Shear Stress-Mediated Regulation of Thermolysin-Like Zinc Metallopeptidase Expression levels in Vascular Endothelial Cells

A dissertation submitted for the degree of Ph.D
By

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Declaration:

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

Signiture: Paul Fitzgerald

Student I.D: 53146336

Date: 21/9/07

Dedication

To my sisters,
Rosemary, whom I never knew and Lorraine, whom I love to know.

Acknowledgments

In an effort to be as succinct as possible I will restrict my thanks to those without whom, this thesis could not have been made possible. There are a great many people to whom I owe a great deal, but firstly I want to extend my thanks to my ever vigilant supervisor, Dr. Phil Cummins, whose guidance was invaluable and whose enthusiasm managed to sustain my own through the very very long hours.

I offer my warmest thanks to my family, especially my parents, Teresa and Michael, for their constant support and for listening to my many complaints over the years.

Thanks to my brothers, John and Pat, laughing at you two got me through many a stressful patch. To my big sister, Lorraine, constant thanks will never be enough!

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Abstract

Blood flow confers on the blood vessel, both biochemical and biophysical stimuli, resulting in a number of signalling cascades leading to morphological and biochemical changes in the cells that comprise the vessel wall. Among these stimuli are the mechanical forces, cyclic strain and shear stress. Cyclic strain is the outward pulsatile pressure of blood flow, which stretches the cells of the vessel wall in a perpendicular direction to flow. Shear stress is the frictional force generated by flow as blood drags against the innermost cell layer of the vessel, the endothelium. In our study, we examined the role played by laminar shear stress in the regulation of expression levels of a number of vasoactive peptidases. Vasoactive peptidases of the *Thermolysin-Like Zinc Metallopeptidase* family, namely NEP, ACE, ECE, EP24.15 and EP24.16, act on a number of important peptides that directly control vessel tone and function to balance the vasoconstrictive and vasodilatory effectors endogenous to the endothelium. We evidenced the ability of applied shear stress (10 dynes/cm^2, 24 h) to alter mRNA and protein levels of some of these peptidases, with a significant *attenuation* of NEP, ACE, ECE and EP24.16 levels and an *increase* in EP24.15 levels in bovine aortic endothelial cells (BAECs) *in vitro*. We hypothesized that this regulation by shear stress was mediated via alteration of the cellular oxidative state. To test this, we induced an acute artificial oxidative state in static BAECs with known ROS and ROS-inducing agents and again evidenced a similar trend in expression levels as to that induced by shear stress. It was then sought to link these results by confirming through the use of antioxidants the role played by ROS in the shear-induced changes in metallopeptidase expression. Incubation of BAECs with antioxidants (N-acetylcysteine, superoxide dismutase and catalase) completely blocked the shear-induced changes in metallopeptidase expression, further supporting our hypothesis. We also evidenced the ability of applied shear stress to induce a non-toxic transient burst of ROS in the endothelium and hence supply the oxidative state needed for modulation of metallopeptidase expression levels. Through the use of a specific pharmacological inhibitor, apocynin, we identified the source of this ROS burst to be NAD(P)H oxidase, a known shear-sensitive ROS producer. Finally we sought to identify the signalling pathway mediating these shear-dependent events, using NEP as a study model. To this end, we examined expression levels of NEP (neprilysin, neutral endopeptidase) under applied shear stress in the presence and absence of pharmacological and molecular inhibitors. We determined for the first time that the shear-dependent attenuation of NEP expression levels in vascular endothelial cells proceeds via a $G\beta\gamma$/Rac1/NAD(P)H oxidase signalling axis. This study confirms and extends the therapeutic value of these enzymes as possible targets in the treatment of vascular pathologies characterised by flow-dependent endothelial dysfunction (e.g. atherosclerosis).

Abbreviations

Ap-1	Activating protein-1
AC	Adenylate cyclase
ACE	Angiotensin converting enzyme
ANG	Angiotensin
ANP	Atrial natriuretic peptide
BAEC	Bovine aortic endothelial cell
BASMC	Bovine aortic smooth muscle cell
BCA	Bicinchoninic acid
BK	Bradykinin
BNP	Brain natriuretic peptide
CAT	Catalase
CNP	c-type natriuretic peptide
cDNA	Complementary DNA
cGMP	Cyclic Guanosine monophosphate
CHD	Coronary heart disease
CT	Cholera toxin
CVD	Cardiovascular disease
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
ECE	Endothelin converting enzyme
ECM	Extra cellular matrix

EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
FAK	Focal adhesion Kinase
FBS	Fetal bovine serum
FDA	Federal drug administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAPDH	Glyceraldehyde phosphate dehydrogenase
GC	Guanyalate cyclase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GNRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GPI	Glycophosphatidylinositol
Grb-2	Growth factor binding protein-2
GTP	Guanosine triphosphate
HBSS	Hanks buffered saline solution
HPF	High power field
HRP	Horseradish peroxidase

ICAM-1	Intracellular adhesion molecule-1
IκB	Inhibitor kappa B
ILGF	Insulin-like growth factor
ILGFR	Insulin-like growth factor receptor
JNK	c-jun N-terminal kinases
LB	Luria Bertrani
LDH	Lactate dehydrogenase
LHRH	Leuteinizing hormone releasing hormone
MAPK	Mitogen activated protein kinases
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
mRNA	Messanger Ribonucleic acid
MT-MMP	Membrane Type-MMP
NAC	N-Acetyl –L-Cysteine
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NEP	Neprilysin, Neutral Endopeptidase, EP24.15
NPR	Natriuretic peptide receptor
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
OxLDL	Oxidised Low density lipoprotein

PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI-3 kinase	Phosphoinositide 3 kinase
PTK	Protein Tyrosine Kinase
PTX	Pertussis toxin
RAS	Rennin angiotensin system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
Shc	Src homology
SiRNA	Small interfering RNA
SOD	Superoxide dismutase
SSRE	Shear stress response element
SVR	Systemic vascular resistance
TBS	Tris buffered saline
TF	Tissue factor
TGF-β	Transforming growth factor-β
TLZM	Thermolysin-like zinc metallopeptidase
TNF-α	Tumour necrosis factor-alpha

TRX	Thioredoxin
Txnip	Thioredoxin interacting protein
t-PA	Tissue plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Publications

Peer-reviewed journals

von Offenberg Sweeney N, Cummins PM, Cotter EJ, Fitzpatrick PA, Birney YA, Redmond EM and Cahill PA. Cyclic strain-mediated regulation of vascular endothelial cell migration and tube formation. *Biochemical & Biophysical Research Communications* 2005;**329:**573-582.

Cotter EJ, Fitzpatrick PA, Wu TJ, Glucksman MJ, Murphy RP, Meade G, Cahill PA, and Cummins PM. Cyclic strain-induced regulation of metalloendopeptidase EC3.4.24.15 influences peptide hydrolysis and cell fate in vascular endothelial cells. *American Journal of Physiology - Heart Circulation Physiology* 2006; In Preparation.

Fitzpatrick PA, Killeen MT, Murphy RP, Glucksman MJ, Cahill PA, and Cummins PM. Shear stress-mediated regulation of neutral endopeptidase expression and function within vascular endothelial cells: a role for reactive oxygen species. *Circulation Research* 2007; In Preparation.

Posters/Abstracts

Fitzpatrick PA, Killeen MT, Birney YA, Glucksman MJ, Cahill PA, and Cummins PM. Shear stress regulation of NEP is mediated via reactive oxygen species in endothelial cells. *7th Annual Conference on Arteriosclerosis, Thrombosis, and Vascular Biology* 2006; Denver CO, USA.

Fitzpatrick PA, Cotter EJ, Birney YA, Glucksman MJ, Cahill PA, and Cummins PM. Expression of thermolysin-like zinc metalloendopeptidases in vascular endothelial cells is regulated by shear stress and reactive oxygen species. *6th Annual Conference on Arteriosclerosis, Thrombosis, and Vascular Biology* 2005; Washington DC, USA.

Cotter EJ, Fitzpatrick PA, Birney YA, Wu TJ, Glucksman MJ, Cahill PA, and Cummins PM. Cyclic strain induces changes in EP24.15 subcellular localization and peptide cleavage: possible implications for endothelial cell fate decisions. *6th Annual Conference on Arteriosclerosis, Thrombosis, and Vascular Biology* 2005; Washington DC, USA.

Oral presentations

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Chapter 1 Introduction

1.0 Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) is a general diagnostic category consisting of several distinct diseases of the heart and circulatory system. Cardiovascular diseases affect the heart and blood vessels and include conditions such as arteriosclerosis, coronary artery disease, heart valve disease, arrhythmia, heart failure, hypertension, orthostatic hypotension, endocarditis, diseases of the aorta and its branches, disorders of the peripheral vascular system, and congenital heart disease. According to the Irish Heart Foundation, approximately 10,000 people die each year from CVD, including coronary heart disease (CHD), stroke and other circulatory diseases. CVD is the most common cause of death in Ireland, accounting for 36% of all deaths, with the largest number of these deaths relating to CHD, mainly heart attacks at 5,000 per annum. 22% of premature deaths (under age 65) in Ireland are caused by CVD.

In a study carried out by Leal *et al.*, CVD was estimated to cost the EU €169 billion annually, with healthcare accounting for 62% of costs (Leal *et al.*, 2006). Productivity losses and informal care represented 21% and 17% of costs, respectively. CHD represented 27% and cerebrovascular diseases 20% of overall CVD costs. The study also found significant variations in different countries' spending on CVD, with the UK and Germany expending 17% and 15% of their respective healthcare budgets on the treatment of CVD while Ireland was shown to be investing a mere 4.4% of the total healthcare budget.

The target set by the Heart Foundation's Strategic Plan 2005-2009 was to reduce overall mortality from CVD from 39% in 2003 to 34% in 2009. By 2005 36% of all deaths were due to CVD, indicating that we remain on target to achieve these aims. However, in a report by the Irish Heart Foundation on the implementation of the Cardiovascular Health Strategy, it was highlighted that only €60m of the outlined €220m had been invested, leading the Heart Foundation to conclude that while the overall trend shows a decrease in mortality rates due to CVD, this may not be sustainable in the future without further investment and research. The Heart Foundation highlighted Ireland's shortage of cardiologists, with only 11 per million as compared to the European average of 35 per million. It is clear from these reports that further investment and research is required if we are to meet the proposed guidelines for the reduction of CVD mortality rates by 2009. In-line with this, the Health Board has sought to increase public awareness as to the risk factors that can impact on the development of CVD.

These risk factors include diet, exercise and alcohol and drug ingestion, including smoking. In addition to these modifiable risk factors, there are a number which cannot be varied such as gender, age, ethnicity and family history. Of the factors that influence the development of CVD, many of those listed above may have a direct or indirect impact on the regulation of hemodynamic forces. Hemodynamic forces associated with blood flow through the vasculature play a pivotal role in the initiation and progression of CVD, including atherosclerosis, hypertension and pathological vascular remodeling. Endothelial cells form the innermost lining of the entire vascular system and hence are the primary recipients of these hemodynamic forces.

It is through biochemical alteration in vasoactive peptidases found in the endothelium that these impact factors and consequently their linked hemodynamic forces can have a direct influence on the initiation and progression of CVD.

1.2 The Endothelium

The endothelium is a thin monocellular layer that completely covers the entire inner surface of the blood vessels that comprise the vascular system. It is composed solely of endothelial cells, a squamous cell type that is structured to sense and respond to a number of biochemical and biomechanical stimuli. The endothelium is uniquely positioned at the interface between the blood and the vessel wall. The entire lining represents an organ of 1.5 kg in an average adult, which is distributed throughout the body. As such, the endothelial layer performs multiple functions: it is involved in the regulation of coagulation, inflammation processes, vessel tone, and vascular smooth muscle cell growth, and also acts as a barrier to transvascular flux of liquids and solutes. Far from being a passive participant in these events, it is a dynamic tissue, influencing the behavior of other cell types, and regulating extracellular matrix production and composition.

Endothelial cells are a major source of physiologically relevant molecules synthesized therein and secreted into the blood and/or the sub-endothelial extracellular matrix. Since its initial discovery, the endothelium has been the focus of intense experimental work, resulting in the evolution of a new appreciation of its potential role in vascular disease (Griendling *et al.*, 1996, Bassenge *et al.*, 1996).

At the base of the endothelial layer is a thin layer of spongy connective tissue that secretes a layer of elastic collagen. This supportive layer forms the "basement membrane" for the endothelium. These two layers together are known as the tunica intima. The surrounding layer of smooth muscle cells is quite thick in arteries, and can stretch to accommodate passing blood, or contract to restrict blood flow. This muscle layer is also underlain by a spongy layer of connective tissues that produces elastic collagen fibers. These fibers allow the vessel to be stretched as required and yet return to their original shape. Together these two layers are known as the tunica intermedia. Surrounding the tunica intermedia is a layer of connective tissues that produces both elastic and rigid collagen fibers. This layer will stretch with limitations. This outermost layer is called the tunica adventitia (See Fig 1.1).

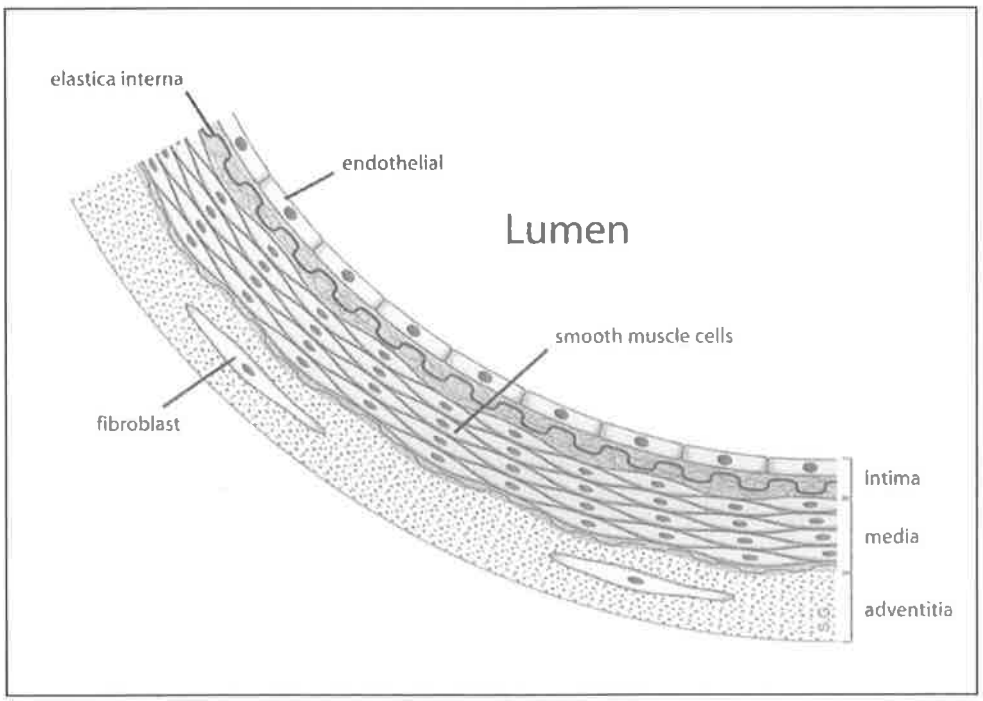


Fig 1.1 Structure of differing layers of the vessels found in the vasculature (http://www.applesnail.net)

Dysfunction of the endothelial layer has been shown to be an accurate precursor for many of the pathological conditions associated with CVD, with signs of these pathological conditions pre-dating any clinically obvious vascular pathologies by many years. Dysfunction of the endothelium can be loosely described as an impairment of the normal biochemical processes indigenous to the cell type, mainly a decrease in synthesis, release, and/or activity of endothelial-derived nitric oxide (NO), a potent vasodilator (Ogita *et al.*, 2004). A number of causative agents, such as infection, injury and hypoxia, of endothelial dysfunction and consequentially CVD have been reported, chief among them is blood flow and the effects of modulation of both velocity and pattern of that flow.

1.3 Blood Flow

Due to the unique position of the endothelium, it is constantly in contact with circulating blood and the milieu of components therein. Of the multitude of factors both biochemical and biomechanical that will impact on the endothelial layer, of primary importance are the hemodynamic forces of cyclic strain and shear stress. As blood flows through the vessel, it imparts a number of mechanical forces on the cells that constitute said vessel. These include; i) cyclic circumferential strain and hydrostatic pressure, a tangential force which acts outwardly on the vessel walls. Blood pressure creates strain on the vessel wall in a direction perpendicular to the endothelial surface and is the major determinant of vessel stretch via rhythmic distension of the vessel wall.

The magnitude of this circumferential force is dependent on vessel geometry, and position within the vessel wall, and ii) fluid shear stress, a "dragging" or frictional force that acts on the intraluminal membrane of the endothelial layer in the direction of flow. This latter force is described in terms of fluid velocity and viscosity (Lehoux and Tedgui, 2003). See Fig 1.2.

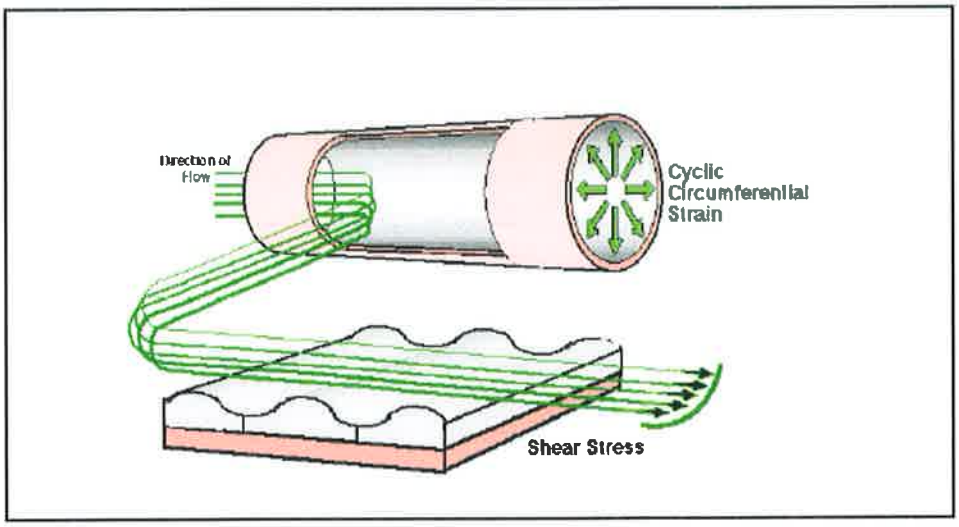


Fig 1.2 Schematic diagram of a blood vessel and the hemodynamic forces affecting the vessel wall

1.3.1 Cyclic Circumferential Strain

Cyclic strain can be defined as the tension or stress exerted on the wall of the vessel through which blood is flowing under pressure. It has been evidenced to act tangentially on the vessel causing a rhythmic deformation of the arterial wall and is closely associated with systolic-diastolic pressure changes. Normal levels of blood pressure are taken to be between 100/65 mm Hg and 130/85 mm Hg, levels of blood pressure above or below this range are taken as hypertensive

and hypotensive respectively. There are a number of physiological and pathophysiological forces that regulate the level of blood pressure experienced by an artery and hence the degree of cyclic strain. Alteration in blood pressure can be an acute or chronic event. Transient acute alterations in pressure levels can be caused by factors such as diet and physical and emotional stresses, while chronic changes are more commonly attributed to genetic pre-disposition, advancing age, poor lifestyle and secondary effects of a pre-existing condition such as renal damage.

The outward force of blood pressure induces a gentle stretching of the endothelium and the cells therein, causing a morphological alignment of cells supported by actin fiber restructuring in the direction of stretch (Iba and Sumpio 1991); (Iba *et al.* 1991). Cyclic strain therefore acts not only on the endothelial layer but also on the sub-endothelial matrix and surrounding smooth muscle cells inducing a milieu of resultant effects. Primarily, it is an important determinant of vessel thickness, and concomitantly luminal space. Thoma originally commented in 1893, that the diameter of the blood vessel was regulated by the magnitude of the blood flowing through it, while vessel thickness was dependent on blood pressure (Schaper 1967). This observation was confirmed by Leung and co-workers, when they compared the thickness of the pulmonary artery and aorta pre- and post-birth. Both vessels experience similar pressures *In utero* and both are almost identical in size; however, after birth as systemic pressure increases, the aorta thickens proportionally, while the pulmonary artery undergoes atrophy following the fall in pressure post-partum (Leung *et al.*, 1977).

1.3.1.1 Effect of Circumferential Strain on Endothelial and Smooth Muscle Cells

Cyclic strain, as previously evidenced, has a dramatic influence on the condition and activity of the vessels of the vasculature. This influence is mediated through a number of biochemical alterations, both subtle and otherwise, in the endothelial and smooth muscle cells that comprise the vessel in question. A number of factors important in the vasculature have been shown through *in vitro* experiments to be regulated via cyclic strain. Applied cyclic strain *in vivo* has been shown to increase nitric oxide synthase (NOS), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-14 (MMP-14), platelet derived growth factor-B (PDGF-B), endothelin-1 (ET-l), intracellular adhesion molecule-1 (ICAM-1), and plasminogen activator inhibitor-1 (PAI-1) and fibroblast growth factor-2 (FGF-2), (Awolesi *et al.*, 1995; Cheng *et al.*, 1996a; de Jonge *et al.*, 2002; Wang *et al.*, 2003; Wung *et al.*, 2001; Cheng *et al.*, 1996b).

The complete pathways through which these complex cyclic strain-induced events are transduced have not yet been completely elucidated, but the ability of cells to respond to cyclic strain is believed to play a role in a number of pathologies including atherosclerosis, hypertension and in particular restenosis following balloon angioplasty (Li and Xu, 2000; Zou *et al.*, 1998).

Cyclic strain is a powerful stimulus and can also regulate cell fate decisions. Exposure of vascular smooth muscle cells to cyclic strain leads to apoptosis via a p53-dependent pathway. Conversely, cyclic strain can suppress endothelial cell apoptosis via Akt phosphorylation (Mayr *et al.*, 2002; Persoon-Rothert *et al.*, 2002; Haga *et al.*, 2003). Similarly cyclic strain has been linked to inhibition of proliferation in addition to increases in angiogenesis associated with TGF-ß, MMP-2 and VEGF (Rivilis *et al.*, 2002; Zheng *et al.*, 1999; Vailhe and Tranqui, 1996; Banai *et al.*, 1994).

These studies clearly demonstrate the importance of cyclic strain in coordinating and regulating cell function by mediating changes in gene transcription, activation of signaling molecules and release of vasoactive compounds. Cyclic strain also functions by increasing the sensitivity of cells to other hemodynamic forces, namely shear stress (Zhao *et al.*, 1995).

1.3.2 Shear Stress

Under normal physiological conditions the endothelium is continuously exposed to mechanical shear stress due to blood flow. Blood flow exerts a frictional force on the luminal surface of the endothelial membrane. This tractive force is determined by vessel geometry, blood viscosity and velocity. Shear stress is estimated using fluid dynamics models and is expressed in units of dynes/cm^2.

Laminar blood flow within a vessel can be described by the equation:

$$t = 4\mu Q / pr^3$$

Where t = shear stress

μ = blood viscosity

Q = flow rate

r = vessel radius.

It is worth noting that the term r is raised to the third power. Thus, where Q is constant, a small change in r, the vessel radius, will result in a large change in t, the shear stress experienced by the endothelium (Lehoux and Tedgui., 2003) (Cunningham and Gotlieb., 2005). Vascular shear stress of large conduit arteries, such as the aorta, typically varies between 5 and 20 dynes/cm^2; however, significant instantaneous values range from negative measures to nearly 40 dynes/cm^2 during states of increased cardiac output (Cunningham and Gotlieb., 2005).

This continuous exposure to a physiological range of shear stress, especially in relatively long, straight sections of the artery, which are exposed to unidirectional pulsatile laminar flow, promotes an anti-inflammatory, anti-thrombotic, anti-coagulative, pro-fibrinolytic and anti-hypertensive state. Furthermore, shear stress inhibits apoptosis specifically of endothelial cells in response to various stimuli, demonstrating a potent atheroprotective effect (Berk, Abe *et al.* 2001).

At certain positions, such as bifurcations in the vessel or points of extreme curvature, the vessel may be exposed to turbulent flow, oscillatory shear stress and eddy currents, all of which can abrogate the protective effects of laminar shear. Hence, atherosclerotic plaques tend to form at these points. The mechanisms by which hemodynamic forces such as shear stress are transduced into cellular signaling are not yet fully known. *In vitro* studies in which the endothelial monolayer is been subjected to defined levels of shear stress have been essential to our understanding of shear stress-related molecular responses. The complexity of the shear stress response is only now being elucidated; some of the best-characterized responses include, reorganization of actin-containing stress fibers, alterations in metabolic activities and changes in cell cycle kinetics (Davies, 1995; Davies and Tripathi, 1993).

Shear stress modulates cellular structure, function and endothelial gene expression (Lehoux and Tedgui, 2003; Chiu *et al.*, 2004; Bartling *et al.*, 2000). For example, shear stress regulates gene expression of various proteins, including vasoactive substances (e.g., nitric oxide synthase and endothelin-1) (Kuchan and Frangos, 1993), growth factors (e.g., transforming growth factor-ß1 and platelet-derived growth factor) (Negishi *et al.*, 2001; Resnick *et al.*, 1993), adhesion and chemoattractant molecules (e.g., intercellular adhesion molecule-1, vascular cellular adhesion molecule-1, and monocyte chemoattractant protein-1) (Chiu *et al.*, 2004; Cheng *et al.*, 1996), coagulation factors (e.g., tissue factor) (Lin *et al.*, 1997), proto-oncogenes (Bartling *et al.*, 2000), and antioxidant enzymes (e.g., superoxide dismutase) (Wung *et al.*, 2001).

Shear-related effects can be broadly categorized into two responses; a) re-organization or regulation of pre-existing proteins and b) *de novo* protein synthesis and gene expression; the latter is usually associated with delayed or chronic shear-mediated responses.

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

Endothelial cell gene expression in response to shear stress is known to be a function of the magnitude of the force. For example, tissue plasminogen activator (t-PA) expression is only increased at shear stresses above 5 dynes/cm^2 (Diamond *et al.*, 1990), whereas secretion of the vasoconstrictor ET-1 is increased in HUVECs at shear stresses less than 5 dynes/cm^2 (Kuchan and Frangos, 1993).

This may be explained by the fact that the magnitude of shear stress may vary depending on the location within the vasculature. Therefore in situations where shear stresses may be low, increases in ET-1 will promote vasoconstriction to increase blood flow rate, and thus shear, through that section of the vessel. Much work has recently sought to identify the multitude of genes possessing a shear stress response element. Hence, the number of genes shown to contain an SSRE is ever increasing. Recently, fifty-two flow-sensitive genes were identified in HUVECs, with prostaglandin and cytochrome p_{450} being the most strongly up-regulated, while ET-1 and MCP-1 were the most strongly down-regulated (McCormick *et al.*, 2001). 143 genes have been identified in HUVECs, which are differentially expressed in the presence of static, laminar, or turbulent flow (Garcia-Cardena *et al.*, 2001). *In vivo*, the expression of a number of genes, such as transforming growth factor-ß (TGF-ß) (Negishi *et al.*, 2001), PDGF-A, PDGF-B (Tulis and Prewitt, 1998), and urokinase plasminogen activator (uPA) (Essig, Terzi *et al.* 2001) have been found to be shear sensitive. Of the multitude of studies carried out to identify those genes in receipt of an SSRE, some of the more important have focused on those genes that regulate vessel tone and, hence, have a direct impact on vascular health.

1.4 Shear Stress and Vessel Tone

Vascular tone as defined by the level of constriction that a blood vessel experiences relative to it's maximally dilated state. This state of contraction/dilation is controlled by the surrounding layers of smooth muscle cells. The smooth muscle layer is itself under the control of a number of other factors, chief among them, the endothelium. All vessels under basal conditions exhibit some degree of smooth muscle contraction which in turn determines the diameter, and hence tone, of the vessel. Differing organs experience variations in basal vascular tone. Those organs possessing a large vasodilatory capacity (e.g., myocardium, skeletal muscle, skin, splanchnic circulation) have a high vascular tone, whereas organs having relatively low vasodilatory capacity (e.g., cerebral and renal circulations) have a low vascular tone. It is for this reason that systemic vascular tone and total resistance to flow through the system needs to be considered. Systemic vascular resistance (SVR) refers to the total resistance to blood flow offered by all of the systemic vasculature. It is generally assumed that small arterioles form the major site of vascular resistance. However, studies in the skeletal and cardiac muscle have revealed that 40±55% of the total network resistance resides in large arterioles and small arteries with diameters >100 μm (Smaje *et al.*, 1970; Bohlen *et al.*, 1977; Chilian *et al.*, 1986; Meininger 1987). Changes in the vascular tone of these vessels therefore have considerable impact on overall organ and systemic blood flow. There are a number of CVD states that are initiated by, or result in, an alteration of the ability of the endothelium to regulate vessel tone.

