sample was then stored at –80°C until used. The concentration of total RNA was determined by UV spectrophotometry as outlined in section 2.3.2.

2.3.2 Spectrophotometric Analysis of Nucleic Acids

DNA or RNA concentrations were determined by measuring the absorbance at 260 nm, the wavelength at which nucleic acids absorb light maximally (λ max). A 50 μg/ml solution of DNA or 40 μg/ml solution of RNA has an absorbance of reading of 1.0 at this wavelength. In order to calculate the concentration of DNA/RNA in samples the following calculations were used;
DNA; Abs \(_{260\text{nm}}\) x 50 x 200 (dilution factor, i.e. 5 \(\mu\)l of sample in 995 \(\mu\)l H\(_2\)O) = \(\mu\)g/ml

RNA; Abs \(_{260\text{nm}}\) x 40 x 200 (dilution factor, i.e. 5 \(\mu\)l of sample in 995 \(\mu\)l H\(_2\)O) = \(\mu\)g/ml

The purity of the DNA or RNA samples was established by reading the absorbance at 260 nm and the absorbance at 280 nm and then determining the ratio between the two (ABS\(_{260}/\text{ABS}_{280}\)). 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, higher ratios imply the presence of organic reagents.

2.3.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Real Time PCR

PCR has over the last 20 years proved to be an important and powerful tool for amplifying small quantities of DNA for analysis. RT-PCR is a modification of this technique in which small quantities of specific messenger RNA (mRNA) are analysed. Total RNA is converted to copy DNA (cDNA) using the oligo d\(_{12-18}\) primers enzyme reverse transcriptase. cDNA of interest can subsequently be amplified by PCR using gene specific primers. The PCR was semi-quantitative. GAPDH (a house-keeping gene) expression was monitored in tandem with the gene of interest, the ratio of gene X to GAPDH served as a means of determining relative amounts of the target genes in each reaction.
A recent development in PCR technology has been Real-Time PCR technology. This allows monitoring of DNA amplification throughout the polymerase chain reaction by the use of a fluorescent dye (SYBER green), which binds to the double stranded DNA product. The advantages of this technique over standard RT-PCR is that it is fully quantitative, allowing either quantification using an appropriate DNA standard curve or relative quantification by comparison with GAPDH expression. In addition automatic melt curve analysis at the end of the reactions indicates if non-specific product has been formed during the reaction.

2.3.4 Reverse Transcription

Reverse transcription was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) in accordance with manufacturers specifications with some minor modifications (Roth et al., 1985; Sambrook et al., 1989). 0.5 µg of total RNA (isolated as described in section 2.1.9) was mixed with 0.125 µg oligo dT\textsubscript{12-18} primers and the reaction mixture brought to a final volume of 12 µl with DEPC water. This mixture was heated for 10 min at 70°C to allow annealing of oligo dT\textsubscript{12-18} primers to polyA tail of mRNA. Following this, tubes were immediately cooled on ice and the remaining components of the reaction were added as follows:

- MMLV 5X Reaction Buffer 5 µl
- 10mM dNTP 3 µl
- MMLV-RT 200 units
The mixture was then made up to a final volume of 25 μl using DEPC water and incubated for 60 min at 42°C. Contaminating RNA was subsequently removed by the addition of 1μl of RNase H (2 units/μl) at 37°C for 20 min. cDNA samples were then either used immediately or stored at -80°C until required.

For real-time PCR reverse transcription was also carried out using the proprietary iScript™ cDNA synthesis kit (BioRad, Ca, USA). Using 0.5 μg of total RNA template the reaction was performed as per manufacturers directions:

15 μl DEPC water/RNA
1 μl iScript reaction buffer
1 μl Reverse Transcriptase

The mixture was held at 25°C for 5 min to allow for oligo dT 12-18 annealing, a 30 min incubation at 42°C to allow for cDNA formation. cDNA samples were either used immediately or stored at -80°C until required for analysis.
2.3.5 Polymerase Chain Reaction

A. Standard PCR

A 50 µl PCR reaction mixture was prepared as follows:

- RNase-free water: 36.5 µl
- 10X reaction buffer: 5 µl
- 10 mM dNTP: 1 µl
- 25 mM MgCl: 3 µl
- 10 µM Forward primer: 1 µl
- 10 µM Reverse primer: 1 µl
- Taq Polymerase: 0.5 µl
- cDNA sample: 2 µl

The mixture was overlaid with 50 µl of mineral oil and then placed in a Hybaid PCR Thermocycler. Samples were subjected to an initial incubation of 92°C for 2 min followed by 30 cycles comprising of the following steps: 92°C for 1 min, annealing temperature for 2 min and 72°C for 3 min. PCR products were removed from beneath the mineral oil and placed in fresh tubes before being subjected to agarose gel electrophoresis.
B. Real-Time PCR

Reactions for real time PCR were prepared as follows as per manufacturers directions;

- RNase -free water 8.5 μl
- SYBERgreen Jump Start™ Taq Ready Mix 12.5 μl
- cDNA 2 μl
- 10 μM Forward primer 1 μl
- 10 μM Reverse primer 1 μl

The sample were made in triplicate and placed in a Rotor-Gene RT3000 real time PCR machine, which subjected them to the following regime;

Denaturation step; 95°C 15min

Cycling (up to 40 cycles)
- denaturation 95°C 10 sec
- primer annealing 55°C 30 sec
- elongation/data acquisition 72°C 30 sec

Hold 60°C 1min

Melt Curve Generation; increase temperature from 50°C to 90°C at a rate of 1°C/5 sec.

The excitation and emission wavelengths of SYBER green are 470 nm and 510 nm respectively.
Table 1 Primers used with standard and Real Time PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Product size</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THOP (EP24.15)</td>
<td>For. 5'-tga agg tca ccc tca agt a-3' Rev. 5'-tcc acc tgg ttc atg tag ta-3'</td>
<td>401bp</td>
<td>55°C</td>
</tr>
<tr>
<td>Neurolysin (EP24.16)</td>
<td>For.5'-tgg aaa ctg act ttg tag agg-3' Rev.5'-aaa agt agc tgg cat att tgt-3'</td>
<td>308bp</td>
<td>55°C</td>
</tr>
<tr>
<td>GAPDH</td>
<td>For. 5'agg tca tcc atg acc act tt 3' Rev. 5'ttg aag tgc cag gag aca a 3'</td>
<td>337 bp</td>
<td>54°C</td>
</tr>
<tr>
<td>COXII</td>
<td>For. 5'-ttt gaa gaa ctta cag gag-3' Rev. 5'-tat tgc aga tga gag act ga-3'</td>
<td>280bp</td>
<td>55°C</td>
</tr>
</tbody>
</table>

2.3.6 Agarose Gel Electrophoresis

Agarose gels were prepared by boiling the appropriate quantity of agarose in 100 ml of 1X TAE buffer (40 mM Tris-Acetate pH 8.2, 1 mM EDTA), gels were generally 1-2% (w/v) depending on the size of the DNA fragment being visualised. Gels contained 0.5 μg ethidium bromide per 1 ml of agarose for visualization of DNA within the gel. When the gel was hand-hot the gel was cast in a Gibco-BRL Horizion-20.25 gel electrophoresis apparatus.

Samples were mixed with 6X gel loading buffer (40% w/v sucrose, 0.25% w/v bromophenol blue). 12.5 μl of PCR product from standard RT-PCR was mixed with 3 μl
of loading buffer and subsequently loaded. The gel was run at 100 V in 1X TAE buffer until the blue dye front was approximately 0.5 cm from the end of the gel. DNA was visualized on a transilluminator and photographed for densitometric analysis using the Kodak 1D gel documentation system (Scientific Imaging Systems, Eastman Kodak Group, Rochester, NY).

2.4 Polyacrylamide Gel Electrophoresis

2.4.1 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:

<table>
<thead>
<tr>
<th>Resolving Gel:</th>
<th>1.5 ml</th>
<th>Buffer A (1.5M Tris pH8.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml</td>
<td>40% bisacryl stock</td>
<td></td>
</tr>
<tr>
<td>3 ml</td>
<td>distilled water</td>
<td></td>
</tr>
<tr>
<td>60 μl</td>
<td>10% (w/v) SDS</td>
<td></td>
</tr>
<tr>
<td>30 μl</td>
<td>10% (w/v) ammonium persulphate</td>
<td></td>
</tr>
<tr>
<td>7 μl</td>
<td>TEMED</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stacking Gel:</th>
<th>0.75 ml</th>
<th>Buffer B (0.5 M Tris pH6.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375 ml</td>
<td>40% bisacryl stock</td>
<td></td>
</tr>
</tbody>
</table>
1.85 ml distilled water
30 μl 10% (w/v) SDS
15 μl 10% (w/v) ammonium persulphate
7 μl TEMED

Samples were mixed with 4X loading buffer (8% SDS, 20% β-mercaptoethanol, 40% glycerol, Brilliant Blue R in 0.32 M Tris pH6.8) and boiled at 95°C for 5 min, then immediately placed on ice. Typically 2-10 μg of protein per well was added, with equal amounts of protein added to each well. The gel was electrophoresed in reservoir buffer (0.025 M Tris pH 8.3; 0.192 M Glycine; 0.1% (w/v) SDS) 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 min in cold transfer buffer (0.025 M Tris pH8.3; 0.192 M Glycine; 15% v/v methanol). During this time nitrocellulose membrane and 16 sheets of Whatmann filter paper were cut to the same size as the gel and soaked in transfer buffer. Apparatus was set up according to manufacturers protocol. Proteins were transferred to the membrane for 30 min at 100V in an ATTO semi-dry 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 between lanes.

Membranes were blocked for 1 hour in blocking solution [5% (w/v) skimmed milk in Tris Buffered Saline [TBS]; 10mM Tris pH8.0; 150mM NaCl]]. Membranes were
then incubated either overnight at 4°C or for 3-4 h at room temperature, with primary antibody diluted according to manufacturers instructions in blocking solution. Primary antibodies used were anti-Gi$_{a1}$, anti-Gi$_{a2}$, and anti-Gi$_{a3}$ rabbit polyclonal IgG (1:500) (CN Biosciences - Nottingham, UK) and anti-endothelial nitric oxide synthase (eNOS) rabbit polyclonal IgG (1:1000) (Cayman Chemicals - Ann Arbor, MI. The blots were then vigorously washed in three changes of TBST (0.05% (v/v) Tween in TBS) and then incubated for 2 h at room temperature with a suitable HP linked secondary antibody diluted in blocking solution. Following incubation in secondary antibody, the blots were again washed in three changes of TBST.

Antibody-antigen complexes were detected by incubation in West Pico Supersignal reagent (Pierce Chemicals). Briefly, an equal volume of solution A and B were mixed and the incubated with blot for 5 min at room temperature. Blots were exposed to autoradiographic film (Amersham Hyperfilm ECL) 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 min.
2.5 DNA Preparation Methods

2.5.1 Transformation of Competent Cells

100 µl of competent JM109 and/or XL1-Blu E.Coli. cells were placed in a sterile microfuge tube, to which approximately 10 ng of plasmid DNA, typically encoding ampicillin resistance was added. The cells and plasmid were mixed by moving a sterile pipette tip gentle though the cells, after which the cells were subsequently heat shocked by placing a tube in a waterbath at 42°C for 45-50 sec. Cells were then immediately placed on ice for 2 min.

Cells were grown for 1 h at 37°C with agitation (200 rpm) in 1ml of sterile Luria Bertrani (LB) broth (1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl; pH 7.5). The cells were then centrifuged at 12000 g for 1 min and the supernatant removed. The resultant pellet was resuspended in 0.2 ml of LB broth and spread plated either 100 µl and 50 µl on LB-Amp agar. The plates were incubated at 37°C overnight and for no longer than 18 h to prevent colasence of colonies. As a control for each transformation a mock transformation was included, in which no DNA was added to the competent cells.

N.B. Plasmid maps of all vectors used are included in the appendix
2.5.2 Plasmid DNA Mini-Preparation

Plasmid DNA was isolated as specified by Qiagen Plasmid Kit protocol (Sambrook et al., 1989; Ausubel et al., 1991; Birnboim et al., 1983). Single colonies of transformed cells were removed from plates and grown in 3 ml of LB broth supplemented with 35µg/ml ampicillin. These mini-cultures were grown at 37°C for 8 h with gently agitation (<200 rpm). 1.5ml of the final culture was used for the generation of glycerol stocks. The remainder was diluted in 100 ml of LB-amp broth and grown at 37°C overnight at 250 rpm. The following day the cells were harvested by centrifugation at 6000rpm for 15 min at 4°C.

The pellet was resuspended in 4 ml of Buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A). The resuspended cells were lysed by gentle inversion with 4 ml of Buffer P2 (200 mM NaOH; 1% SDS), and was incubated at room temperature for 10 min. Protein was precipitated by the addition of 4 ml of pre-chilled Buffer P3 (3M potassium acetate, pH5.5), gentle mixing and incubation on ice for 5 min. Protein precipitates were removed by high-speed centrifugation, 13000 g for 30 min at 4°C. The supernatant was removed and centrifuged at 13,000 g for 30 min 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 has been equilibrated with Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% triton X-100). The sample was allowed to enter the column by
gravity flow and the column washed with 2 x 10 ml washes of Buffer QC (1M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol). Finally, DNA was eluted from the column using 5 ml of Buffer QF [1.25M NaCl; 50mM Tris-HCl, pH 8.3; 15% isopropanol].

DNA was precipitated from the eluate by the addition of 3.5ml of isopropanol at room temperature. The mixture was centrifuged at 12,000 g for 30 min 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 2 ml of 70% ethanol, (to remove precipitated salts and to make reconstitution of the DNA easier) and then centrifuged at 12,000rpm for 10 min. The DNA was air-dried for 5–10 min after removal of the supernatant and then redissolved in sterile Tris-EDTA (TE) buffer (10mM Tris-HCl pH8.0; 1mM EDTA).

2.5.3 Restriction Digests

DNA was digested with restriction endonucleases for identification of purified plasmid DNA. 1 µg of DNA was prepared in a reaction solution containing 10X reaction buffer (supplied with each enzyme, by the manufacturer) along with 1 µl of restriction enzyme. The digest was incubated at a suitable temperature (enzyme-specific) for 30 min to one hour, the reaction was typically stopped by heating the reaction mixture to 72°C. In the case of multiple digests, following incubation with the first enzyme, linearised DNA was precipitated and then exposed to subsequent enzymes to overcome problems with differing reaction buffers. The products of the digest were then resolved by electrophoresis on a 2% agarose gel with appropriate size standards.
2.5.4 Transient Transfection

Lipofectamine™ reagent is a polycationic liposome as such it is suitable for transfection of DNA into eukaryotic cells (Invitrogen-Groningen, Netherlands). The day prior to transfection, cells were seeded into a bioflex plate at a density of approximately 6X10^5 cells/well, and grown overnight in RPMI-1640 supplemented with serum and antibiotics.

When cells had reached approximately 70% confluency they were transfected with plasmid DNA. For transfection purposes plasmid DNA was diluted in 150 μl of DMEM without FCS or antibiotics such that there would be 1 μg of DNA per 10 cm² of surface area (In the case of a six well plate, 1 μg DNA per well). In a separate tube 10 μl of lipofectamine reagent (4 μl per 10 cm²) was diluted in 150 μl of DMEM without FCS or antibiotics. The diluted DNA was then mixed with diluted lipofectamine reagent and incubated at room temperature for 30 min. This time permits the formation of DNA-liposome complexes.

While the DNA complexes were forming the cells were washed three times in HBSS followed by one wash in DMEM. This was to remove any antibiotics from the flask, which may impede transfection efficiency. The DNA/lipofectamine mixture was made up to a final volume of 0.5 ml, which is just enough media to cover the surface area of the well. The contents of the tube were then added to the well. The cells were incubated for 4 h in transfection media, following which the media was removed and
replaced with normal RPMI-1640 growth media. Cells were subsequently allowed to recover overnight before they were exposed to experimental conditions. Cells were routinely co-transfected with either a Lac Z or green fluorescent protein (GFP) encoding plasmid as a means of estimating approximate transfection efficiency.

2.5.5 Monitoring of Transfection Efficiency

Co-transfection with either Lac Z, a plasmid encoding β-galactosidase, or a plasmid encoding GFP was used to monitor transfection efficiency.

Increased levels of β-galactosidase activity was attributed to successful transfection of the gene of interest. Following transfection and cell lysis, a 30 μl sample was added to 3 μl of 100X Mg solution [0.1 M MgCl₂ and 4.5 M β mercaptoethanol], 66 μl of 1X OPNG (o-nitrophenyl-β-D-galactopyranosidase)[4 mg/ml ONPG in 0.1 M sodium phosphate, pH 7.5] and 201 μl of 0.1M sodium phosphate. The reaction was incubated for 4-6 h at 37°C until a yellow colour developed. The reaction was subsequently stopped with 500 μl of Na₂CO₃, and optical density read at 420 nm. Suitable positive and negative controls were included in this assay.

Transfection efficiency was monitored using co-transfection with a plasmid encoding GFP. Cells successfully transfected with this plasmid, and expressing GFP, could be viewed under using a fluorescent microscope (excitation 395 nm/ emission 508
nm). Comparison of fluorescent and bright field images allowed degree of transfection to be approximated. Typically 50-60% of the cells were seen to express GFP.

2.6 Transfection with siRNA

siRNA mediated gene silencing has grown rapidly in popularity as a method of analyzing gene function. This procedure involves the transfection of cells with small interfering RNA (siRNA) duplexes. These duplexes are 21 nucleotides in length and are designed to specifically target an individual mRNA of interest, in our case that of EP24.15. siRNA are effective at much lower quantities than other gene silencing methods including antisense and ribozyme based protocols (Yu et al., 2002; Yang et al., 1999). siRNA used in these studies was supplied as a 2’-deprotected, annealed oligonucleotide in a desalted form.

This method of gene silencing is derived from a cellular defense mechanism probably evolved to deal with RNA viral infection. Mammalian cells typically show a characteristic “acute phase response” when transfected with small amounts of viral dsRNA. This involves the interferon-mediated activation of dsRNA responsive protein kinase (PKR) leading to a cascade that ends in global non-specific gene silencing and apoptosis. However this process can be circumvented to prevent the acute phase response and still attain specific gene silencing by altering the length of dsRNA. In the cell gene specific silencing begins with the RNase III activity of the enzyme DICER. This enzyme cleaves the dsRNA molecules into 19 base pair fragments in a random fashion. These
small interfering RNAs (siRNAs) induce the formation of the RISC (RNA induced silencing complex) complex of proteins. This RISC complex with bound siRNA is capable of interacting with the specific mRNA complementary to the anti-sense strand of the siRNA. This interaction of RISC/siRNA complex to the specific mRNA induces an endonuclease specific cleavage of mRNA in the siRNA targeted sequence leading to the full-length degradation of the mRNA (see Fig 2.1).

2.6.1 Design of siRNA Duplex

A sequence is selected in the open reading frame of the cDNA which is at least 75-100 bp downstream of the start codon. Untranslated regions near the start codon may be richer in regulatory protein binding sites, which may interfere with binding of the siRNA endonuclease complex. The first AA dimer is located and the next 19 nucleotides are recorded. The G/C content of the AA-N_{19} base sequence is determined. Ideally the G/C content must be greater than 30% and less than 70%. If the sequence does not meet these criteria, a sequence further downstream starting with an AA dimer is analysed. This is continued until a sequence is found which meets all of the above conditions. Following this the 21 base sequence is subjected to a BLAST search to ensure that only one gene is targeted.
Figure 2.1 Mode of action of siRNA; introduction of siRNA duplex leads to the formation of RNA induced silencing complexes (RISC), which bind specifically to the mRNA of interest leading to its degradation by endonucleases. (www.mwg.com)

2.6.2 Transfection with siRNA Duplex:

siRNA was transfected into BAECs using lipofectamine™ 2000. siRNA was diluted in 5X universal buffer (200 mM KCl; 30 mM HEPES-KOH pH 7.5; 1mM MgCl₂) and RNase-free water to a final concentration of 20 |M (20 pmoles/μl). The following procedure is for transfection in a 6-well format. 8X10⁵ cells were plated per well to achieve 70–80% confluency at time of transfection. siRNA/lipofectamine™ 2000 complexes were prepared as follows:
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence of SiRNA Duplex</th>
<th>GC Content</th>
<th>Mol. Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP24.15 I</td>
<td>5'-CCUG CUG AAG GAG UAC UUC 3'</td>
<td>48%</td>
<td>13,323.2g/mol</td>
</tr>
<tr>
<td></td>
<td>3'-UUG GAC GAC UUC CUC AUG AAG 5'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP24.16</td>
<td>5'-CUGA CUU GAA CCA CGA GAA UU 3'</td>
<td>42%</td>
<td>13,373.0g/mol</td>
</tr>
<tr>
<td></td>
<td>3'-UUG ACU GAA CUU GGU GCU CUU 5'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP24.15 II</td>
<td>5'-C UGU CGC UGC AAG GAG GAG UU 3'</td>
<td>57%</td>
<td>13,353.0g/mol</td>
</tr>
<tr>
<td></td>
<td>3'-UUG ACA GCG ACG UUC CUC CUC 5'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>scrambled</td>
<td>5'-AUU CUA UCA CUU GCG UGA C UU 3'</td>
<td>42%</td>
<td>13,373.0g/mol</td>
</tr>
<tr>
<td></td>
<td>3'-UU UAA GAU AGU GAU CGC ACU G 5'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Firstly, 5μl (100 pmoles/10 cm²) of siRNA was diluted in 395 μl of DME medium without serum or antibiotic (the presence of antibiotics during transfection causes cell death). The mixture was then gently mixed. Lipofectamine™ 2000 was mixed prior to use and subsequently 5 μl was diluted in 395 μl of DME medium and incubated for 5 min at room temperature. The two mixtures were then combined and mixed gently. The mixture was incubated for 30 min at room temperature to allow the formation of siRNA/lipofectamine™ 2000 complexes. The 800 μl of siRNA/lipofectamine™ 2000 complexes were added to the well and mixed gently by slowly rocking the plate back and forward to ensure complete coverage of the cells. Cells were incubated for 3 h at 37°C in a CO₂ incubator. After the 3 h incubation the media was removed and replaced with
growth medium and cells were recovered overnight. Following recovery, media was replaced with fresh growth media and cells and media were harvested 24 h later for analysis.

A commercially available non-specific control siRNA sequence (scrambled) (MWG-Biotech) was transfected into BAECs.

2.7 Enzymatic Assays

2.7.1 Fluorometric Plate Assay for EP24.15/EP24.16

Enzymatic activities of EP24.15 and EP24.16 were routinely determined using a quenched fluorescent substrate (QFS), 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys(2,4-dinotrophenyl) (Bachem - Meyerside, UK), according to the method of Crack and co-workers with modifications for assay in 96-well format (Crack et al., 1999). Briefly, subcellular fraction (5 μL) or media (50 μL) was incubated at 37 C with 4.28 μmol/L QFS in reaction buffer to a final volume of 218 μL. Specific inhibitors of both EP24.15 and EP24.16 were incorporated into the assay to delineate the relative contributions of each enzyme to total QFS hydrolysis. These included the active site-directed inhibitor, N-[1-(R,S)-carboxyl-2-phenylethyl]-Ala-Ala-Phe-p-amino-benzoate (cFP-AAF-pAB) (4.00 μmol/L) (Orlowski et al., 1988), which selectively inhibits both endopeptidase activities, and the dipeptide, Pro-Ile (Bachem - Meyerside, UK)(4.82 mM), an EP24.16-specific inhibitor (Dauch et al., 1995). Reactions were terminated after
60 min with 32 μL of 0.5 M sodium formate, pH 3.5. Liberated 7-methoxycoumarin-4-acetyl-Pro-Leu was subsequently measured on a Perkin Elmer LS-50B fluorescence spectrophotometer plate reader at excitation and emission wavelengths of 328 and 393 nm, respectively. Enzyme activities were determined from a standard curve prepared under identical assay conditions with the aforementioned product. Suitable negative controls were included in each assay. Total substrate hydrolysis was typically <10% in all assays.

2.7.2 Whole Cell in situ Cleavage Assays

2.7.2.1 Quenched Fluorescent Substrate Assay

For monitoring QFS cleavage by BAECs a modified version of the assay described by Norman and co-workers was employed (Norman et al., 2003). Approx 6.5X10⁵ BAECs per well were plated onto Bioflex™ plates and left for 24 h. The media was then removed and the cells washed with HBSS. Cells were incubated with QFS (7.4 μM, 100 μL added to give a final volume of 2 ml) in the presence and absence of cFP-AAF-pAB (3.4 μM), which selectively inhibits EP24.15 and EP24.16. All assays included phosphoramidon (10 μM) and captopril (10 μM) to selectively inhibit ACE, ECE and NEP, metalloendopeptidases known to degrade QFS. All assays were carried out in RPMI(-serum/-antibiotics). Following addition of substrate, straining commenced (5%, 24 h). Unstrained plates were included as controls. Prior to addition of substrate all plates were incubated with inhibitor for 1h. At each time point (3, 6, 12 and 24 h) 100 μL
of media from three identical wells was harvested and combined, centrifuged to remove cell debris, and snap frozen in liquid NO₂ and stored at –80°C until required for analysis. At t=24h the cellular protein was also harvested and assayed for normalization purposes. To monitor QFS hydrolysis 109 µL of each sample was added to 16 µL of sodium formate in a 96-well plate and its fluorescence read on a Perkins Elmer fluorimeter at excitation 328nm/emission 392 nm. All samples were added in duplicate.

2.7.2.2 BK/Ang-I Assay

For monitoring BK and Ang-I cleavage by BAECs a modified version of the assay described by Norman and co-workers was employed (Norman et al., 2003). Approx 6.5X10⁵ BAECs per well were plated onto Bioflex™ plates and left for 24 h. The media was then removed and the cells washed with HBSS. Cells were incubated with BK or Ang-I (100 µM, 100 µL added to give a final volume of 1 ml) in the presence and absence of cFP-AAF-pAB (3.4 µM), which selectively inhibits EP24.15 and EP24.16, or following prior transfection with EP24.15-FLIP or mock transfection. EP24.15-FLIP is an antisense construct against EP24.15 consisted of a vector containing 45 nucleotides of the EP24.15 promoter sequence “flipped” in an antisense conformation and cloned into a pMV12 vector containing two long terminal repeats with ampicillian resistance to propagate the plasmid in bacterial cells (Glucksmann and Roberts, 1995). All assays included phosphoramidon (10 µM) and captopril (10 µM) to selectively inhibit ACE, ECE and NEP, metalloendopeptidases known to degrade BK/Ang-I. The aminopeptidase inhibitor bestatin (10 µM) was also included. All assays were carried out in RPMI (-
serum/-antibiotics). Following addition of substrate, straining commenced (5%, 24h). Unstrained plates were included as controls. Prior to addition of substrate all plates were incubated with inhibitor for 1 h. At time point (t= 24 h) 300 µL of media from three identical wells was harvested and combined, centrifuged to remove cell debris, and snap frozen in liquid N₂ and stored at −80°C until required for analysis (see Fig 2.2).

**Figure 2.2** Diagram of Experimental Approach for BK/Ang-I Degradation Assays

incubated with inhibitor for 1 h. At time point (t= 24 h) 300 µL of media from three identical wells was harvested and combined, centrifuged to remove cell debris, and snap frozen in liquid N₂ and stored at −80°C until required for analysis (see Fig 2.2).

**2.7.2.3 HPLC Analysis for Peptide Substrate Hydrolysis**

HPLC analysis was carried out using a Varian Inert 9012 pumping system with a Varian 9050 UV/VIS detector and Varian Star Workstation analysis/control software.
Prior to analysis harvested media samples were thawed and acidified by addition of TFA to a final concentration of 0.1% v/v. 50 μL of each sample were loaded in triplicate on a Waters Symmetry C-18 reverse phase column at room temperature in a running buffer composed of 1.8% v/v acetonitrile, 0.1% TFA, 0.02% Acetic Acid at 1 ml/min and eluted with a 30min linear gradient to 60% acetonitrile/0.1% TFA. Peptides and peptide fragments were detected at 208 nm.

Experimental peptide concentrations were determined with reference to appropriate standard curves of peak area versus concentration for 0-100 μM BK/Ang-I. Generally less than 10% of the exogenous peptide was degraded under assay conditions.

2.8.3 Scratch-Wound Healing Assay

Endothelial cell migration was observed using the scratch-wound healing assay, modified from the method of de Jonge and co-workers (de Jonge et al., 2002). Briefly, post-strain approximately 2x10^5 BAECs were harvested and transferred into 24 well plates, 2 wells per treatment. Plates were incubated for 12 h (37°C/5% CO₂), and then scratched with a modified yellow pipette tip across the diameter of the well. The cells were then washed with Hanks to removed resultant cell debris. At t=0 scratch was photographed using an Olympus microscope DP50 controlled with AIM imaging software at 3 distinct fields (top, middle and bottom of scratch) at 100X magnification. Fresh media containing serum (10% FBS) was then added and the cells incubated for 4 h. Following incubation the cells were washed and the scratch closure monitored by digital
photography as described. NIH image was used to calculate the distance between the wound edges at T=0 and T=4 and the migration judged to be the reduction in the width of the scratch. The contribution of cell proliferation to the wound healing involved was controlled for by serum starving cells for 24 h in order to quiesce them, and then performing assay as described above in both serum free and serum containing media. The presence of serum, a known endothelial cell mitogen, was not seen to significantly increase the rate of wound closure in the time period observed. This indicated that cell proliferation did not significantly contribute to the wound closure observed.

Figure 2.3 Representative micrographs (100X) of wound healing assay at t=0 and t=4. The reduction in scratch width can be clearly seen.
2.9 *In vitro* Tube Formation Assay

2.9.1 Preparation of Three-Dimensional Collagen Gels

Collagen gels were prepared as previously described (Yang *et al.*, 1999; Zheng *et al.*, 2001). Briefly, a neutralized collagen mixture was prepared by mixing stock (1.5 mg/ml in 10 mM acetic acid) type I rat tail collagen (Sigma, Dorset, UK), with growth medium and 1 M NaOH. Typical mixture: 600 μl of collagen + 60 μl of culture medium + 50 μl of 1 M NaOH. The mixture was poured into 24-well tissue culture plates (100 μl/well) and allowed to solidify in an incubator containing 5% CO$_2$-95% ambient air at 37°C for 1 h. After polymerization the gels were then incubated with growth medium overnight at 37°C before use.

![Unstrained Strained](image)

Figure 2.4 Representative micrographs (200X) showing increased endothelial cell tubule formation in response to exposure to cyclic strain. The tubules are clearly marked with arrows in the "strained" panel.
2.9.2 Tube Formation Assay

Following treatment under differing experimental conditions, ie unstrained versus strained, BAECs were trypsinized as previously described. Cell counts were performed and $1.5 \times 10^4$ cells were seeded per well in RPMI 1640 supplemented with 10% FBS. Cells were incubated overnight for 16-18 h before assessment of tube formation. Cells were photographed at 3 distinct fields using an Olympus microscope DP50 controlled with AIM imaging software. Four random fields of vision were photographed (200X) from each gel. Endothelial cell tubule formation was determined using NIH image. Briefly the length of the cell was determined at its longest point by drawing a line, which was subsequently measured using NIH image. Three such cells were analysed per image, and an average value for endothelial cell tubule length determined.

2.10 Immunocytochemical Staining

2.10.1 Preparation of Cover Slips

Experimental samples (i.e. unstrained vs. strained cells) were trypsinised post treatment and transferred to sterile 6 well plates into which sterilized glass cover slips had been placed (1 slide per well). Cells were allowed to adhere for 10-12 h. The cells were then stained as described below. Cover slips were precoated with gelatin to aid cell adhesion as follows; briefly glass cover slips were acid washed (1 M HCl, 10 min) to remove any other coatings, washed several times in HBSS, sterilized by washing in IMS
and then transferred to sterile 6 well plates. 1ml of sterile 2% gelatin was added to each well and left for 10 min at room temperature. Excess gelatin was then removed and cover slips left to air dry for approximately 1 hour. Plates/cover slips were then stored at 4°C until required. If unused within 48 h plates/cover slips were discarded and fresh ones prepared.

2.10.2 Whole Solubilised (Dead) Staining

This staining protocol was designed to stain all the protein present in the cell. Cover slips of experimental samples were prepared as described above and stained using the following protocol; (all solutions/antibodies were prepared in PBS unless otherwise indicated, and, where possible, procedures were carried out on ice). Cells were first washed several times in 5% bovine serum albumin (BSA). Following washing cells were fixed by treatment with 3% formaldehyde for 15 min, washed again in 5% BSA solution, and solublised using 2% Triton-X for 5min. Cells were again washed before being incubated with primary antibody for 1 h (rabbit anti-EP24.15 polyclonal IgG 1/1000, rabbit anti-eNOS polyclonal IgG 1/2000). Cells were washed stringently to remove any unbound primary antibody, and then incubated in secondary antibody for 30 min (AlexaFluor 488 conjugated anti rabbit IgG 1/800). Cells were also treated solely with secondary antibody as a control for non-specific staining. After washing cells were counter-stained with the nuclear-specific DAPI (Molecular Probes, Oregon USA) stain for 2 min. Cover slips were then washed once more, partially dried by holding at an angle and allowing excess liquid to be absorbed by tissue held at the bottom edge of the cover
slip and finally mounted on glass slides using Dako (Molecular Probes, Oregon USA) mounting media. Slides were kept at room temperature for 1 hour to allow Dako to set before being refrigerated until imaged.