Indeed CVD has been classified as an inability of the vasculature to synthesize, release and respond to the vasodilatory effects of nitric oxide (NO) (Ogita *et al.*, 2004). Shear stress exerts an atheroprotective force, that modulates reaction of vessel tone to appropriate stimuli through the eNOS-dependent production of NO and the actions of ACE to produce AngII, an inability or dysfunction of the endothelium to carry out these important functions is an indication of a disease state.

1.4.1 Regulation of Vessel Tone

Vascular tone is determined by many different competing vasoconstrictor and vasodilatory influences acting on the blood vessel. Hence the expression of the various vasoactive mediators of these events and the forces that regulate their expression are of utmost importance when understanding the regulation of vascular tone. These influences will be discussed in greater detail in relation to shear stress and the role played in control of vessel tone.

1.4.1.1 Nitric Oxide (NO) as a Mediator of Vessel Tone

The actions of NO and it's effects on the vasculature have been comprehensively studied. It was one of the originally isolated mediators of vessel tone and has been shown to be a potent vasodilator. The endothelium constitutively produces nitric oxide and thus maintains the vasculature in a steady state of vasodilation.

In addition to prostacyclin, NO is responsible for endothelium-dependent tonic relaxation of all types of blood vessels by stimulating soluble guanylate cyclase and increasing guanosine 3',5'-cyclic monophosphate (cGMP) in smooth muscle cells.

The increase in intracellular cGMP concentration leads to a relaxation via a decrease in intracellular Ca^{2+} and dephosphorylation of myosin light chains (Luscher *et al.*, 1990). NO also functions as a neurotransmitter in the central and peripheral nervous system, and contributes to the anti-microbial activity of macrophages as well as to hormone release and platelet inhibition. Thus, NO is found to be active in the neural, cardiovascular and immune systems. Both type II (or inducible) and type III (or endothelial nitric oxide synthase, NOS), which catalyse the conversion of L-arginine to nitric oxide, have been found in endothelial cells (Galley and Webster 2004) See Fig 1.3.

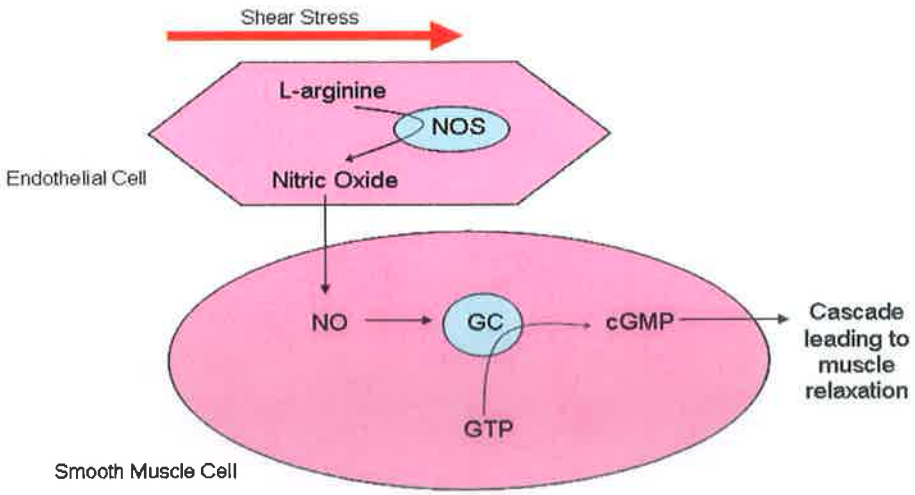


Fig 1.3 Diagrammatic representation of the expression and production of eNOS

The ability of laminar flow to up-regulate NOS expression has been widely studied. Indeed NOS is so positively regulated by flow that to date six repeats of the GAGACC SSRE have been identified in the 5' promoter region of the gene encoding eNOS (Cooke and Dzau, 1997).

Dysfunction of the actions of NO within the vessel wall is an indicative measure of CVD states. Indeed loss of NOS activity and hence available NO within the endothelial cell has been linked to a number of conditions such as hypercholesterolemia, atherosclerosis and hypertension. Studies have linked the actions of NO with regulation of VCAM-1 and MCP-1 within the endothelium, thus elucidating a role for NO in atherogenesis (Cooke and Dzau, 1997). In addition to the ability of NO to modulate vascular health via vessel tone, it has also been shown to posses a strong anti-inflammatory capacity. Nuclear factor kappa B (NFκB) is a redox-sensitive transcription factor which has the ability to regulate, at least in part, gene expression of many factors involved in inflammatory responses, namely cytokines, growth factors, and adhesion molecules. NFκB is maintained in the cytoplasm, in a non-activated state by association with an inhibitor subunit, IκB. Constitutive nitric oxide production in the endothelium inhibits adhesion molecule expression through a stabilization of IκB, thus attenuating pro-inflammatory responses (Galley *et al.*, 2004).

1.5 Thermolysin-Like Zinc Metallopeptidases (TLZM)

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 Zn2+, 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 oligo peptidase 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 (Lew, 2004).

As previously mentioned, there are a number of physiologically relevant members of this clan namely ACE, ECE, NEP, Neurolysin and THOP; all of which have been shown to play roles in the control of vascular tone. Given this, the possibility that the expression and function of these metallopeptidases might be sensitive to hemodynamic stimuli has been considered. Each peptidase will be discussed in greater detail in relation to shear stress and vessel tone.

1.5.1 Angiotensin Converting Enzyme (ACE)

Angiotensin-I-converting enzyme (ACE, also known as peptidyl-dipeptidase A or kininase II) was first isolated in 1956 and shown to be a chloride-dependent metalloenzyme that cleaves a dipeptide from the carboxyl terminus of the decapeptide angiotensin I to form the potent vasopressor (blood vessel constrictor) angiotensin II. In addition, it inactivates the vasodilator bradykinin by sequential removal of two carboxy-terminal dipeptides. Indeed, it is a broadspecificity dipeptidyl carboxypeptidase and may also act on non-vasoactive peptides. There are two forms of ACE in humans, encoded by a single gene located on chromosome 17 at q23; it is 21 kb in length and contains 26 exons and 25 introns.

The longer form, known as somatic ACE (sACE), is transcribed from exons 1-12 and 14-26, whereas the shorter form, known as germinal or testicular ACE (gACE), is transcribed from exons 13-26 (Corvol and Williams, 1998). Human sACE is a type-I membrane-bound protein. It consists of a 28-residue carboxy-terminal cytosolic domain, a 22-residue hydrophobic transmembrane domain and a 1227-residue extracellular domain that is heavily glycosylated. The extracellular domain is further divided into two homologous domains, a 612-residue N domain at the amino terminus linked by a 15-residue sequence to a 600-residue C domain. Each of the extracellular domains contains an HEXXH sequence in which the two histidine residues serve as zinc-binding ligands; together with a glutamine located 23-24 residues toward the carboxyl terminus and a water molecule, they provide the metal with tetrahedral coordination geometry (Wei, Alhenc-Gelas *et al.* 1991); (Jaspard, Wei *et al.* 1993).

Human sACE is expressed strongly in many types of endothelial cells, especially in the capillaries of the lung, as well as in epithelial cells in the kidney, small intestine and epididymis. Indeed, microarray analysis of gene expression indicates that sACE mRNA is expressed in virtually all mammalian tissues (Coates, 2003). As previously mentioned, ACE plays an important role in the vasculature, mediating vessel tone through action on vasomotive peptides. The specific role played by ACE in relation in vessel tone and the impact of shear stress will be discussed in greater detail.

1.5.1.1 Angiotensin Converting Enzyme (ACE) as a Mediator of Vessel Tone

Angiotensin II (Ang II) is a well known and highly characterized vasoconstrictor. It is considered as the biologically active component of the rennin angiotensin system (RAS). The RA system is a hormonal system which helps to regulate blood pressure and extracellular volume in the body.

The system comprises of a number of organs and cell types producing/releasing and responding to a number of factors. Primary among them is endothelium-derived Ang II (See Fig 1.4) (Isenovic *et al.*, 2004).

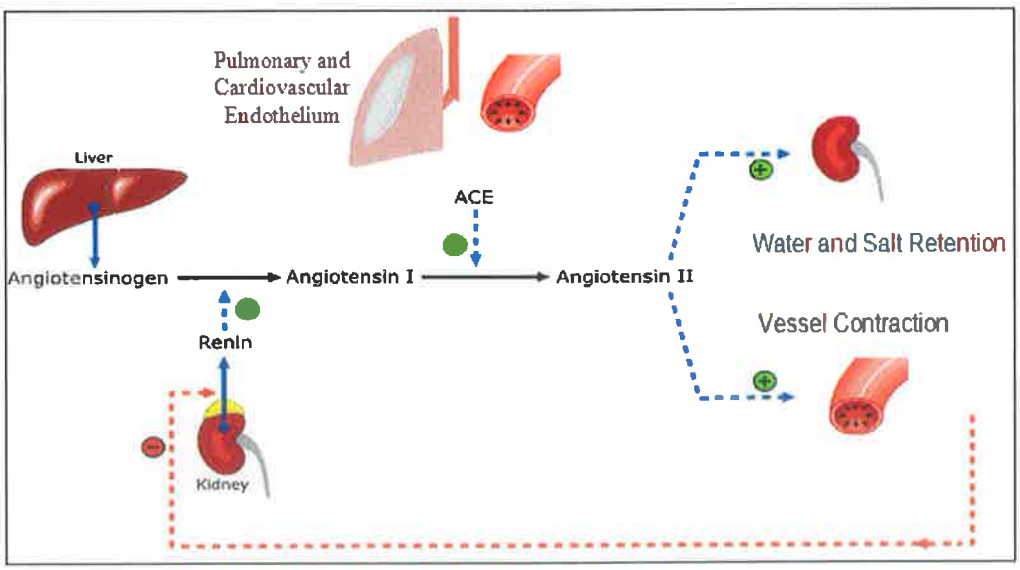


Fig 1.4 The Renin-Angiotensin System

Studies have shown that the actions of Ang II are modulated via the AT_1 and AT_2 receptors. All classic physiological effects of Ang II, such as vasoconstriction, cell proliferation, aldosterone and vasopressin release, sodium and water retention, and sympathetic facilitation, are mediated by the AT_1 receptor (De Gasparo, 2002, Kaschina and Unger, 2003). Pueyo *et al*, showed vasoconstriction in SMC via the AT_1 receptor (Pueyo *et al.*, 1998) . The AT_2 receptor, in contrast, is up-regulated in pathological conditions, and it counteracts several of the growth responses initiated by the AT_1 and growth factor receptors.

Ang II is formed by the actions of ACE on Angiotensin I (Ang I). ACE was first isolated from plasma and identified as the primary peptidase responsible for the production of Ang II (Skeggs *et al.*, 1954, 1956). Since the publication of those studies, much work has concentrated on ACE expression and has isolated this important peptidase to a number of cell and tissue types (Cushman and Cheung, 1971). Due to the role played by ACE in the regulation of vessel tone via Ang II, it was hypothesized that ACE expression in the vasculature was modulated by fluid flow in a similar fashion to that of eNOS. Following investigation, ACE expression and activity was shown to be shear responsive, with levels of activity reduced by 49.5% after 18 hr of shear stress at 20 dynes/cm^2. The promoter region of the gene encoding for ACE was also shown, through the use of reporter gene assays, to contain the classic SSRE typically responsible for sensitivity to shear stress (Rieder *et al.*, 1997). Incidentally, upon closer study, this same group has shown that the classic SSRE, while present in the promoter region, was not active under their shear stress experimental paradigm.

In an effort to explain their previous finding of shear stress attenuation of ACE levels, the group identified two *cis*-acting elements, Barbie and GAGA boxes, and demonstrated their responsiveness to fluid flow and their role in the observed shear-induced effects (Miyakawa *et al.*, 2004)

While it is clear from previous studies that shear stress plays an important role in the regulation of this vasoactive peptidase, little work has been carried out to fully elucidate the mechanisms by which hemodynamic force exerts its effects. Work has mainly focused on ACE as a clinical target for the treatment of CVD states such as hypertension. Of great significance, the two pathophysiological alterations associated with hypertension (coronary ischemia and fibrosis) are shown to be improved significantly by the anti-hypertensive agents, ACE inhibitors and AT_1 receptor antagonists. In addition, prolonged co-administration of an ACE inhibitor with L-arginine, a source of NO has been demonstrated to result in additive hemodynamic and anti-fibrotic improvements (Frohlich, 2001).

1.5.2 Endothelin Converting Enzyme (ECE)

Two isoenzymes of ECE (ECE-1 and ECE-2) have so far been identified. Both are type II integral membrane proteins, belonging to a family of membrane-bound metalloproteases. They have a short cytoplasmic tail in the NH2-terminus, followed by a membrane-spanning region and a large extracellular domain containing a zinc-binding motif essential for enzymatic activity.

Both ECE-1 and ECE-2 catalyze the conversion of big ET-1 most efficiently among the three big ETs in vitro, with a neutral and acidic optimum pH of 6.8 and 5.6 for ECE-1 and ECE-2, respectively. Conversion of big ETs are hence proteolytic activation is carried out by ECE1/2 via cleavage at the common Trp21 residue. The distribution of ECE-1 mRNA in bovine and rat tissues has been investigated by Northern blot and RT-PCR analyses (Ikura *et al.* 1994; Xu *et al.* 1994; Shimada et al. 1995). They demonstrated particularly high levels of ECE-1 mRNA in the lung, adrenal gland, ovary, testis, and heart. In situ hybridization confirmed that ECE-1 mRNA is found in various bovine tissues, with the largest amounts in the endothelial cells and certain parenchymal cells (Xu *et al.* 1994). Takahashi *et al.* (1995) performed immunofluorescence studies with rat tissue sections and monoclonal antibodies (MAbs) raised against purified rat ECE. They detected ECE-1 protein in the endothelial cells of the aorta, lung, kidney, liver, and heart, and in certain endocrine cells, such as the chromaffin cells of the adrenal gland and the b-cells of the pancreatic islets.

The distribution of human ECE-1 has been analyzed by Northern blotting (Schmidt *et al.* 1994; Valdenaire *et al.* 1995) and RT-PCR (Rossi *et al.* 1995), showing that ECE-1 mRNA is expressed in various tissues. However, these studies detected mRNA in tissue homogenates, so they provide little information about the cellular distribution of ECE-1 in humans.

1.5.2.1 Endothelin Converting Enzyme (ECE) as a Mediator of Vessel Tone

Endothelin (ET) is a highly potent vasoconstrictive peptide that was initially isolated from the conditioned media of cultured endothelial cells. Indeed, by 1988, details of the isolation, identification, amino acid sequence, cDNA sequence and pharmacology of ET had been published (Yanagisawa *et al.*, 1988). To date, a number of additional isoforms have also been identified.

Of primary importance in the vascular system are the endogenous isoforms of ET-1, ET-2 and ET-3. These isoforms have been shown to possess a highly similar amino acid sequence, with similarities in their receptor affinity and site of action. Whilst they have been isolated from a number of cell and tissue types, ET-1 has been shown to be the predominantly expressed, and hence active, isoform in endothelial cells (Masaki, 2004).

Two receptor types have also been identified for these three isoforms, ETa and ETb. ETa receptors will preferentially bind the ET-1 and ET-2 isoforms, whilst the ETb receptor binds all three isoforms with equal affinity. Studies have identified the presence of both ET receptors in vascular SMCs. The ETa receptor, which preferentially binds ET-1 has been shown to regulate SMC contraction in a Ca^{2+}-dependent manner (Summer *et al*, 1992). The ETb receptor has also been demonstrated to play a role in ET-1 dependent mediation of vascular tone (See Fig 1.5).

Whilst the predominant function of ET-1 is to effect a potent and sustained contraction in SMCs, evidence has been shown to indicate an auto-regulation or possible negative feedback mechanism via the ETb receptor. The ETb receptor has been shown to facilitate vasodilation of the artery via NO and prostaglandin release (Verharr *et al.*, 1998).

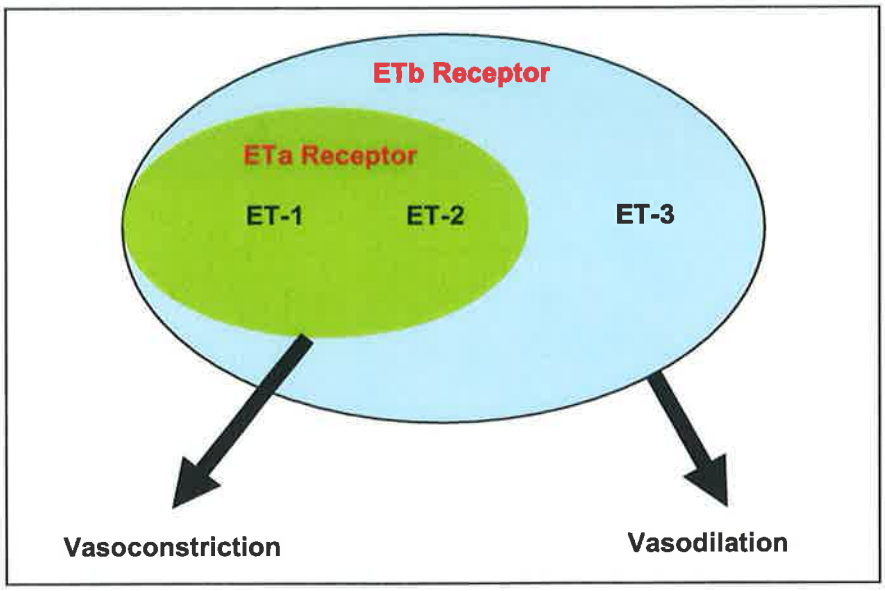


Fig 1.5 Diagram of ET Isoforms and the Receptors they Preferentially Bind

ECE, primarily expressed in the endothelium, is the peptidase responsible for the production of ET from the inactive proET or big-ET (Luscher and Barton, 2000). The ETs are expressed constitutively by vascular endothelial, smooth muscle and other cell types. Their biosynthesis involves a series of proteolytic steps in the processing of the initial preproET product. The processing pathway from preproET to active ET involves the cleavage of the biologically inactive intermediate big-ET by ECE.

Hence ECE plays a pivotal role in ET-mediated vasomotive events, in that it is required for their conversion to an active state (Yanagisawa *et al.*, 1988). Publication of the complete exon/intron structure of human ECE (Valdenaire *et al.*, 1995), allowed for the identification of four classic GAGACC SSREs in the promoter region, thus indicating a possible role for shear stress as a mediator of ECE expression. This is further supported by the finding of similar SSRE sites in the promoter region of ET-1 (Resnick *et al.*, 1995; Orzechowski *et al.*, 1997).

A number of studies exploring the shear stress responsiveness of ECE have followed these publications and have shown a marked down-regulation of ECE expression due to shear, with attenuation ranging from 34% to 75% of control levels (Morawietz *et al.*, 2000, Masatsugu *et al.*, 2003).

Due to the role played by the endothelin system in the regulation of vessel tone, as evidenced by previously mentioned studies, this system has been the focus of interest as a possible clinical target for the treatment of CVD. Elevated ET levels have been linked to a number of CVD states including, chronic heart failure, hypertension, atherosclerosis, pulmonary hypertension and chronic renal failure (Masaki., 2004). To date a number of ET receptor antagonists have been successfully employed to reduce overall blood pressure. The ETa receptor antagonists BQ123 and Darusentan were shown to decrease mean arterial pressure in animal models (Leslie *et al.*, 2001). Indeed the Food and Drug Administration (FDA), in 2001, approved the use of Bosentan for the treatment of pulmonary hypertension, with a 1-year report describing the long term beneficial effects of this treatment (Sitbon *et al.*, 2003).

Whilst more work has focused on the receptor antagonists for ET receptors, there is much evidence to indicate a possible role for ECE inhibitors as part of clinical treatment. ECE expression has been found to be elevated in a number a CVD states, namely atherosclerosis and hypertension. In studies employing rat balloon-injured arteries, ECE expression was found to be elevated above the norm. This finding was interesting, as balloon angioplasty destroys the endothelium, thus indicating an alternative expression site (Wang, Douglas et al. 1996).

Neointimal SMCs have been identified as a possible location for ECE expression in this pathological state. Blockage of ECE in this occasion was successful in reducing neointimal formation post injury. Work in this area is still in an early stage, due in part to the lack of understanding of the exact mechanisms that underlie ECE expression and activity and an inability to isolate an inhibitor capable of selectively targeting ECE. To this end, employment of ECE inhibitors has been mainly in conjunction with inhibitors of other vasoactive peptidases, namely ACE and Neprilysin (Turner and Tanzawa, 1997).

1.5.3 Neprilysin/Neutral Endopeptidase (NEP)

Neutral endopeptidase (NEP) is an endothelial, membrane-bound zinc metallopeptidase, that cleaves endogenous peptides at the amino side of hydrophilic residues. The membrane-bound metalloproteinase has a catalytic unit similar to that of ACE and indeed is classed as a fellow member of the same metalloendopeptidase family.

Although expression of NEP is greatest in the kidney, it was from studies on the brain's metabolism of enkephalins that the first physiological roles were identified. NEP is located on neuronal cells as a synaptic ectoenzyme, where it can inactivate released enkephalins, hence its original name of "Enkephalinase". Subsequent studies have shown it capable of terminating the actions of other neuropeptides, such as substance P in the tachykinin family (Turner *et al.*, 2001). NEP has also been found to have profound activity outside of the nervous system. It is widely distributed in endothelial cells, smooth muscle cells, cardiac myocytes, renal epithelial cells, and fibroblasts. NEP is also found in the lung, gut, adrenal glands, and heart. It catalyzes the degradation of vasodilator peptides, including ANP, BNP, CNP and bradykinin, as well as vasoconstrictor peptides, including endothelin-1 and Ang II (Corti *et al.*, 2001).

1.5.3.1 Neprilysin (NEP) as a Mediator of Vessel Tone

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family, which is produced in vascular endothelial cells and may play an important paracrine role in the vasculature. Other members of this family include atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), both found to be produced predominantly by the cells of the heart and secreted into circulating blood plasma, with BNP also serving a role as a regulator of neuropeptides in the nervous system. Members of this family have been shown to act on a specific family of receptors bound to cGMP (Cho, Somer *et al.* 1999).

These receptors have been found in a number of cell and tissue types, indicating the possibility of a role for the natriuretic peptides in a variety of biological systems. Work in this area has been somewhat hampered by differences in expression and function of these receptors in differing species studied. Although all three members of the natriuretic peptide family mediate their effects through the cGMP linked receptors, ANP and BNP activate natriuretic peptide receptor–A (NPR-A), whereas CNP is the specific ligand for NPR-B. A third receptor exists which is non-guanylyl cyclase-linked, NPR-C and acts as a clearance receptor to mediate the effects of the other two (Dickey, Flora *et al.* 2007).

The natriuretic peptide family, exert a wide range of effects on both cardiovascular and renal function. Both ANP and BNP are circulating peptides of cardiac origin, with recent studies indicating a high degree of sequence and functional homology between them. Acute administration of ANP elicits a potent and brisk natriuresis and diuresis and reduced arterial blood pressure in humans. The acute hypotensive effect of ANP is mediated primarily by a decrease in cardiac output, brought about by a reduction in intravascular volume (Fenoy, Salazar *et al.* 1990). This reduction in intravascular volume is independent of the diuretic effects of the hormone, and appears to be due largely, to direct effects on capillary permeability (Hempel, Noll *et al.* 1998). CNP also produces a significant hypotensive effect in the vasculature, albeit through a distinctly dissimilar mechanism than that used by both ANP and BNP.

CNP is present in endothelial cells of arteries and veins as a peptide of some 53 amino acids. Recent studies focusing on the mechanism by which it exerts its vasoactive effect have indicated a role for SMC hyperpolarization. After stimulation of endothelial cells by a vasoconstrictive factor, for instance, bradykinin, CNP is secreted, after which it can react with its specific guanylate cyclase receptor NPR–B on the vascular smooth muscle cell. NPR-B stimulation increases intracellular cGMP, with subsequent stimulation of potassium efflux and inhibition of calcium influx, resulting in hyperpolarization of the smooth muscle cell membrane and a closing of the Ca^{2+} pump (Honing *et al.*, 2001, Ramon de Berrazueta, 1999).

From the previously mentioned studies, it is clear that NEP plays a significant role in vasomotive regulation, with interactions in the RAS and ET systems. Due to the evidence supporting hemodynamic regulation of important members of those systems (e.g. ACE and ECE), it was proposed that NEP expression may also be sensitive to hemodynamic forces. The promoter region of the NEP gene was examined for the presence of sequences conferring blood flow sensitivity. A classic SSRE sequence identical to those present in the ACE and ECE promoter regions was found, indicating that NEP could also be regulated via shear stress (Kim *et al.*, 2003, Li *et al* 1995). Furthermore, the presence of two SSRE sequences in the promoter region of the NPR-A, strongly indicate a role for shear stress in the regulation of the natriuretic peptide system (Nakayama., 2005).

To date however, no studies have been published to exclusively examine the role that shear stress may play in the regulation of NEP expression. Conversely, much work has focused on the employment of NEP inhibitors in the treatment of CVD states such as hypertension.

Studies have shown that ANP infusion reduces blood pressure while increasing urine volume, urinary excretion of sodium, and cyclic GMP. It also inhibits renin and aldosterone secretion, and increases the hypotensive effect of BNP. Moreover, ANP inhibits endothelin production and proliferation of vascular smooth muscle cells and myocardial hypertrophy. Because of its biological effect (as an antagonist to Ang II), ANP is an endogenous inhibitor of the RA system (See Table 1.1).

ANP and BNP production in the myocardium is induced by increased atrial pressure, as may occur with increased sodium intake and by ventricular dysfunction. A hallmark of ventricular remodeling, secondary to heart failure or left ventricular hypertrophy is the increase in plasma ANP and BNP. Indeed, measurement of circulating levels of ANP is currently employed in a clinical setting as a clear indicator of a CVD state such as CHF (Burnett *et al.*, 1984, 1986, Seymour *et al.*, 1995, Azevedo *et al.*, 2000). The many diverse biological roles of the peptides of the natriuretic peptide system and subsequently NEP, as a regulator of these peptides has brought the system under examination as a potential clinical target. Currently, a number of NEP inhibitors are employed in the treatment of hypertension, namely candoxatril and thiorphan, and succeed in lowering blood pressure.

These inhibitors however, have limitations in long-term treatment, as members of the RA system tend to offset the hypotensive effects over time. NEP inhibitors have also been employed with some success in the treatment of CHF, however to achieve maximum efficiency, they have often been combined with an ACE and/or ECE inhibitor to prevent compensation by anti-diuretic and hypertensive agents. Currently a number of dual-inhibitors are available such as omapatrilat and fasldotrilat (Corti *et al.*, 2001). It is worth noting however, that a number of questions have been raised as to the possible side effects of such inhibitors. NEP located on the surface of neutrophils and in the lung, limits neurogenic inflammation initiated by a variety of stimuli, including tachykinins, cigarette smoke and allergen exposure, inhibition of these processes may promote inflammation and contribute to the development other pathologic states.

It also plays a pivotal role in various cancers, with downregulation of NEP linked to small cell carcinoma formation (Turner *et al.*, 2001). Finally, NEP has also been indicated as a neuroprotective agent against the formation of ß-amyloid peptides associated with Alzheimer's disease (Shirotani *et al.*, 2001). Thus, it is evident that a critical balance of NEP is required to ensure the stability of a number of systems and distortion of that balance in either a positive or negative direction can lead to the progression of pathological states.