2.10.3 Extracellular Unsolublised (Live) Staining

All solutions/antibodies were prepared in PBS unless otherwise indicated, and, where possible, procedures were carried out on ice. Cells were first washed several times in 5% bovine serum albumin (BSA). Cells were then incubated with primary antibody for 1 h (rabbit anti-EP24.15 polyclonal IgG 1/1000). Following washing cells were fixed by treatment with 3% formaldehyde for 15 min. Cells were washed stringently to remove any unbound primary antibody, and then incubated in secondary antibody for 30 min (Alexa 488 conjugated anti rabbit IgG 1/400). Cells that were untreated with primary antibody were also treated with secondary antibody as a control for non-specific staining. After washing cells were counterstained with the nuclear specific DAPI stain for 2 min. Cover slips were then washed once more, partially dried by holding at an angle and allowing excess liquid to be absorbed by tissue held at the bottom edge of the cover slip and mounted on glass slides using Dako mounting media. Slides were kept at room temperature for 1 hour to allow Dako to set before being refrigerated until imaged.
2.10.4 Imaging

2.10.4.1 Fluorescent Microscopy

Whole cell staining preparations were photographed with Olympus microscope DP50 fitted with an Olympus U-RFLT (Olympus Corporation, USA) fluorescent light source and controlled with StudioLite™ imaging software. The microscope was fitted with two filters Green (Excitation Wavelength 460-490 nm, Emission Wavelength 515 nm) used for alexafluor 488 labelled antibodies (Excitation Wavelength 488 nm, Emission Wavelength 515 nm), and a blue filter (Excitation Wavelength 360-370 nm, Emission Wavelength 420 nm) used for DAPI stained cells.

Typically cells were located using the DAPI filter, which allowed visualization of the cell nucleus, and photographed (typically 1/60 sec, sensitivity 400), before changing to the green filter in order to visualize and photograph alexafluor 488 staining (1/1.5 sec, sensitivity 400). Cells were photographed at 200x and 1000x. Photographs were saved as .jpeg files for the purpose of reporting and image overlay.

2.10.4.2 Confocal Microscopy

Confocal microscopy of whole and extracellularly stained preparations was carried out on a Zeiss LSM-510 META Axioplan 2 upright confocal microscope with a Plan NeoFluor 40x/1.3 oil DIC objective lens. Cells were located and photographed
under bright field before being photographed at excitation 488nm/emission 515nm to visualize immunostaining. Resulting images were analysed and overlayed in Zeiss LSM Image Browser. Confocal microscopy was carried out at the Royal College of Surgeons, Dublin.
Chapter 3; Results Section 1
Examination of the Effects of Cyclic Strain on EP24.15 and EP24.16 mRNA Levels
and Both Cellular and Secreted Enzymatic Activity in BAECs
3.1 Introduction:

Blood flow results in the generation of hemodynamic forces, namely cyclic strain and shear stress (see section 1.2). Blood vessels are chronically exposed to these stresses and, as such, both of these forces are required for maintaining vascular homeostasis. It has been demonstrated that hemodynamic forces can induce changes at the expression and functional level for a number of physiologically relevant genes (Traub et al., 1998; Papadaki et al., 1997).

The closely related members of the clan MA family of zinc metallopeptidases, EP24.15 and EP24.16, are known to be expressed within the vasculature and to cleave a number of vasoactive peptides, such as BK and Ang-I. Other members of the Clan MA family (ECE and ACE) involved in the processing of vasoactive peptides have been shown to be regulated by hemodynamic forces. Moreover, EP24.15 and EP24.16, as well as the latter enzymes have a single copy shear stress response element (SSRE) [GAGACC] in their promotors (McCool et al., 1998; McCool et al., 2000; Li et al., 1995; Shinoki et al., 1998). As such, EP24.15 and EP24.16 are prime candidates for regulation by hemodynamic forces.

The aim of this chapter was to examine the effects of physiological levels of cyclic strain on the expression, subcellular localization and activity of EP24.15 and EP24.16 in BAECs.
3.2 Results

3.2.1 Hemodynamic regulation of EP24.15 and EP24.16 mRNA expression

The regulatory effects of 5% equibiaxial cyclic strain (24 h) on EP24.15 and EP24.16 mRNA expression in BAECs was monitored using semi-quantitative RT-PCR and agarose gel electrophoresis with gene-specific primers for EP24.15, EP24.16 and cyclooxygenase II (COX-II). The levels of the housekeeping gene GAPDH, unaffected by hemodynamic forces, were included to act as a control and for normalization purposes for start mRNA concentration. The levels of EP24.15 and EP24.16 (Fig 3.1 a, b) increased in response to cyclic strain by 1.41 ± 0.2 and 1.55 ± 0.3 fold respectively relative to untreated control (n=3, *P*<0.05). The levels of COX-II were also increased in response to exposure to strain (1.91 ± 0.4 fold relative to control, *P*<0.05).

Both EP24.15 and EP24.16 PCR primers were checked for specificity and potential cross-reactivity using pGEX plasmid constructs containing both genes (Rioli, et al., 1998). Both sets of primers produced product of correct size when appropriate construct was used as DNA template in a PCR reaction, while neither primer pair showed any cross reactivity (see Fig. 3.1 c).
Figure 3.1 Cyclic strain-dependent increase in EP24.15 and EP24.16 mRNA expression in BAECs. Cyclic strain (0% or 5%) was applied to BAECs for 24 h. Following mRNA extraction and RT-PCR (as described in METHODS), all amplified products were analysed by agarose gel electrophoresis on 2.5% gels and visualized by ethidium bromide staining. Representative gels (inverted) shown are as follows; Lanes 1-4 for each of the three gels are GAPDH. Lanes 5-8 are EP24.15, EP24.16 and COX-II (a). Densitometric analysis of individual gels is summarized in the graph beneath the gels (b). GAPDH was used for normalization of gel loading. Statistical differences were
established at $*P \leq 0.05$ versus unstrained control. Data are the result of 3 sets of experiments. Primers for EP24.15 and EP24.16 were checked for specificity using pGEX plasmid constructs containing the gene for each enzyme. Representative gels are shown; Left hand gel shows opGEXEP24.15 and pGEXEP24.16 constructs probed with EP24.15 primers. Right hand gel shows pGEXEP24.16 and pGEX24.15 constructs probed with EP24.16 primers (Fig 3.1 c). Images are representative of a number of independent experiments.
Figure 3.2 Cyclic strain does not significantly alter levels of lactate dehydrogenase (LDH) activity in experimental media samples. BAECs were exposed to cyclic strain (5%, 24 h) and samples of media were collected and assayed for LDH activity. Graph represents fold change in LDH activity in experimental media samples relative to unstrained control. All data is the result of two independent experiments ± SEM.
3.2.2 Hemodynamic regulation of EP24.15 cellular and membrane-associated protein levels as monitored by immunocytochemistry

Following exposure to cyclic strain (5%, 24 h) BAECs were immunocytochemically stained by incubation with a rabbit primary antibody specific to either EP24.15 or eNOS, followed by incubation with an Alexafluor 488 conjugated antirabbit secondary antibody, using techniques for both whole cell and extracellular staining. The cells were also counterstained with the nuclear-specific DAPI stain, which allowed confirmation of cell position. A control for non-specific staining was also carried out by incubation of cells with secondary antibody only (Fig 3.3 a). The cells were imaged using fluorescent and confocal microscopy (Fig 3.3, Fig 3.4).

A clear increase in the total cellular levels of EP24.15 can be observed in response to cyclic strain (Fig 3.3 b, c; Fig 3.4 a, b). This increase was particularly evident in the cytoplasm. The cellular levels of eNOS can also be seen to increase significantly in response to exposure to strain (Fig 3.3 d, e).

Using confocal microscopy in conjunction with live (non-fixed) staining techniques, an increase in extracellular levels of EP24.15 in response to cyclic strain was observed, with an evident increase in punctate staining at the cell extremities (Fig 3.3 c, d). Due to limitations on the amount of EP24.15 antisera available it was only possible to perform this procedure once.
Due to the lack of suitable antisera, we were unable to ascertain the effects of cyclic strain on EP24.16 protein expression in BAECs.
Figure 3.3 Immunocytochemical staining shows an increase in BAEC protein expression levels of EP24.15 in response to cyclic strain. Following exposure to cyclic strain (5%, 24 h) cells were stained with antisera for EP24.15 (b, c) and eNOS (d, e), before being stained with Alexafluor 488-labelled antibody. Cells were also strained with secondary antibody only as a control for non-specific staining (a). The nuclear specific DAPI stain was also included. Nuclear and cytoplasmic associated areas of staining are indicated with yellow and red arrows respectively. Cells were then photographed using a fluorescent microscope. All images shown were photographed at 1000x magnification. All images are representative of at least three individual experiments.
Figure 3.4 Immunocytochemical staining and confocal microscopy reveal an increase in total and extracellular membrane-associated EP24.15 in BAECs in response to cyclic strain. Following exposure to cyclic strain (5%, 24 h) cells were stained with antisera for EP24.15, using both whole (dead a, b) and extracellular (live c, d) techniques, before being stained with alexafluor 488-labelled antibody. Cells were also strained with secondary antibody to remove, non-specific background fluorescence. Cells were then photographed using confocal microscopy. Areas of cytoplasmic, nuclear and extracellular membrane associated areas of staining are indicated with red, yellow and blue arrows respectively. All images shown were photographed at 1600x magnification. Images are representative of one experiment.
3.2.3 Exposure to cyclic strain causes a time- and force- dependent increase in EP24.15 and EP24.16 cellular and secreted activity in BAECs:

The regulatory effects of cyclic strain on EP24.15 and EP24.16 activity in cell lysate and conditioned media was routinely determined using a 96 well fluorometric assay. A standard curve for MCA-Pro-Leu, the fluorescent product of QFS cleavage by EP24.15/EP24.16, is shown in Fig 3.5. Both endopeptidase activities were routinely detected in the soluble fraction of BAECs cultured under static (unstrained) conditions. Basal levels were determined to be 4.4 ± 0.6 and 6.6 ± 0.6 nmoles QFS hydrolyzed.h⁻¹.mg⁻¹ protein for EP24.15 and EP24.16, respectively. Initial experiments clearly demonstrated a strain-dependent up-regulation of soluble EP24.15 and EP24.16 activity in BAECs, with maximal inductions of 2.3 ± 0.35 and 1.9 ± 0.35 fold respectively at 10% strain (P<0.05) (Fig 3.6 a). eNOS protein expression was also monitored and found to increase significantly (1.9 fold) in response to chonic cyclic strain (Awolesi et al., 1995). Strain-dependent up-regulation of both endopeptidase activities was also found to be time-dependent, with induction of activity observed after 3 h strain, both increasing to maximal levels at 24 h for EP24.16 and 12 h EP24.15 (1.69 ± 0.13 and 1.9 ± 0.46 respectively) (Fig 3.6 b).

The effect of cyclic strain on the levels of secreted enzyme activity was also determined. Exposure of BAECs to 5% cyclic strain led to a time-dependent increase in EP24.15 and EP24.16 activity in BAEC-conditioned media, increases which were significantly in excess of constitutive endopeptidase release (0%). Differences between
constitutive and force-dependent endopeptidase release were apparent for both enzymes after 3h of strain and, after 24 h, fold differences of 2.6 ± 0.02 and 3.6 ± 0.2 fold were observed for EP24.15 and EP24.16, respectively (P≤0.05) (Fig 3.7 a, b). To determine if the increase in secreted activity observed was due to a strain-mediated loss of cell membrane integrity the levels of lactate dehydrogenase (LDH) were also determined in the media. Analysis of media from control and strained samples showed no increase in levels of LDH activity as a result of straining (Fig 3.2).
Figure 3.5 Fluorescent standard curve for 7-methoxycoumarin-4-acetyl-Pro-Leu (MCP-Pro-Leu). A standard curve for MCP-Pro-Leu was prepared in the concentration range 0-130 nM, both in the presence and absence of the EP24.16 inhibitor Pro-ile (4.82 mM). Graph represents fluorescence units versus MCP-Pro-Leu concentration. Graph is representative of two experiments.
Figure 3.6 Cyclic strain-dependent increase in EP24.15 and EP24.16 enzymatic activity in BAECs. (a) Force-dependent elevation of endopeptidase activities in BAECs following exposure to varying levels of cyclic strain (0-10%) for 24 h. Insert shows a representative gel displaying 1.9 fold up-regulation of eNOS protein expression in BAECs in response to cyclic strain (5%, 24 h), as monitored by Western blotting (7 μg/lane). (b) Time dependence of cyclic strain-mediated elevation of endopeptidase activities in BAECs following exposure to cyclic strain (0 or 5%) for varying times (0-24 h).
h). Results are expressed as fold change in specific activity relative to control (unstrained cells). Statistical differences were established at \*\(P \leq 0.05\), **\(P \leq 0.01\), and ***\(P \leq 0.001\) versus unstrained control. Data are the result of 3-4 sets of experiments.
Figure 3.7 Cyclic strain-dependent increase in EP24.15 and EP24.16 secretion from BAECs. Elevation of (a) EP24.15 and (b) EP24.16 enzymatic activities in BAEC-conditioned media following exposure to cyclic strain (0 or 5%) for varying times (0-24 h). Results are expressed as pmoles substrate hydrolyzed.min⁻¹.mL⁻¹.μg⁻¹ protein. Statistical differences were established at *P≤0.05 and **P≤0.01 versus unstrained controls. Data are the result 3 sets of experiments.
3.3 Discussion

Peptidase-mediated metabolism of bioactive peptide hormones plays a vital role in endothelial cell-mediated vascular events. As shear stress and cyclic strain likely represent the principle regulatory stimuli influencing vascular homeostasis and associated pathologies, we postulated that metalloendopeptidase expression and activity in vascular endothelial cells (ECs) may be regulated by these hemodynamic variables. To further investigate this hypothesis, we examined the relationship between cyclic strain and the mRNA expression, protein levels, and enzyme activity of both EP24.15 and EP24.16, homologous members of the thermolysin-like zinc metalloendopeptidase family, within vascular ECs. A flexwell *in vitro* culture system which uses vacuum to mechanically strain or deform cells cultured on a flexible, matrix-bonded growth surface was subsequently employed for culturing vascular ECs under conditions of defined cyclic strain (Banes *et al*., 1985).

Initially it was decided to investigate if exposure to physiological levels of cyclic strain (5%, 24h) affected changes at the level of the transcription of EP24.15 and EP24.16 mRNA. It was observed that exposure to cyclic strain significantly increased the levels of mRNA for both enzymes relative to unstrained controls. In addition the levels of cyclooxygenase II (COX-II) mRNA were seen to increase relative to controls, a well-characterized response to strain (Kito *et al*., 1998).

To our knowledge no other studies have examined on the effects of hemodynamic forces on the expression levels of EP24.15/EP24.16 in the vasculature. Numerous studies
have demonstrated the ability of hemodynamic forces to regulate the mRNA expression of ECE-1 and ACE, which are structurally related to EP24.15/EP24.16 (Masatsugu et al., 2003; Reider et al., 1997).

This result suggested that cyclic strain might also alter levels of EP24.15/EP24.16 protein and enzymatic activity. In this regard, exposure to cyclic strain altered the levels of EP24.15 protein, as measured immunocytochemically using fluorescent and confocal microscopy. Total levels of EP24.15-specific immunostaining were dramatically increased in both the cytoplasm and nucleus. In addition, a robust increase in the levels of eNOS staining, a well-characterized response to cyclic strain was observed (Awolsei et al., 1995). Immunostaining was also carried out with non-permeabilised (non fixed) BAECs at 4°C, in which internalization of the antibody was prevented. This appeared to indicate the presence of EP24.15 on the extracellular plasma membrane surface, albeit at very low levels. The levels of extracellular staining for EP24.15 were also increased in response to strain, suggesting a putative influence on the enzymes extracellular function.

Crack and co-workers have employed similar immunostaining strategies in their investigations of EP24.15 in mouse neuronal AtT-20 cells. Their studies also revealed the presence of membrane-bound and cytoplasmic EP24.15 (Crack et al., 1999). However their studies did not indicate significant levels of nuclear EP24.15. In contrast our studies revealed that in BAECs much of the EP24.15 staining occurs in the nuclear region. This may be due to cell/species specificity, and the different roles of EP24.15 in both tissue types.
Both endopeptidase activities were routinely detected in the soluble fraction of BAECs cultured under static (unstrained) conditions. Basal levels were determined to be 4.4 ± 0.6 and 6.6 ± 0.6 nmoles QFS hydrolyzed.h⁻¹.mg⁻¹ protein for EP24.15 and EP24.16, respectively. Initial experiments clearly demonstrated a strain-dependent up-regulation of soluble EP24.15 and EP24.16 activity in BAECs, with maximal inductions of 1.9 ± 0.4 and 2.3 ± 0.4 fold, respectively (at 10% strain). In concurrence with previous reports, eNOS protein expression was also monitored and found to increase significantly (1.9 fold) in response to chronic cyclic strain. Strain-dependent up-regulation of both endopeptidase activities was also found to be time-dependent, with induction of activity observed after 3 h strain, both increasing to maximal levels at 24 h. These observations lead us to conclude that cyclic strain can potentially regulate the levels of EP24.15 and EP24.16 activity within BAECs in a dose- and time-dependent manner.