Table 1.1 Contrasting Effects of AngII and NP

Ang II	Effect	NP
+	Blood Pressure	–
–	Sodium Secretion	+
+	Aldosterone	–
–	Rennin Secretion	–
+	Cell Proliferation	–
+	Hypertrophy	–

1.5.4 Thimet Oligopeptidase (THOP, EP24.15) and Neurolysin (EP24.16)

EP24.15 and EP24.16 are closely related members of the large metallopeptidase family in the MA clan of peptidases. The primary sequence and substrate specificity of these enzymes are extremely similar, with the only biochemical distinctions being the cleavage site within neurotensin, and sensitivity to selectively designed peptidomimetic inhibitors (Massarelli *et al.*, 1999). Indeed, the primary sequence for both peptidases has been shown to be at least 65% identical (Oliveira *et al.*, 2003). For this reason, they shall be discussed in tandem. Both peptidases were originally isolated and purified from rat brain homogenates and found to act on a number of neuroactive substances such as neurotensin and gonadotropin-releasing hormone (GNRH).

Further studies have indicated a greater tissue distribution for the peptidases, with activity detected in cells of the testes, lung and vasculature (Orlowski *et al.*, 1989; Yang *et al.*, 1998; Cotter *et al.*, 2004). A wider range of substrates has also been identified, with studies indicating a capacity to act on luteinizing hormone releasing hormone (LHRH), dynorphin A 1-8, metorphinamide, bradykinin (BK) and Ang I (Rioli *et al.*, 1998).

Whilst both are classified as largely soluble endopeptidases, due predominantly to an apparent lack of a signal sequence and membrane-spanning domain, studies incorporating confocal microscopy and percoll gradient analysis, have shown that these enzymes can be secreted and indeed some co-association of THOP with the plasma membrane and extracellular activity has been reported (Crack *et al.*, 1999). The mechanism of release is not fully understood, however a study by Jeske *et al.* 2003 suggests that enzyme secretion may occur through association of the enzyme with lipid rafts.

Whereas roles for the previously mentioned members of the metalloendopeptidase family have been well established and characterized, the distinct physiological functions of EP24.15 and EP24.16 are still emerging, with mounting evidence pointing to a significant role in the vasculature and the regulation of vessel tone.

1.5.4.1 Thimet Oligopeptidase (THOP, EP24.15) and Neurolysin (EP24.16) as Mediators of Vessel Tone

Both endopeptidases have been reported to cleave vasoactive peptides such as Ang I, neurotensin and BK (Schrimpton *et al.*, 2002). Specific site-directed inhibitors of both enzymes have been shown to potentiate BK-induced vasodilation (Smith *et al.*, 2000) and to decrease myocardial ischemia/reperfusion injury following 3 and 7 days of reperfusion in rabbits (Schriefer *et al.*, 2001). More recent studies by Norman *et al* and Rioli *et al* strongly suggest roles for both endopeptidases in the metabolism of vasodilatory peptides in peripheral and cerebral vascular beds. Furthermore, EP24.15 has been shown to hydrolyze Ang I in vascular smooth muscle cell cultures at the Pro7-Phe8 position to generate the peptide Ang-(1-7), thereby subverting formation of Ang II by ACE. Furthermore, Ang-(1-7) has itself been shown to possess bioactivity in the vasculature. It has been shown that Ang-(1-7) produces relaxation in several vascular beds, including canine and porcine coronary arteries, canine middle cerebral artery, piglet pial arterioles, feline systemic vasculature, rabbit renal afferent arterioles, rat aortic rings, and mesenteric microvessels of normotensive and hypertensive rats (Ferreira and Santos 2005).

Many studies have shown that the vascular actions of Ang-(1-7) appear to involve increased production of vasodilatory prostanoids, NO and endothelium-derived hyperpolarizing factor. Moreover, some vascular effects involve simultaneous participation of a number of these vasodilator mediators.

A cross-talk between a number of receptors such as the Ang-(1-7)-specific receptor and AT_2 have also been shown to activate vasomotive pathways in blood vessels (Ferreira and Santos, 2005). These cumulative observations clearly point to a putative role for these related enzymes in regulating the balance between pressor and depressor peptides within the vasculature, thereby contributing significantly to the maintenance of vascular tone and homeostatic mechanisms. As members of the same endopeptidase family as ACE, ECE and NEP, it was thought that they may possibly be regulated in a similar flow-dependent manner.

Studies examining the effect of cyclic strain on endothelial cells have shown a significant regulatory effect of this force on these enzymes. Work carried out in our lab alone has shown that cyclic strain mediates expression levels of these peptides, with EP24.15 and EP24.16 displaying marked increases of 2.3 and 1.9 fold respectively, furthermore, through the use of pharmacological and molecular inhibitors, the strain induced up-regulation of these peptides was shown to be Gi_a- and $G_{\beta\gamma}$-dependent (Cotter *et al.*, 2004). Furthermore, the classic SSRE sequence GAGACC has been identified in the promoter region of EP24.15 (Kim, Grum-Tokars *et al.* 2003). Little work however, has centered on the regulation of either of these peptidases by shear stress.

1.6 Mechanotransduction and Vessel Tone

Signal transduction refers to any process by which the cell converts one form of signal or stimulus into another, most often involving ordered sequences of biochemical reactions inside the cell. They are carried out by a number of active molecules and linked through second messengers resulting in what is thought of as a second messenger cascade pathway (See Fig 1.6). Vascular cells are equipped with numerous receptors that allow them to detect, respond and subsequently transduce the mechanical forces generated by pressure and shear stress. These mechanosensitive structures will be discussed in turn.

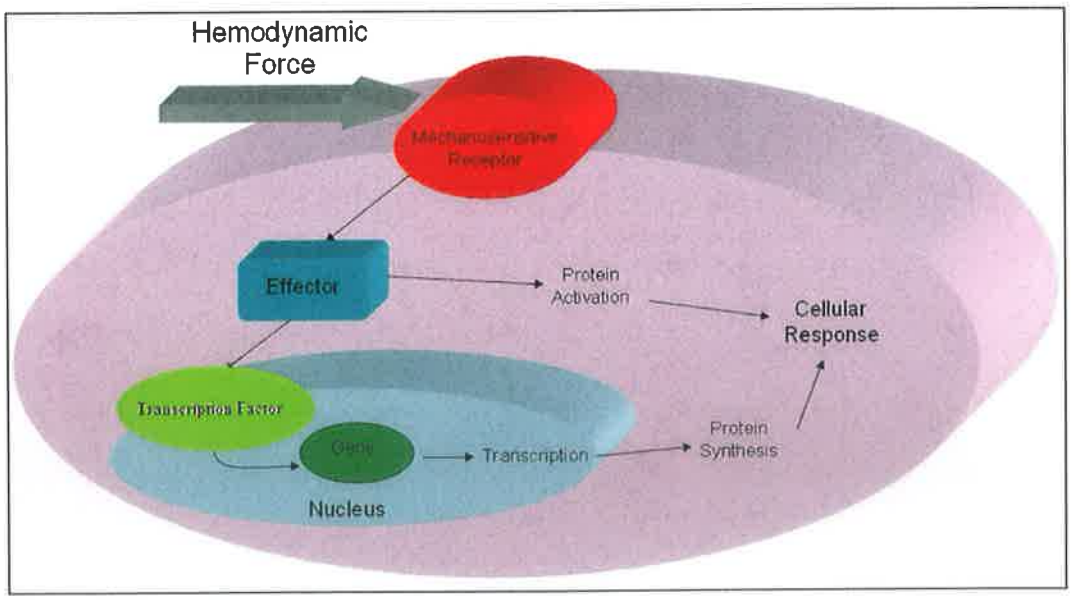


Fig 1.6 Schematic representation of signal transduction in the endothelial cell

1.6.1 The Cytoskeleton

The cytoskeleton and other structural components have an established role in mechanotransduction, being able to transmit and modulate tension within the cell via focal adhesion sites, integrins, cellular junctions and the extracellular matrix. The cytoskeleton is composed of three major types of protein filaments; microtubules, microfilaments, and intermediate filaments. Microfilaments are polymers of actin that, together with a large number of actin-binding and associated proteins, form a continuous dynamic connection between nearly all cellular structures.

The cytoskeletal network changes in response to extracellular stimuli and participates in transmembrane signaling, providing a scaffold for organizing or translocating signaling molecules and organelles (Lehoux and Tedgui, 2003). Evidence now points towards an important role for transduction of the frictional force of shear stress through the cytoskeleton from focal adhesion sites on the basal side of the cell, implying that cell-matrix interactions may be pivotal in the detection process. The dramatic re-alignment of filamentous actin (F-actin) stress fibres, which occurs 12-15 hr after the onset of increased shear, implicates cytoskeletal proteins as playing a large role as a force transmission structure of endothelial cells. In the presence of low shear, the intermediate filament, F-actin, is predominantly located in the periphery of the cell, particularly at cell-cell junctions, where it is thought to play a role in cell permeability.

In the presence of higher levels of shear however, thicker strands of F-actin are observed centrally in association with cell elongation in the direction of flow (Davies, 1995, Noria *et al.*, 1999). It should be noted that this is a force- and time-dependent response, with variability in the rapidity of the response observed in differing species. Beyond the cytoskeletal modifications incurred, and possibly incorporating them as part of the transduction pathway, mechanical forces can also initiate complex signal transduction cascades leading to functional changes within the cell, often triggered by activation of a number of cell surface complexes. Most predominantly studied among them are integrins, G-protein receptors, tyrosine kinase receptors and ion channels (See Fig 1.6). Each of the major signal transduction structures and their function in regulation of vessel tone shall be discussed in turn.

1.6.2 Integrins

Endothelial cells reside in a protein network, the extracellular matrix (ECM), which they secrete and mold into the intercellular space. This matrix exerts profound control over the cells with which it is in contact. The effects of the matrix are primarily mediated by integrins, a family of cell surface receptors that attach cells to the matrix and mediate mechanical and chemical signals from it. These signals regulate the activities of cytoplasmic kinases, growth factor receptors, and ion channels and control the organization of the intracellular actin cytoskeleton (Rupp and Little 2001).

Integrin-dependent physiological processes include tissue morphogenesis, inflammation, wound healing, and regulation of cell growth and differentiation. They are composed of two subunits, a and ß, with each aß combination having its own binding specificity and signaling properties. Nineteen different integrin a-subunits and 8 different ß-subunits have been reported in vertebrates, forming at least 25 aß-heterodimers and perhaps making the integrins the most structurally and functionally diverse family of cell adhesion molecules (Giancotti and Ruoslahti 1999), see Fig 1.7.

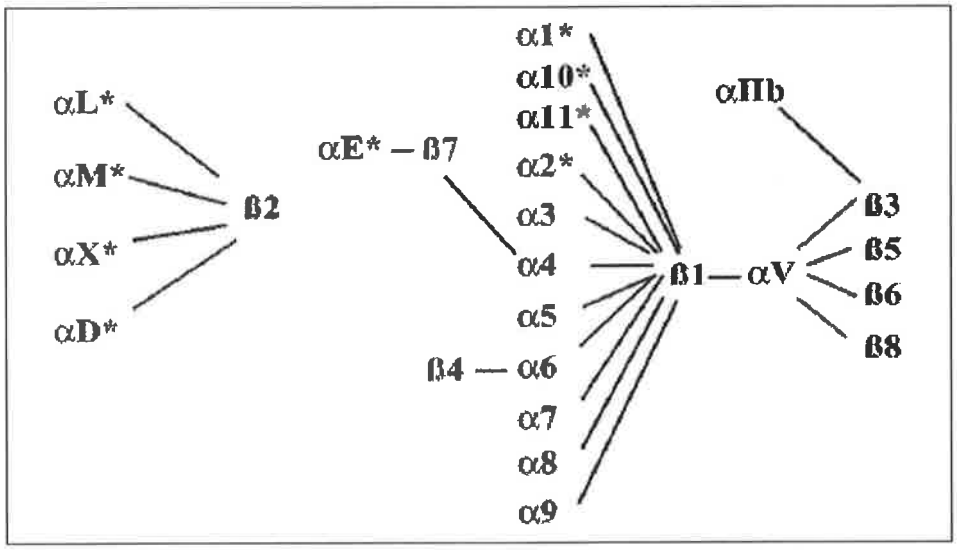


Fig 1.7 Integrin aß subunit interaction (*indicates presence of I domain)

Most integrins recognize several ECM proteins. Conversely, individual matrix proteins such as fibronectin, laminins, collagens and vitronectin, bind to several integrins.

Integrins can signal through the cell membrane in either direction: the extracellular binding activity of integrins is regulated from the inside of the cell (inside-out signaling), while the binding of the ECM elicits signals that are transmitted into the cell (outside-in signaling).

The a and ß subunits are type I transmembrane glycoproteins with extracellular domains of >940 and >640 residues, respectively. The intracellular domains are shorter, except for the ß4 cytoplasmic domain, which is specialized to connect to the keratin cytoskeleton and contains fibronectin type III domains. Studies involving electron microscopy have revealed the shape of the extracellular domain to be that of a ligand-binding globular headpiece, from which two long stalk regions protrude from both the a and ß subunits. These stalk regions are transmembrane and serve to connect the extracellular to the cytoplasmic domains (Giltay and van Mourik 1988).

1.6.2.1 a-Integrin Subunit

The N-terminal region of the integrin a -subunit contains seven segments of about 60 amino acids each with weak homology to one another, which have been predicted to fold into a seven-bladed ß-propeller domain. The heterotrimeric G protein ß-subunit contains a ß-propeller domain with the same topology. Mutagenesis studies show that ligand-binding residues cluster to one portion of the top and side of the ß-propeller. About half of the integrin a-subunits contain no I domain (Mould, Askari *et al.* 2000).

The I domain of about 200 amino acids, known as an I domain or a vonWillebrand factor A domain is the major ligand-binding site in integrins that contain I domains. The I domain is inserted between ß-sheets 2 and 3 of the ß-propeller domain. In integrins lacking an I domain, the ß-propeller domain appears to directly participate in ligand binding. In integrins that contain I domains, the ß-propeller domain can cooperate in binding to some but not other ligands, or play no direct role.

1.6.2.2 ß-Integrin Subunit

The ß-subunit contains an inactivating subunit, that of the PSI domain. This cysteine-rich domain is predicted to contain seven cysteines and two a-helices. The first of these seven cysteines has been shown to form a long range disulphide bond to the C-terminal cysteine rich region of the ß-subunit and in this way keep the integrin in an inactive confirmation. The ß-subunit also contains a highly conserved region of about 240 residues, which has weak but detectable homology with the I domain of the a-subunit. This I-like domain serves to directly bind ligands in integrins lacking an I domain. (Luo, Carman et al. 2007)

1.6.2.3 Integrins and Shear Stress

The extracellular matrix is an important contributor to the process of mechanotransduction, containing glycoproteins which are displaced by stretch and shear forces and which interact with integrins.

Integrins have been shown to participate not only in cell attachment but also in intracellular transmission of mechanical signals. Mechanical stresses stimulate conformational activation of cell integrins and increase cell binding to the extracellular matrix (Jalali *et al.*, 2001). Indeed, conformational activation of integrins is required for stretch- or shear-induced mechanotransduction, as evidenced through blocking of integrin binding sites with specific antibodies (e.g. WOW-1) or RGD peptides, resulting in inhibition of intracellular signaling induced by mechanical forces (Jalali *et al.*, 2001; Wilson *et al.*, 1995; Tzima *et al*, 2001).

A study by Chen *et al.* has shown that shear stress activates EC integrins, as evidenced by their clustering association with the adapter proteins Shc and FAK, both of which have been shown to mediate the activation of downstream mitogen-activated protein kinases (Chen *et al.*, 1999).

1.6.2.4 Integrins and Shear Stress Regulation of Metallopeptidase Expression

Many studies have shown shear stress activation of a number of integrins in endothelial cells, e.g. aVß3 and a6ß1 (Lehoux and Tedgui, 2003), and have also linked integrin activation to downstream signaling events such as MAPK pathways (Chen *et al.*, 1999). While there have been a number of studies that link TLZM family members and their associated products, namely Ang II, with integrin activation and function. To date, however, no studies have shown a conclusive role for integrins in the shear stress-dependent modulation of expression of TLZM family members.

Recently, studies have shown that Ang II has the ability to induce integrin subunit expression in rat cardiac fibroblasts, namely ß3 and aV (Kawano *et al.*, 2000) and thus play a role in the pro-fibrotic actions of Ang II. Integrins have also been shown to play a role in the Ang II mediated vascular inflammatory response and associated monocyte adhesion. Ang II is evidenced to up-regulate ICAM-1 and the integrin receptors aLß2 and a4ß1, as part of its pro-inflammatory mechanisms (Mervaala *et al.*, 1999). ET-1 has also been linked to integrin activation. Studies on ovarian cancer cell adhesion have shown that ET-1 and its receptor ETa are linked to downstream activation of the a2ß1 and a3ß1 integrins and subsequently downstream kinase activation (Rosano *et al.*, 2006).

Of more relevance to the vasculature is a recent study which linked activation of integrins with ET-1 expression. A novel study has employed magnetic twisting cytometry, a method that applies cell surface ligands bound to ferromagnetic beads and through the use of magnetic fields can "twist" the receptors (e.g. integrins) once bound to the magnetic ligands into an active conformation. This study showed a dramatic up-regulation of ET-1 mRNA expression following 2 hr of integrin activation twisting in HUVECs (Chen *et al.*, 2001).

1.6.3 Heterotrimeric G-Proteins

All cells possess transmembrane signaling systems that allow them to receive information from extracellular stimuli like hormones, neurotransmitters, or sensory stimuli. This fundamental process allows cells to communicate with each other.

All transmembrane signaling systems share two basic elements, a receptor which is able to recognize an extracellular stimuli as well as an effector which is controlled by the receptor and which can initiate an intracellular signal cascade. Many transmembrane signaling systems like receptor tyrosine kinases incorporate these two elements in one molecule. In contrast, the G-protein signaling systems are relatively complex, consisting of a receptor, a heterotrimeric G-protein and an effector. This modular design of the G-protein signaling system allows convergence and divergence at the interfaces of receptor and G-protein, as well as of G-protein and effector.

In addition, each component, the receptor, the G-protein and the effector can be regulated independently by additional proteins, soluble mediators or at the transcriptional level (Wettschureck and Offermanns, 2005). The relatively complex organization of the G-protein-mediated transmembrane signaling system provides the basis for a huge variety of transmembrane signaling pathways that are tailored to serve particular functions in distinct cell types. These proteins represent the largest group of cell surface receptors encoded by the mammalian genome (> 1% of human genes), and in the cardiovascular system, G-protein coupled receptors (GPCRs) are implicated in more or less every regulatory event. Indeed, some 865 genes in man encode GPCRs, whilst G-proteins themselves have been shown to derive from 35 genes, 16 encoding a, 5 β and 14 γ subunits (Milligan and Kostenis, 2006). With G-proteins and their associated receptors being so widely spread though diverse systems and tissues, they have been linked to a number of physiologically distinct processes, including over half of known GPCRs playing a role as taste and olfactory

receptors (Wettschureck and Offermanns, 2005). They have, however, been linked to a number of other systems including the cardiovascular system. Indeed they have been implicated in nearly every regulatory event in the vasculature (Wheeler-Jones, 2005).

The heterotrimeric G-protein consists of an a-subunit that binds and hydrolyzes GTP as well as a ß- and a γ-subunit that form an undissociable dimeric complex. Several subtypes of a-, ß-, and γ-subunits have been described.

To dynamically couple activated receptors to effectors, the heterotrimeric G-protein undergoes an activation-inactivation cycle (See Fig 1.8). In the basal state, the βγ-complex and the GDP-bound a-subunit are associated, and the heterotrimer can be recognized by an appropriate activated receptor. Coupling of the activated receptor to the heterotrimer promotes the exchange of GDP for GTP on the G protein a-subunit. The GTP-bound a-subunit dissociates from the activated receptor as well as from the βγ-dimer, and both the a-subunit and the βγ-dimer are now free to modulate the activity of a variety of effectors such as ion channels or enzymes (e.g, adenylate cyclase). Signaling is terminated by the hydrolysis of GTP by the GTPase activity, which is inherent to the G protein a-subunit. The resulting GDP-bound a-subunit re-associates with the βγ-dimer to enter a new cycle if activated receptors are present (Wettschureck and Offermanns., 2005).

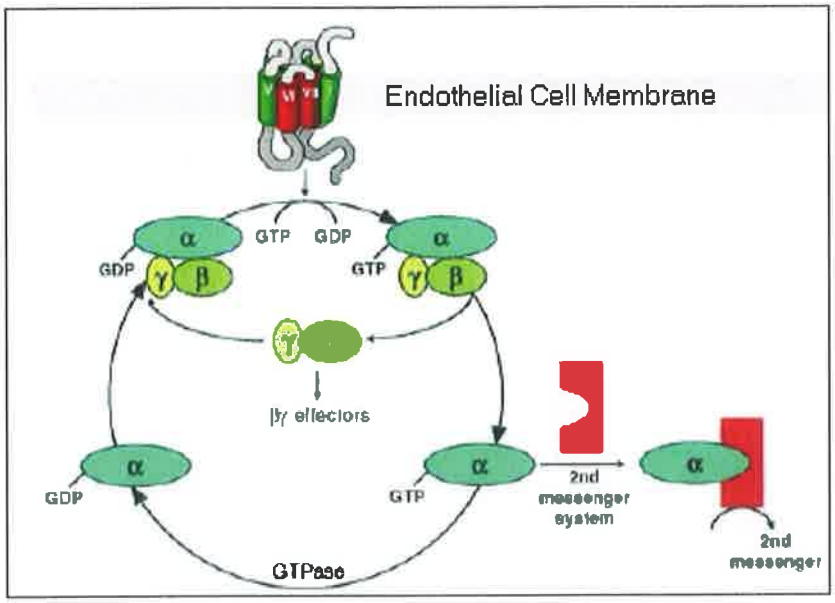


Fig 1.8 Schematic representation of signal transduction via G proteins (Milligan and Kostenis, 2006)

1.6.3.1 G-Protein a-Subunit

There are four principal classes of G-protein a-subunit families (Gas, Gai/o, Gaq/11 and Ga12/13), each identified by a subunit that preferentially regulates specific classes of effectors (Cabrera-Vera *et al*, 2003). The Gas subunit is ubiquitously expressed and stimulates adenylate cyclase (AC), leading to increased cAMP levels and activation of calcium channels. It has a similar isoform, Ga_{olf}, whose expression is limited to neuronal cells associated with olfactory processes. Gai inhibits AC and activates K^+ channels, and has a number of similar isoforms with a high degree of homology for each other. They include ai_1, ai_2, ai_3, ao_1, ao_2, $a_{t\text{-}rod}$, $a_{t\text{-}cone}$, a_{gust} and a_z. They share the ability to inhibit AC, but some are largely thought to serve redundant functions.

All Gai/o subunits are inhibited by pertussis toxin (PTX), except for the a_z subunit, which can continue to inhibit AC in the presence of PTX. It has been identified in a number of tissues but its physiological role is as of yet unidentified. Gaq/11 stimulates phospholipase C (PLC) but its expression is limited to a small number of sites such as kidney, lung and testis. Members of Ga12/13 are principally involved in the small G-protein Rho-mediated responses, but have been shown to play a role in Gaq-linked signaling as well (Cabrera-Vera *et al*, 2003). This arrangement, whereby a large number of GPCRs that transmit a diverse array of stimuli operate via only a limited number of known G-protein subtypes and effectors, cannot fully account for the specificity in responses that is generally observed.

The demonstration that GPCRs can interact with a variety of additional proteins has a bearing on such issues. With this new knowledge, there is an emerging theme that these additional proteins, by providing a scaffold, form signaling complexes in association with individual GPCRs, resulting in compartmentalization of receptors and signaling elements that enable the regulation of specificity, intensity and duration of the signaling process (Tilakaratne and Sexton, 2005).

1.6.3.2 G-Protein βγ-Subunit

At least 5 different ß- and 12 γ-subunits have been described, although only a limited number of pairings have identified. However, a number of possible pairings have been indicated "not" to form, and tissue expression patterns may further limit the actual number of pairings in particular cells and tissues. There is still the potential for co-expression of a substantial number of pairs for which further study is needed to identify. Initially, the βγ-dimer was considered to act as little more than a binding partner for the Ga-subunit to suppress spontaneous signaling and because it was significantly more hydrophobic, to potentially provide a membrane anchor for the Ga-subunit. The various βγ-complexes were also though to be functionally interchangeable in their associated role with the Ga-subunit. Recent work however, has identified a number of roles for the βγ complexes, including effects on AC, phospholipase C, potassium channels, calcium pumps and phosphoinositide 3-kinase (PI-3 kinase) (Exton, 1996; Yamada *et al.*, 1998; Lotersztajn *et al.*, 1992).

1.6.3.3 G-Proteins and Shear Stress

Shear stress stimulates numerous responses in endothelial cells. These include; (i) elevated production of the second messengers IP_3 and cGMP; (ii) increased release of the vasoactive compounds PGI_2, NO, and ET-1; (iii) increased mitogen-activated protein kinase activity (MAPK); and (iv) elevated levels of platelet-derived growth factor and c-*fos* gene expression.

Many of these flow-mediated events are mediated via G-protein activation, as demonstrated in studies examining the effect of G-protein inhibitors. These inhibitors include GDP-ßS, a non-hydrolyzable analogue of GDP and PTX. GDP-ßS has been shown to inhibit shear-induced MAPK activity in endothelial cells (Tseng *et al.*, 1995) whilst PTX inhibited PGI_2, without having any effect on NO or cGMP production, indicating that both PTX-sensitive and -insensitive G-proteins are activated by flow (Gudi *et al.*, 1996).

1.6.3.4 G-Proteins and Shear Stress Regulation of Metallopeptidase Expression

Numerous studies have linked TLZM family members with G-proteins. TLZM family members produce a variety of peptide products whose receptors can be G-protein associated. ET receptors, which bind the potent vasoconstrictive peptide ET-1, are G-protein-coupled, resulting in activation of phospholipase C and generation of two second messengers, inositol triphosphate and diacylglycerol, which respectively stimulate calcium release and protein kinase C activation, respectively.

Phospholipase D activation with generation of diacylglycerol, phospholipase A2 stimulation with release of arachidonic acid, activation of the Na^+/H^+ exchanger, and activation of tyrosine kinases and MAP kinases, are other pathways that contribute to contraction and growth induced by ET receptor stimulation. ET receptors and hence G-proteins, may be downregulated by ET-1, especially under conditions in which large amounts of ET-1 are being produced in the vasculature.

This has been demonstrated in some models of experimental hypertension and in human hypertension (Schiffrin and Touyz, 1998). Interactions of G-proteins and members of the RAS system have also been examined, with studies indicating a role for ACE inhibitors in the down-regulation of cardiac G-proteins (Pandey and Anand-Srivastava, 1996; Makino *et al.*, 2003). Other members of the RAS system have also been shown to mediate their effects via G-proteins, namely Ang-(1-7), an important vasopeptide with contrasting effects to the potent vasoconstrictor Ang II (Ferreira and Santos, 2005). Recently studies have also shown that cyclic strain, can differentially regulate expression of members of the TLZM family, namely EP24.15 and EP24.16 in a G-protein dependent manner. Incubation with Ga_i inhibitors (PTX and NF023) strongly attenuated the strain-induced increases in both of these peptidases. Further examination using dominant negative mutants to selectively attenuate $Ga_{i1,2,3}$ evidenced more specific results, with both EP24.15 and EP24.16 increases displaying a sensitivity to blockade of $Ga_{i2,}$ whilst, blockade of Ga_{i3} and Ga_{i1} selectively inhibited the strain induced increases in EP24.15 and EP24.16 respectively. Moreover EP24.16 displayed a greater sensitivity to $\beta\gamma$ blockade (Cotter *et al.*, 2004).

1.6.4 Small G-Proteins (GTPases)

The small G-protein superfamily contains over 100 members that are generally classified, by structural similarity, into five sub-families: Ras family GTPases (Ras, Rap and Ral), Rho family GTPases (Rho, Rac and cdc42), Arf family GTPases (Arf1–Arf6, Arl1–Arl7 and Sar), Rab family GTPases (>60

members, e.g. Rab5) and Ran family GTPases (Mackay and Hall, 1998). The small GTPases are monomeric G-proteins with molecular masses over the range 20–30 kDa. The functions of the many small G-proteins are still being elucidated. In general, Ras family GTPases regulate cell signaling events that lead to alterations in gene transcription; Rho family GTPases function as regulators of the actin cytoskeleton and can also influence gene transcription; Rab and Arf family GTPases control the formation, fusion and movement of vesicular traffic between different membrane compartments of the cell; and Ran GTPases regulate both microtubule organization and nucleocytoplasmic protein transport.

All of these GTPases function as molecular switches that control eukaryotic cell function by cycling between two interconvertible forms, a GDP-bound 'inactive' form and a GTP-bound 'active' form. The rate-limiting step of the GDP/GTP exchange, which is the dissociation of GDP, is promoted by the association of a guanine nucleotide exchange factor (GEF), the activity of which may be regulated by an upstream signal such as the activation of a heterotrimeric G-protein. The binding of GTP eventually leads to a conformational change in the downstream effector-binding domain of the G-protein such that this region interacts with either one or many downstream effector(s), which function to either activate or inactivate a variety of signaling pathways. The GTP-bound form of the small G-protein is converted into the GDP-bound form due to its intrinsic GTPase activity, which is stimulated by GTPase-activating proteins. Of particular interest in the vasculature is the Rho GTPase family which shall be discussed further.

1.6.4.1 Rho GTPase Small G-Protein Family

The Rho GTPase family of small G-proteins consists of at least 20 members, subdivided into the Rho (RhoA, RhoB and RhoC), Rac (Rac1, Rac2 and Rac3) and Cdc42 (Cdc42Hs and G25K) families. These GTPases have been shown to have at least 30% sequence homology with other GTPases, namely the Ras family. This is thought to be the source of their commonality in ability to regulate gene expression (Mackay *et al.*, 1998; Takaishi *et al.*, 2000). Like Ras and other GTPases, the Rho GTPases cycle between GTP-bound active states and GDP-bound inactive states. They are activated by PI-3 kinase-dependent activation of guanine nucleotide exchange factors (GEFs) that stimulate GTP exchange. GTPases are inactivated by GAPs, which stimulate their intrinsic GTPase activity. Rho GTPases perform important functions in controlling gene transcription, cell cycle control and apoptosis. However, their best understood and conserved function is to regulate filamentous actin and thereby regulate the actin cytoskeleton.

Through regulation of actin, Rho GTPases are able to coordinate the intracellular responses to extracellular stimuli thus regulating cell movement, cell shape, cell polarity, matrix adhesion, and cell to cell adhesion (Fryer and Field 2005). Of primary importance in the Rho GTPase family is Rac1. Rac1 is required at the apical surface of the cell to regulate actin polymerization and membrane protrusion and assembly of membrane ruffles depends on Rac1 activity. Rac1 therefore plays a pivotal role in the shear stress response of endothelial cells.

Studies incorporating biochemical and immunofluorescence studies in ECs indicate that Rac1 GTPase is activated by shear stress. Using pull-down assays, it has been shown that shear stress can transiently activate Rac1 within 30 minutes followed by a return to basal levels.

Rac1 activation by shear stress has also been shown to be upstream of a number of physiologically important processes in the vasculature, namely, the activation of the transcription factor, NF-κB via the production of Rac1-dependent reactive oxygen species (Tzima, 2006). Rac1 has also been shown to act as part of a larger signaling network of GTPases, in which GPCR activate one or more GTPases which then work in conjunction with each other, namely Rho, Rac and Cdc42 to affect downstream targets such as MAPK (Matozaki *et al.*, 2000).

1.6.5 Protein Tyrosine Kinase

Tyrosine phosphorylation of numerous proteins is an important step in the signal transduction of cellular pathways that control various activities, such as proliferation, migration, differentiation and survival. PTKs are integral components of such signal transduction cascades. They can be separated into two distinct sub-families, receptor and non-receptor linked, that act at a membrane and cytoplasmic level, respectively.

1.6.5.1 Receptor PTK

These PTKs are distinguishable 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 a shift in cell 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). 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 (see Fig 1.9), creating binding sites for intracellular adaptor molecules such as 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, 1998; Sugden and Clerk, 1997).

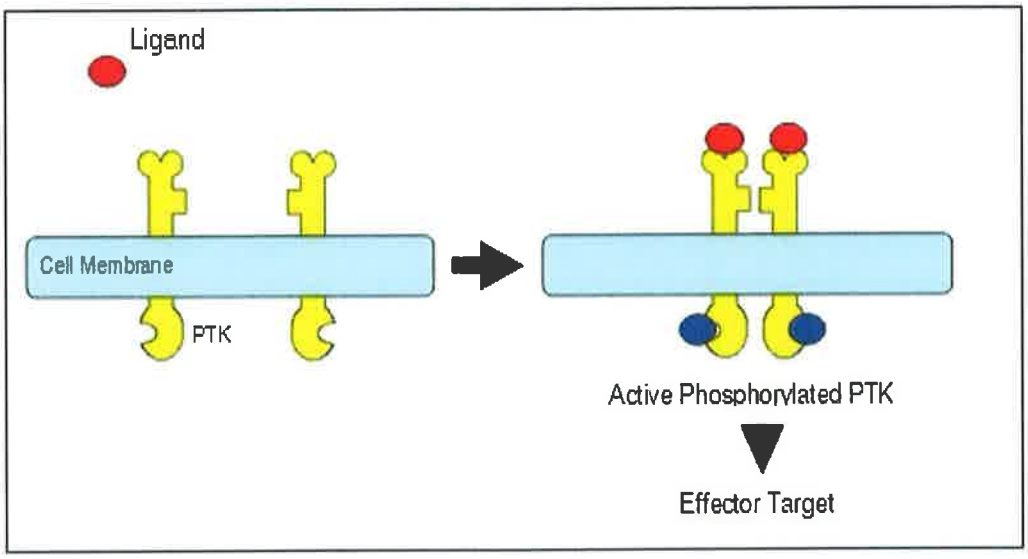


Fig 1.9 Schematic representation of signal transduction via PTK

1.6.5.2 Non-receptor PTKs

Included in the sub-family of non-receptor PTKs are 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 and Parsons, 1994). Focal adhesion-associated kinases such as FAK and c-Src are rapidly activated in ECs by shear stress (Li *et al.,* 1997).

1.6.5.3 Protein Tyrosine Kinases and Shear Stress

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 pharmacological PTK inhibitor, can attenuate the shear stress activation of ERK and JNK (Li *et al.,* 1997; Jo *et al.,* 1997; Takahashi and Berk, 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 and Izumo, 1997, Ishida *et al.,* 1996). It has been shown that shear stress induces 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, integrins and tyrosine kinases (Lopez-Ilasaca, 1998).

1.6.6 Caveolae

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 and Berk, 1998). A number of members of the TLZM family peptidases and their associated peptides and receptors have been shown to be clustered in the plasma membrane, namely EP24.15, ACE, ECE, ET-1 and ETa (Jeske *et al.*, 2003, Ryan *et al.*, 1976, Takahashi *et al.*, 1995, Chun *et al.*, 1994).

1.6.7 Ion Channels

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 and Gaub, 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, Olesen *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 smooth muscle cells or atrial myocytes (Papadaki and Eskin, 1997). The hyperpolarization 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 Ca^{2+} (Luckhoff and Busse, 1990). However, it has since been shown that NO-mediated relaxation in endothelial cells is largely calcium independent (Ayajiki *et al.,* 1996, Poston, 2002). Further study is therefore required in order to fully elucidate the possible roles played by ion channels in mechanotransduction pathways.

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, as indicated by shear-mediated ERK1/2 stimulation through addition of the Na^+ channel inhibitor, tetrodoxin.

1.7 Reactive Oxygen Species

Reactive oxygen species (ROS) were classically regarded as accidental by-products of metabolism and were considered largely detrimental due to their reactivity with membranes, DNA, macromolecules and proteins. ROS include free radicals such as $O_2^{-\bullet}$ (Superoxide), $NO^{\bullet}$ (nitric oxide), $ONOO^{-\bullet}$ (peroxynitrite) and $OH^{\bullet}$ (hydroxyl) and non-radicals such as H_2O_2. Ground-state diatomic oxygen (more commonly, O_2), despite being a radical species, and the most important oxidant in aerobic organisms, is only very sparingly reactive itself. This lesser reactivity is due to the fact that its two unpaired electrons are located in different molecular orbitals and possess ''parallel spins.'' As a consequence, if O_2 is simultaneously to accept two electrons, these must both possess anti-parallel spins relative to the unpaired electrons in O_2, a criterion which is not satisfied by a typical pair of electrons in atomic or molecular orbitals (which have opposite spins according to the Pauli Exclusion Principle). As a result, O_2 preferentially accepts electrons one at a time from other radicals (such as transition metals in certain valences). Thus, *in vivo*, typical two- or four-electron reduction of O_2 relies on coordinated, serial, enzyme-catalyzed one-electron reductions. One- and two-electron reduction of O_2 generates $O_2^{-\bullet}$ and H_2O_2, respectively, both of which are generated by numerous routes *in vivo*. In the presence of free transition metals (in particular iron and copper), $O_2^{-\bullet}$ and H_2O_2 together generate the extremely reactive $OH^{\bullet}$ (Fig 1.10). Ultimately, $OH^{\bullet}$ is assumed to be the species responsible for initiating the oxidative destruction of biomolecules (Beckman and Ames 1998; Poli *et al.*, 2004).

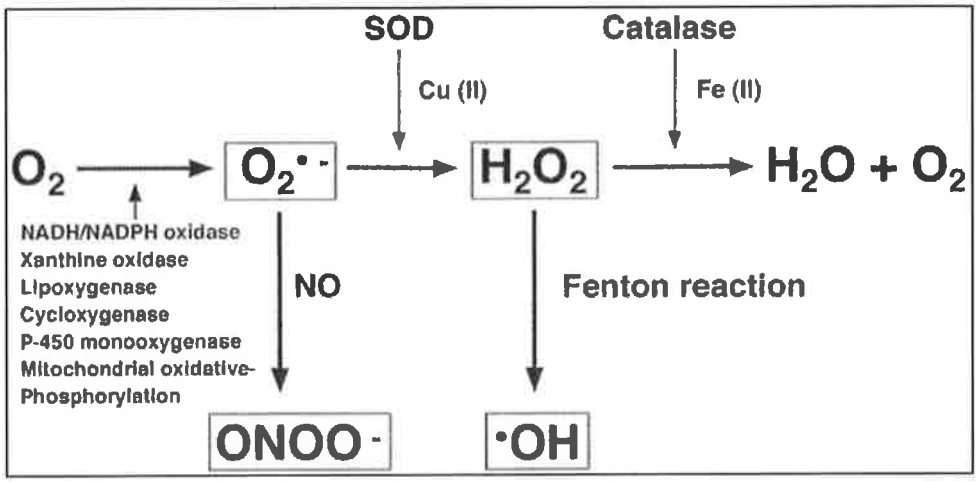


Fig 1.10 Reactive oxygen species present in the vasculature

1.7.1 Reactive Oxygen Species Within the Vasculature

The presence of ROS in a cellular environment is referred to as the oxidative state of the cell. This oxidative state has a number of wide ranging impacts on cellular and tissue function as a whole. Any significant increase in the levels of ROS present, which outstrips endogenous anti-oxidant systems, is termed oxidative stress. Whilst it has been shown that differing ROS are required for normal cellular function, when levels of ROS exceed that which is tolerable by the cell and approach oxidative stress, ROS then contribute to a number of pathophysiological conditions. Oxidative stress plays a major role in the development of endothelial dysfunction by direct and indirect mechanisms. An important direct mechanism of EC dysfunction is inactivation of endothelial derived NO by $O_2^{-\bullet}$. NO has been shown to react with $O_2^{-\bullet}$ to form $ONOO^{-\bullet}$ (Masumoto *et al.*, 1996).

Unlike NO that readily activates guanylyl cyclase and increases cyclic GMP formation in vascular smooth muscle, $ONOO^{-\bullet}$ is a much weaker agonist for guanylyl cyclase (Tarpey *et al.*, 1995). Thus, any reaction of NO with $O_2^{-\bullet}$ will impair NO-induced activities such as vasodilation.

$ONOO^{-\bullet}$ has been shown to alter other enzyme systems that are important in vascular homeostasis. For example, $ONOO^{-\bullet}$ causes tyrosine nitration of prostacyclin synthase leading to inactivation of its activity (Zou and Ullrich, 1996). Endothelial prostacyclin release causes potent vascular relaxation. Thus, loss of prostacyclin will further contribute to endothelial dysfunction under conditions of increased oxidative stress. There is also evidence that tyrosine nitration of prostacyclin synthase is particularly important in diabetic states, as $ONOO^{-\bullet}$ formation is induced in ECs by hyperglycemia, leading to inactivation of the enzyme (Zou *et al.*, 2002). More detrimental actions of $ONOO^{-\bullet}$ have been reported, such as its potent oxidant properties. $ONOO^{-\bullet}$ oxidizes tetrahydrobiopterin (or the zinc cluster of eNOS), processes that may cause eNOS uncoupling, further limiting the production and action of NO (Milstien and Katusic, 1999). Increased ROS production may also affect vascular homeostasis by a number of indirect mechanisms. Many studies have indicated a pro-atherogenic effect of ROS mediated through their actions of oxidation of low density lipoproteins (LDL). This is evidenced through the role of oxidized LDL in cell toxicity, adhesion of leukocytes to the endothelium and migration of leukocytes into the sub-endothelial space and impairment of receptor-induced NO production (Schulz *et al.*, 2004).

Among the oxidation products of the lipid moiety of LDLs are oxysterols, 27-carbon products of cholesterol oxidation, are by far those quantitatively more represented. Oxysterols have been indicated as possible mediators of the structural and functional changes that occur in the vessel wall during the atherosclerotic process. They have been shown to exert a wide variety of effects, including enhancement of chronic inflammation, fibrosis and programmed cell death (Guardiola *et al.*, 2004). Of note, the parent molecule, non-oxidized cholesterol, is far less reactive than oxysterols, so that oxidation of cholesterol appears to be a necessary step for the sterol to exert its pro-atherogenic action.

The introduction of an oxygen function, such as a hydroxyl or ketone group, on the sterol nucleus or on the side chain of the molecule, confers to the resulting compounds the ability to pass lipophilic membranes at a high rate, and in this way, to interfere with cell signaling at a higher rate than cholesterol (Smondyrev and Berkowitz, 2001, Meaney *et al.*, 2002). Recent studies have indicated a number of possible methods through which oxysterols exert their effects. Studies have shown that oxysterols can affect expression of monocyte chemotactic protein-1 (MCP-1) in a PKC-dependent manner. Oxysterols have also been shown to induce a pro-inflammatory effect. Studies have shown that 7-ketocholesterol induces IL-1ß, an important cytokine which mediates inflammatory processes. Indeed in HUVECs studies, 7-ketocholesterol, 7a-hydroxycholesterol and 7ß-hydroxycholesterol were all shown to induce a 10-20 fold increase in IL-1ß secretion (Lemaire *et al.*, 1998). These three sterols also induce expression of VCAM-1, ICAM-1 and E-selectin, important mediators of inflammation in the vasculature.

Cholesterol oxidation products such as 25-hydroxycholesterol, have also been indicted to play a role in pathogenic processes in the vasculature, with 25-hydroxycholesterol inducing mRNA expression of pro-fibrogenic basic fibroblast growth factor (Kraemer *et al.*, 1993).

Whilst ROS were originally considered as simply deleterious and toxic substances involved in tissue injury, recent studies have examined the roles played by sub-toxic levels of ROS, namely their apparent ability to act as signaling molecules in a number of important pathways within the vasculature.

ROS initially seemed unlikely members of signal transduction pathways, due mainly to the fact that they lack some of the characteristics traditionally associated with signaling molecules; they are small and do not have structural complexity. Furthermore they are highly, and hence randomly, reactive. They interact very rapidly and within very short distances, often with the first organic molecule they encounter, entirely by chance. Growing evidence however, firmly places an important role on sub-toxic levels of ROS in normal physiological signaling within the vasculature. Ligands that induce an increase in intracellular concentration of H_2O_2 include peptide growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and ET-1 and cytokines such as transforming growth factor-ß1 (TGF- ß1) and tumor necrosis factor-a (TNF-a) (Poli *et al.*, 2004). H_2O_2 and $O_2^{-\bullet}$ have been shown to induce tyrosine phosphorylation of several PTKs, namely Src and JAK2, in different cell types, including fibroblasts, SMCs and T and B lymphocytes (Abe and Berk, 1999; Esposito *et al.*, 2003).

Oxidative state has also been shown to modulate activity of transcription factor NF-κB through the ROS-induced phosphorylation of I-κB, the NF-κB inhibitor molecule. Activator protein-1 (AP-1), a transcriptional complex formed by the dimerisation of Fos-Jun or Jun-Jun proteins, has also been demonstrated to be redox sensitive. The activity of AP-1 is regulated by redox effects on single cysteine residues located in the DNA-binding domain of each subunit, in that oxidation reduces binding activity while reduction enhances it (Sun and Oberley, 1996). Sub-toxic levels of ROS have also been demonstrated to act in an atheroprotective manner.

Studies carried out in HUVECs, have shown that low doses of H_2O_2 can induce expression of thioredoxin-1 (Trx-1). Trx-1 not only acts as a ROS scavenger but also binds and inhibits the actions of apoptosis signaling kinase-1 (ASK-1) (Haendeler *et al.*, 2004). These studies, when considered collectively, not only indicate an important role for sub-toxic levels of ROS in a number of physiologically relevant processes but highlight the need for further characterization of the precise interactions that mediate those processes.

1.7.2 Sources of Reactive Oxygen Species in the Vasculature

There are several mechanisms that can induce and regulate the oxidative state of the cell. These mechanisms have been shown to be active in normal physiological roles as well as in pathogenic processes. There have been several potential sources of ROS identified in the endothelium, with differing sensitivities to stimuli and differing levels of productivity.

Sources of ROS include the mitochondrial electron transport chain, xanthine oxidase, cytochrome P_{450} enzymes, uncoupled NO synthases and NAD(P)H oxidase.

The mitochondrial electron transport chain can be a significant source of ROS. Indeed, increased mitochondrial ROS generation has been implicated in diabetic vasculopathy and in ischaemia/reperfusion. The mitochondria itself is quite susceptible to oxidative damage which can result in yet further ROS production (Duchen 2004). Enzymes of the inner mitochondrial matrix space use the electron transfer chain to generate a proton gradient that is used by ATP synthases to phosphorylate ADP to yield ATP. However, under normal conditions, up to 2% of the electrons involved may "leak" from the chain and react with molecular oxygen to produce $O_2^{-\bullet}$ and H_2O_2. These mitochondrial-derived ROS have been shown to play a role in the pathogenesis of hypoxia and diabetes, via interleukin-6 release and PKC activation respectively (Shulz *et al.*, 2004).

Xanthine oxidase has also been implicated as a ROS producer. It is expressed on the luminal surface of the endothelium in many organs and serves to catalyse the conversion of hypoxanthine into urate in a process that generates $O_2^{-\bullet}$ and H_2O_2. The enzymes is normally present as xanthine dehydrogenase, which does not generate $O_2^{-\bullet}$, but is converted into xanthine oxidase either through oxidation or by proteolytic cleavage of a segment of xanthine dehydrogenase, in a reversible and irreversible manner, respectively (Ray and Shah 2005).

eNOS has also been shown to be a contributor of ROS to the oxidative state of the endothelium. NO synthases normally generate NO, but can become "uncoupled", usually in a deficiency of the essential cofactor H_4B (tetrahydrobiopterin). The "uncoupling" causes NOS to carry out the reduction of molecular oxygen, instead of the production of NO. ROS generation by uncoupled eNOS is reportedly involved in the pathophysiology of diabetic vasculopathy, atherosclerosis, hypertension and hypercholesterolemia.

Of interest, H_4B is highly susceptible to oxidative degradation by $O_2^{-\bullet}$ or $ONOO^{-\bullet}$; thus, initial degradation of H_4B by ROS derived from other sources can induce NOS uncoupling and further add to the oxidative state (Landmesser *et al.*, 2003). In experimental hypertension, it has been demonstrated that initial ROS generation by NAD(P)H oxidase leads to the initiation of further ROS production from the previously mentioned sources.

1.7.2.1 NAD(P)H Oxidase

Considerable work has focused on the NAD(P)H oxidase dependent release of $O_2^{-\bullet}$. Originally, NAD(P)H oxidases were described and characterized by their role in phagocytic immune cells. Neutrophils and other phagocytic cells produce ROS during phagocytosis through activation of NAD(P)H oxidase, this is referred to as the respiratory burst. This burst is responsible for $O_2^{-\bullet}$ and H_2O_2 production, with these oxygen-derived species playing a major role in the bactericidal activity of neutrophils (Babior *et al.*, 2002).

The phagocytic NAD(P)H oxidase is a multi-component enzyme, consisting of two main structures, cytosolic and membranous. The cytosolic component is composed of $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the small G-protein Rac1, with "phox" indicating its initial identification as phagocyte oxidase.

The membrane complex is made up of $gp91^{phox}$ and $p22^{phox}$, and termed cytochrome b_{558}, with $gp91^{phox}$ as the major catalytic subunit (Parkos *et al.*, 1987). Upon activation of the enzyme, the GDP on the G-protein Rac1 is exchanged for GTP and $p47^{phox}$ is PKC phosphorylated. These two events trigger a conformational change in the cytosolic components which facilitates association with the membrane bound cytochrome b_{558}. Once all components have translocated to the membrane the active complex is formed. NAD(P)H, the reduced substrate of the enzyme, binds to $gp91^{phox}$ subunit, with a subsequent transfer of two electrons to two molecules of oxygen to produce two $O_2^{-\bullet}$ radicals (see Fig 1.10).

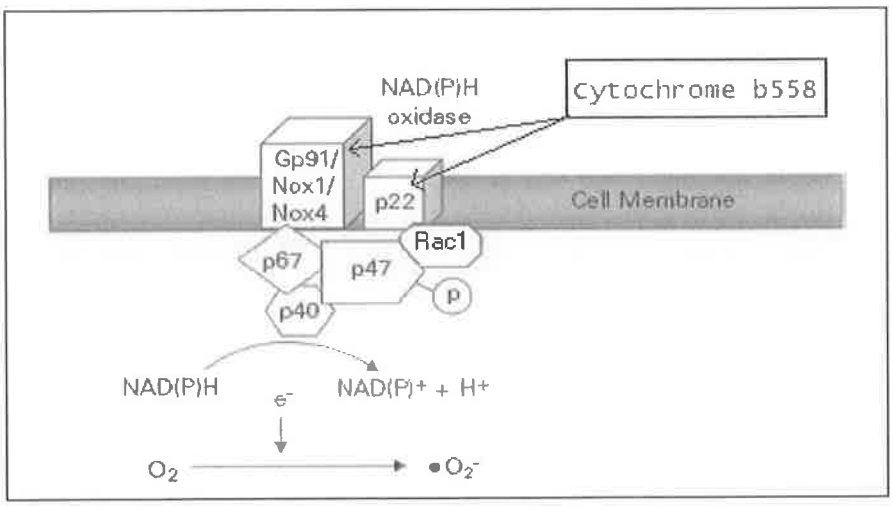


Fig 1.10 Schematic representation of the active NAD(P)H oxidase complex

Recently it has become evident that the expression of the major phox subunits is not restricted to phagocytic cells, with many vascular cell types also exhibiting similar oxidases. The $p22^{phox}$, $p67^{phox}$ and $p47^{phox}$ components were found to be ubiquitously expressed in the major cell types of the vasculature. In contrast, the catalytic subunit, $gp91^{phox}$, has been found to be differentially expressed as slightly dissimilar isoforms in differing cell types of the vasculature. The family of isoforms of $gp91^{phox}$, carry out the same function, in that they catalyse the transfer of electrons to molecular oxygen and are referred to as Nox1-5. ECs express Nox4 and Nox2 predominantly whilst, SMCs have been shown to express Nox4 with negligible levels of Nox2 present (Schulz *et al.*, 2004).

1.7.3 Reactive Oxygen Species and Shear Stress

Among the many stimuli that regulate the production of ROS in the vasculature, recent studies have highlighted a role for hemodynamic forces. Cyclic strain has been evidenced to regulate oxidative state in endothelial cells in an NAD(P)H oxidase-dependent manner, with downstream implications for PTK pathways (Cheng *et al.*, 2002). Moreover, shear stress has come recently to the fore as a major regulator of ROS production in the endothelium. As discussed in section 1.3.2, the tractive force exerted by blood flow on the endothelium causes a number of physiological changes in the cell. Of these changes ROS production plays a pivotal role. Previous studies have indicated that not only does the levels of shear stress experienced by the endothelium, but also the duration and form of flow, influence the initiation and progression of ROS production.

It has been shown that oscillatory forms of flow, described as a back and forth motion of the fluid, found principally in the lesion-prone areas of vessel bifurcation, cause a lasting increase in oxidative state, and hence contribute to the increasing oxidative stress of the cells of that area and can lead to pathogenesis (Hwang *et al.*, 2003). Continuous or laminar shear stress has also been shown to modulate oxidative state, albeit in a different manner than that of oscillatory shear. Laminar shear, as experienced by long vessels without bifurcations, has been shown to act as an athero-protective force which maintains vessel tone and homeostasis. One of the mechanisms through which laminar shear does this is through tight control on the oxidative state of the endothelium. It has been evidenced that laminar shear produces a transient, but distinct, increase in ROS production, in an NAD(P)H oxidase-dependent manner (De Keulenaer *et al.*, 1998). Through this sharp rise and fall of ROS, the endothelium may control a number of shear-sensitive signaling pathways, as discussed in section 1.7.1. Indeed a number of important vasoactive peptides and peptidases have shown sensitivity to oxidative state, namely ECE, ET-1 and CNP.

1.7.4 Reactive Oxygen Species and Shear Stress Regulation of TLZM Expression

With recent studies focusing on the mechanisms that regulate expression of TLZM family members, namely shear stress, a role for ROS has been implicated. As discussed in section 1.7.2, ROS has been shown to modulate expression of a number of important vasoactive molecules, namely MCP-1, PKC, AP-1, NFκB and Trx-1.

In this regard, work has been carried out to explore the roles of ROS and shear stress in the regulation of peptides and their associated peptidases that regulate vessel tone, specifically ACE, ECE, ET-1 and CNP.

ECE, the enzyme responsible for production of the potent vasoconstrictor ET-1, has been shown to be sensitive to regulation by shear stress and oxidative state. A study carried out in 2003 by Masatsugu *et al*, clearly indicated the role played by shear-induced ROS in the attenuation of ECE expression levels in endothelial cells, at both a transcriptional and translational level (Masatsugu *et al.*, 2003). These workers clearly demonstrated a shear-induced attenuation of ECE and ET-1 mRNA and protein expression by 40-70%, a trend that was mimicked by H_2O_2 incubation and could be blocked by the broad-spectrum antioxidant, NAC. They also confirmed a shear-induced ROS burst as is consistent with previous studies.

1.8 Thesis Overview

The data presented in the following chapters examines the relationship between the hemodynamic force of laminar shear stress and expression levels of clinically important metallopeptidases, ACE, ECE, NEP, EP24.15 and EP24.16 in cultured BAECs. It details the role played by shear stress, oxidative state and the possible signaling pathways involved. Through the elucidation of these pathways we hoped to identify additional targets for future therapeutic treatments of a number of disease states associated with CVD. The data has been divided into three main chapters.

Chapter 3: Examination of the role of shear stress in regulating expression of the TLZM family members, ACE, ECE, NEP, EP24.15 and EP24.16 in BAECs.

Chapter 4: Examination of shear stress-induced ROS production in BAECs and subsequent investigation of the effects of artificially-induced oxidative states on TLZM mRNA expression and the influence of antioxidants on shear-induced changes in mRNA expression in BAECs.

Chapter 5: Examination of signalling pathways involved in shear stress-induced ROS production and regulation of NEP expression in BAECs.