Although predominantly cytosolic, secreted forms of EP24.15 and EP24.16 have also been widely reported for various cell types, including endothelial cells, subsequently enabling them to function extracellularly (Acker et al., 1987; Crack et al., 1999; Ferro et al., 1999; Cotter et al., 2004). Recently Jeske and co-workers have demonstrated EP24.15 association with and extracellular release from lipid rafts in mouse neuronal GT1-7 cells, indicating a putative mechanism for its constitutive release. (Jeske et al., 2003; Jeske et al., 2004). Several studies have previously detailed the effects of cyclic strain on secretory processes in vascular cells. Considering the regulatory influence of cyclic strain on soluble levels of EP24.15 and EP24.16 activity described here, it was decided to examine its potential influence on endopeptidase release from BAECs. Exposure of
BAECs to 5% cyclic strain led to a time-dependent increase in EP24.15 and EP24.16 activity in BAEC-conditioned media increases which were significantly in excess of constitutive endopeptidase release. Differences between constitutive and force-dependent endopeptidase release were apparent for both enzymes after 3 h of strain and, after 24 h, fold differences of 2.6 and 3.6 fold were observed for EP24.15 and EP24.16, respectively. Based on these observations, we can conclude that cyclic strain potentially modulates release of EP24.15 and EP24.16 from BAECs, suggesting a putative influence on their extracellular functions.

In conclusion therefore, it was our intention to examine the effects of cyclic strain on the mRNA/protein expression and enzymatic activity of EP24.15 and EP24.16. We observed a clear up-regulation of EP24.15/EP24.16 mRNA expression and both cellular and secreted activity of both enzymes in response to cyclic strain. The increase in activity was found to be time- and force-dependent with maximum increases observed at 24 h and at 10% strain. In addition strain was seen to clearly increase the total cellular levels of EP24.15 immunostaining, both nuclear and cytoplasmic, and to increase the amount of membrane-bound EP24.15. These changes occurred in parallel with increases in the levels of eNOS protein, and of COX-II mRNA, both of which are well characterized responses to exposure to cyclic strain. It is our conclusion therefore that both EP24.15 and EP24.16 are putatively regulated by physiological levels of equibiaxial cyclic strain.
Chapter 4; Results Section 2
The Involvement of $G_{i\alpha}$ and $G_{\beta\gamma}$ Signaling Pathways in the Strain Mediated Regulation of EP24.15 and EP24.16 Activity in BAECs.
4.1 Introduction

Having demonstrated the regulatory effects of cyclic strain on EP24.15 and EP24.16 expression and specific enzymatic activity in BAECs, it was decided to delineate the specific cell signaling pathways involved in the transduction of this stimulus.

The intracellular transduction of hemodynamic forces in vascular ECs, manifested though changes in gene expression and protein function, remains poorly understood. Several protein candidates have been implicated in this process and include PTX-sensitive (Gi) and -insensitive (Gq) heterotrimeric G-proteins, G-protein-coupled receptors (GPCRs), ion channels, integrins and receptor tyrosine kinases (RTKs) (Traub et al., 1998; Papadaki et al., 1997; Redmond et al., 1998). Inhibitory guanine nucleotide regulatory G-protein (Gi) signalling pathways are of particular interest. Activation of Gi-protein subunits and increased GTPase activity occur immediately (within sec) following mechanical loading, suggesting that these ubiquitous regulatory proteins are strategically located within vascular ECs to facilitate transduction of mechanical stimuli.

The objective of this section was to investigate the putative role of Gi\textsubscript{a} and G\textsubscript{f\textbeta} subunits in putatively mediating the cyclic strain-dependent effects observed for EP24.15 and EP24.16 in BAECs.
4.2.1 Pharmacological inhibition of Giα protein signaling attenuates the strain dependent increase in EP24.15 and EP24.16 activity in BAECs.

Cells were subjected to 5% cyclic strain (24 h) in the presence and absence of pharmacological inhibitors of Giα signaling, namely pertussis toxin (PTX-100 ng/ml) and NF023 (100 μM). Pertussis toxin, which catalyzes the ADP-ribosylation of the α-subunit in the intact heterotrimer, thereby uncoupling the G-protein from its receptor, and NF023, a suramin analogue which blocks GDP dissociation from the α-subunit, have been extensively employed by others for the inhibition of Gi-protein mediated signaling (Labrador et al., 2003). In our studies both agents were demonstrated to strongly attenuate strain-dependent up-regulation of activity for both endopeptidases, suggesting a role for Gi-protein signaling in these events.

Specifically PTX treatment was seen to considerably attenuate the strain dependent increase in activity of both EP24.15 and EP24.16 in BAECs, by 74.4 ± 34.9%, and 89.9 ± 36.8% respectively (P≤0.01). Treatment with NF023 was also seen to attenuate the strain dependent increase in EP24.15 and EP24.16, with a 91.2 ± 32.3% attenuation observed with EP24.15 and 91 ± 37.7% attenuation observed with EP24.16 (P≤0.01) (Fig. 4.1 a, b).
Figure 4.1 Attenuation of the cyclic strain-dependent increase in EP24.15 and EP24.16 activity by pharmacological inhibition of Gi-protein function. BAECs were strain in the presence or absence of either PTX (a) or NFO23 (b) (0 or 5%) for 24 h. Results are expressed as fold change in specific activity relative to unstrained control. Statistical differences were established at \(^*P\leq 0.05\), \(^{**}P\leq 0.01\), and \(^{***}P\leq 0.001\) versus unstrained control. Significant differences were also established at \(^{\ddagger}P\leq 0.05\) and \(^{\ddagger\ddagger}P\leq 0.01\) versus strained control (no PTX/NF023, cells at 5%). Data are the result of 3 sets of experiments.
4.2.2 Attenuation of Cyclic Strain-Dependent Increases in EP24.15 and EP24.16 Activity in BAECs by Molecular Inhibitors of Giα-Subunit Function

Following transient transfection of BAECs with plasmid constructs encoding wild type and dominant-negative mutant Giα1,3 subunits; cells were exposed to cyclic strain (either 0 or 5%) for 24 h. A consistently high (approx. 40-50%) transfection efficiency was achieved throughout these studies and over-expression of transfected Gi-protein subunits (both wild type and mutant) was subsequently confirmed by Western immunoblotting (data shown for Giα2, Fig 4.2). Cyclic strain-dependent elevation of cellular EP24.15 activity in BAECs was found to be attenuated by Giα2-G203T (81.9 ± 36.3%) and Giα3-G202T (102.9 ± 21.8%), whereas Giα1-G202T had no significant effect (P=0.46). Interestingly, force-dependent increase in cellular EP24.16 activity was strongly attenuated by Giα1-G202T (69.6 ± 18.6%) and Giα2-G203T (101.7 ± 26.7%), whereas Giα3-G202T had no significant effect (P=0.29) (Fig 4.3) (Inhibition data for above experiments is tabulated in Table 4.1). In contrast to the dominant negative mutants, over-expression of the Giα1,3 wild type subunits had no negative impact on cyclic strain-dependent increases in the activity of either endopeptidase in BAECs, and in some instances actually resulted in further, albeit slight, increases in activity.
Figure 4.2 representative western blot confirming protein over-expression following transient transfection of either wild type or mutant Giα-subunit constructs. Immunoblotting was performed using an anti-Gi_{α2} rabbit polyclonal IgG selective for both wild type and mutant forms of the Gi_{α2}-subunit. Protein loading was 10 μg/lane. Over-expression of Gi_{α2}-G204A and Gi_{α2}-G203T (null mutant and dominant negative forms of Gi_{α2}, respectively) are shown in lanes 2 and 3. Over-expression of wild type Gi_{α2} is shown in lane 5.
Figure 4.3 Attenuation of the cyclic strain-dependent increase in EP24.15 and EP24.16 activity by molecular inhibition of Giα-protein function. Following transient transfection of BAECs with plasmid constructs encoding Giα-G202T, Giα-G203T or Giα-G202T, dominant-negative mutant forms of the associated wild type subunits, cells were allowed to recover overnight before applying cyclic strain (0 or 5%) for 24 h. Results are expressed as fold change in specific activity relative to unstrained control (mock transfected cells at 0%). Statistical differences were established at *P≤0.05, **P≤0.01, and ***P≤0.001 versus unstrained control. Significant differences were also established at 6P≤0.05 and 66P≤0.01 versus strained control (mock transfected cells at 5%). Data are the result of 3-4 sets of experiments.
4.2.3 Attenuation of Cyclic Strain-Dependent Increases in EP24.15 and EP24.16 Activity in BAECs by Molecular Inhibitors of Gβγ-Dimer Function

Following transfection of BAECs with βARK.ct, cells were exposed to cyclic strain (either 0 or 5%) for 24 h. Cyclic strain-dependent elevation of EP24.15 activity in BAECs was only slightly attenuated in cells (12.7 ± 7.3%) by βARK.ct. In contrast, strain-dependent increases in EP24.16 activity were substantially attenuated in BAECs (86.4 ± 2.6%) by βARK.ct (Fig 4.4; Table 4.1).
Figure 4.4 Attenuation of the cyclic strain-dependent increase in EP24.15 and EP24.16 activity by molecular inhibition of Gβγ-protein function. Following transient transfection of BAECs with βARK.ct, cells were allowed to recover overnight before applying cyclic strain (0 or 5%) for 24 h. Results are expressed as fold change in specific activity relative to unstrained control (mock transfected cells at 0%). Statistical differences were established at ***P<0.001 versus unstrained control and at *P<0.05 and **P<0.01 versus strained control (mock transfected cells at 5%). Data are the result of 3 sets of experiments.
Table 4.1 Summary of findings of molecular inhibition studies. Figures represent percentage attenuation of cyclic strain-dependent increase in enzyme activity for both endopeptidases.

<table>
<thead>
<tr>
<th></th>
<th>$G_{a1}$-G202T</th>
<th>$G_{a2}$-G203T</th>
<th>$G_{a3}$-G202T</th>
<th>$\beta$ARK-ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP24.15</td>
<td>No significant inhibition</td>
<td>81.9±36.3</td>
<td>102.9±21.8</td>
<td>12.7±7.3</td>
</tr>
<tr>
<td>EP24.16</td>
<td>69.6±18.6</td>
<td>101.7±26.7</td>
<td>No significant inhibition</td>
<td>86.4±2.6</td>
</tr>
</tbody>
</table>
4.3 Discussion

The observation in the previous chapter that exposure to cyclic strain could putatively up-regulate EP24.15/EP24.16 expression and activity in BAECs raised the issue as to which signaling pathway was involved in the transduction of this response.

A number of pathways have been implicated in the transduction of hemodynamic forces in the endothelium. These include ion channels, integrins, tyrosine kinases and G-protein coupled receptors (GPCR) (Papadaki et al., 1997). G-protein signaling represents a highly sophisticated molecular system with the ability to receive, integrate, and process information from extracellular stimuli. G-protein coupled receptors (GPCRs) constitute a large family of single polypeptide chain receptors, with seven trans membrane α-helices composed primarily of hydrophobic residues looping between the extracellular and intracellular phases. GPCRs couple to heterotrimeric G-proteins, which consist of the α, β, and smaller γ subunits (Seifert et al., 2003). A role for G-protein involvement was first suggested after it was found that intracellular messengers associated with G-protein pathways were also activated in response to flow. These included inositol triphosphate (IP₃), diacylglycerol (DAG), protein kinase C (PKC) and a rise in intracellular calcium. Involvement of the Gi₃α subunits was suggested by the pertussis toxin sensitivity of shear induced NO release in some experiments (Poston, 2002; Ohno et al., 1993). Later studies by Gudi and co-workers demonstrated the rapid activation of endothelial Gαᵣ₃α₁₁ and Gαᵣ₃α₀ proteins by shear, with activation occurring as little as one second after onset of flow (Gudi et al., 1996). This group also demonstrated that treatment of endothelial cells
with antisense G\(_{a4}\) oligonucleotides inhibited shear stress-induced ras-GTPase activity, while scrambled oligonucleotide treatment had no effect. Further studies on G-proteins reconstituted on liposomes in the absence of protein receptors showed an increase in activity in response to shear stress (Gudi et al., 1997; Frangos et al., 1997). In addition co-culture studies, using both ECs and SMCs, by Redmond and co-workers demonstrated a shear strain induced increase in Gi\(_{a2}\) expression in ECs coupled with a decrease in Gi\(_{a1,2}\) expression in SMCs (Redmond et al., 1998).

Our study focused on the effects of both pharmacological and molecular inhibition of Gi-a-subunit and G\(\beta\gamma\)-mediated signaling on the strain-induced increase in cellular activity of both enzymes. Pharmacological inhibitors of Gi-protein signaling were initially employed to investigate this role. Pertussis toxin, which catalyzes the ADP-ribosylation of the \(\alpha\)-subunit in the intact heterotrimer, thereby uncoupling the G-protein from its receptor, and NF023, a suramin analogue which blocks GDP dissociation from the \(\alpha\)-subunit, have been extensively employed by others for this purpose (Labrador et al., 2003; Beindl et al., 1996). Both agents were demonstrated to strongly attenuate strain-dependent up-regulation of activity for both endopeptidases, suggesting a role for Gi-protein signaling in these events. The use of pharmacological tools to delineate G-protein-mediated signaling events must be interpreted with caution however, as potential effects on Gi-protein-independent pathways cannot be ruled out.

Further inhibition studies therefore focused on selective ablation of individual Gi-protein subunits at the molecular level. Plasmids encoding dominant negative mutants of
Gi<sub>α1,2,3</sub> subunits (i.e. Gi<sub>α1</sub>-G202T, Gi<sub>α2</sub>-G203T, and Gi<sub>α3</sub>-G202T) were subsequently employed to selectively ablate the function of their endogenous wild type counterparts in BAECs (Cullen et al., 2002). A plasmid encoding the carboxyl terminus of β-Adrenergic Receptor Kinase (βARK.ct), a well-known Gβγ-subunit scavenger (Koch et al., 1994), was also employed to selectively inhibit Gβγ signaling pathways. Strain-dependent up-regulation of both endopeptidase activities was found to be strongly attenuated (80-100%) following over-expression of Gi<sub>α2</sub>-G203T. By contrast, only strain-dependent elevation of EP24.15 activity was inhibited by Gi<sub>α3</sub>-G202T (100%), whilst EP24.16 activity was significantly inhibited by Gi<sub>α1</sub>-G202T (69.6 ± 18.6%). A putative role for Gβγ-signaling is also implicated in these events. βARK.ct, whilst only marginally blocking the strain-dependent increase in EP24.15 activity in BAECs (12.7 ± 7.3%), substantially blocked increase in EP24.16 activity (86.4 ± 2.6%). These findings confirm our earlier observations with PTX and NF023, and reinforce the involvement of multiple Gi-protein subunits in transduction of these strain-induced responses.

Interestingly, the differential requirements of Gi-protein subunits for the strain-dependent changes in either endopeptidase suggests distinct signal transduction pathways. The reason(s) for this divergence are presently unclear, however, significant differences exist between EP24.15 and EP24.16, despite their structural and functional similarities, which may underlie this divergence. In addition to differences in their substrate specificities, both enzymes are separate gene products (approx. 60% homology) under the control of distinct promoters, with additional evidence to indicate that EP24.16 expression is subject to alternative promoter usage, thereby enabling targeting of some
EP24.16 to the mitochondrial compartment (Kato et al., 1997). Mechanoregulation in vascular endothelial cells by divergent signaling pathways has also been reported by other workers; Jo and co-workers demonstrated that expression of βARK-ct inhibits the shear stress activation of HA-JNK, whereas blockade of Gαi2 with the dominant negative mutant Gαi2-G203T or antisense Gαi2 prevented shear dependent activation of HA-ERK (Jo et al., 1997). Also significant is the ability of hemodynamic forces to regulate expression of vasoactive enzymes such as eNOS by divergent signaling pathways (Davies et al., 2001). Hemodynamic forces also modulate the expression and function of G-proteins themselves (Redmond et al., 1998).

In conclusion, having demonstrated that exposure of BAECs to cyclic strain up-regulates the mRNA levels and activity of EP24.15 and EP24.16, and increases EP24.15 protein levels, the aim of the studies detailed in this section was to ascertain the mechanism by which the effects of cyclic strain on these enzymes are transduced.