Chapter 2 Materials and Methods

2.1 Materials

AGB Scientific (Dublin, Ireland)

Whatmann Chromatography paper

Alpha Diagnostic International (Texas, USA)

Anti-NEP monoclonal antibody

Amersham Pharmacia Biotech (Buckinghamshire, UK)

Anti-mouse 2^{o} antibody, HRP-conjugated

Anti-rabbit 2^{o} antibody, HRP-conjugated

Anti-rat 2^{o} antibody, HRP-conjugated

ECL Hybond nitrocellulose membrane

ECL Hyperfilm

Rainbow molecular weight marker, broad range (6-175kDa)

Bachem UK Ltd. (St. Helens, UK)

Linear RGD peptide

Cyclic RGD peptide

Bio Sciences Ltd (Dun Laoghaire, Ireland)

DMEM

dNTP's

DEPC-treated water

Trizol® reagent

Calbiochem (San Diego, CA)

Apocynin

Genistein

NSC23766 Rac1 Inhibitor

Pertussis toxin

Cayman Chemical Company (Michigan, USA)

eNOS polyclonal antibody

Chemicon (Temecula, CA)

Anti-ACE antibody

Corriell Cell Repository (NJ, USA)

Bovine Aortic Endothelial Cells (BAECs)

Dako Cytomation (UK)

Dako mounting media

Invitrogen (Groningen, The Netherlands)

Dihydroethidium

Lipofectamine

Lipofectamine 2000

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

Kodak 1D image analysis software

Molecular probes (Oregon, USA)

Alexa-conjugated anti-rabbit IgG

Alexa-conjugated anti-rat IgG

Alexa-conjugated anti-mouse IgG

Alexa-Phalloidin 568

MWG Biotech (Milton Keynes, UK)

ACE primer set

ECE primer set

eNOS primer set

GAPDH primer set

NEP primer set

Neurolysin (EP24.16) primer set

THOP (EP24.15) primer set

PALL Corporation (Dun Laoghaire, Ireland)

Biotrace nitrocellulose membrane

Pierce Chemicals (Cheshire, UK)

BCA protein assay kit

Supersignal West Pico chemilumescent substrate

Plasmids

β-ARK-ct was the generous gift of Dr. John Cullen (University of Rochester Medical Centre, Rochester NY)

Promega (Madison, WI)

Taq DNA Polymerase

MLV-RT

RNase H

Oligo dT

Sarstedt (Drinagh, Wexford, Ireland)

T25 tissue culture flasks

T75 tissue culture flasks

T175 tissue culture flasks

6-well tissue culture plates

2,5,10 and 25ml serological pipettes

Sigma Chemical Company (Tallaght, Dublin, Ireland)

2-mercaptoethanol

Acetic Acid

Acetone

Agarose

Ammonium Persulphate

Angiotensin II

Apocynin

Bisacrylamide

Bovine Serum Albumin

Brightline Haemocytometer

Bromophenol blue

Chloroform

Catalase

Dapi Nuclear Stain

EDTA

EGTA

Ethidium Bromide

Fibronectin

Ferricytochrome C

Foetal Calf Serum

Bovine Gelatin

Glycerol

Glycine

Hanks Balanced Salt Solution

Hydrochloric acid

Hydrogen Peroxide

Isopropanol

Leupeptin

Methanol

N-Acetyl Cysteine

Penicillin-Streptomycin (100x)

Ponceau S

Potassium Chloride

Potassium Iodide

Potassium Phosphate (Dibasic)

Phosphatase Inhibitor Cocktail

RPMI-1640

Sodium Chloride

Sodium Hydroxide

Sodium Orthovanadate

Sodium Phosphate

Sodium Pyrophosphate

SDS

Superoxide Dismutase

Syber Green Jumpstart Taq-ready PCR Mix

TEMED

Tris Acetate

Tris Base

Tris Cl

Triton X-100

Trypsin-EDTA solution (10x)

Tween 20

Upstate Cell Signaling Solutions (Chicago, USA)

Anti-THOP antibody

Zymed Laboratories Inc (California, USA)

Anti-ECE antibody

2.2 Cell Culture Methods

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

2.2.1 Culture of bovine aortic endothelial cells (BAECs)

Differentiated BAECs were obtained from Coriell Cell Repository, New Jersey, USA. (CAT NO: AG08500): The cells are derived from a one-year-old male Hereford bull. 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. Cells were cultured in T175 cm^2, T75 cm^2, T25 cm^2 and 6 well plates. Cells between passage 8 – 14 were used in these experiments.

BAECs possess a distinct cobblestone morphology when grown to confluence. They form a contact-inhibited monolayer of strongly adherent cells. As such trypsinisation was necessary for sub-culturing or harvesting of cells. For trypsinisation, growth media was removed from the flask and the cells were gently washed three times in Hanks Buffered Saline Solution (HBSS) to remove α-macroglobulin, a trypsin inhibitor present in FBS.

A suitable volume of trypsin/ethylenediamine tetracetic acid (EDTA) (10% v/v trypsin EDTA in HBSS) was added to the flask and incubated for 3 min or until the cells began to detach from the flask surface. Trypsin was inactivated by the addition of FBS containing-growth medium and the cells were removed from suspension by centrifugation at 3500 rpm for 5 min. Cells were then re-suspended in culture medium and typically diluted 1:5 into culture flasks, or cryogenically preserved. Cells were incubated in a humidified atmosphere of 5% v/v CO_2 at 37°C.

2.2.2 Cryogenic preservation and recovery of cells

For long-term storage of cells, BAECs were maintained in liquid nitrogen in a cryofreezer unit. Cells to be stored were centrifuged following trypsinisation and the resultant pellet was re-suspended in 20% v/v FBS containing dimetylsulphoxide (DMSO) at a final concentration of 10% v/v. 1ml aliquots were transferred to sterile cryovials and frozen in a –80°C freezer at a rate of –1°C/minute using a Nalgene cryo-freezing container. Following overnight freezing at –80°C, the cryovials were transferred to a cyrofreeze unit (Thermoylen Locator Jr. Cryostorage System). Cells were recovered from long-term storage by rapid thawing at 37°C and re-suspension in 5 ml of growth medium followed by centrifugation at 3500 rpm for 5 min. The resultant cell pellet was carefully re-suspended in fresh medium and transferred to a culture flask. The following day, media was removed and cells were washed in HBSS and fresh culture media added.

2.2.3 Cell counting

Cells counting was 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, the mixture was left to incubate for 2 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:

$$\textit{Average Cell No. x Dilution Factor x } 1x10^{4} \textit{ (area under cover slip } mm^{3}\textit{)} = \textit{Viable cells/ml}$$

2.2.4 Applied shear stress

For laminar shear stress studies, BAECs were seeded onto cell culture grade 6-well plates and allowed to adhere for 24 h and grown to 90% confluency. Following this, media was removed and replaced with 4 ml fresh culture media before exposure to laminar shear stress. Plates were then firmly fixed to the rotating platform of an orbital shaker (Stuart Scientific Mini Orbital Shaker SO5) using autoclave tape. The exact measurement of applied shear stress was not taken but could be estimated from the following equation which relates fluid viscosity and rotation to shear stress in the measurement of dynes/cm^2.

A suitable RPM to produce an estimated shear stress of 10 dynes/cm^2 was determined from the equation (Hendrickson *et al.*, 1999):

$$\textit{Shear Stress} = \alpha v \rho n (2\pi f)^3$$

Where α = radius of rotation in cm

ρ = density of liquid in g/l

n = liquid viscosity 7.5 X 10^{-3} dynes/cm^2 @ 37°C

f = rotation per second

Control plates containing un-sheared "static" endothelial cells were cultured in the same incubator but on a differing shelf to avoid vibrations caused by the orbital shaker. Studies were carried out for differing periods of time (0-xh, 0 - 10dynes/cm^2). Following shearing, the media was removed and stored for analysis, and cells either; (i) harvested for Real-Time PCR measurement of mRNA expression levels; (ii) harvested for Western blotting to determine protein expression levels; (iii) or fixed *in situ* for immunocytochemical analysis of protein expression and localization. Cells were also examined for intra- and extra-cellular ROS production under the above sheared and un-sheared conditions (see section 2.2.5).

2.2.5 Measurement of reactive oxygen species

Cells were analysed for the production of superoxide anion in response to shear stress, a potent, if short-lived signaling molecule and the precursor to most reactive oxygen species (ROS) found in the endothelial cell.

Extracellular release of superoxide was measured by the assessment of the reduction of ferricytochrome C to ferrocytochrome C with the reaction monitored spectrophotometrically at 550nm. Briefly, cells were cultured in phenol red-free RPMI-1640, so as to avoid false positive readings. Upon commencement of shear, 2 ml samples of the media were extracted every ten minutes for up to 4 h. Ferricytochrome C was added to each extracted sample to a final concentration of 65 μM. This was incubated at 37°C for 5 min to allow sufficient time for the production of ferrocytochrome C. The resultant solution was read in the spectrophotometer at 550 nm. Results were expressed as a change in absorbance at 550 nm as compared to that of static control cells.

Intracellular production of ROS was assessed through the use of dihydroethidium, a fluorescent dye which is superoxide anion-specific. This cell-permeable dye produces a weak blue-fluorescent cytoplasm in its reduced state. Upon oxidation by superoxide anion, the strong red/orange-fluorescent ethidium is produced, which permanently binds to the nucleus.

In order to assess the production of ROS over time using this method, cells were visualized using standard fluorescent microscopy (Olympus BX50). Briefly, cells were cultured in 6-well plates until confluent. At t = 0, one well from both the static and shear plates was visualized for the presence of superoxide anion. Media was then removed, cells were washed twice with 1 X PBS and then incubated with 10 μM dihydroethidium for 15 min. Cells were then washed again and immediately visualized. Cells were subsequently exposed to shear at 10 dynes/cm^2 with ROS visualization at t=1,2,3 and 4 h. An increase in the prevalence of strongly red/orange-fluorescent nuclei was taken as an increase in superoxide anion production.

2.2.6 Induction of reactive oxygen species

In order to examine the role played by ROS in the shear-induced regulation of TLZM expression, the production of differing oxygen species were induced under static conditions. For oxidative state studies, BAECs were cultured to a minimum of 80% confluence. Immediately prior to induction of the oxidative state, cells were gently washed once in HBSS to remove any ROS that may have accumulated in the media during incubation. Working solutions of ROS inducers were made up in culture media supplemented with FBS and antibiotics.

Hydrogen peroxide is a readily available and highly stable source of ROS within the endothelial cell. It not only contributes to the production of other less stable ROS, namely superoxide anion, but is itself classed as a reactive oxygen molecule. In order to produce a transient oxidative state to mimic the "burst" of ROS production typically observed upon commencement of shear, cells were incubated with 10 μM Hydrogen peroxide for 24 h. The hydrogen peroxide-induced sub-toxic oxidative state dissipates within hours due to antioxidant defense mechanisms endogenous to the endothelial cell thus accurately mimicking an oxidative burst. Following ROS induction, cells were harvested after 24 h for Real-Time PCR measurement of mRNA expression levels.

To specifically induce the production of superoxide anion, cells were incubated with 0.1 μM Angiotensin II (Ang II). Ang II is a known activator of NAD(P)H oxidase, an important source of ROS in the endothelium, via the Ang II AT 1 receptor. In order to accurately mimic an oxidative burst, cells were incubated with Ang II for 8 h, a shorter incubation time was required as Ang II is not as readily broken down as is hydrogen peroxide. Following ROS induction, cells were harvested for Real-Time PCR measurement of mRNA expression levels.

2.2.7 Treatment with anti-oxidants and pharmacological inhibitors

Prior to treatment with anti-oxidants or pharmacological inhibitors, cells were cultured for at least 2 passages. For these studies, BAECs were grown until approximately 70-80% confluent, after which the growth media was removed and cells rinsed 3 times in HBSS. Inhibitors and anti-oxidants were reconstituted in a suitable diluent. Working concentrations were then made in RPMI-1640 supplemented with FBS and antibiotics. For DMSO-soluble compounds, a suitable stock concentration was prepared so that the final concentration of DMSO in working solutions was less than 0.5%.

For anti-oxidant studies, working solutions were made up fresh and adjusted for pH immediately prior to incubation. N-Acetyl-L-cysteine, a broad spectrum anti-oxidant was applied at a concentration of 5 mM, whilst the hydrogen peroxide-specific catalase and the superoxide anion-specific superoxide dismutase (SOD) were applied at 1000 U/ml and 100 U/ml respectively. All anti-oxidants were prepared in complete culture media and applied to the pre-washed cells for 1 h immediately prior to the commencement of shearing and subsequently for the duration of the experiment.

Studies to explore shear-dependent signal transduction pathways employed the following pharmacological inhibitors: pertussis toxin (100 ng/ml) to inhibit Gi_a-subunit activation, apocynin (10 mM) which inhibits activation of NAD(P)H oxidase, NSC23766 to inhibit activation of Rac1 (50 μM), linear RGD (0.5 mM), cyclic RGD (100 μM) peptides which inhibit signal transduction through integrins and genistein (50 μM) to inhibit protein tyrosine kinases. These studies mirrored those of the anti-oxidants, in that inhibitors were prepared in complete culture media and applied to the pre-washed cells for 1 h immediately prior to the commencement of shearing and subsequently for the duration of the experiment. Concentrations used were gained from both current literature and manufacturers recommendations.

2.3 Western Blotting Analysis

2.3.1 Preparation of whole cell lysates

Cells were trypsinised as described in section 2.2.1, the cell pellet was washed in 1 X 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; 1 mM Na_2EDTA; 1 mM EGTA; 1% Triton X-100 v/v; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM sodium orthovanadate; 1 μg/ml leupeptin).

The resulting lysates were frozen and thawed three times followed by three cycles of ultrasonication for 5 seconds on ice using a sonic disembrator (Vibra Cell, Sonics and Materials Inc). Samples were stored at –20°C for short-term storage or –80°C for long-term storage.

2.3.2 Bicinchoninic Acid (BCA) protein microassay

In this assay, Cu^{2+} reacts with the 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 96-well plate was incubated at 37°C for 30 min and the absorbance read at 560 nm using a microtitre plate reader.

2.3.3 Western blotting

SDS–PAGE was carried out on prepared whole-cell lysates as described by Laemmli *et al.* using 10% polyacrylamide gels (Laemmli *et al.*, 1970). The protein concentration of each sample was determined by BCA assay and equal amounts of total protein were resolved in each well of the gel.

Firstly, a 4X loading buffer (8% SDS w/v, 20% β-mercatoethanol v/v, 40% glycerol v/v, Brilliant Blue R in 0.32 M Tris pH 6.8) was added to the protein samples, which were then boiled at 95°C for 5 min, and immediately placed on ice. The gel was subjected to electrophoresis in reservoir buffer (0.025M Tris pH 8.3; 0.192M Glycine; 0.1% (w/v) SDS) at 40 milliamps (mA) per gel using an Atto vertical mini-electrophoresis system until the samples were sufficiently separated in the gel, as indicated by the movement of the molecular weight marker.

10% resolving and 5% stacking gels were prepared as follows:

Resolving Gel:	1.5 ml	Buffer A (1.5 M Tris pH 8.8)
	1.5 ml	40% acrylamide stock
	3 ml	distilled water
	60 µl	10% (w/v) SDS
	30 µl	10% (w/v) ammonium persulphate
	7 µl	TEMED
Stacking Gel:	0.75 ml	Buffer B (0.5 M Tris pH6.8)
	0.375 ml	40% acrylamide stock
	1.85 ml	distilled water
	30 µl	10% (w/v) SDS
	15 µl	10% (w/v) ammonium persulphate
	7 µl	TEMED

Following electrophoresis the resolving gel was soaked for 15 min in cold transfer buffer (0.025 M Tris pH 8.3; 0.192 M Glycine; 15% v/v methanol). Nitrocelluose membrane and 16 sheets of Whatmann filter paper were cut to the same size as the gel and soaked in transfer buffer. Proteins were transferred to the membrane for 1 h at 100 V 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 any possible variations in protein loading.

Following washing, membranes were blocked for 2 h in blocking solution (5% w/v skimmed milk or BSA in Tris Buffered Saline (TBS), 10 mM Tris pH 8.0; 150 mM NaCl). Membranes were then incubated overnight at 4°C with primary antibody.

Primary antibodies were diluted in appropriate blocking solutions according to manufacturer's recommendations, with some modifications of the dilution factors used following optimization steps. The blots were then vigorously washed in two changes of TBST (0.05% v/v Tween in TBS) and then incubated for 3 h at room temperature with a suitable HRP-linked secondary antibody diluted in TBST. Following incubation in secondary antibody, the blots were again washed in two changes of TBST. Antibodies were diluted as indicated in Table 1.

Table 1. Primary and secondary antibody dilution factors

Target Protein	Primary Antibody Dilution Factor	Secondary Antibody Dilution Factor	Blocking Solution
eNOS	1/2000	1/5000	BSA
ACE	1/100	1/1000	Milk
ECE	1/100	1/200	Milk
NEP	1/50	1/500	BSA
EP24.15	1/100	1/1000	BSA

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 blot was incubated for 5 minutes at room temperature. Blots were exposed to autoradiographic film (Amersham Hyperfilm ECL) to visualize bands present on the blot and developed (Amersham Hyperprocessor Automatic Developer). Bands of interest were identified either by use of a commercial recombinant protein control or based on molecular weight markers. Exposure times varied depending on the antibody being used but were typically between 1-2 minutes. Results are expressed as fold change over static control as Ponceau S revealed equal loading between all samples.

2.4 Immunocytochemistry

In order to visually monitor the expression and/or subcellular localization of proteins, cells were prepared for immunocytochemical analysis as previously described by Groarke *et al* with some minor modifications (Groarke *et al.*, 2001). Cells were washed twice in phosphate buffered saline (PBS) and fixed with 3% formaldehyde for 15 min. Cells were subsequently washed, permeabilised for 15 min with 0.2% Triton X-100 and blocked for 30 min in 5% BSA solution. Following blocking, cells were incubated with the appropriate primary antiserum or stain as indicated in the below table, followed by 1 h incubation with 1:400 dilution of either Alexa Fluor 488 anti-mouse, anti-rabbit, or anti-rat fluorescent secondary antiserum. Nuclear DAPI staining was routinely performed by incubating cells with 0.5×10^{-6} µg/ml DAPI for 3 min. Cells were sealed with coverslips using DAKO mounting media (DAKO Cytomation, Cambridgeshire UK) and visualized by standard fluorescent microscopy (Olympus BX50).

Table 2. Immunocytochemical antibody dilution factors

Primary Antiserum/Stain	Concentration/Dilution	Time (h)
ACE	1/100	3
ECE	1/50	3
NEP	1/50	3
EP24.15	1/100	3
Rhodamine Phallodin	1/400	0.6

2.5 Nucleic Acid Studies

2.5.1 RNA isolation

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

Cells were lysed directly in culture plates by the addition of 1 ml of Trizol per 10 cm^2. A volume less than this can result in contamination of the RNA with DNA. To ensure complete homogenization, cells were lysed by passing through a pipette a number of times. The samples were then incubated for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complexes. 0.2ml of chloroform was added per ml of Trizol reagent used and was then mixed vigorously for 15 seconds before being incubated for 5 minutes at room temperature. Samples were then centrifuged at 12,000xg for 15 minutes 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 1 ml of Trizol used.

Samples were incubated for 15 min at room temperature and then centrifuged at 12,000xg for 10 minutes 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 7,500xg 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 below.

2.5.2 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 50μg/ml solution of DNA or 40μg/ml solution of RNA has an absorbence 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 - $Abs_{@260nm}$ x50x200 (dilution factor, i.e. 5μl of sample in 995μl H_2O) = μg/ml

RNA - $Abs_{@260nm}$ x 40 x 200 (dilution factor, i.e. 5μl of sample in 995μl H_2O) = μg/ml

The purity of the DNA or RNA samples was established by reading the absorbence at 260nm and the absorbence at 280nm and then determining the ratio between the two (ABS_{260}/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.

2.5.3 Reverse Transcription

Initially reverse transcription was preformed 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 was mixed with 0.125 μg oligo dT 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 to oligo dT 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:

MLV 5X Reaction Buffer	5 μl
10 mM dNTP	3 μl
MLV-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 1 μl of RNase H (2 units/μl) at 37°C for 20 min.

Samples generated through this method were used for primer optimization using standard PCR as described in section 2.5.4.1.

Copy DNA (cDNA) was also synthesized from messenger RNA (mRNA) using the commercially available iScript™ cDNA synthesis kit (Bio-Rad Laboratories, California, USA). Samples generated by this kit are generally of higher quality than those generated through the previous method and were used in all analysis of gene expression. Each RT reaction was made up to a total of 20µl as follows:

5X reaction buffer	4 µl
Reverse transcriptase enzyme	1 µl
mRNA	2 µg

The reaction solution was gently mixed to ensure complete homogenization of the reaction solution without causing damage to the delicate mRNA. The reaction mixture was then subjected to one RT cycle using a PCR Sprint Thermo-cycler (Thermo Electron Corporation – Massachusetts, USA). The RT cycle consisted of three phases as follows:

i) 25°C for 5 min

ii) 42°C for 30 min

iii) 85°C for 5 min

2.5.4 Polymerase Chain Reaction (PCR)

2.5.4.1 Semi-quantitative standard PCR

Standard PCR was used to optimize primer sets and to rule out primer dimerisation before their use in quantitative Real-Time PCR. 50 µl PCR reaction mixtures were 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 (SPRT 001). 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 (55-65°C) 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.

2.5.4.2 Quantitative Real-Time PCR

Quantitative Real-Time PCR was carried out using a Real-Time Rotor-GeneRG-3000^{TM} Lightcycler (Corbett Research) in conjunction with Syber Green Jumpstart Taq-Ready PCR kit. SYBR Green is a fluorescent dye which, when bound to the minor groove of double stranded DNA, emits fluorescence. The fluorescence is directly proportional to the amount of cDNA product formed during the PCR cycles. cDNA was amplified for the target sequences of interest, as indicated in the table below. All data was normalized against the untreated GAPDH sample in that GAPDH is known to be non-responsive to shear stress and hence was an indicator of equal cDNA loading in all other samples. Results were expressed as a fold change in the expression level of the target gene of interest under applied shear stress as compared to static un-sheared controls of that same gene.

Target Gene	Forward and Reverse Primer Sequences	Product size	Annealing Temp (°C)
ACE	5` aca aga ctg cc acct gct gg 3` 5` agg cat gga ggt tca ggt ag 3`	350 bp	60°C
ECE	5` gga cct tca gca acc tct gg 3` 5` gtg tcc tgg aag ttg tcc tt 3`	385 bp	60°C
NEP	5` caa agc caa aga aga aac ag 3` 5` cat ctc tta aaa tgt caa ag 3`	325 bp	55°C
EP24.15	5` tga agg tca ccc tca agt a 3` 5` tcc acc tgg ttc atg tag ta 3`	401 bp	55°C
EP24.16	5` tgg aaa ctg act ttg tag agg 3` 5` aaa agt agc tgg cat att tgt 3`	308 bp	55°C
GAPDH	5` agg tca tcc atg acc act tt 3` 5` ttg aag tcg cag gag aca a 3`	337 bp	54°C

Each reaction was set up in triplicate as follows:

SYBR Green total reagent	12.5 μL
DEPC Water	8.5 μL
Forward Primer	1.0 μL
Reverse Primer	1.0 μL
cDNA	2.0 μL

The following PCR cycle program was used with the appropriate annealing temperature inserted for the gene of interest.

Denature		95^0C	15 min	
Cycling	Denature	95^0C	20 s	45 cycles
	Annealing	59^0C	30 s	
	Extension	72^0C	30 s	
Hold		60^0C	1 min	
Melt		50 – 100^0C		

Immediately following all reactions, melt curve analysis was carried out to ensure single product formation and to rule out possible contamination of product by non-specific binding and primer dimerization. Samples from both standard and real-time PCR reactions were then analysed for same using agarose gel electrophoresis.

2.5.5 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 being visualised. Gels contained 0.5 μg ethidium bromide per 1 ml of agarose for DNA visualization. When the gel mixture was hand-hot the gel was cast in a GibcoBRL 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 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, typically 2-3 h. 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).

2.6 DNA preparation methods

2.6.1 Transformation of competent cells

10 ng of plasmid DNA of interest was placed in a sterile microfuge tube. To this was added 100 µl of competent JM109 E.C*oli*. The mixture was gently mixed and placed on ice for 30 min. The cells were heat-shocked by placing the tube in a water-bath at 42°C for 45-50 seconds after which they were placed on ice for 2 min.

Transformed cells were grown for 1 h at 37°C with agitation (200 rpm) in 1 ml 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 6000 rpm for 1 min and the supernatant removed. The resultant pellet was resuspended in 0.2 ml of LB broth and spread-plated either 150 µl or 50 µl on LB agar plus ampicillin (LB medium containing 1.5% w/v agar plus 35 µg/ml ampicillin). 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.

2.6.2 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 35 μg/ml ampicillin. These mini-cultures were grown at 37°C for 8 h with gentle agitation (<200rpm). 1.5 ml of the final culture was used for the generation of glycerol stocks. The remainder was diluted in 100 ml of LB broth supplemented with 35 μg/ml ampicillin and grown at 37°C overnight at 250 rpm. The following day the cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C.

Pellets were 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 gently 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 (3 M potassium acetate, pH 5.5), gentle mixing and incubation on ice for 5 min. Protein precipitates were removed by high-speed centrifugation, (13000rpm for 30 min at 4°C). The supernatant was removed and re-centrifuged at 13,000 rpm for 30 min at 4°C to ensure complete removal of all protein.

Final supernatant was applied to a Qiagen tip-100 pre-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 (1 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol). Finally, DNA was eluted from the column using 5 ml of Buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.3; 15% isopropanol).

DNA was precipitated from the eluate by the addition of 3.5 ml of isopropanol at room temperature. The mixture was centrifuged at 12,000 rpm for 30 minutes at 4°C, to yield a glassy pellet. The supernatant was removed carefully so as not to disturb the isopropanol/DNA pellet. The pellet was then washed in 2 ml of 70% ethanol, to remove precipitated salts and to make reconstitution of the pellet easier; this was then centrifuged at 12,000 rpm for 10 min. The final pellet was air-dried for 5–10 min after removal of the supernatant and then re-dissolved in sterile Tris-EDTA (TE) buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA).

2.6.3 Restriction digests

DNA was digested with restriction endonucleases for identification of purified plasmid DNA. 1µg of DNA was prepared in a reaction solution containing 10X reaction buffer (supplied with each enzyme, by the manufacturer) along with 1µl of restriction enzyme.

The digest was incubated at a suitable temperature (enzyme-specific) for 30 minutes 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.

2.6.4 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, $1.5x10^6$ cells were plated on a T25cm^2 flask 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, ß-Ark plasmid DNA was diluted in 150 µl of DMEM without FCS or antibiotics such that there would be 1 µg of DNA per 10 cm^2 of surface area. In a separate tube 10 µl of lipofectamine reagent (4 µl per 10 cm^2) 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 2 ml, which is just enough media to cover the surface area of the flask. The contents of the tube were then added to the culture flask. The cells were incubated for 4 h in transfection medium, following this, the medium was removed and replaced with normal RPMI-1640 growth medium.

The cells were allowed to recover overnight, following which they were exposed to experimental conditions. Cells were routinely co-transfected with Lac Z encoding plasmid as a means to determine approximate levels of transfection.

2.6.5 β-galactosidase assay

Lac Z a plasmid encoding β-galactosidase was used to monitor transfection levels. 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 $MgCl_2$ 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 0.1 M 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 Na_2CO_3, and optical density read at 420 nm.

Chapter 3 Results - Section 1

3.1 Introduction

As previously discussed, the endothelium is a dynamic cell interface between the vessel wall and the blood stream. The unique position of the endothelium confers on it the ability to sense and respond to a variety of blood-borne components. Among these are included peptide hormones, neurotransmitters and growth factors, acting in a paracrine, autocrine or endocrine fashion. Associated peptidases that have the ability to regulate vasoactive peptide levels are of primary importance in the endothelium and its many roles in the vasculature. These components effect both biophysical and biochemical changes on the endothelium which are, in turn, translated into a pronounced response within the vessel wall (Cummins *et al.*, 2004).

In addition to circulatory components, blood flow itself imparts a strong influence on the vessel wall. Of particular importance are the mechanical forces of cyclic strain and shear stress. Cyclic strain is a circumferential force that acts outwardly on the vessel wall as a result of pulsatile blood flow, resulting in a stretching of the cells of the endothelium. This stretch has been shown to influence a number of EC functions, namely eNOS and ET-1 expression (Awolesi *et al.*, 1995; Cheng *et al.*, 1996). Furthermore, cyclic strain also has the ability to regulate cell fate decisions, playing a role in apoptotic pathways, through regulation of p53 and Akt phosphorylation (Mayr *et al.*, 2002; Persoon-Rothert *et al.*, 2002; Haga *et al.*, 2003).