It was observed that pharmacological and molecular inhibition of Gi-protein subunit signaling differentially attenuated the cyclic strain-dependent increase in cellular EP24.15 and EP24.16 activity; with the increase in EP24.15 activity displaying greater sensitivity to inhibition of Gαi1/Gαi3 signaling, while the increase in EP24.16 activity was significantly attenuated by inhibition of Gαi1/Gαi2 and Gβγ subunit mediated signaling.

These findings lead us to the conclusion that Gi-protein mediated signaling plays a significant role in the transduction of the regulatory effects of cyclic strain on both
Chapter 5; Results Section 3
5.1 Introduction

Having demonstrated that EP24.15 and EP24.16 are regulated at the functional and transcriptional levels by cyclic strain putatively via a G-protein dependent pathway in BAECs our attention turned to the analysis of the effects of strain on the EP24.15- and EP24.16- mediated cleavage of exogenously added substrates. Although primarily cytosolic, both secreted and membrane bound forms of both enzymes have been identified (Crack et al., 1999; Acker et al., 1987; Ferro et al., 1999; Cotter et al., 2004). EP24.15, and EP24.16 specific extracellular cleavage of exogenously added substrates, including BK, Ang-I and QFS, has also been reported by Norman and co-workers (Norman et al., 2003).

Ang-I and BK are physiologically relevant peptide substrates of EP24.15 and EP24.16 (Cummins et al., 2004; Shrimpton et al., 2002). Both enzymes convert Ang-I to Ang-(1-7) and hydrolyse BK to inactive fragments (Rioli et al., 1998; Shimpton et al., 2002). Both BK and Ang-(1-7) are considered to have an important regulatory functions in the vasculature, impacting on a number of vascular processes, including the maintenance of blood pressure (Duffy et al., 2004) and the regulation of angiogenesis (Machado et al., 1999; Ishihara et al., 2002).

The objective of this section was to examine the effects of exposure to cyclic strain on the ability of BAECs in culture to cleave both synthetic (QFS) and natural peptide (BK, Ang-I) in an EP24.15/EP24.16 dependent manner.
5.2 Results

5.2.1 Exposure to cyclic strain increases EP24.15 and EP24.16 specific cleavage of an exogenously added fluorescent substrate in a time-dependent fashion in BAECs.

BAECs were exposed to cyclic strain (5%, 24 h) in the presence of an inhibitor cocktail containing 10 μM Captopril/Phosphoramidon (to inhibit non-specific cleavage of QFS) and the fluorometric substrate (QFS) to a final concentration of 7.4 μM. cFP-AAF-pAB (3.4 μM) was also used to specifically inhibit EP24.15/16. Unstrained cells were included as controls. Media samples were collected at 0, 3, 6, 12 and 24 hour and assayed for fluorescence (Fig 5.1 a, b).

Strain was seen to induce a time-dependent increase in QFS cleavage over unstrained controls in the time period examined (Fig. 5.1 a), with an almost two-fold strain-induced increase in cleavage relative to unstrained control after 24h; 20.1 ± 4.77 pmole/μl/μg protein for strained, versus 10.5 ± 2.45 pmole/μl/μg for unstrained cells (*P<0.05). This increase in cleavage was significantly attenuated (74%, b P<0.05) with the addition of cFP-AAF-pAB (Figure 5.1 b).
Figure 5.1 Exposure to cyclic strain increases EP24.15 and EP24.16 specific cleavage of an exogenously added fluorometric substrate. (a) BAECs were subjected to cyclic strain (5%, 24 h) in the presence of an inhibitor cocktail containing 10 μM Captopril/Phosphoramidon (to prevent non-specific QFS cleavage) and the fluorometric substrate (QFS) to a final concentration of 7.4 μM. cFP-AAF-pAB (3.4 μM) was also used to specifically inhibit EP24.15/16 (b). Unstrained cells were included as controls.
(a) Graph represents QFS hydrolysis by strained and unstrained cells over time. (b) Graph represents QFS hydrolysis by strained and unstrained cells at 24 h, in the presence and absence of cFP-AAF-pAB. Results are expressed as QFS hydrolysis (pmoles/ml/μg protein) ± SEM. Statistical differences were established at *P≤0.05 versus unstrained control, significant differences were also established at *P≤0.05 versus strained control. All data are the mean of 4 independent experiments.
5.2.2 HPLC analysis of BK and Ang-I concentration

The levels of BK and Ang-I in experimental samples were routinely determined by reverse phase HPLC using a method adapted from Norman and co-workers (Norman et al., 2003). Typical chromatograms for BK and Ang-I are shown in Fig 5.2 a. and Fig 5.2 b respectively. The peak for BK typically eluted at 22-23 min, while that for Ang-I typically eluted at 16-17 min.

The concentrations of BK and Ang-I in experimental samples was calculated with use of previously prepared standard curves of peak area versus concentration. The standard curves for BK (Fig 5.2 c) and Ang-I (Fig 5.2 d) are shown.
Figure 5.2 HPLC analysis of BK and Ang-I concentration. BK and Ang-I concentration was routinely determined in experimental samples by the use of reversed phase HPLC with appropriate standards. (a, b) representative chromatograms for BK and Ang-I (100 µM). BK/Ang-I elution peaks are clearly labeled. Data are expressed at Absorbance Units (AU) versus retention time (min). (c, d) HPLC standard curves for BK and Ang-I for the concentration range 0-100 µM. Data are expressed as Peak Area versus Concentration (µM).
5.2.3 Transfection with the antisense FLIP significantly reduces EP24.15 mRNA expression and cellular protein levels in BAECs.


The effect of Flip-ci transfection on EP24.15 protein and mRNA levels in BAECs is clearly displayed in Fig 5.4 a, b. The reduction in protein levels is demonstrated immunocytochemically in Fig 5.4 a; both cytoplasmic and nuclear staining decreases significantly from mock transfected control cells to FLIP transfected cells. This attenuation is also evident at the mRNA level; transfection with FLIP was seen to significantly reduce the levels of EP24.15 mRNA (>65% attenuation, *P≤0.05) (Fig 5.4 b), while no significant reduction of EP24.16 mRNA levels was observed (Fig 5.4 c).

The molecular inhibition of both EP24.15 and EP24.16 was also attempted using siRNA (Fig 5.3 a, b) (see section 2.6). However none of the siRNA duplexes designed were seen to inhibit EP24.15 or EP24.16 mRNA expression significantly.
Figure 5.3 Transfection with siRNA targeted to EP24.15 and EP24.16 fails to significantly reduce mRNA levels for either enzyme in BAECs. BAECs were transfected with either EP24.16, EP24.15 I, EP24.15 II, siRNA duplexes or a scrambled (control) sequence. 24 h post-transfection cells were harvested for mRNA, and EP24.15/EP24.16 mRNA levels subsequently determined using Real-Time with EP24.15 and EP24.16 specific primers. (a) Graph represents fold change in EP24.16 mRNA levels relative to scrambled transfected control in BAECs transfected with siRNA targeted to EP24.16. (b) Graph represents fold change in EP24.15 mRNA levels relative to scrambled transfected control in BAECs transfected with two different siRNA s targeted
to EP24.15, EP24.15 I and EP24.15 II. All data are expressed as the mean of two independent experiments ± SEM.
Figure 5.4 Transfection with the antisense FLIP significantly reduces EP24.15 mRNA expression and cellular protein levels in BAECs. BAECs were transfected with a specific antisense construct (EP24.15 FLIP) targeted to the promoter region of EP24.15, and allowed to recover for 48 h before being immunocytochemically stained for EP24.15. Post recovery, BAECs were also harvested for RNA and EP24.15 mRNA levels determined using Real-Time PCR with EP24.15 specific primers. (a) Representative fluorescent micrographs (1000x) of mock and FLIP transfected BAECs stained with
rabbit anti-EP24.15 primary antibody and goat-anti-rabbit Alexafluor488 conjugated secondary antibody, and the nuclear specific DAPI stain. Areas of staining are indicated with red arrows. Pictures of secondary only control for non specific staining are also included. (b) Real-Time PCR data showing EP24.15 mRNA levels in both mock and FLIP transfected cells. (c) Real-Time PCR data showing EP24.16 levels in both mock and FLIP transfected cells. Data is expressed as fold change relative to mock-transfected controls. Significance was established at \(*P \leq 0.05\) versus mock transfected control. Data represent the mean of two experiments ± SEM.
5.2.4 Exposure to Cyclic Strain Increases EP24.15/EP24.16-specific Degradation of Exogenously Added BK and Ang-I—Inhibition by cFP-AAF-pAB

After exposure of BAECs to cyclic strain (5%, 24 h) in the presence and absence of cFP-AAF-pAB and also in the presence of an inhibitor cocktail containing 10 μM Captopril/Phosphoramidon/Bestatin, to prevent non-specific peptide hydrolysis, media samples were collected and monitored for hydrolysis of exogenously added BK and Ang-I by HPLC.

Cyclic strain (5%, 24 h) was observed to significantly increase hydrolysis of exogenously added BK in BAECs by 2.22 ± 0.1 fold relative to control (*P<0.05, n=3). This strain-dependent increase in BK degradation was significantly attenuated by addition of cFP-AAF-pAB (>100%, *P<0.05). Addition of cFP-AAF-pAB, did not however significantly alter the levels of BK hydrolysis in unstrained BAECs (Fig 5.5 a).

Cleavage of exogenously added Ang-I in BAECs was also significantly up-regulated by exposure to cyclic strain (5%, 24 h), 1.45 ± 0.09 fold relative to control (*P<0.05, n=3). As was the case with BK, this strain-dependent increase in Ang-I degradation was significantly attenuated by addition of cFP-AAF-pAB (>90%, *P<0.05). cFP-AAF-pAB was also seen to reduce the levels of Ang-I hydrolysis by unstrained cells by 35 ± 10.6% (*P<0.05, n=3) (Fig 5.5 b).
Figure 5.5 Exposure to cyclic strain increases EP24.15/EP24.16 specific degradation of exogenously added BK and Ang-I. BAECs were subjected to cyclic strain (5%, 24 h) in the presence of an inhibitor cocktail containing 10 μM Captopril/Phosphoramidon/Bestatin, to prevent non specific peptide hydrolysis and either BK (100 μM) or Ang-I (100 μM). cFP-AAF-pAB (3.4 μM) was used to specifically inhibit EP24.15/16. Unstrained cells were included as controls. After treatment media samples were analysed for BK and Ang-I concentration using HPLC with appropriate BK and Ang-I standards. (a) Cyclic strain induced increase in EP24.15/EP24.16 specific
degradation of BK. (b) Cyclic strain induced increase in EP24.15/EP24.16 specific degradation of Ang-I. All data are expressed as fold change in peptide degradation relative to unstrained control. Statistical differences were established at *$P\leq 0.05$ versus unstrained control. Significant differences were also established at $^bP\leq 0.05$ versus strained control. All data are the mean of 3 independent experiments ± SEM.
5.2.5 Exposure to cyclic strain increases EP24.15-specific Degradation of Exogenously Added BK and Ang-I – Inhibition by FLIP

After exposure of BAECs to cyclic strain (5%, 24 h) after either prior transfection with FLIP or mock transfection, and also in the presence of an inhibitor cocktail containing 10 μM Captopril/Phosphoramidon/Bestatin, to prevent non-specific peptide hydrolysis, media samples were collected and monitored for hydrolysis of exogenously added BK and Ang-I by reverse phase HPLC.

Cyclic strain (5%, 24 h) was observed to significantly increase hydrolysis of exogenously added BK in BAECs by 1.73 ± 0.02 fold relative to control (*P≤0.05, n=3). This strain-dependent increase in BK degradation was significantly attenuated by prior transfection with FLIP (>100%, °P≤0.05). Prior transfection with FLIP, did not however significantly alter the levels of BK hydrolysis in unstrained BAECs (Fig 5.6 a).

Cleavage of exogenously added Ang-I in BAECs was also seen to be significantly up-regulated by exposure to cyclic strain (5%, 24 h), 1.49 ± 0.02 fold relative to control (*P≤0.05, n=3). As was the case with BK, this strain-dependent increase in Ang-I degradation was significantly attenuated by prior transfection with FLIP (>100%, °P≤0.05). Prior transfection with FLIP was also seen to reduce the levels of Ang-I hydrolysis by unstrained cells by 45±15% (*P≤0.05, n=3) (Fig 5.6 b).
Figure 5.6 Exposure to cyclic strain increases EP24.15 specific degradation of exogenously added BK and Ang-I. BAECs were subjected to cyclic strain (5%, 24 h) in the presence of an inhibitor cocktail containing 10 μM Captopril/Phosphoramidon/Bestatin, to prevent non specific peptide hydrolysis and either BK (100 μM) or Ang-I (100 μM). Prior tranfection with FLIP was used to specifically inhibit EP24.15. Unstrained cells were included as controls. After treatment media samples were analysed for BK and Ang-I concentration using HPLC with appropriate BK and Ang-I standards. (a) Cyclic strain induced increase in EP24.15 specific degradation of BK. (b) Cyclic strain induced increase in EP24.15 specific degradation of Ang-I. All data are expressed as fold change in peptide degradation relative to unstrained control.
Statistical differences were established at *$P \leq 0.05$ versus unstrained control. Significant differences were also established at $^5P \leq 0.05$ versus strained control. All data are the mean of 3 independent experiments ± SEM.
5.3 Discussion

The demonstration in previous chapters that both EP24.15 and EP24.16 cellular and secreted activity is up-regulated by cyclic strain combined with the observed increase in both cellular and membrane-associated EP24.15 immunostaining after exposure of BAECs to strain led us to examine the potential effects of cyclic strain on the extracellular hydrolysis of exogenously added substrates by BAECs in culture.

Both the synthetic substrate, QFS, and the natural peptides BK and Ang-I were employed. Both BK and Ang-I are important regulators of vascular function. BK is inactivated by EP24.15/EP24.16, resulting in the specific peptide fragments BK-(1-5) and BK-(6-9). Ang-I is also cleaved by both endopeptidases to produce the vasoactive peptide Ang-(1-7) (Chappell et al., 1998), which numerous studies have shown to have opposing actions to Ang-II, the main product of Ang-I hydrolysis by ACE (Chappell et al., 1998; Rioli, et al., 1998; Machado et al., 1999; Shimpton et al., 2003).

The effects of strain on endopeptidase-specific cleavage of QFS were examined over a 24 h period. cFP-AAF-pAB was used to specifically inhibit activity of both endopeptidases. Inhibitors specific to other related peptidases were also included to prevent non-specific QFS cleavage. In unstrained cells, QFS cleavage increased over the 24 h period, while cFP-AAF-pAB significantly attenuated any cleavage, indicating strong involvement of EP24.15/EP24.16. This finding was in agreement with the observations by Norman and co-workers who observed that under basal conditions Ea.hy926 cells...
could cleave exogenously added QFS in an EP24.15/EP24.16 specific manner (Norman et al., 2003). Exposure to cyclic strain was seen to significantly increase the levels of QFS cleavage relative to unstrained cells, while the ability of cFP-AAF-pAB to significantly decrease cleavage indicated that the observed QFS cleavage was EP24.15/EP24.16 mediated. Thus it appears that cyclic strain can putatively alter the ability of EP24.15/EP24.16 to cleave exogenous synthetic peptide.

We next decided to extend these investigations by determining the effects of strain on the ability of EP24.15/EP24.16 to hydrolyse exogenously added natural substrates BK and Ang-I. BAECs were strained for 24 h in the presence of either peptide with the inclusion of inhibitors appropriate to prevent non-specific cleavage of these peptides. cFP-AAF-pAB was included to specifically inhibit both EP24.15 and EP24.16, while prior transfection with FLIP, an antisense construct specifically targeted to the EP24.15 promoter, was used to selectively inhibit EP24.15.

A significant increase in the hydrolysis of both exogenously added BK and Ang-I was observed in response to BAEC exposure to cyclic strain. Treatment with cFP-AAF-pAB, and prior transfection with EP24.15 specific FLIP, reduced these strain-dependent increases in cleavage to basal levels in the case of BK, and to below basal levels in the case of Ang-I. This would appear to indicate that the increase in BK and Ang-I hydrolysis observed in response to strain is most likely due to the increase in EP24.15 expression and activity. It was also observed that both cFP-AAF-pAB and EP24.15 knockout with FLIP significantly reduced the amount of Ang-I cleavage by unstrained BAECs.
However no similar reduction in levels of BK cleavage in unstrained cells was observed following EP24.15/EP24.16 inhibition. This may potentially be due to the relative affinity of the extracellular forms of both endopeptidases for BK and Ang-I. It has been previously demonstrated by Rioli and co-workers that recombinant forms of rat EP24.15 and porcine EP24.16 display a greater affinity for BK than Ang-I (Rioli et al., 1998); however in the whole cell assay format employed by our study it may be that the relative affinities of the bovine endothelial forms both endopeptidases for BK and Ang-I differ significantly from those previously described.