Concomitantly cyclic strain functions to increase the sensitivity of the endothelium to another important hemodynamic force, shear stress. Shear stress is a frictional or tractive force caused by blood flow that acts directly on the EC membrane. Due to the action of shear stress on the cellular membrane, it allows for the rapid transfer of extracellular signals into the cellular space and the induction of appropriate cellular responses. Of primary importance among these responses is the ECs ability to modulate expression of a number of vasoactive molecules.

Vascular tone, referred to as the level of constriction versus dilation that a vessel experiences, is regulated by a number of competing vasoconstrictor and vasodilatory influences (e.g. NO and peptide hormones). The circulating levels of these molecules have been evidenced to be modulated by the mechanical force of shear stress (Cooke *et al.*, 1997; Morawietz *et al.*, 2000) indicating a primary role for shear stress in the regulation of vessel tone, although further study is clearly required to ascertain the precise mechanisms involved. Of the numerous endothelial systems that can influence vessel tone, the TLZM family of peptidases have recently fallen under the spotlight as possible clinical targets in the management of endothelial dysfunction and CVD. However, despite application of TLZM-specific pharmacological inhibitors in clinical treatments, little or no work has been done to fully elucidate the mechanisms by which the expression of these important peptidases is regulated in endothelial cells by blood flow-associated forces (a fundamental source of endothelial dysfunction).

This study will therefore focus on investigating the impact of shear stress on the expression of TLZM family members in the endothelium. With the exception of ECE and ACE, to our knowledge, no studies have explored the role played by shear stress in the regulation of other closely related members of this family namely, EP24.15, EP24.16 and NEP. It is hoped that, through the examination of these peptidases and the forces that putatively regulate their expression, we will obtain a greater understanding of their potential roles in endothelial dysfunction and vascular remodelling.

The aim of this chapter was to firstly characterise the cell type studied as endothelial. Secondly, we wanted to ascertain that the cells are indeed responsive to shear stress in the model employed. Finally we wanted to explore the role of shear stress in regulating expression of the TLZM family members;

- ACE (Angiotensin Converting Enzyme)
- ECE (Endothelin Converting Enzyme)
- NEP (Neprilysin)
- EP24.15 (Thimet Oligopeptidase)
- EP24.16 (Neurolysin)

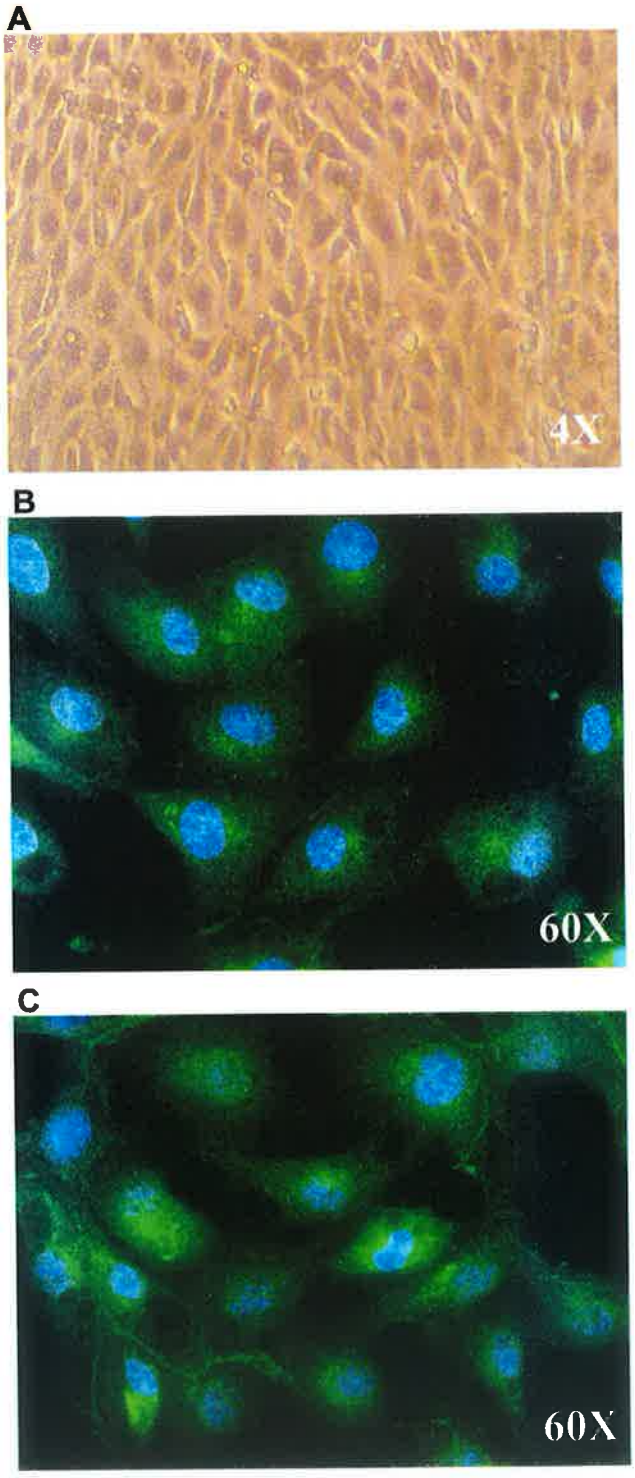
3.2 Results

3.2.1 Endothelial cell characterization

Upon commencement of this study, it was firstly important to correctly identify the cell type examined. In order to correctly identify the cells as those of the endothelium, three cell-specific characteristics were studied.

1. Gross cellular morphology
2. Endothelial cell specific marker, Von Willebrand Factor
3. eNOS

Using standard light microscopy, gross morphology of cells in culture was examined at 100% confluency. As can be seen in Fig 3.1 A, the cells displayed a distinct "cobblestone" morphology consistent with endothelial cells. Secondly, cells were cultured on glass coverslips in 6-well tissue culture plates, as described in section 2.2.1. Cells were then examined immunocytochemicaly as described in section 2.4, for the presence of two endothelial cell-specific markers, Von Willebrand Factor and eNOS. As evidenced in Fig 3.1 B and C, cells were positive for both markers, clearly indicating the cell type as that of endothelial cells.



Cobblestone Morphology

Von Willebrand Factor

eNOS Expression

Fig 3.1 Characterization of BAECs. Static BAECs were examined for endothelial cell-specific markers including; (A) Cobblestone morphology, (B) VonWillebrand Factor and (C) eNOS expression.

3.2.2 Endothelial cell response to shear stress

In order to validate the shear stress model (orbital rotation) employed in this study and to ensure that the ECs were indeed capable of sensing and responding to the level of applied shear. Cells were monitored after 24 h of applied shear estimated at 10 dynes/cm^2. In this regard, a number of previously published and well documented responses of the endothelium to shear stress were monitored. These included gross morphological and F-actin filament re-alignment and eNOS expression.

Control randomly aligned, uniformly polygonal BAECs were exposed to an applied shear stress (10 dynes/cm^2, 24 h) and examined under the light microscope for gross morphological realignment in the direction of the shear vector. As can been seen in Fig 3.2 B, cells realigned in the direction of flow, in contrast to the static cells found in Fig 3.2 A. Further examination focused on structural changes in the actin cytoskeleton of the cells. Using Alexia Phalloidin to stain the F-actin filaments of the cytoskeleton, static and sheared cells were examined using fluorescence microscopy (60X). The static cells in Fig 3.2 C show a disorganised cell wide distribution pattern of F-actin. The sheared cells in Fig 3.2 D show a dramatic realignment of the cytoskeleton in the direction of flow with cortical actin filaments clearly located at the periphery of the cell.

Finally cells were examined for shear-dependent changes in expression of eNOS. Many studies have shown a dramatic increase in both mRNA and protein expression of eNOS due to laminar shear.

Following applied shear stress (10 dynes/cm^2, 24 h), cells were examined by immunocytochemistry for eNOS, (Fig 3.2 E,F). Both static and shear samples were monitored. As can be seen in Fig 3.2 F, shear stress did produce a dramatic increase in eNOS within the endothelial cells as compared to static controls. To further examine the effects of shear stress in this context, eNOS mRNA and protein levels were examined. Following exposure to shear stress (10 dynes/cm^2, 24 h), eNOS mRNA expression, as measured by Real-Time PCR, increased by 2.3±0.2 fold compared to static controls (Fig 3.3 A). A similar increase in eNOS protein expression was observed by western blotting (2.1±0.02 fold, Fig 3.3B).

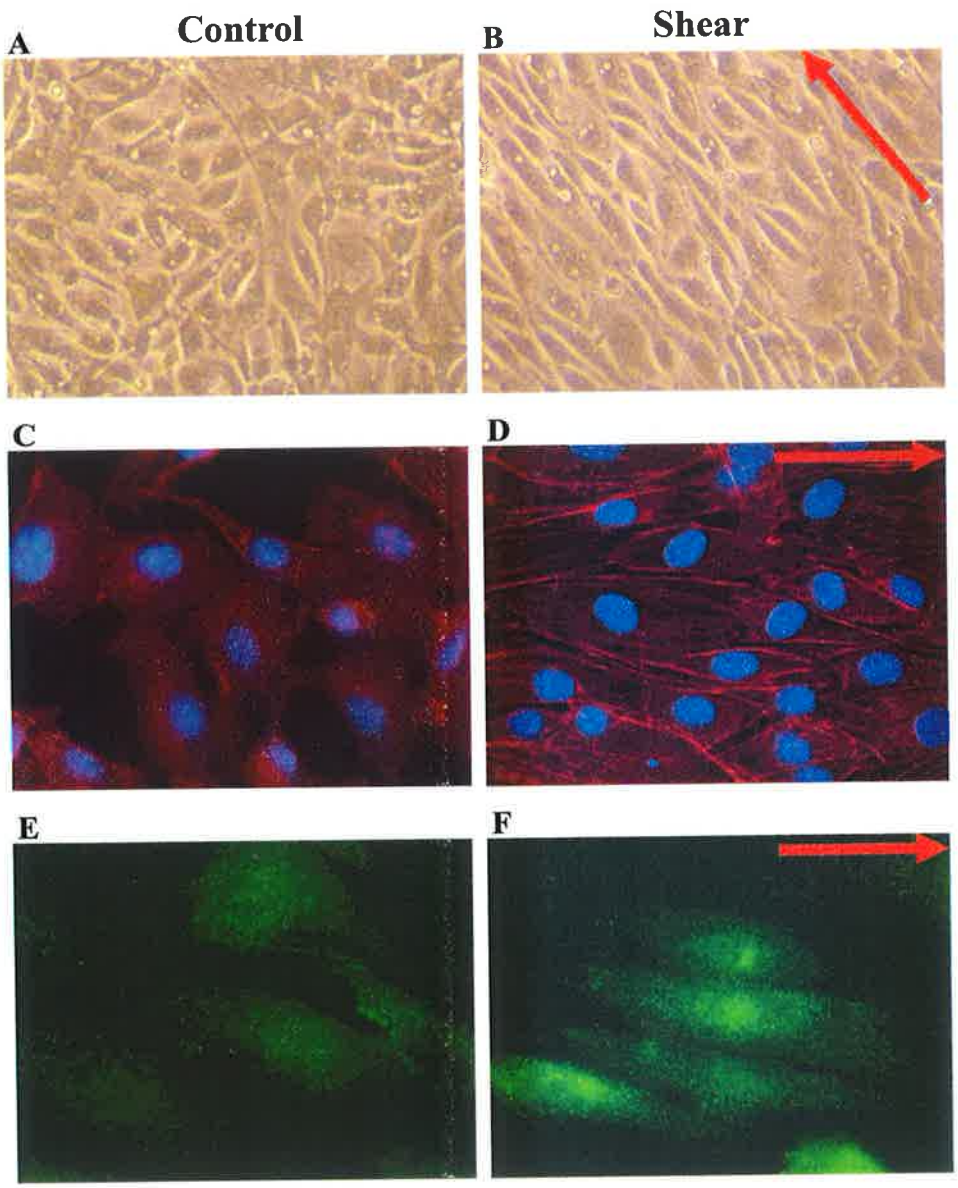


Fig 3.2 Imaging of shear stress responses in BAECs. Following shear stress (24 h 10 dynes/cm^2) cells were imaged to assess shear-dependent characteristics. Brightfield imaging of (A) static and (B) sheared cells. F-Actin staining of (C) static and (D) sheared cells. eNOS immunocytochemical staining of (E) static and (F) sheared cells. Red arrows indicate the direction of flow. Images are representative of three independent experiments.

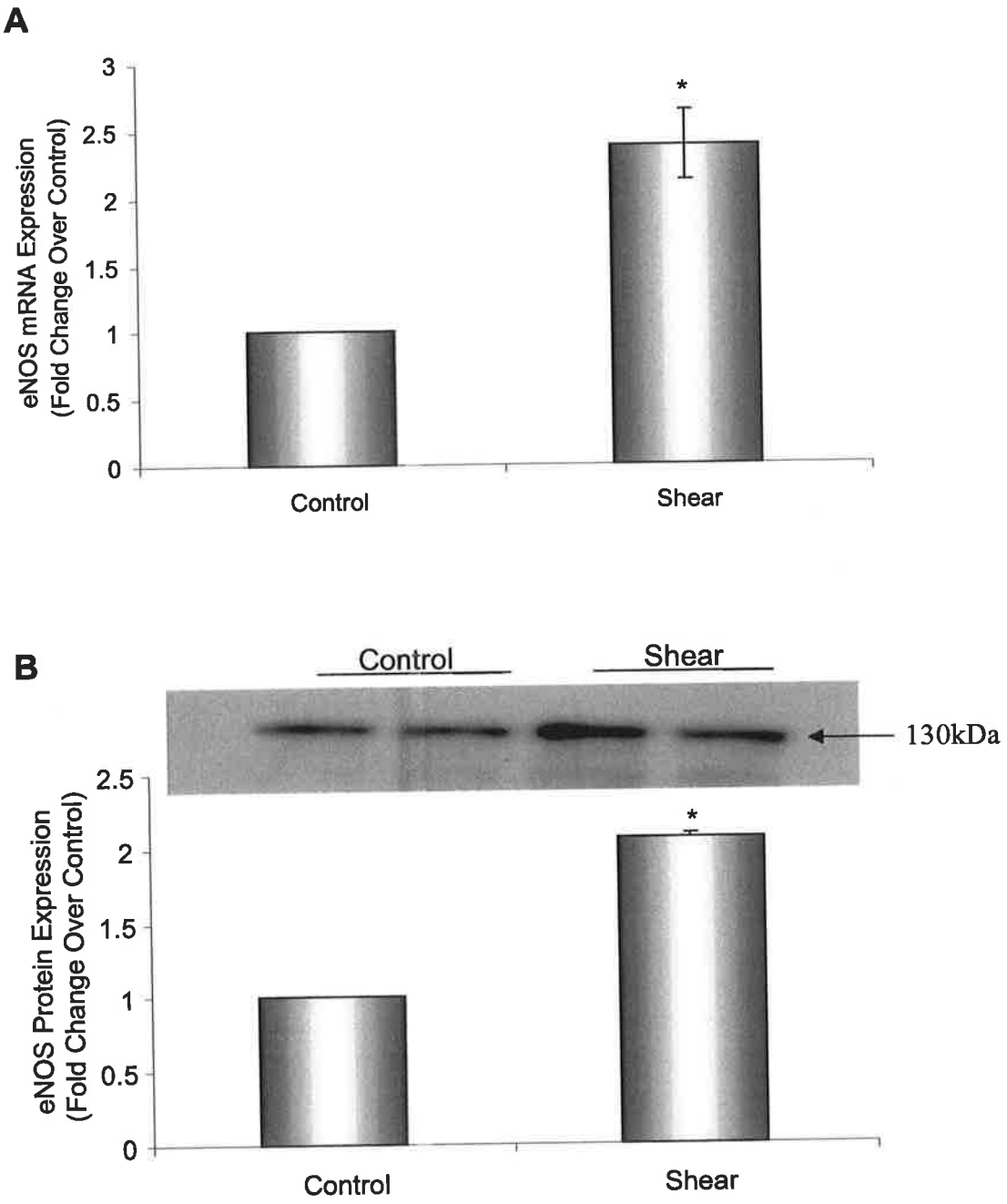


Fig 3.3 Shear-dependent modulation of eNOS mRNA and protein expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) and monitored for (A) mRNA expression using Real-Time PCR and (B) protein expression by Western blotting. Histogram in (A) represents fold change in mRNA expression level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control. Histogram in (B) represents fold change in band intensity level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control.

3.2.3 Shear stress-dependent changes in endothelial TLZM expression

The effect of laminar shear stress (10 dynes/cm^2, 24 h) on expression of a number of Thermolysin-like zinc metallopeptidases in BAECs was examined. These included the ectoenzymes ACE, ECE, NEP and the soluble peptidases EP24.15 and EP24.16.

Following 24 h shear, ACE mRNA levels were measured using Real-Time PCR and gene specific primers and were found to be attenuated to 0.7±0.03 fold of control (Fig 3.4A). A similar finding was observed for ACE protein levels, as measured by Western blotting, with attenuation to 0.65±0.01 fold of control (Fig 3.4B).

Following 24 h shear, ECE mRNA levels were measured using Real-Time PCR and gene specific primers and were found to be 1.109±0.05 fold of control (Fig 3.5A). Western blotting however showed a significant attenuation in protein levels to 0.69±0.04 fold of control (Fig 3.5B).

Following 24 h shear, NEP mRNA levels were measured using Real-Time PCR and gene specific primers and were found to be attenuated to 0.39±0.04 fold of control (Fig 3.6A). A similar finding was observed for NEP protein levels, as measured by Western blotting, with attenuation to 0.51±0.04 fold of control (Fig 3.6B).

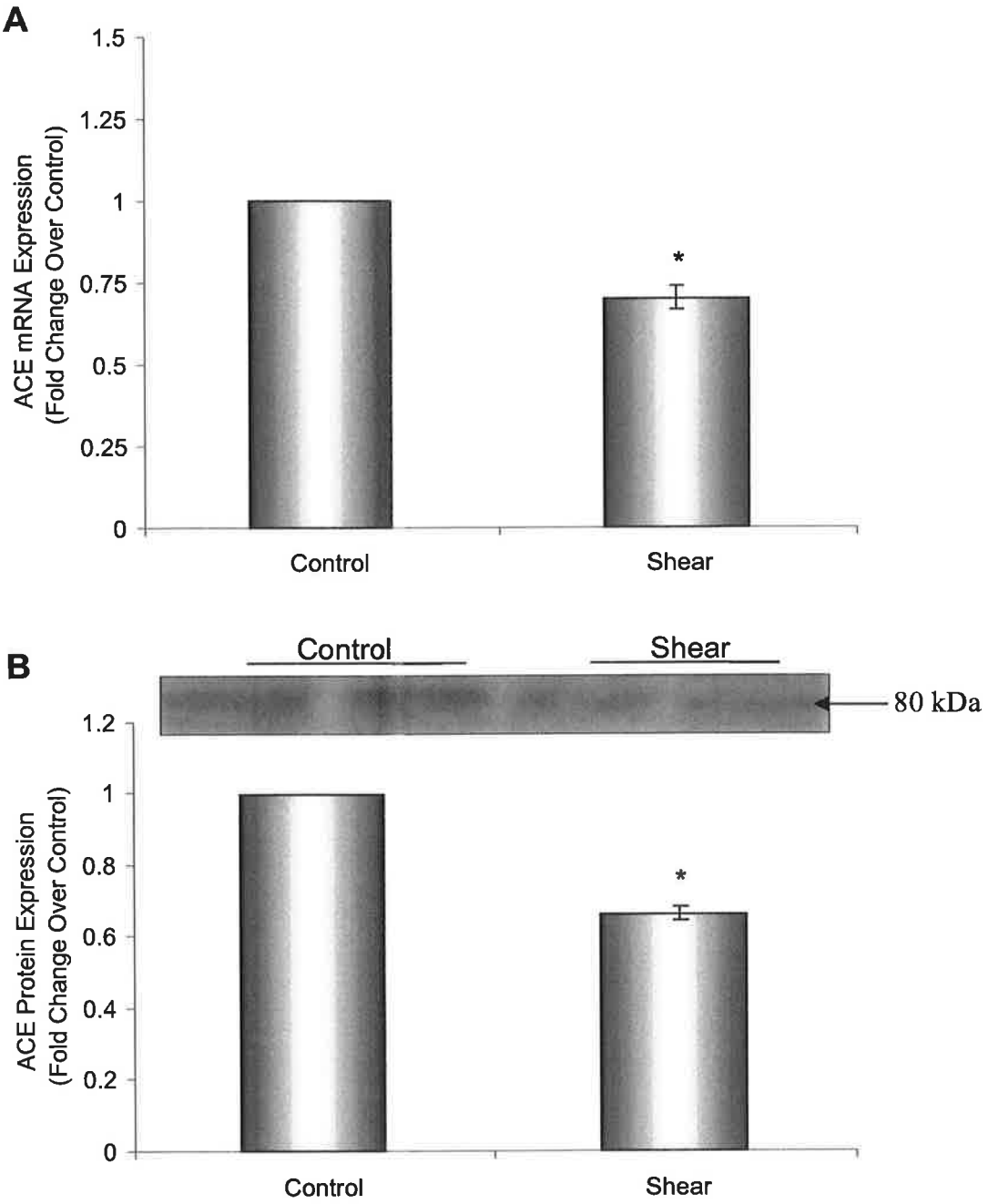


Fig 3.4 Shear-dependent modulation of ACE mRNA and protein expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) and monitored for (A) mRNA expression using Real-Time PCR and (B) protein expression by Western blotting. Histogram in (A) represents fold change in mRNA expression level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control. Histogram in (B) represents fold change in band intensity level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control.

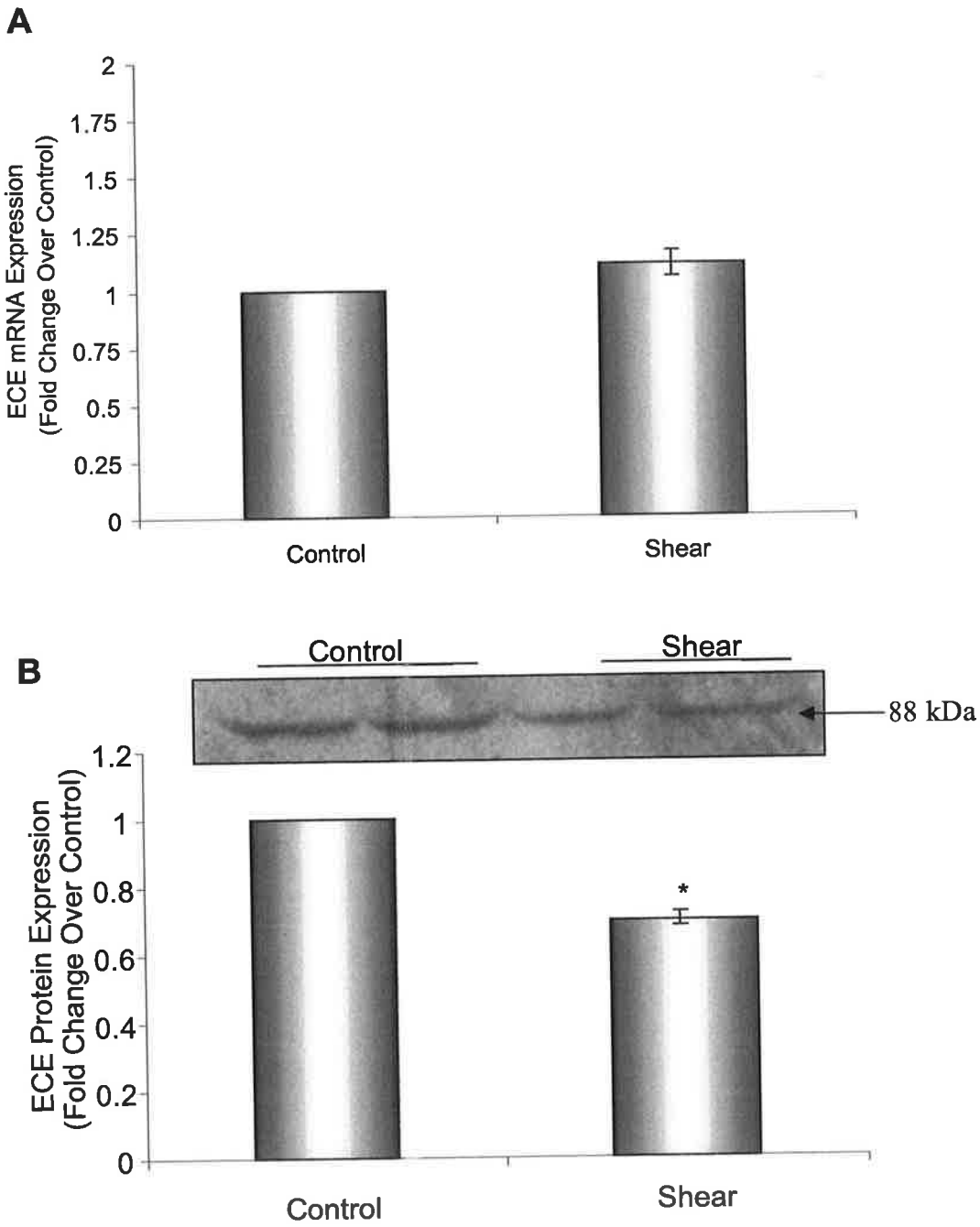


Fig 3.5 Shear-dependent modulation of ECE mRNA and protein expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) and monitored for (A) mRNA expression using Real-Time PCR and (B) protein expression by Western blotting. Histogram in (A) represents fold change in mRNA expression level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control. Histogram in (B) represents fold change in band intensity level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control.

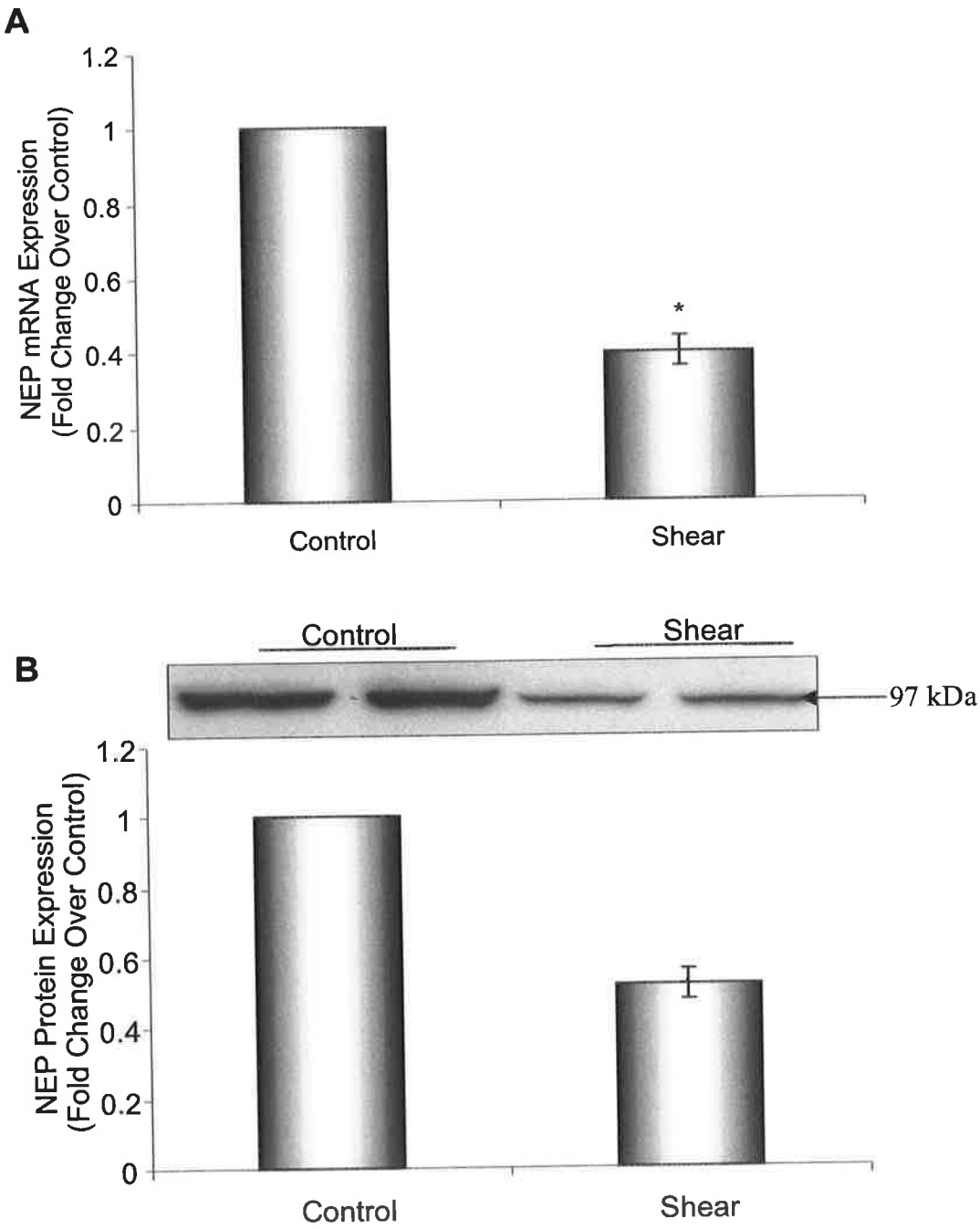


Fig 3.6 Shear-dependent modulation of NEP mRNA and protein expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) and monitored for (A) mRNA expression using Real-Time PCR and (B) protein expression by Western blotting. Histogram in (A) represents fold change in mRNA expression level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control. Histogram in (B) represents fold change in band intensity level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control.