The complete attenuation of these increases by FLIP suggested, that despite the broadly similar levels of cellular and secreted activity of EP24.15 and EP24.16, the attenuation observed with cFP-AAF-PAB was largely due to the inhibition of EP24.15 rather than EP24.16. It is possible however that in the experimental paradigm employed, (i.e. living whole cell studies versus studies with harvested cellular proteins), that cFP-AAF-PAB inhibits only EP24.15. cFP-AAF-pAB was initially developed as an EP24.15 specific inhibitor, but was later shown to inhibit EP24.16, albeit at a higher concentration. (Orlowski et al., 1988; Shimpton et al., 2002). It has since been shown to be unstable in vivo (Telford et al., 1995) and has been superceded by its more stable analogue, JA2, which has been successfully employed as an inhibitor of both enzymes in whole cell cleavage assays (Shimpton, et al., 2002; Norman et al., 2003). Although attempts were made to inhibit EP24.16 expression using siRNA transfection, no significant reduction in EP24.16 mRNA levels were observed. This may be due to the fact that because the bovine EP24.16 sequence is unpublished it was necessary to design
this siRNA by looking for regions of consensus between the, rat, mouse and human EP24.16 gene. Also, pharmacological inhibitors for this enzyme proved commercially unavailable. Moreover their synthesis was beyond the scope and resources of this project. In the absence of a method of specifically inhibiting either EP24.16 expression or activity it is impossible to completely ascertain its precise contribution to the strain-induced peptide cleavage events observed. Also, although we were able to demonstrate an apparent increase in extracellular membrane association of EP24.15 in response to cyclic strain, the lack of availability of a suitable antiserum against EP24.16 meant we were unable to ascertain if the basal levels of extracellular membrane-bound EP24.16 (if any) increase in response to cyclic strain. It is wholly possible that this ability to associate with the extracellular plasma membrane affects the ability of the endopeptidase to cleave peptide substrates extracellularly. It remains to be determined, however, if this is a significant factor in these events.

The apparent involvement of EP24.15 in strain-mediated BK and Ang-I hydrolysis is in contrast somewhat with the observation by Norman and co-workers that EP24.15 plays a minor role in the extracellular processing of BK relative to that of EP24.16 (Norman et al., 2003). However this disparity in the findings may be explained in part by the different cell types employed, and the fact that the latter study employed only basal "unstimulated" conditions.

In conclusion, having demonstrated that the secreted activity of both EP24.15 and EP24.16, and the degree of extracellular membrane association of EP24.15 increases in
response to exposure cyclic strain in BAECs, the effects of cyclic strain on the ability of the two enzymes to cleave exogenous substrates in BAECs in culture was investigated. It was clearly demonstrated that cyclic strain induced significant increases in the cleavage of QFS, BK, and Ang-I, increases that could be attenuated by both cFP-AAF-pAB, in the case of all three, and prior transfection with FLIP, in the case of the natural substrates BK and Ang-I. These observations lead us to conclude that exposure of BAECs to chronic cyclic strain putatively leads to an increase in cleavage of exogenously added substrates in an EP24.15-specific manner.
Chapter 6; Results Section 4
6.1 Introduction

Having investigated the putative effect of cyclic strain on the ability of EP24.15 to hydrolyse extracellular BK and Ang-I in BAECs it was a logical progression to study the potential phenotypic and functional effects of this phenomenon in BAECs. In this regard, we investigated the involvement of EP24.15 and EP24.16 in two important angiogenic processes, namely endothelial cell migration and tubule formation.

The main product of EP24.15/EP24.16 mediated cleavage of Ang-I is Ang-(1-7) (Chappell et al., 1998), which has been demonstrated to influence angiogenic events in an opposing manner to Ang-II (Machado et al., 1999). Ang-II has frequently been associated with the regulation of angiogenesis both in vitro and in vivo (Munzemaier et al., 1996; Desideri et al., 2003), although its exact role remains unclear. BK, which is cleaved to BK-(1-5) and BK-(6-9) by both EP24.15/EP24.16, has also been shown to have a significant pro-angiogenic role, both in vivo and in vitro. However, unlike the products of Ang-I cleavage, the cleavage products of BK are generally considered to be functionless with regard to the regulation of angiogenesis (Hu, et al., 1993; Ishihara, et al., 2002).

*The hypothesis examined in this chapter is that cyclic strain influences endothelial cell phenotype (and potentially function) in BAECs via endopeptidase-mediated cleavage of peptide natural substrates such as BK and Ang-I.*
6.2 Results

6.2.1 Inhibition of EP24.15 attenuates cyclic strain induced tube formation in BAECs.

BAEC tubule formation was measured using a collagen gel matrix assay as previously described. Following exposure to cyclic strain (5%, 24 h), either in the presence or absence of cFP-AAF-pAB or after prior transfection with the EP24.15 antisense FLIP, BAECs were harvested and plated onto collagen and incubated for 24 h. Tube formation was subsequently recorded and quantified by digital image capture and NIH image analysis.

It was observed that BAECs exhibit a significant strain-induced increase in tubule formation (1.97 ± 0.18 fold relative to unstrained control, **P≤0.01). Treatment of cells during straining with the EP24.15/EP24.16 specific inhibitor cFP-AAF-pAB led to a significant attenuation of strain-induced tube formation (>80%, °P≤0.05) (Fig 6.1 a, 6.2). Prior transfection with the EP24.15 specific antisense FLIP was also seen to significantly attenuate the strain dependent increase in tube formation (>80%, °°P≤0.01) (Fig 6.1 b, 6.2). Neither inhibition strategy induced any significant changes on the basal levels of tube formation (Fig 6.1, 6.2).
Figure 6.1 Inhibition of EP24.15 attenuates cyclic strain induced tube formation in BAECs. Cells were exposed to cyclic strain (5%, 24 h) either in the presence or absence of the EP24.15/EP24.16 specific inhibitor cFP-AAF-pAB (3.4 μM) or following transfection with the EP24.15 specific antisense FLIP. Unstrained plates were included as controls. Following exposure to strain cells were harvested, plated onto collagen matrixes, and incubated for 24 h before being analysed using digital image capture and NIH image analysis. Representative pictures are shown for cFP-AAF-pAB and FLIP treated cells (a, b).
Figure 6.2 Inhibition of EP24.15 attenuates cyclic strain induced tube formation in BAECs. Cells were exposed to cyclic strain (5%, 24 h) either in the presence or absence of the EP24.15/EP24.16 specific inhibitor cFP-AAF-pAB (3.4 μM) or following transfection with the EP24.15 specific antisense FLIP. Unstrained plates were included as controls. Following exposure to strain cells were harvested, plated onto collagen matrixes, and incubated for 24 h before being analysed using digital image capture and NIH image analysis. Graph represents fold changes in BAEC tubule length relative to unstrained control. Data are expressed as the mean of three independent experiments ± SEM. Statistical differences were established at **P ≤ 0.01 relative to unstrained, untreated control, and significant differences established at ^P ≤ 0.01 and ^P ≤ 0.05 relative to strained, untreated control.
6.2.2 Inhibition of EP24.15 attenuates cyclic strain induced migration in BAECs.

BAEC migration was measured using a wound-healing (scratch) assay as described previously. Following exposure to cyclic strain (5%, 24 h), either in the presence or absence of cFP-AAF-pAB or after prior transfection with the EP24.15 antisense FLIP, BAECs were harvested and plated into 24-well plates, allowed to adhere for 12 h and then scratched. BAEC migration was subsequently recorded and quantified by digital image capture and NIH image analysis.

In response to exposure to cyclic strain BAEC migration increased by 1.58 ± 0.09 fold relative to unstrained control (***P≤0.005). Treatment of BAECs with the EP24.15/EP24.16 specific inhibitor, cFP-AAF-pAB, led to a significant attenuation of the strain induced increase in migration ("P≤0.01), as well as inducing a smaller (>10%) decrease in the baseline levels of migration (Fig. 6.3 a, 6.4). Prior transfection of BAECs with FLIP completely attenuated the strain-induced increase in migration. However this attenuation, though total, was not deemed to be statistically significant (P=0.08) (Fig. 6.3 b, 6.4).
Figure 6.3. Inhibition of EP24.15 attenuates cyclic strain induced migration in BAECs. Cells were exposed to cyclic strain (5%, 24 h) either in the presence or absence of the EP24.15/EP24.16 specific inhibitor cFP-AAF-pAB (3.4 μM) or following transfection with the EP24.15 specific antisence FLIP. Following exposure to strain cells were harvested, plated onto 24 well plates, incubated for 12 h and then scratched. The scratch was photographed immediately (t=0) and after 4 h incubation. Photographs were analysed using NIH image software, with the degree of migration judged to be the extent of reduction of the width of the scratch. Representative pictures are shown for cFP-AAF-pAB and FLIP treated cells (a, b).
Figure 6.4. Inhibition of EP24.15 attenuates cyclic strain induced migration in BAECs. Cells were exposed to cyclic strain (5%, 24 h) either in the presence or absence of the EP24.15/EP24.16 specific inhibitor cFP-AAF-pAB (3.4 μM) or following transfection with the EP24.15 specific antisence FLIP. Following exposure to strain cells were harvested, plated onto 24 well plates, incubated for 12 h and then scratched. The scratch was photographed immediately (t=0) and after 4 h incubation. Photographs were analysed using NIH image software, with the degree of migration judged to be the extent of reduction of the width of the scratch. Graph represents fold changes in BAEC migration. Data are expressed as the mean of thee independent experiments ± SEM. Statistical differences were established at ***P≤0.005 and *P≤0.05 relative to unstrained control and significant differences established at #P≤0.01 relative to strained control.
6.2.3 Incubation of BAECs with Ang-(1-7) and BK increases endothelial tubule formation.

BAECs were incubated for 24h in the presence of BK-(1-5), Ang-II and Ang-(1-7) (10 μM). Untreated cells were included as controls. Post-incubation cells were harvested and assayed for tube formation as previously described.

BK-(1-5) had no significant effect on BAEC tubule formation. In contrast Ang-(1-7) and, the product of cleavage of Ang-I by EP24.15/EP24.16, and BK to significantly increased BAEC tubule formation (1.63 ± 0.61 fold and 1.49 ± 0.64 fold respectively relative to untreated control, **P≤0.01). In contrast incubation of the cells with Ang-II, was seen to induce a small but significant decrease in BAEC tubule formation (Fig 6.5 a, b).
Figure 6.5. Effects of the addition of various vasoactive peptides on BAEC tube formation. BAECs were incubated for 24 h with either BK, BK-(1-5), Ang-I, Ang-II or Ang-(1-7) (10 μM), with an inhibitor cocktail containing 10 μM captopril/phosphoramidon/bestatin and 3.4 μM cFP-AAF-pAB added to prevent peptide degradation. Untreated cells were also included as controls. Following treatment cells were harvested, plated onto collagen matrixes, and incubated for 24 h before being photographed. Representative photographs are shown in (a), with examples of significant
tube formation indicated by arrows. Tubule length was determined using NIH image software. Graph (b) represents fold change in tubule formation relative to untreated control. Data are expressed as the mean of three independent experiments ± SEM. Statistical differences were established at ***$P \leq 0.005$ and **$P \leq 0.01$ and *$P \leq 0.05$ relative to untreated control.
6.2.4 Addition of Ang-(1-7), but not Ang-II, reverses the attenuation of strain induced tubule formation in BAECs by EP24.15 inhibition.

Having monitored the effect of addition of Ang-II and Ang-(1-7) on tubule formation in static BAECs, it was decided to investigate what effects, if any, these peptides had on the blockade of the strain-dependent increase in tube formation following EP24.15 inhibition by transfection with the antisense EP24.15-Flip. Addition of BK was not considered for this study as, although its addition significantly increases levels of BAEC tubule formation, it is cleaved and inactivated by EP24.15. BK-(1-5) and Ang-1 were also omitted from this study as they were shown to exert no influence on tubule formation in BAECs.

Briefly cells were subjected to strain (5%, 24 h) in the presence of either Ang-II or Ang-(1-7) (10 μM). An inhibitor cocktail of bestatin/captopril/phosphoramidon (10 μM) was also included to prevent non-specific peptide hydrolysis. Prior transfection with the antisense FLIP was used to inhibit EP24.15 expression. Untreated and unstrained plates were included as controls. After straining the cells were harvested and assayed for tubule formation as previously described.

In all experiments a somewhat lower than normal strain-induced increase in tubule formation was observed in untreated, mock transfected BAECs (1.51 ± 0.69 fold increase relative to control versus 1.98 ± 0.18 fold increase in previous experiments, Fig 6.2). This was most likely due to the effect of the blockade of ACE, ECE, NEP and
aminopeptidases (necessary to prevent unwanted peptide degradation) on basal vasoactive peptide processing. However in all experiments prior transfection of FLIP was seen, as before, to significantly attenuate strain induced tubule formation (>80% attenuation, $^5P\leq0.05$).

In mock transfected BAECs the addition of Ang-II was seen to attenuate the strain dependent increase in tubule formation (>70% attenuation), while only marginally decreasing levels of tubule formation in unstrained cells. In the case of BAECs transfected with FLIP the addition of Ang-II neither significantly altered the levels of tubule formation in unstrained cells nor had any significant effect on the attenuation of the strain dependent increase in tubule formation resulting from FLIP transfection (Fig. 6.6 b). By contrast the addition of Ang-(1-7) induced a significant increase in levels of tubule formation in unstrained mock and FLIP transfected BAECs (1.42 ± 0.1 fold and 1.34 ± 0.14 fold respectively relative to control, $^*P\leq0.05$) (Fig 6.7). In cells exposed to strain addition of Ang-(1-7) did not significantly alter levels of tubule formation in mock transfected cells but induced an increase in tubule formation in FLIP transfected BAECs (1.63 ± 0.03 fold versus 1.09 ± 0.04 fold relative to unstrained control $^{***}P\leq0.005$).
Figure 6.6. Ang-II addition does not reverse the reduction in cyclic strain induced tube formation resulting from EP24.15 inhibition. Cells were exposed to cyclic strain (5%, 24 h) following transfection with the EP24.15 specific antisense FLIP, and in the presence of 10 μM Ang-II (and an inhibitor cocktail containing 10 μM phosphoramidon/captopril/bestatin to prevent non specific peptide degradation). Following exposure to strain cells were harvested, plated onto collagen matrices, and incubated for 24 h before being analysed using digital image capture and NIH image.
analysis. Representative pictures are shown in (a), with arrows indicating examples of BAEC tubule formation. Graph (b) represents fold change in BAEC tubule formation. Data are expressed as the mean of three experiments ± SEM. Statistical differences were established at *$P \leq 0.05$ relative to unstrained control and at **$P \leq 0.01$ relative to strained control.
Figure 6.7 Addition of Ang-(1-7) reverses the reduction in cyclic strain induced tube formation resulting from EP24.15 inhibition. Cells were exposed to cyclic strain (5%, 24 h) following transfection with the EP24.15 specific antisense FLIP, and in the presence of 10 μM Ang-II (and an inhibitor cocktail containing 10 μM phosphoramidon/captopril/bestatin to prevent non-specific peptide degradation). Following exposure to strain cells were harvested, plated onto collagen matrices, and incubated for 24 h before being analysed using digital image capture and NIH image analysis. Representative
pictures are shown in (a), with arrows indicating examples of BAEC tubule formation. Graph (b) represents fold change in BAEC tubule formation relative to unstrained control. Data are expressed as the mean of thee experiments ± SEM. Statistical differences were established at \( *P \leq 0.05 \), and \( ***P \leq 0.005 \) relative to unstrained control and significant differences established at \( ^\wedge P \leq 0.01 \) relative to strained control.
6.3 Discussion

Vasoactive peptides such as Ang-II, Ang-(1-7) and BK have been well characterized as modulators of a wide variety of vascular processes, including the control of blood pressure by invoking vasodilation/vasoconstriction and also in the regulation of new vessel formation (Duffy et al., 2004; Chappell et al., 2000). Our observation that the exposure of BAECs to cyclic strain leads to an apparent increase in the EP24.15 specific cleavage of exogenous BK and Ang-I led us to examine the possible implications this phenomenon may have on endothelial cell function.

Angiogenesis, the formation of new vessels, is a complex process initiated at the level of the endothelium. Endothelial cells “sprout” forming tubules into the extracellular matrix which grow to form capillary-like structures both though endothelial cell migration and proliferation. In the final stages, smooth muscle cells adhere to the newly forming vessel (Quarmbry et al., 2002).

Exposure to hemodynamic forces has been previously shown to increase both endothelial cell migration and tubule formation in vitro (Hsu et al., 2001; Von Offenberg-Sweeney et al., 2005). Our baseline findings of the effects of cyclic strain on migration and tube formation in BAECs reflect the findings of others, with chronic exposure to cyclic strain inducing a significant increase in migration and tube formation, as measured by a wound healing (scratch) assay and collagen matrix tube formation assay respectively (see Fig 6.1/ 6.2/6.3/6.4).
Both the dual endopeptidase inhibitor, cFP-AAF-pAB, and the specific antisense construct targeted to the EP24.15 promoter, FLIP, were used to delineate the contribution, if any, of these enzymes to the observed increase in endothelial cell migration and tubule formation in response to strain.

Both cFP-AAF-pAB and FLIP attenuated strain-induced increases in both tubule formation and migration to near baseline levels. (The attenuation of strain-induced BAEC migration by FLIP, although total, was deemed to be statistically insignificant). The complete attenuation of these increases by FLIP suggested that, despite the broadly similar levels of cellular and secreted activity of EP24.15 and EP24.16, the attenuation observed in migration and tubule formation with cFP-AAF-pAB was largely due to the inhibition of EP24.15 rather than EP24.16. However it may be that in the experimental paradigm employed, (i.e. living whole cell studies versus studies with harvested cellular proteins), that cFP-AAY-pAB inhibits only EP24.15 to a significant degree. As previously discussed (section 5.4), although cFP-AAF-pAB can inhibit both enzymes, its inhibitory concentration is significantly higher for EP24.16 (K, [EP24.15]= 19nM, K, [EP24.16] = 700 nM (Dauch et al., 1991)). Cytosolic EP24.15 and EP24.16 also have been shown to display different affinities for a range of natural peptide substrates (Rioli et al. 1998), and it may be possible that secreted or extracellularly bound EP24.16 and EP24.16 display significantly different substrate affinities (with regard to Km and Kcat) for the test peptide substrates. Although attempts were made to inhibit EP24.16 expression using siRNA transfection, no significant reduction in EP24.16 mRNA levels were observed. In the absense of a method of specifically inhibiting either EP24.16 expression or activity it
is impossible to completely ascertain its precise contribution to the strain-induced cell fate events observed.

Having shown that transfection with antisense FLIP significantly and specifically reduces EP24.15 mRNA and protein levels, and that FLIP significantly inhibits the increase in tubule formation and migration in response to cyclic strain, it appears that EP24.15 plays an important role in strain-induced endothelial cell fate decisions.

Our previous observation of a strain-dependent increase in the cleavage of exogenously added BK and Ang-I by EP24.15 in BAECs (Fig. 5.5, 5.6) led us to hypothesize that EP24.15 may be exerting its effects on strain-induced BAEC migration and tubule formation through its cleavage of vasoactive peptides. EP24.15 cleaves Ang-I to produce Ang-(1-7), which has been demonstrated to have a number of important functions in the vasculature, often acting in opposition to the effects associated with Ang-II, the product of cleavage of Ang-I by angiotensin converting enzyme (ACE) (Shimpton et al., 2002; Chappell et al., 2000; Fetterik et al., 2000). Machado and co-workers have demonstrated that Ang-II had a pro-angiogenic effect in vivo, while Ang-(1-7) reduced angiogenesis. Other studies have demonstrated that Ang-(1-7) induces the release of NO, which is known both to promote angiogenesis and to inhibit ACE activity (Machado et al., 1999; Fetterik, et al., 2000; Ackermann et al., 1998). The exact role of Ang-II in regulation of angiogenesis also remains unclear, with many studies indicating that it has a negative effect on angiogenic processes (Silvestre et al., 2001, Desideri et al., 2003). In contrast, BK, which is generally considered to be inactivated by cleavage by EP24.15,
has a definite pro-angiogenic effect, inducing the release of pro-angiogenic NO (Ishihara, et al., 2002; Duffy et al., 2004).

Consequently, we examined the effects of addition of the products of Ang-I and BK cleavage by EP24.15 on the basal levels of tubule formation in BAECs. Cells were treated with Ang-I, Ang-(1-7), BK and BK-(1-5). Ang-II, the product of Ang-I cleavage by ACE, was also included.

Ang-(1-7) and BK were seen to significantly increase the levels of tubule formation in BAECs, while Ang-II induced a small but statistically significant drop in the levels of tubule formation relative to untreated control. BK-(1-5) however induced no significant change in the levels of tubule formation. These findings suggested a putative means by which the strain induced increase in EP24.15 activity and tubule formation/migration may be linked. Increased EP24.15 activity would not only lead to an increase in the levels of Ang-(1-7), but would also reduce the amount of Ang-I available for conversion to Ang-II by ACE, with the net effect of promoting angiogenesis. The reduction of ACE expression in the endothelium by hemodynamic forces has previously been demonstrated (Reider et al., 1997), while a co-worker has demonstrated that shear stress reduces the levels of ACE mRNA in BAECs (Fitzpatrick et al., 2005). These observations support this hypothesis; as reduced ACE activity would not only reduce Ang-II levels but would also reduce ACE-mediated degradation of Ang-(1-7). Given that BK is degraded by EP24.15, it was judged that, although it stimulated tubule formation, it
played no significant role in the EP24.15-dependent increase in tubule formation observed in response to cyclic strain.

The possibility that EP24.15 derived Ang-(1-7) was responsible for the strain-induced increase in BAEC tube formation was further investigated by determining the effects of Ang-(1-7) addition to both FLIP- and mock-transfected cells exposed to cyclic strain. The effects of Ang-II addition were also investigated. Addition of Ang-(1-7), while increasing basal levels of tubule formation in both FLIP-transfected and mock-transfected cells did not significantly alter tubule formation in strained, mock-transfected cells. In contrast, addition of Ang-II actually attenuated the strain-induced increase in tubule formation. Moreover, in FLIP-transfected cells Ang-(1-7) reversed the effects of EP24.15 blockade on the strain-induced increase in tubule formation, again in contrast to Ang-II. These findings were in line with our hypothesis that EP24.15 influences endothelial tubule formation both directly through the production of Ang-(1-7), and indirectly through reduction in availability of Ang-I for conversion to Ang-II by ACE. As previously discussed, the possibility that ACE activity, and therefore Ang-II production, might be decreased in response to cyclic strain leading to an increase in available Ang-I, suggests that tubule formation (and other endothelial cell fate decisions) may be regulated, in part, by a balance between Ang-II and Ang-(1-7) production.

The apparent contribution of Ang-(1-7) to strain-induced endothelial tubule formation may not be solely due to its increased production by EP24.15. The number and activity of receptors for Ang-(1-7) may alter in response to strain. The number and
activity of the receptors of a number of vasoactive peptides have been shown to alter in response to hemodynamic forces. For example the expression of the endothelin-1 ET\textsubscript{b} receptor was seen to increase at the level in response to laminar shear stress (Moraweitz \textit{et al.}, 2000). Studies have shown that although Ang-(1-7) mediates some of its effects in the vasculature though the BK B2 receptor, it also has its own distinct receptor, characterised by sensitivity to D-[Ala 7]-Ang-(1–7) (Neves \textit{et al.}, 2003; Fetterik \textit{et al.}, 2000). Although yet to be determined, it is quite possible that cyclic strain increases the number of membrane bound receptors for Ang-(1-7), thus potentiating the effects of Ang-(1-7) production by EP24.15.

In conclusion, our studies have shown that exposure to cyclic strain induces a clear and significant increase in both BAEC migration and tubule formation in \textit{vitro}. These increases are significantly attenuated by pharmacological and molecular inhibition of EP24.15. In addition, Ang-(1-7), the product of EP24.15 hydrolysis of Ang-I, induces an increase in tubule formation in static BAECs an is also able to reverse the attenuation of the strain induced increase in tubule formation resulting form FLIP-mediated EP24.15 blockade. Therefore it is our conclusion that the increase in endothelial tubule formation and migration, observed in response to exposure of BAECs to cyclic strain, may putatively be caused by increased production of Ang-(1-7) from Ang-I by EP24.15.
Chapter 7; Final Summary and

Conclusions
7.1 Final Summary

Hemodynamic forces, namely shear stress and cyclic strain, are considered to be important regulators of a number of vascular endothelial functions such as hemostasis, angiogenesis and the maintenance of blood pressure. The regulation of these events by hemodynamic forces is partly effected by inducing peptide synthesis and release, by inducing changes in the expression of peptide receptors, and by regulating both the expression and activity of peptide-degrading enzymes. These effects are mediated by a number of mechanotransducers, including Gi-protein coupled receptors, integrins and receptor tyrosine kinases (Duffy et al., 2004; Papadaki et al., 1997).

EP24.15 and EP24.16 are members of the Clan MA family of peptidases, the thermolysin-like zinc metallopeptidases which are known to be expressed in the vasculature and have been shown to cleave a number of peptides relevant to vascular endothelial function, including Ang-I and BK (Shimpton et al., 2003, Norman et al., 2003). EP24.15 and EP24.16 are also closely related to ECE and ACE, both of which have been shown to be subject to mechanoregulation by hemodynamic forces (Reider et al., 1997; Moraweitz et al., 2000).

Angiogenesis, the formation of new vessels, is a complex process, initiated at the level of the endothelium. Endothelial cells “sprout”, forming tubules into the extracellular matrix, which grow to form capillary-like structures both though endothelial cell migration and proliferation. In the final stages smooth muscle cells adhere to the newly
forming vessel (Quarmbry et al., 2002). Exposure to hemodynamic forces has been previously shown to increase both endothelial cell migration and tubule formation in vitro (Hsu et al., 2001; Von Offenberg-Sweeney et al., 2005). Peptidases, including aminopeptidase and the zinc metalloendopeptidase ACE, have been implicated in the regulation of angiogenic cell fate decisions in the endothelium via their cleavage of a number of vasoactive peptide substrates (Sato, 2004; Desideri et al., 2003).

Both EP24.15 and EP24.15 cleave Ang-I to produce Ang-(1-7), which has been demonstrated to have a number of important functions in the vasculature, often acting in opposition to Ang-II, the product of cleavage of Ang-I by ACE (Shimpton, et al., 2002; Chappell et al., 2000). Both enzymes also degrade BK to BK-(1-5). BK is an important mediator of a number of important endothelial functions, including maintenance of vascular tone and endothelial cell migration.

It was our hypothesis that cyclic strain regulated EP24.15/EP24.16 expression and activity in BAECs putatively via a G-protein dependent pathway, with consequences for endothelial cell-mediated peptide hydrolysis and cell fate decisions such as migration and tubule formation.

We have demonstrated that exposure to cyclic strain increases levels of EP24.15 and EP24.16 mRNA in BAECs. A significant time- and force-dependent increase in cellular enzyme activity was also observed for both enzymes, along with an increase in secreted enzyme activity. Using immunocytochemical staining techniques with
fluorescent and confocal microscopy we also demonstrated the apparent ability of strain to increase the levels of cytoplasmic, nuclear and extracellular membrane-associated EP24.15.

Although, to our knowledge, we are the first to have demonstrated the hemodynamic regulation of these enzymes in endothelial cells, other researchers have demonstrated the ability of hemodynamic forces to regulate the closely related peptidases ECE-1 and ACE. Masatsugu and co-workers demonstrated a significant time- and force-dependent decrease in expression of both ECE-1α and ECE-1β mRNA in response to shear stress in both human umbilical vein endothelial cells (HUVECs) and BAECs cultured in a parallel plate flow chamber (Masatsugu et al., 2003). Similar studies by Morawietz and co-workers showed a 63.8% and 37.8% decrease in levels of ECE-1α and ECE-1β mRNA in HUVECs in response to chronic laminar shear stress (Morawietz et al., 2000). Rieder and co-workers have investigated the mechanosensitive properties of ACE by employing a cone-plate viscometer to expose bovine pulmonary artery endothelial cells (BPAECS) to chronic laminar shear stress (20 dynes/cm², 18 h), ACE activity was reduced by 49.5% and ACE mRNA expression by 82% (Rieder et al., 1997). Our studies suggest, that unlike those concerning ACE and ECE-1, the expression and activity of EP24.15 and EP24.16 up-regulated by increasing hemodynamic force. However the degree of sensitivity to cyclic strain exhibited by EP24.15 and EP24.16 is comparable with that observed for ACE/ECE-1 with respect to shear stress in the above mentioned studies.
Having determined that EP24.15 and EP24.16 expression and activity can be up-regulated in BAECs by chronic exposure to cyclic strain, we next tried to ascertain if the phenomenon was mediated by G-protein signaling. Our study focused on the effects of both pharmacological and molecular inhibition of Giα- and Gβγ-mediated signaling on the increase in cellular activity of both enzymes. Pharmacological inhibitors of Gi-protein signaling were initially employed to investigate this role; pertussis toxin and NF023. Both agents were demonstrated to strongly attenuate strain-dependent up-regulation of activity for both endopeptidases, suggesting a role for Gi-protein signalling in these events. Further inhibition studies therefore focused on selective ablation of individual Gi-protein subunits at the molecular level. Plasmids encoding dominant negative mutants of Giα1,2,3 subunits (i.e. Giα1-G202T, Giα2-G203T, and Giα3-G202T) were subsequently employed to selectively ablate the function of their endogenous wild type counterparts in BAECs (Cullen et al., 2002). A plasmid encoding the carboxyl terminus of β-Adrenergic Receptor Kinase (βARK. ct), a well known Gβγ-subunit scavenger (Koch et al., 1994), was also employed to selectively inhibit Gβγ signaling pathways. The cyclic strain dependent increase in cellular EP24.15 was shown to be strongly attenuated by inhibition of Giα2/Giα3 signaling, while the increase in cellular E24.16 activity was significantly attenuated by inhibition Giα1/Giα2 and Gβγ subunit mediated signaling. The observation that EP24.15 and EP24.16 are up-regulated by cyclic strain via divergent G-protein signaling pathways is not with precedence; the ability of hemodynamic forces to differentially regulate signaling molecules by PTX-sensitive and insensitive pathways has been previously demonstrated; Jo and co-workers demonstrated that expression of βARK-ct inhibits the shear stress activation of HA-JNK, whereas blockade of Giα2 with
the dominant negative mutant $G_{i2}\text{-G203T}$ or antisense $G_{i2}$ prevented shear dependent activation of HA-ERK (Jo et al., 1997). Also although closely related, EP24.15 and EP24.16 are significantly different. In addition to differences in their substrate specificities, both enzymes are separate gene products (approx. 60% homology) and are under the control of distinct promoters, with additional evidence to indicate that EP24.16 expression is subject to alternative promoter usage, thereby enabling targeting of some EP24.16 to the mitochondrial compartment (Kato et al., 1997).

Although we can now state that G-protein signaling is involved in the transduction of the effects of cyclic strain on EP24.15 and EP24.16, the remaining downstream and upstream signaling mechanisms remain to be elucidated, and as such remain an area for possible future study.

The demonstration that cyclic strain could both increase the levels of secreted activity for both EP24.15 and EP24.16 and could putatively increase the amount of membrane-bound EP24.15 led us to examine the possibility that exposure to cyclic strain could affect the levels of endopeptidase-specific cleavage of extracellular synthetic and natural peptide substrates. The ability of cultured bovine and ovine endothelial cells to cleave exogenously added peptide substrates in an EP24.15/EP24.16-specific manner has previously been demonstrated by Norman and co-workers (Norman et al., 2003). We observed that exposure to cyclic strain increases EP24.15/EP24.16 specific cleavage of exogenously added QFS in BAECs. By employing both a pharmacological inhibitor of both enzymes (cFP-AAF-pAB) and an antisense construct to inhibit EP24.15 expression
we also demonstrated that cyclic strain increases the EP24.15 specific cleavage of exogenously added BK and Ang-I in BAECs in culture. Our observations also suggest that EP24.16 appears to have no significant role in the strain-induced extracellular processing of BK and Ang-I. This contrasts somewhat with the findings of Norman and co-workers, who showed that EP24.16 had a greater role in the cleavage of extracellular peptides than EP24.15. However this divergence in findings may be explained in part by the different cell types used, and the experimental paradigm employed (Ovine versus Bovine endothelial cells, static versus unstrained cells). Although possessing broadly similar substrate specificities, EP24.15 and EP24.16 have been shown to display different affinities for a range of natural substrates, and it may be possible that the secreted or membrane-associated form of EP24.16 in BAECs displays significantly different substrate affinities than those displayed by EP24.15. Also, although an increase in extracellular membrane association for EP24.15 in response to cyclic strain was suggested by immunocytochemical studies, the unavailability of suitable anti-EP24.16 antisera made it impossible to observe if a similar increase occurs in the case of EP24.16. A further barrier to assessing the precise role (if any) played by EP24.16 in strain-induced extracellular peptide cleavage in BAECs was the unavailability of a suitable inhibition strategy specific for EP24.16 as stable, highly specific inhibitors of EP24.16 are commercially unavailable and attempts to inhibit EP24.16 by transfection with siRNA proved unsuccessful.