Following 24 h shear, EP24.15 mRNA levels were measured using Real-Time PCR and gene specific primers and were found to be increased to 1.35±0.09 fold of control (Fig 3.7A). A similar finding was observed for EP24.15 protein levels, as measured by Western blotting, with protein levels increased to 1.6±0.05 fold of control (Fig 3.7B).

Following 24 h shear, EP24.16 mRNA levels were measured using Real-Time PCR and gene specific primers and were found to be attenuated to 0.67±0.06 fold of control (Fig 3.6A). In the absence of a commercially available antisera, we relied entirely on mRNA measurements gathered from Real-Time PCR.

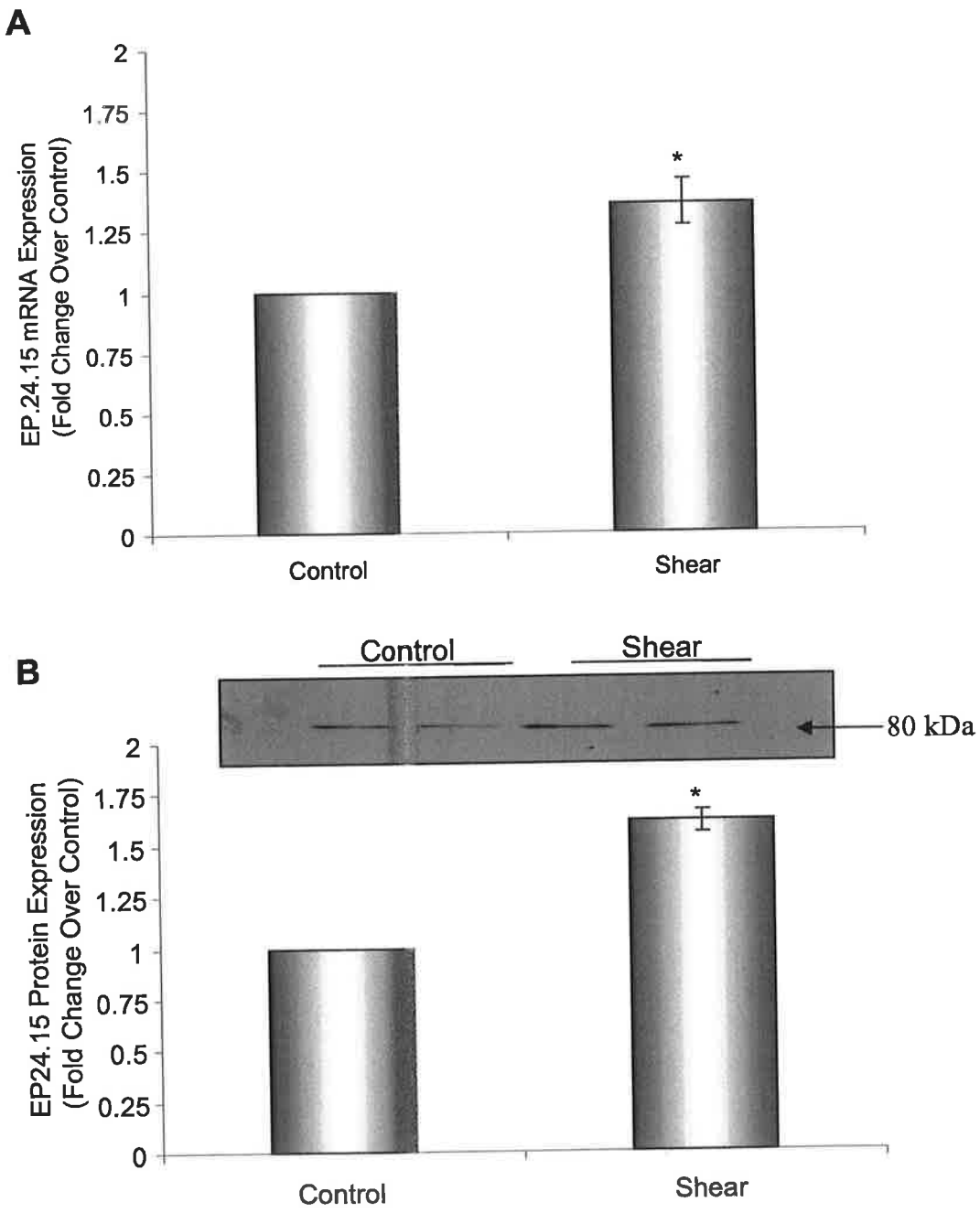


Fig 3.7 Shear-dependent modulation of EP24.15 mRNA and protein expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) and monitored for (A) mRNA expression using Real-Time PCR and (B) protein expression by Western blotting. Histogram in (A) represents fold change in mRNA expression level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control. Histogram in (B) represents fold change in band intensity level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control.

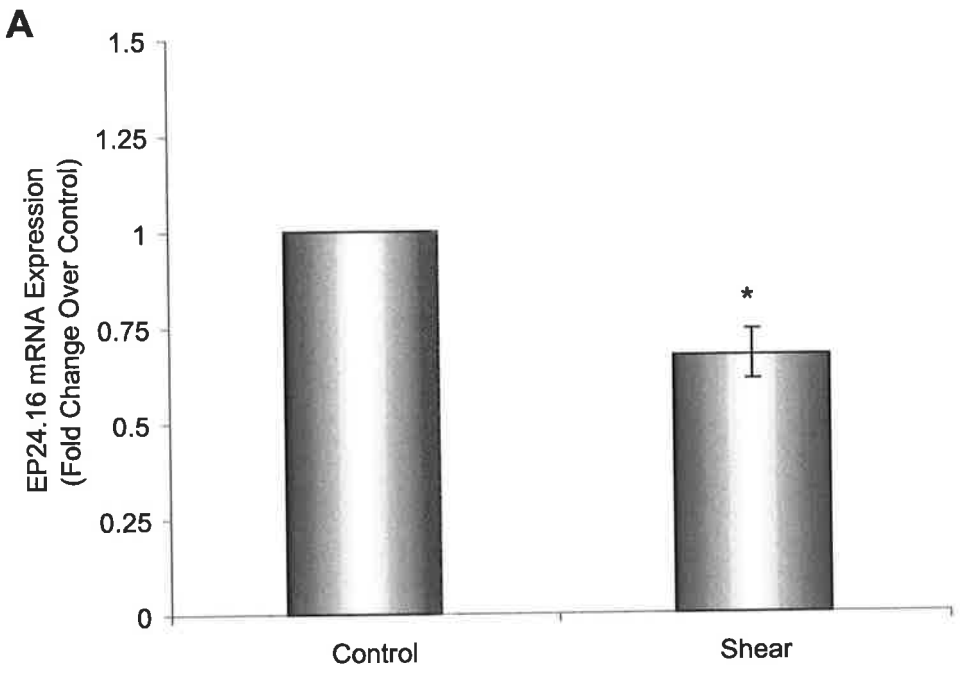


Fig 3.8 Shear-dependent modulation of EP24.16 mRNA expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) and monitored for (A) mRNA expression using Real-Time PCR. (A) represents fold change in mRNA expression level over static control and is averaged from three independent experiments ±SEM; *p=0.05 versus control.

3.3 Discussion

Our initial study focused primarily on establishing a shear stress model, which had the ability to impart upon cultured ECs a level of shear stress that was detectable by the cells, as evidenced by morphological and biochemical characterization. In this regard previous studies have demonstrated the responses of endothelial cells to similar levels of shear stress as employed in our model and have clearly evidenced actin cytoskeletal-driven morphological realignment in the direction of flow, with concomitant upregulation in the expression of shear sensitive genes such as those encoding eNOS (Noria *et al.*, 2004).

Application of shear stress (10 dynes/cm^2, 24 h) induced a clear morphological realignment of cells in the direction of flow was observed. With the aid of Alexia Phalloidin to stain the F-actin filaments of the cytoskeleton, we demonstrated that shear-induced morphological realignment occurred in parallel with actin realignment indicating the importance of cytoskeletal reorganisation in cellular responses to flow. It was concluded at this point that not only could the cultured cells sense the levels of shear stress being applied to them by our model, but also that they responded in an expected manner.

Upon commencement of shear (10 dynes/cm^2, 24 h), expression levels of both mRNA and protein for the known shear stress-responsive enzyme eNOS were measured.

After 24 h of shear stress, over a two-fold increase in both mRNA and protein expression of eNOS was observed. Again this confirms that the levels of shear stress applied in our model were sufficient to mimic other *in vitro* studies and induced responses also seen in *in vivo* models (Searles, 2006).

Next, we turned our attention to shear-mediated regulation of TLZM family members. A limited number of previous studies have shown shear stress responsiveness in members of this family, namely ECE and ACE (Morawietz *et al.*, 2000; Masatsugu *et al.*,2003; Rieder *et al.*, 1997). Indeed, the promoter region of the ECE gene is known to house at least four classic SSRE sequences. Studies following the publication of the ECE promoter region focused on possible regulation of expression by shear stress. Work carried out by Morawietz *et al.*, on HUVECs, have shown a clear shear stress-induced (30 dynes/cm^2, 24 h) attenuation of ECE mRNA to 36.2% of control (Morawietz *et al.*, 2000). Masatsugu *et al.*, have also shown a clear down-regulation in ECE expression due to shear stress. ECE mRNA levels were attenuated by shear stress (15 dynes/cm^2, 24 h) to 15% of control (Masatsugu *et al.*,2003). Following shear stress, a 31% reduction in the levels of ECE protein were measured in our laboratory, however, levels of ECE mRNA remained unchanged for the duration of the experiment. This disparity between studies and indeed between our mRNA and protein results is possibly due to the level of shear stress applied.

These previously mentioned studies employed higher levels of shear stress, thus is can be hypothesized that a higher level of shear stress was needed to alter mRNA expression. In addition protein levels could have been attenuated through a shear-induced increase in protein degradation systems. Attenuation of ECE expression under conditions of shear stress is a logical physiological response, in that ECE functions in the production of endothelin, a potent vasoconstrictive peptide. Lower levels of ECE will result in lower levels of circulating endothelin and hence reduce blood pressure and in turn lessen the level of shear stress experienced by the endothelium.

Previous work has also centred on ACE expression and the role of shear stress in its regulation (Rieder *et al.*, 1997). Like ECE, a classic SSRE was identified in the ACE promoter region. However, upon closer study this element was evidenced to be non-functional. A similar study did however identify two novel SSRE sequences within the promoter region. These sequences are thought to be responsible for shear-induced attenuation of ACE expression and activity levels. In this study, Rieder *et al.*, demonstrated a shear-induced (20 dynes/cm^2,18 h) attenuation of both ACE mRNA and activity of 86% and 40.7% respectively in BPAECs. In our study, exposure of BAECs to laminar shear stress caused a 30% and 35% reduction in ACE mRNA and protein expression levels, respectively.

Whilst greater levels of shear-induced ACE attenuation have been published, we hypothesized that, in a similar fashion to ECE, these disparities were as a result of the differences in the level of shear stress applied and indeed could also be attributable to the differing cell types employed. Again, shear stress induced attenuation of ACE expression is a method by which the vasculature alters levels of vasoconstrictor peptides. ACE functions in the production of Ang II, a potent vasoconstrictor. With shear stress attenuating ACE expression, a concomitant reduction in circulating Ang II would be expected, resulting in an overall reduction of vasoconstrictive peptides and hence lower blood pressure and shear stress experienced by the vasculature.

Expression levels of the vasoactive peptidase NEP was also examined for sensitivity to shear stress. Previously it has been shown that the promoter region of the NEP gene does contain a classic SSRE sequence, similar to those found in the ECE gene promoter region (Kim *et al.*, 2003, Li *et al.*, 1995). In our study, exposure of BAECs to laminar shear stress caused a 61% and 49% reduction in NEP mRNA and protein expression levels, respectively. To our knowledge, this is the first study to indicate a role for shear stress in regulation of NEP expression in the vasculature. NEP has been shown to act by degrading a number of vasodilatory peptides, such as those of the natriuretic peptide family. Hence it is possible to conclude that the reduction in expression of both NEP mRNA and protein is a mechanism through which the endothelium regulates vessel tone, such that, lower levels of NEP will result in potentation of the vasodilatory effects of the natriuretic peptides.

Other members of the TLZM family have also been examined for shear-dependent regulation in BAECs, namely the related endopeptidases, EP24.15 and EP24.16. Recently published studies have shown for the first time that both peptidases are sensitive to regulation by cyclic strain. Circumferential strain causes an outward tension in the vessel wall, stretching the endothelial layer as blood flows through the vessel. Cotter *et al*, in 2003, conclusively demonstrated the sensitivity of EP24.15 and EP24.16 to cyclic strain (5%, 24 h), with increases in both peptidases of 2.3 and 1.9 fold, respectively. In addition, a classic SSRE sequence has been identified in the EP24.15 promoter region allowing us to hypothesize that this peptidase may also be sensitive to the hemodynamic force of shear stress. Following shear stress, we measured an increase of 1.35 fold and 1.6 fold, in expression of EP24.15 mRNA and protein levels respectively, suggesting a role for both hemodynamic forces in the regulation of EP24.15 expression within the vasculature. Indeed EP24.15 has been evidenced to degrade Ang I to Ang-(1-7), resulting in vasodilation (i.e. via a reduction in available Ang I for conversion to Ang II, and in the production of Ang-(1-7), a SMC relaxant).

Shear stress induced an attenuation of 33% in EP24.16 mRNA expression levels. Data relied only on mRNA readings due to the lack of a commercially available EP24.16 antisera. This reduction in expression of EP24.16 is a logical response to shear stress by the vasculature and likely reflects the peptidases' ability to degrade bradykinin (BK) a known potent vasodilator.

A study by Norman *et al,* indicated a role, predominantly for EP24.16, in BK degradation, with inhibition of EP24.16 resulting in potentation of BK-induced vasodilation in rabbits (Norman *et al.*, 2003). Thus a shear-dependent reduction in expression of EP24.16 would be expected to have a vasodilatory effect by maintaining enhanced BK levels. To our knowledge this is the first study to show shear stress regulation of vascular endothelial EP24.15 and EP24.16 expression levels.

3.4 Conclusion

We have clearly demonstrated the ability of cultured endothelial cells to not only sense but also respond to the hemodynamic force of shear stress in the BAEC model employed. We further sought to expand on previously published studies which show a role for shear stress in the regulation of vessel tone via vasoactive peptidases. To this end we measured mRNA and protein expression levels of ACE, ECE, EP24.15, EP24.16 and NEP under static and sheared culture conditions. We conclusively demonstrated a role for shear stress in the regulation of expression of these peptidases. Whilst in some cases the absolute degree of change in expression levels may have differed from those previously published, this can be explained on the basis of differing cell types employed and levels of shear applied. Moreover, we evidenced similar trends to previous studies and identified for the first time, other peptidases (NEP, EP24.15 and EP24.16) within the TLZM family susceptible to regulation by physiological levels of shear stress.

Further chapters will focus on the identification of the cellular signalling mechanisms by which shear stress putatively regulates zinc metallopeptidase expression in vascular endothelial cells and in the case of previously unidentified shear sensitive peptidases, they will show for the first time the processes that regulate their expression.

Chapter 4 Results – Section 2

4.1 Introduction

As shear-dependent regulation of zinc metallopeptidase expression in vascular endothelial cells has been confirmed (chapter 3), our attention turned to the mechanisms through which shear stress mediates its effects. A known intermediate of hemodynamic regulation of vasoactive molecules is both reactive and non reactive oxygen species. Shear stress is known to elicit endothelial ROS production. Oscillatory shear stress, such as that seen at vessel bifurcation and curvatures, has been shown to produce a rapid and prolonged increase in ROS production. Furthermore, this increase in ROS has been evidenced to reach toxic levels under such conditions. ROS levels have been shown to increase 2-3 fold over control under conditions of oscillatory shear within 2 h and remain high for the duration of shear applied (De Keulenaer *et al.*, 1998). The toxic levels of ROS produced by such disturbed blood flow have been shown to lead to endothelial dysfunction, via eNOS disruption, apoptotic pathway activation, lipid oxidation and eventually to lead to activation of pro-inflammatory and pro-atherosclerotic mechanisms.

Conversely, laminar shear stress, such as that seen in long unbranched arterial sections, been shown to produce a rapid, but transient, increase in endothelial ROS. This "ROS burst" in the endothelium is a non-toxic event which dissipates over time (within 3-4 h) due to both a drop in overall ROS production and an increase in expression and activity of anti-oxidant mechanisms (De Keulenaer *et al.*, 1998).

These anti-oxidant mechanisms, such as SOD and catalase expression, thus lead to the stabilization of the oxidative state of the endothelium and hence avoidance of the dysfunctional states associated with the pro-inflammatory and pro-atherosclerotic mechanisms induced by disturbed flow.

Transient ROS bursts in the endothelium have been evidenced to play roles as signalling molecules in a number of pathways, with the ability to influence cell fate decisions. Among the ROS-sensitive mechanisms present in the endothelium, apoptotic pathways are of prime importance. Apoptosis signalling kinase (ASK-1) is a MAP kinase kinase kinase located downstream from a shear-sensitive kinase, which confers on the cell the ability to regulate apoptosis in a shear-dependent manner. ASK-1 has been shown to be inhibited by thioredoxin-1 (Trx-1), with Trx-1 itself being inhibited by thioredoxin interacting protein (Txnip). This latter protein has been evidenced to be shear sensitive, confirming the shear stress regulatory effects on apoptosis. Increased states of oxidative stress have also been shown to release Trx-1 from ASK-1 and, hence, not only implicate shear but also ROS in these apoptotic processes (Harrison, 2005). Indeed, the shear stress regulation of Trx-1 expression has been shown to be a ROS-sensitive phenomenon, with low levels of ROS, such as those transiently produced by laminar shear, increasing Trx-1 expression (Haendeler *et al.*, 2004).

Within the vasculature, the ability of shear-induced ROS to affect expression of zinc metallopeptidases represents a potentially important regulatory mechanism. In this regard, previous studies have evidenced a role for shear stress-induced superoxide anion in the regulation of ECE and ET-1 levels in endothelial cells.

Masatsugu *et al*, have published evidence that shows that not only does laminar shear (15 dynes/cm^2) induce a ROS burst within BAECs but that this burst of ROS is responsible for attenuation of ECE and ET-1 mRNA and protein expression (Masatsugu *et al.*, 2003). This is, to our knowledge, the only published study which clearly links shear stress-induced ROS production and regulation of a member of the TLZM family. Using our experimental paradigm it was decided to examine the potential effects of ROS in the shear stress-dependent regulation of ACE, ECE, NEP, EP24.15 and EP24.16. To this end, we firstly sought to ascertain the ability of our shear stress model to produce a transient ROS burst in response to laminar shear stress. We then sought to examine the effect of distinct oxygen species on TLZM expression in unsheared ECs. Finally, we sought to determine if antioxidants had the ability to inhibit the shear stress-induced changes in expression of the peptidases under study and hence provide conclusive evidence of the role of ROS as mediators in the regulation of TLZM expression by laminar shear stress in vascular endothelial cells.

4.2 Results

4.2.1 Measurement of laminar shear-induced extracellular ROS production

In order to assess the ability of shear stress to alter the oxidative state of the cell, it was decided to examine the production of superoxide anion, a known precursor of many other oxygen species within the endothelium. Levels of superoxide anion released by endothelial cells into the culture medium under static and sheared conditions were measured through use of a spectrophotometric assay. Ferricytochrome C is known to react with superoxide anion to form ferrocytochrome C. This reduction by superoxide anion results in an alteration in the extinction co-efficient of the substance and hence a change in absorbance levels at a fixed wavelength. Thus, a change in absorbance level is taken as a reading of superoxide anion production (Tarpey, Wink *et al.* 2004). Samples of both static and sheared cells were measured at regular time points and the change in absorbance levels at 550 nm was graphed. Shear stress at 10 dynes/cm^2 produced a rapid increase of superoxide anion release, with levels increasing after 10 min of shear and reaching a maximal point of up to an 8 fold difference between static (0.05) and sheared (0.42) samples between 60 and 90 min before beginning to decrease (Fig. 4.1).

4.2.2 Measurement of laminar shear-induced intracellular ROS production

In order to measure the intracellular release of ROS and hence the most readily available source of oxygen species for participation in the shear stress regulation of TLZM expression, dihydroethidium, a superoxide anion specific, cell-permeable fluorescent dye was employed. This dye produces a mildly fluorescent blue/purple cytoplasm in dyed cells. Upon the production of intracellular superoxide anion, the dye will react specifically with this ROS to result in the production of ethidium. Ethidium binds nucleic acid and hence results in the production of a fluorescent red/orange nucleus. Increases in the intensity of nuclear staining are taken as an increase in ROS production (Tarpey, Wink *et al.* 2004). Cells were exposed to shear stress (10 dynes/cm^2, 3 h) and every hour, were dyed with dihydroethidium and observed using standard fluorescence microscopy. An increase in ROS production was evident after 1 h (Fig 4.2, iv) in sheared samples, rising to a maximum after 2 h (Fig 4.2, vi) and dropping once again after 3h (Fig 4.2, viii). These images clearly indicate a rapid but transient increase in ROS production in sheared cells as compared to static controls.

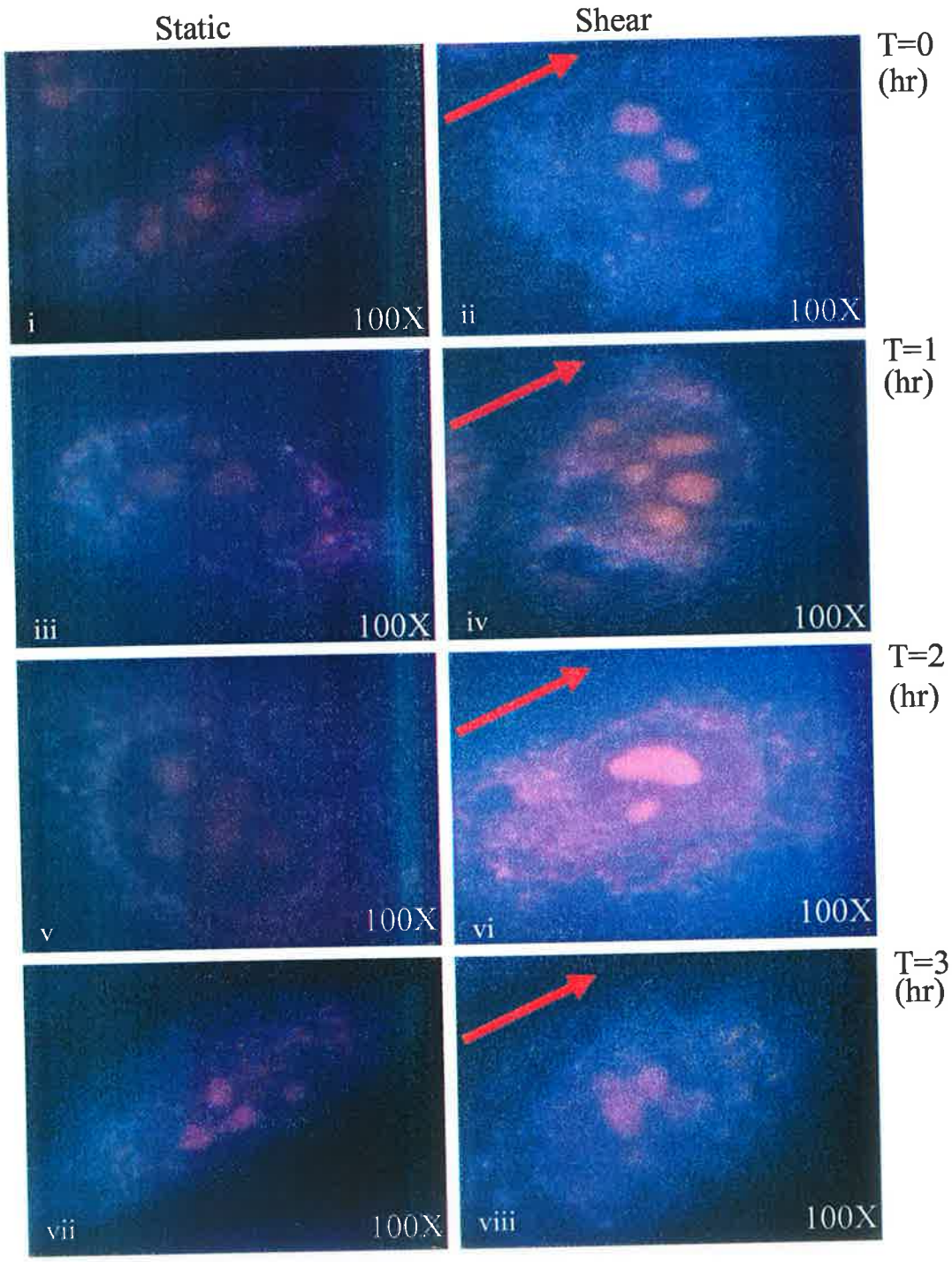


Fig 4.2 Shear stress-induced production of intracellular superoxide anion. BAECs were exposed to shear stress (10dynes/cm^2, 1-3 h), at each hourly interval cells were dyed with dihydroethidium (10 μM, 15 min), washed twice and monitored for ROS production using standard fluorescent microscopy (100X). Red arrows indicate the direction of flow. Images are representative of three separate experiments.

4.2.3 Response in TLZM expression to ROS production

We next sought to elucidate the effects of a change in the oxidative state of the cell on TLZM expression under static or "unsheared" conditions. Specifically, we sought to ascertain the effect of superoxide anion and the significantly more stable hydrogen peroxide on metallopeptidase mRNA expression. To this end, cultured BAECs were incubated with Ang II (0.1 μM), a known superoxide anion inducer, and hydrogen peroxide (10 μM) for 8 and 24 hr respectively. These artificially induced oxidative states were employed to mimic the shear stress induced change in ROS levels with incubation times selected to coincide with the transient nature of the shear induced ROS burst. Following treatment, TLZM family member expression was monitored using Real-Time PCR for changes in mRNA levels.

ACE mRNA expression levels were shown to be attenuated by both Ang II and H_2O_2 stimulation in a similar manner to that produced by shear stress. Ang II incubation resulted in an attenuation to 0.77±0.06 fold of controls (Fig 4.3 A), whilst H_2O_2 stimulation attenuated levels of mRNA to 0.69±0.07 fold of controls (Fig 4.3 B).