Our observation that exposure to cyclic strain can up-regulate the EP24.15 specific cleavage of exogenously added peptide substrates in BAECs led us to investigate
the possible effects of this phenomenon on endothelial cell function. Both Ang-I (and its
derivatives), and BK are known to affect angiogenic cell fate decisions in endothelial
cells. Again, our study used both a dual pharmacological inhibitor of both enzymes (cFP-
AAF-pAB), and an antisense construct to inhibit EP24.15 expression (EP24.15-FLIP) to
assess the contribution of both endopeptidases to cyclic strain-induced BAEC migration
and tubule formation.

Treatment with cFP-AAF-pAB and prior transfection with FLIP significantly
attenuated the strain-dependent increase in both migration and tubule formation in
BAECs. This observation suggested that extracellular processing of peptides by EP24.15
may impact on the strain-mediated cell fate decisions of BAECs in culture. In order to
further investigate this hypothesis we treated static BAECs with Ang-I, Ang-II, Ang-(1-
7), BK, and BK-(1-5), and analysed the effects on their tubule formation ability. Both
Ang-(1-7) and BK were seen to significantly drive BAECs tube formation, while
treatment with Ang-II induced a small but significant decrease in tubule formation. The
ability of Ang-II to inhibit angiogenic processes has been demonstrated previously
(Desideri et al., 2003), while Ang-(1-7) is generally considered to have opposing effects
to those of Ang-II (Fetterik et al., 2000).

If the increased production of Ang-(1-7) from Ang-I by EP24.15 in response to
cyclic strain is responsible, at least in part, for the increase in migration and tube
formation in BAECs, then addition of exogenous Ang-(1-7) should be able to reverse the
effects of EP24.15 inhibition. Although BK also induced a significant increase in BAEC
tubule formation, it is degraded by EP24.15, and unlikely therefore to contribute significantly to the EP24.15-dependent increase in tubule formation observed in response to cyclic strain. We studied the effects of addition of Ang-II and Ang-(1-7) on the strain dependent increase in tubule formation in both mock and FLIP transfected BAECs. We observed that Ang-II addition failed to significantly reverse the effects of EP24.15 inhibition on strain-induced tubule formation. In contrast, addition of Ang-(1-7) was seen to completely reverse the effects of FLIP transfection on strain-induced BAEC tubule formation. These observations suggested that the strain dependent increase observed in EP24.15 activity may putatively contribute to the strain-dependent increase observed in BAEC tubule formation by increasing the levels of production of Ang-(1-7) from Ang-I. Previous studies, demonstrating that shear stress reduces ACE expression and activity in the endothelium (Rieder et al., 1997), and the observation by a co-worker that shear stress reduces the levels of ACE mRNA in BAECs, supports this hypothesis (Fitzpatrick et al., 2005); the combined effect of a reduction in production of Ang-II by ACE, coupled with an increase in production of Ang-(1-7) by EP24.15 would have the net effect of encouraging angiogenic cell fate decisions in our model.

The combined observations of our studies lead us to propose the following model, a generalised version of which is shown in Fig. 7.1. Firstly the exposure of BAECs to cyclic strain leads to the up-regulation of EP24.15 and EP24.16 expression and both cellular and secreted activity. In addition it leads to a significant increase in nuclear, cytoplasmic and membrane-associated EP24.15 protein levels. This up-regulation is dependent on G-protein signaling, with EP24.15 and EP24.16 displaying differences in
the G-protein subtypes involved. Specifically cyclic strain-dependent increase in cellular EP24.15 activity is strongly attenuated by inhibition of Giₐ₂/Giₐ₃ signaling, while the increase in cellular EP24.16 activity is significantly attenuated by inhibition Giₐ₁/Giₐ₂ and Gβγ subunit mediated signaling. Further study remains to be done on determining the down stream effectors of this phenomenon. Also, the possibility that the effects of cyclic strain on EP24.15/EP24.16 may also be mediated in part by other signaling mechanisms (e.g. integrins) remains to be investigated (Fig 7.2).

Figure 7.1 Suggested model of EP24.15/EP24.16 regulation by cyclic strain in BAECs; AP1, adaptor protein complex 1; Ras, small GTPase family; ERK, extracellular regulated kinase; JNK, c-jun terminal kinase; AT₁,Ang-(1-7) receptor.
Cyclic strain also leads to an apparent increase in the extracellular cleavage of BK and Ang-I by EP24.15. In our studies, the contribution of EP24.16 to the extracellular cleavage of these peptides appears negligible. As discussed previously, the lack of a specific inhibition strategy for EP24.16 makes it difficult to conclusively determine its role in the extracellular hydrolysis of BK and Ang-I, and thusly this is an area that warrants further investigation. Also the fact that EP24.16 does not participate to a
significant extent in the strain-mediated extracellular processing of BK and Ang-I in BAECs does not rule out the possibility that EP24.16 may be involved in the extracellular processing of other peptides, such as neurotensin, and this too remains an area for further study.

Our observations that specific inhibition of EP24.15 leads to attenuation of the strain-induced increase in BAEC migration and tubule formation suggests that EP24.15-mediated peptide cleavage plays a role in strain-induced endothelial cell fate decisions. Further studies demonstrated that Ang-(1-7), the main product of EP24.15 cleavage of Ang-I strongly increased BAEC tubule formation in static cells and could reverse the effects of EP24.15 inhibition on strain-dependent tubule formation. This suggested that EP24.15 influences endothelial cell fate decisions though its production of Ang-(1-7) from Ang-I. However, further studies using antagonists against Ang-(1-7) receptors to block Ang-(1-7) stimulation of BAECs are necessary to confirm the role of EP24.15 derived Ang-(1-7) in strain-induced endothelial cell fate decisions.

The functional consequences of these collective findings remain as yet undetermined. They concur however with a likely role for hemodynamic forces in the collective regulation of the expression and function of an entire family of physiologically important metalloendopeptidases, including ACE, ECE and NEP, with consequences for the net vasoactive peptide balance within the endothelium, subsequently leading to downstream effects on vessel wall tone and vascular remodeling. Our findings suggest increased production of Ang-(1-7) by EP24.15 in response to increased hemodynamic
forces. Ang-(1-7) is known to induce the release of NO, a potent vasodilator and pro-angiogenic substance in endothelial cells (Fetterik et al., 2000). In contrast exposure to hemodynamic forces have been shown to reduce the activity and expression of ACE and ECE, leading to a reduction in the levels of Ang-II and ET-1, both of which are potent vasoconstrictors (Reider et al., 1997; Masatsugu et al. 2003). The combination of these synergistic events in vivo would be likely to have a net impact on the vessel wall consistent with an atheroprotective effect imparted typically by hemodynamic forces (Traub et al., 1998).

7.2 Further Work

Although we feel that this study serves its purpose in demonstrating the putative hemodynamic regulation of EP24.15/EP24.16 in the vascular endothelium, and indicating a role for EP24.15 in cyclic strain induced endothelial cell fate decisions, there are a number of areas where further study is warranted. In particular, the intracellular signaling pathways upstream of Gi-protein involved in regulation of EP24.15/EP24.16 requires further study. Also investigation of the involvement of EP24.16 in extracellular peptide cleavage remains to be elucidated. Finally, the exact mechanism by which EP24.15 exerts its influence on cyclic strain mediated endothelial cell fate decisions remains to be identified.

The investigation of the potential role of other mechanosensors, and of the intracellular signaling pathways involved in EP24.15/EP24.16 mechanoregulation
represents what is potentially an extensive area of study. However it is an area of obvious interest for further study.

The first area of focus would be the role of other potential mechanosensors in EP24.15/EP24.16 mechanoregulation. It may be possible that one or more of these mechanisms either contribute directly to EP24.15/EP24.16 mechanoregulation, or lie upstream to Gi-protein activation by cyclic strain (see section 1.3). A relatively simple and straightforward series of experiments could be employed to investigate this hypothesis. There are a number of well-established methods available to block the action of tyrosine kinases, integrins, caveolae and ion channels. For example the use of RGD peptide to inhibit integrin binding to ECM proteins, and the blockage of Na+ channels with tetrodotoxin (Traub et al., 1998; Papadaki et al., 1997). The proposed experiments (much like those involving the use of pharmacological agents to block Gi-mediated signaling) would involve subjecting BAECs to cyclic strain in the presence and absence of the various inhibitory agents and the monitoring of their effects on EP24.15/EP24.16 up-regulation, either by studying mRNA levels or enzyme activity. Using this approach, the involvement of integrins, tyrosine kinases, caveolae and integrins in EP24.15/EP24.16 mechanoregulation could be rapidly determined.

Further study is also required to determine the intracellular signaling molecules, occurring downstream of Gi-proteins, involved in the transduction of the response to cyclic strain. Potential candidates include the PKC family, c-Src kinases, as well as small GTPases such as ras, which feed into the Raf/MEK/ERK/JNK signaling pathway (see
section 1.3.6, 1.3.7) (Traub et al., 1998). As with the potential mecahnosenors, a number of methodologies have been developed and employed to inhibit signaling by these various molecules, including pharmacological inhibitors such as staurosphine (inhibits PKCs), as well as antisense constructs and blocking type antibodies which can specifically inhibit signaling by one particular molecule (e.g. c-Src) (Chen et al., 1999).

As with the potential signal transduction mechanisms lying up-stream of these molecules a logical experimental approach would involve exposing BAECs to cyclic strain in the presence and absence of the various inhibitory agents and the monitoring of their effects on EP24.15/EP24.16 up-regulation, either by studying mRNA levels or enzyme activity. By using this approach, beginning with, for the example inhibition of the PKC family, and working downstream through the MAP kinase signaling pathways to the level of ERK/JNK it would be possible to propose a pathway by which the physical stimulus of cyclic strain is transduced to the level of activation of transcription factors.

However to determine the means by EP24.15 and EP24.16 transcription is activated at the genetic level in response to cyclic strain would involved a different experimental approach, using novel materials and techniques. Although the promoter region of EP24.16 has not been extensively studied, and EP24.16 is considered to be under the control of multiple promoters, the promoter region of EP24.15 has been published and shown to contain the so called shear stress responsive element (SSRE), found in the promoters of a number of mechnosensitive genes (see section 1.4) (McCull et al., 1998). However inactive SSREs have been reported in the promoters of other genes, and it is possible that another motif is responsible for mediating cyclic strain
induced transcription in EP24.15. The role of the SSRE (or indeed of other motifs that may be present) in mediating cyclic strain induced transcription of EP24.15 could be established using constructs containing various truncation mutants of the EP24.15 promoter attached to a reporter gene such a luciferase. Cells transfected with these constructs could be exposed to strain and the levels of luciferase expression monitored. If the SSRE is responsible for mediating cyclic strain induced EP24.15 expression, then cells transfected with constructs containing mutants with an intact SSRE should display increased luciferase expression in response to cyclic strain, while those transfected with constructs containing a partial SSRE, or lacking a SSRE, should not.

Our studies indicated a clear increase in the ability of EP24.15 to cleave exogenous BK and ANG-I in response to cyclic strain. No such increase was observed for EP24.16. However it may be that EP24.16 plays an important role in the processing of BK/Ang-I in unstimulated conditions. It is also possible that EP24.16 may exhibit a strain dependent increase in its ability to cleave other exogenously added peptides such as NT.

As discussed previously, in the absence of a specific inhibition strategy for EP24.15, it is impossible to clarify this. It is clear that attempts to define an effective siRNA against EP24.16 should be continued. Also, alternative inhibition strategies, e.g. antisense, should be explored. In addition the, emerging roles for both enzymes in a variety of biologically relevant processes, such as antigen presentation, and processing of the β-amyloid protein involved in alzheimers disease, make it increasingly likely that, in the near future specific and stable chemical inhibitors for both enzymes may be
commercially available. Use of these inhibitory strategies could be used in whole peptide hydrolysis assays identical to those performed previously, allowing us to pin down the exact role of EP24.16 in extracellular peptide hydrolysis. Also the inclusion of peptides other than BK and Ang-I, such as NT, in these studies would allow us to build up a more complete picture of the role of both enzymes in vasoactive extracellular peptide processing.

The exact mechanism by which EP24.15 effects its role in cyclic strain induced tubule formation also needs to be further investigated. We have preliminary evidence which suggests that the strain dependent increase in EP24.15 activity results in an increase in the production of Ang-(1-7), which induces endothelial tubule formation. However it needs to be confirmed that increased EP24.15 activity increases tubule formation, as does the theory that it does this through increased Ang-(1-7) production. These issues can be clarified with the following series of experiments. Firstly a construct designed to over express EP24.15 could be transfected into BAECs, and endothelial tubule formation monitored. This would allow us to confidently link increased EP24.15 activity with increased tubule formation. Secondly the previous experiment would be repeated in the presence of antagonists against receptors for Ang-(1-7), such as HOE404 (B2 receptor antagonist), and D-[Ala 7]-Ang-(m1–7) (AT_1, antagonist). This would allow us to confirm if it is by increased Ang-(1-7) production that EP24.15 influences endothelial cell tubule formation, and to identify the receptor(s) involved.
If EP24.15 is confirmed to influence angiogenic endothelial cell fate decisions in vitro, there is a possibility of in vivo studies. There are a number of in vivo models available for studying angiogenesis, including the sponge model. This involves implanting a sponge coated in extracellular membrane proteins subcutaneously in the test animals. After a defined time period of time the implants are removed and the degree of angiogenic growth in the sponge ascertained (Machado et al., 1999). The use of such a model, along with inhibitors for EP24.15 and Ang-(1-7) receptor antagonists, would allow us to investigate the in vivo relevance of the phenomenon observed in vitro.

7.3 Possible Clinical Implications

One of the main observation of this thesis is that an increase in EP24.15-mediated Ang-(1-7) production, in response to increasing hemodynamic force, can putatively lead to functional changes in endothelial cell fate decisions. This finding leads to the exciting possibility that this enzyme, like the closely related ACE and ECE plays an important role in the regulation of vascular function through its production of Ang-(1-7), with possible clinical implications. Ang-(1-7) has been shown act as vasodilator in vivo, with Ang-(1-7) infusion potentiating the effects of ACE inhibition and AT1 receptor blockade, a finding with obvious implications for the role of Ang-(1-7) in the treatment of hypertension (Chappell et al, 2000). Elevated levels of Ang-(1-7) have also been detected in the rat myocardium after the experimental induction of myocardial infarction, leading to the suggestion that it may promote recovery by opposing the pressor, proliferative and profibrotic actions of Ang-II (Averill et al., 2003). More recent studies have supported
this hypothesis; Tallant and co-workers have demonstrated that the infusion of Ang-(1-7) following experimentally induced myocardial infarction in rats led to a reduction in myocyte size and attenuated ventricular dysfunction and remodeling (Tallant et al., 2005).

A possible clinical application of our findings relates to cancer treatment. Steward and co-workers have recently demonstrated that treatment with BK receptor antagonists significantly attenuated the growth of prostate and lung tumors in a rat experimental model by attenuating tumor angiogenesis (Steward et al., 2005). BK antagonists also had the advantage of being considerable less toxic than traditional anti-tumor drugs. Our finding that EP24.15 produced Ang-(1-7) putatively encouraged endothelial tubule formation, leads to the possibility that either EP24.15 inhibition or Ang-(1-7) receptor blockade (or a combination of both) alongside BK receptor blockade, may be a possible strategy for cancer treatment.

7.4 Conclusion

In conclusion, our studies have shown that cyclic strain increases EP24.15 and EP24.16 mRNA expression and both cellular and secreted enzyme activity in BAECs. The increases in cellular activity were dependent on G-protein signaling, with the increase in cellular EP24.15 activity shown to be strongly attenuated by inhibition of \( \text{G}_{i2}/\text{G}_{i3} \) signaling, while the increase in cellular E24.16 activity was significantly attenuated by inhibition \( \text{G}_{ia}/\text{G}_{ia2} \) and \( \text{G}_{b\gamma} \) subunit mediated signaling. In addition cyclic
strain induced an apparent increase in the levels of extracellular membrane associated EP24.15. Exposure to cyclic strain was also increased the EP24.15-specific cleavage of exogenously added BK and Ang-I in cultured BAECs. Inhibition of EP24.15 also attenuated cyclic strain induced BAEC tubule formation and migration, with further studies suggesting that EP24.15 may mediate its effects on these strain-induced endothelial cell fate decisions via the increased production of Ang-(1-7) from Ang-I, with possible clinical implications in the treatment of hypertension and cancer.
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Appendix
A.1 Map of pcDNA3.1 Vector; used for Gia₁, Gia₃, wild type and mutant constructs
A.2 Maps of (a) Gia$\alpha_1$ and (b) Gia$\alpha_3$ mutant.
A.1 Maps of (a) G\(\alpha_1\) and (b) G\(\alpha_2\), wild type.
A.3 Map of LCNX2000 Vector. Used for Gia3 wild type and mutant constructs.