ECE mRNA expression levels were shown to be marginally attenuated by both Ang II and H_2O_2 stimulation. Ang II incubation resulted in a slight attenuation to 0.86±0.08 fold of controls (Fig 4.4 A), whilst H_2O_2 stimulation attenuated levels of mRNA to 0.80±0.06 fold of controls (Fig 4.4 B). However, in both, the level of attenuation was not deemed to be statistically significant (Fig 4.4A+B).

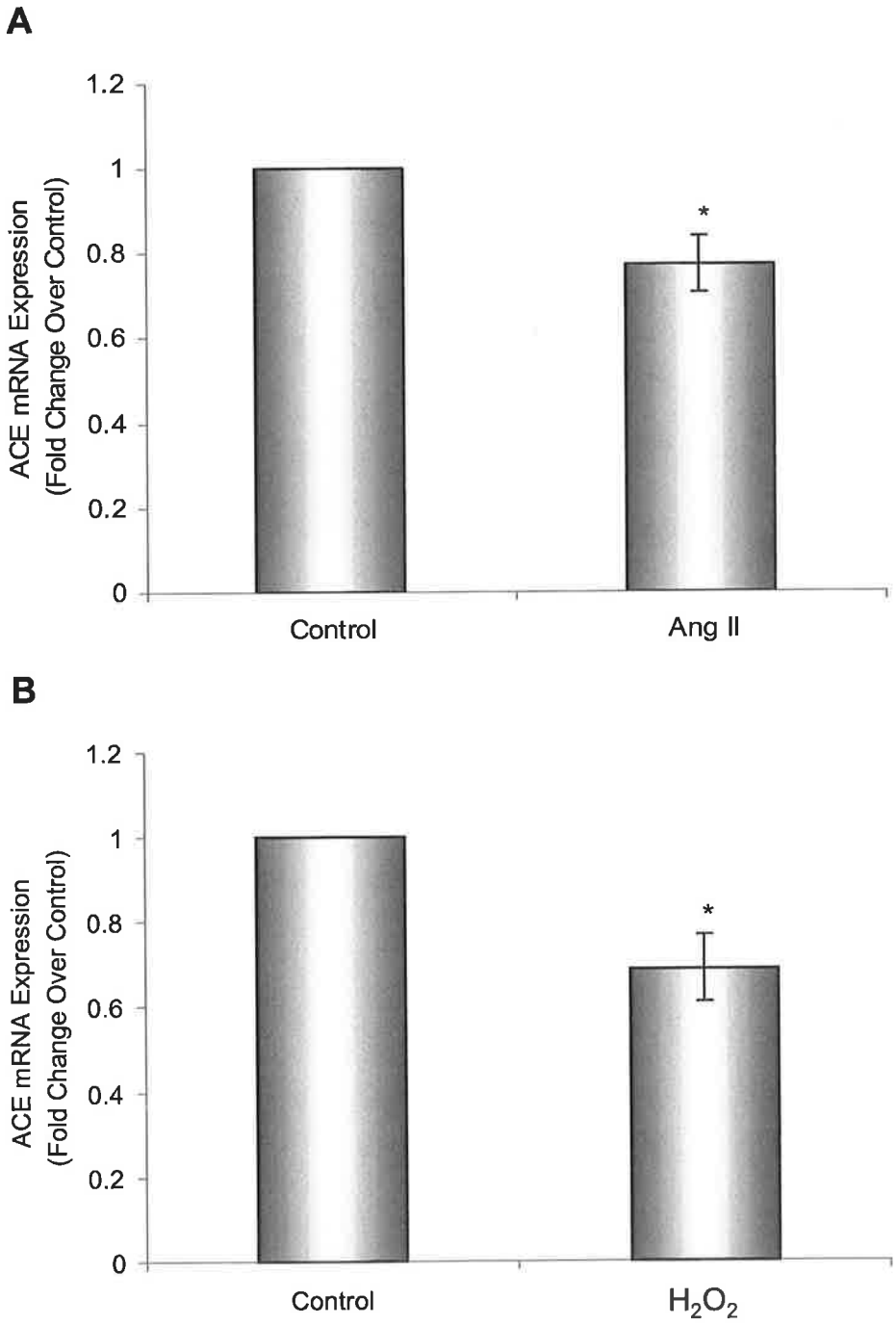


Fig 4.3 ACE mRNA expression in static BAECs following induction of ROS. BAECs were exposed to (A) 0.1 μM Ang II for 8 h, or (B) 10 μM H_2O_2 for 24 h and monitored for ACE mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus untreated controls

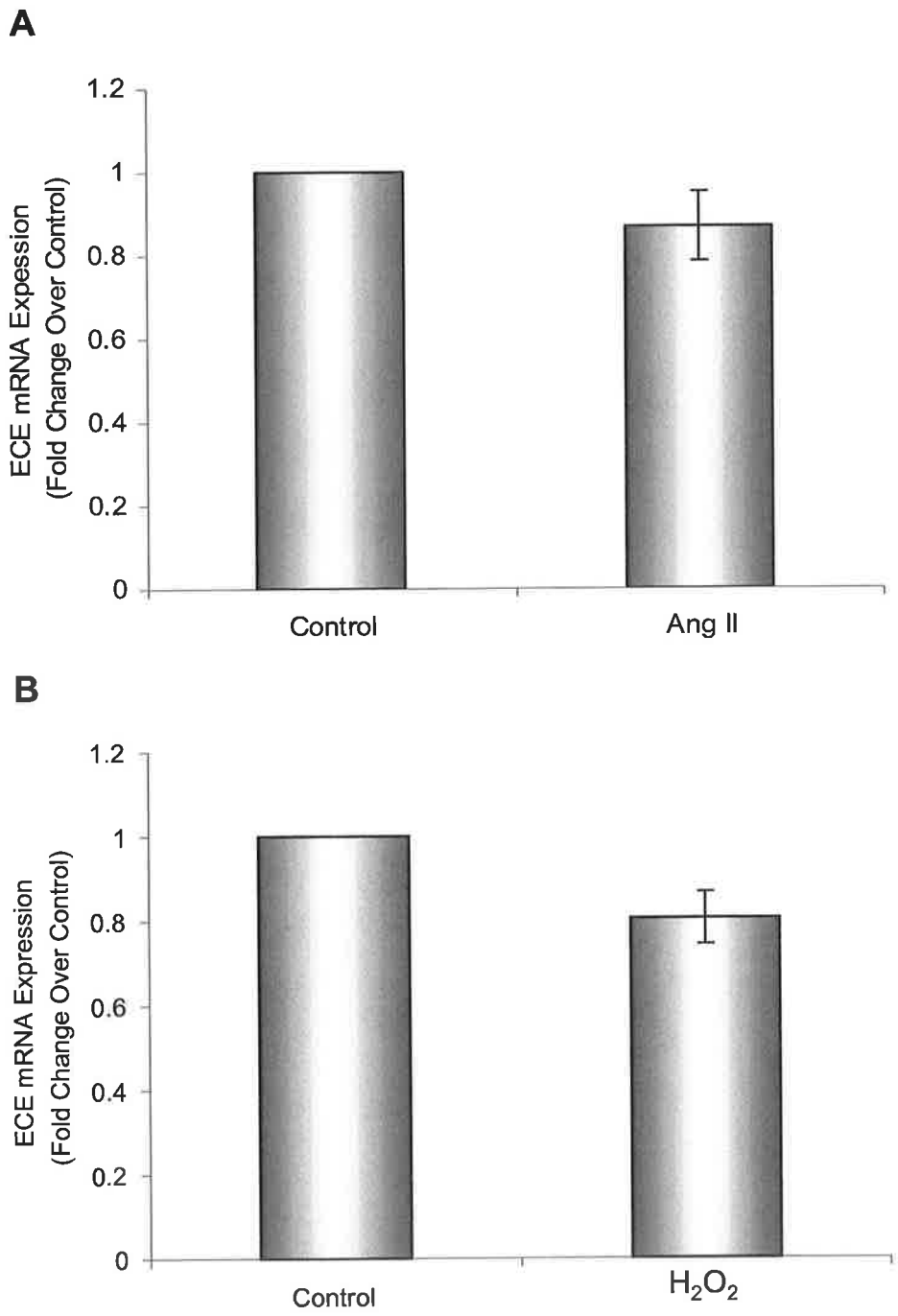


Fig 4.4 ECE mRNA expression in static BAECs following induction of ROS. BAECs were exposed to (A) 0.1 μM Ang II for 8 h, or (B) 10 μM H_2O_2 for 24 h and monitored for ECE mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus untreated controls

NEP mRNA expression levels were shown to be attenuated by both Ang II and H_2O_2 stimulation in a similar manner to that produced by shear stress. Ang II incubation resulted in an attenuation to 0.73±0.05 fold of controls (Fig 4.5 A), whilst H_2O_2 stimulation attenuated levels of mRNA to 0.53±0.12 fold of controls (Fig 4.5 B).

EP24.15 mRNA expression levels were shown to be increased by both Ang II and H_2O_2 stimulation to a similar degree to that produced by shear stress. Ang II incubation resulted in an increase to 1.58±0.16 fold of controls (Fig 4.6 A), whilst H_2O_2 stimulation increased levels of mRNA to 1.49±0.1 fold of controls (Fig 4.6 B).

EP24.16 mRNA expression levels were shown to be attenuated by both Ang II and H_2O_2 stimulation in a similar extent to that produced by shear stress. Ang II incubation resulted in an attenuation to 0.79±0.03 fold of controls (Fig 4.7 A), whilst H_2O_2 stimulation attenuated levels of mRNA to 0.72±0.05 fold of controls (Fig 4.7 B).

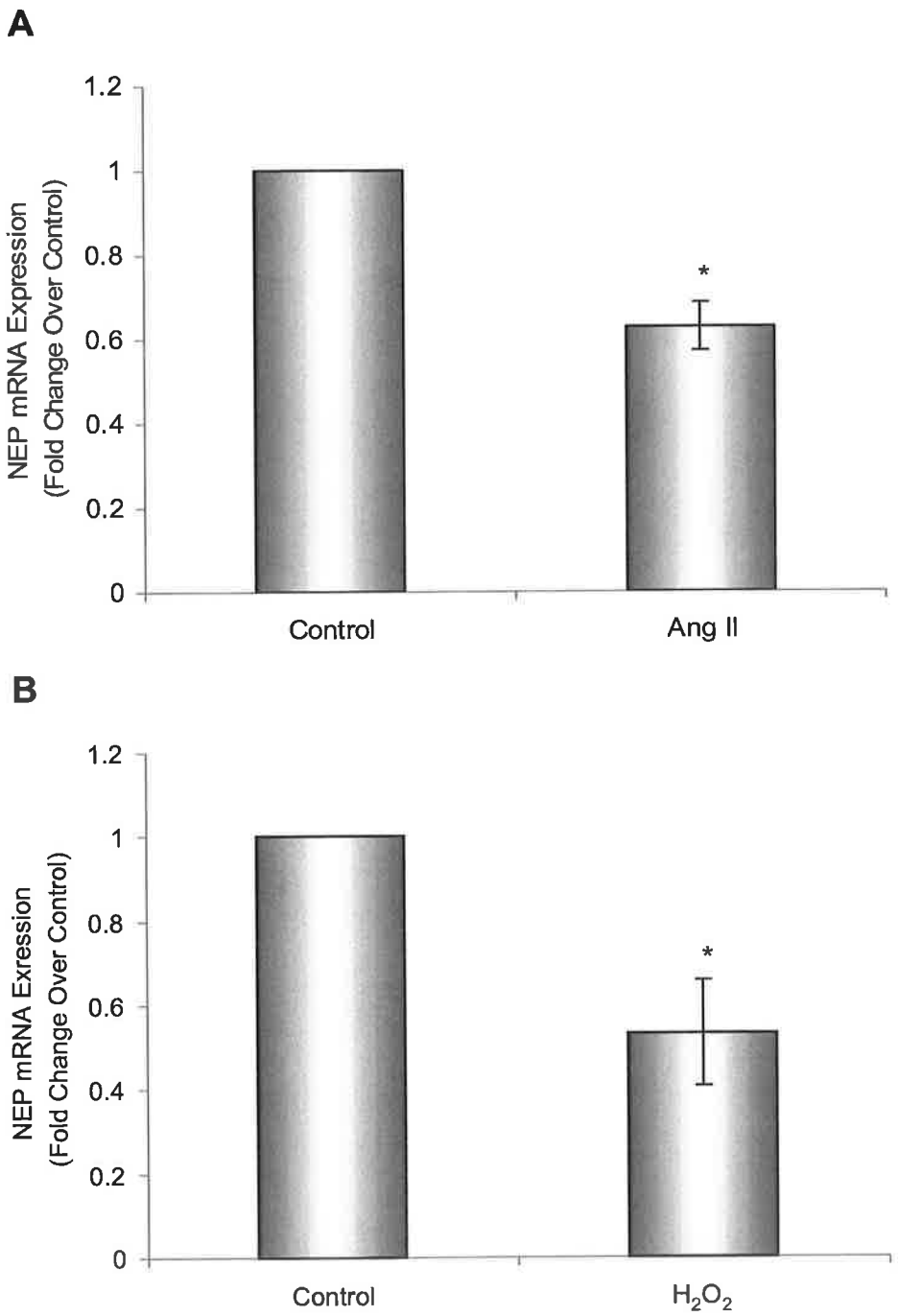


Fig 4.5 NEP mRNA expression in static BAECs following induction of ROS. BAECs were exposed to (A) 0.1 μM Ang II for 8 h, or (B) 10 μM H_2O_2 for 24 h and monitored for NEP mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus untreated controls

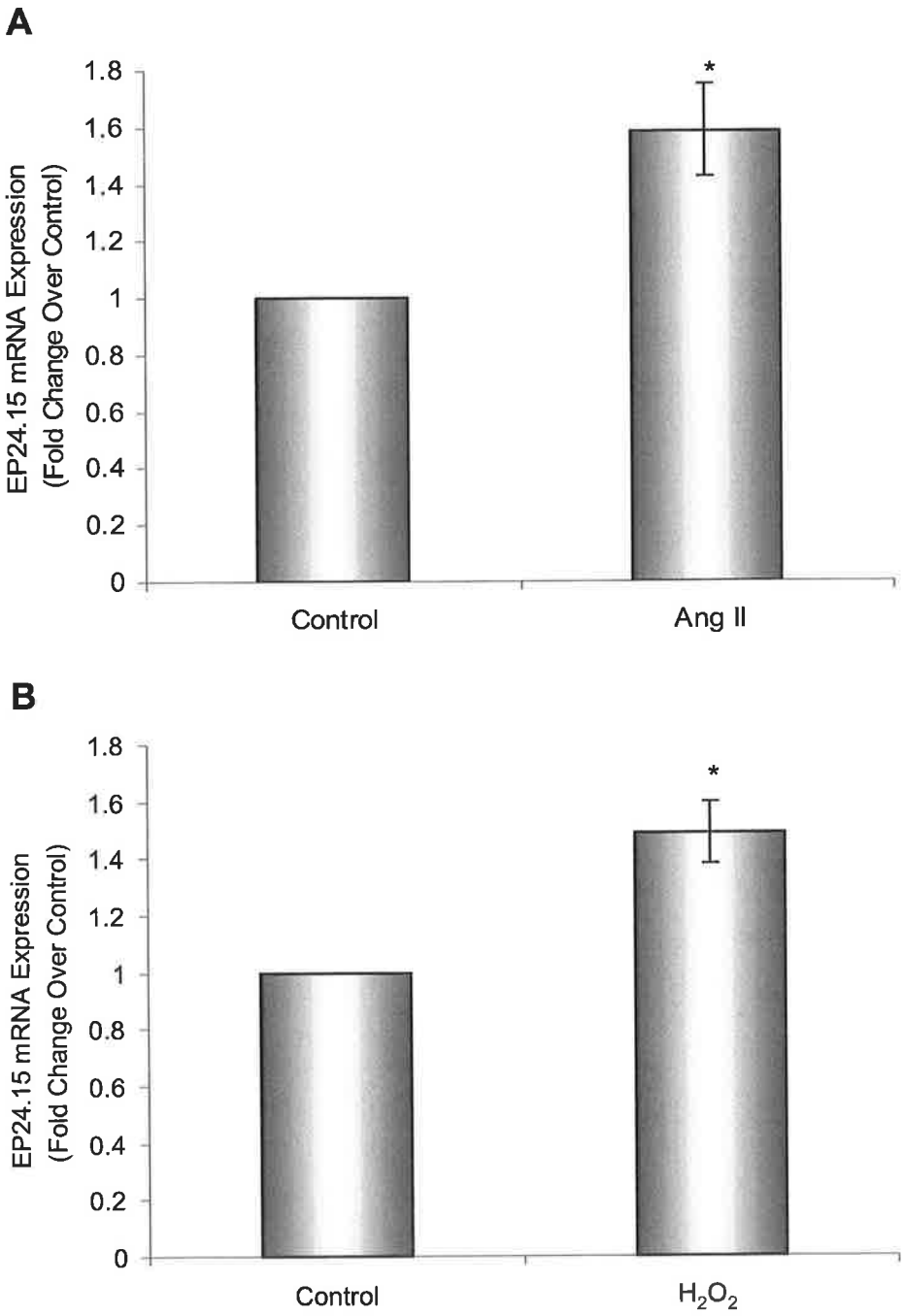


Fig 4.6 EP24.15 mRNA expression in static BAECs following induction of ROS. BAECs were exposed to (A) 0.1 μM Ang II for 8 h, or (B) 10 μM H_2O_2 for 24 h and monitored for EP24.15 mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus untreated controls

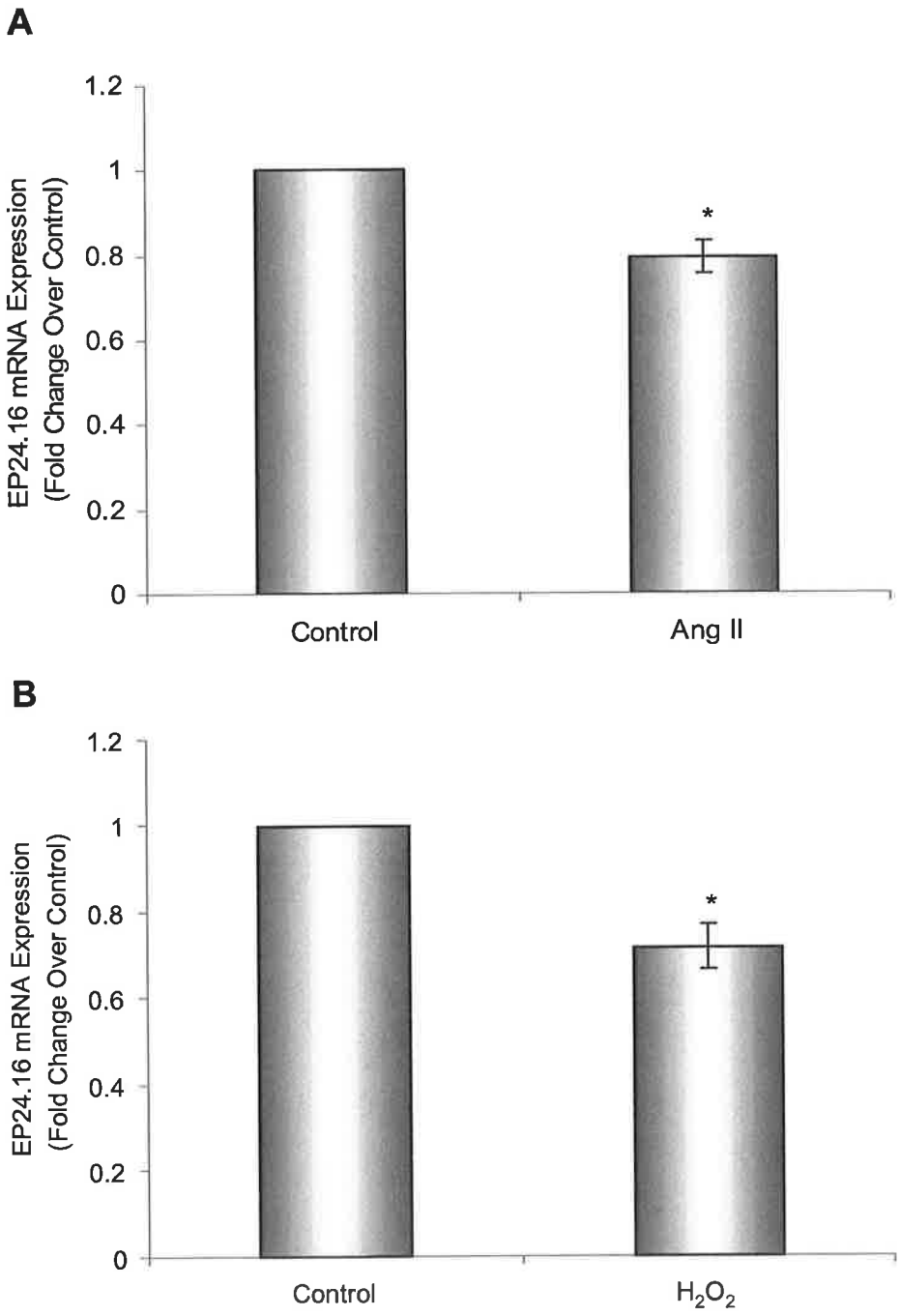


Fig 4.7 EP24.16 mRNA expression in static BAECs following induction of ROS. BAECs were exposed to (A) 0.1μM Ang II for 8 h, or (B) 10 μM H_2O_2 for 24 h and monitored for EP24.16 mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus untreated controls

4.2.4 Effect of antioxidants on shear stress–dependent regulation of TLZM expression

Next it was sought to examine the effects of antioxidants on shear stress-induced regulation of metallopeptidase expression. Antioxidants were employed to scavenge and metabolize oxygen species and in this way attenuate their effects. To this end, mRNA expression levels of ACE, ECE, NEP, EP24.15 and EP24.16 were monitored using Real-Time PCR under shear conditions in the presence and absence of three differentially acting antioxidants.

Antioxidants employed were: 1) N-Acetyl-L-cysteine (NAC), a broad spectrum antioxidant which acts to increase cellular levels of glutathione, a major detoxification enzyme, known to decrease levels of all oxygen species. 2) Catalase (CAT), which catalyzes the breakdown of hydrogen peroxide to oxygen and water. 3) Superoxide dismutase (SOD), which acts specifically on superoxide anion to eventually produce oxygen and water.

4.2.4.1 Effect of antioxidants on shear stress-dependent regulation of ACE expression

The effect of NAC on the shear-induced regulation of ACE mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, ACE mRNA expression was attenuated to 0.67±0.01 fold of control. Levels of ACE mRNA were then raised to 1.03±0.08 fold of control in samples sheared in the presence of NAC (Fig 4.8 A).

The effect of CAT on the shear-induced regulation of ACE mRNA expression was then examined. Following exposure to shear stress, ACE mRNA expression was attenuated to 0.6±0.06 fold of control. Levels of ACE mRNA were then raised to 1.11±0.02 fold of control in samples sheared in the presence of CAT (Fig 4.8 B).

Finally, the effect of SOD on shear-induced regulation of ACE mRNA expression was then examined. Following exposure to shear stress, ACE mRNA expression was attenuated to 0.62±0.01 fold of control. Levels of ACE mRNA were then raised to 0.97±0.01 fold of control in samples sheared in the presence of SOD (Fig 4.8 C).

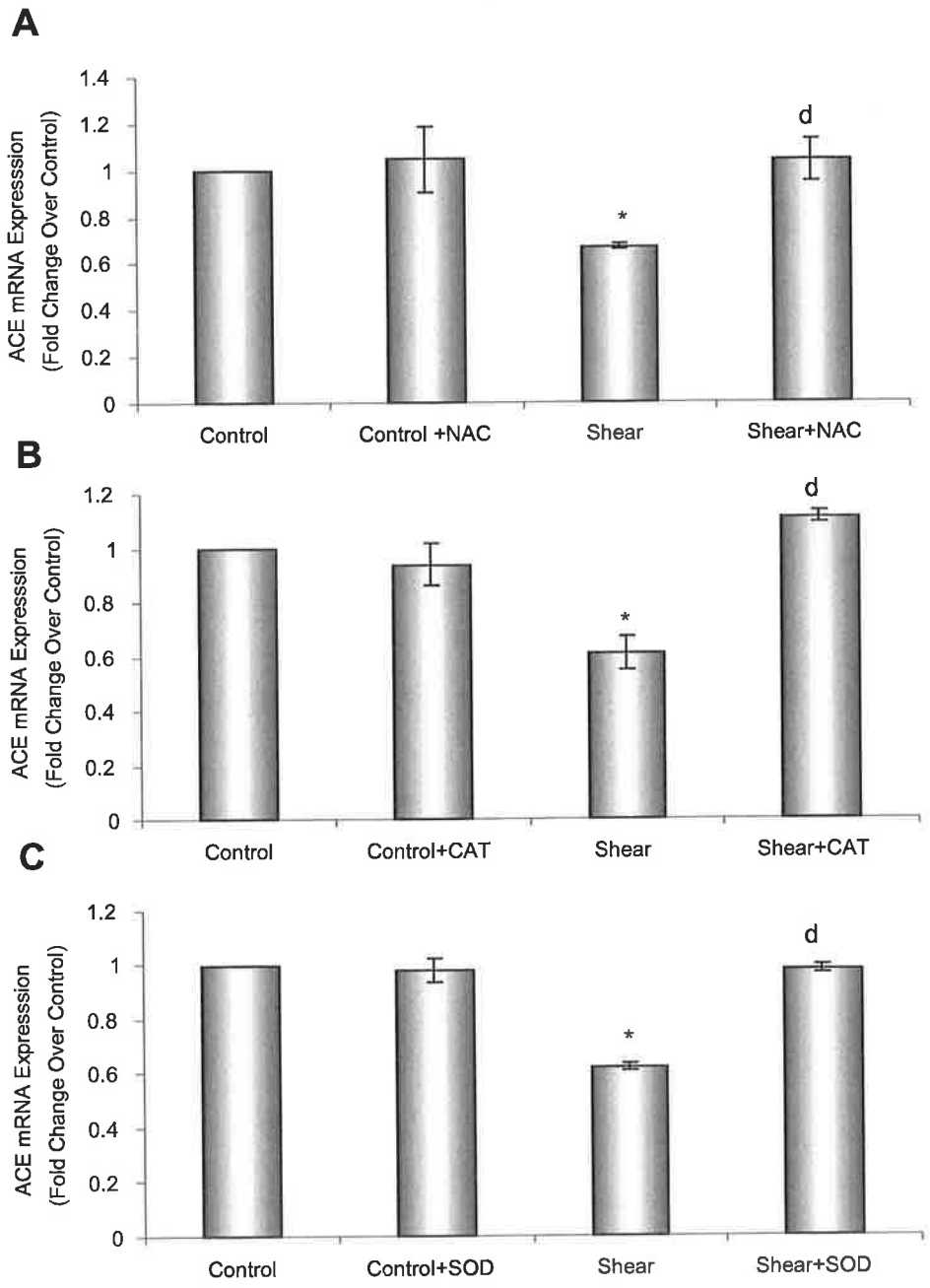


Fig 4.8 Effect of antioxidants on shear-dependent ACE expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence and presence of antioxidants (A) 5 mM NAC or (B) 1000 U/ml CAT or (C) 100 U/ml SOD, and monitored for ACE mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus controls. dp=0.05 versus untreated shear.

4.2.4.2 Effect of antioxidants on shear stress-dependent regulation of ECE expression

The effect of NAC on the shear-induced regulation of ECE mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, ECE mRNA expression was not shown to be significantly altered, 0.94±0.01 fold of control. Levels of ECE mRNA again showed no statistically significant change in samples sheared in the presence of NAC, 0.99±0.05 fold of control (Fig 4.9 A).

The effect of CAT on the shear-induced regulation of ECE mRNA expression was then examined. Following exposure to shear stress, ECE mRNA expression was not shown to be significantly altered, 0.99±0.06 fold of control. Levels of ECE mRNA again showed no statistically significant change in samples sheared in the presence of CAT, 1.04±0.11 fold of control (Fig 4.9 B).

Finally, the effect of SOD on shear-induced regulation of ECE mRNA expression was then examined. Following exposure to shear stress, ECE mRNA expression was not shown to be significantly altered, 0.99±0.06 fold of control. Levels of ECE mRNA again showed no statistically significant change in samples sheared in the presence of SOD, 0.99±0.05 fold of control (Fig 4.9 C).

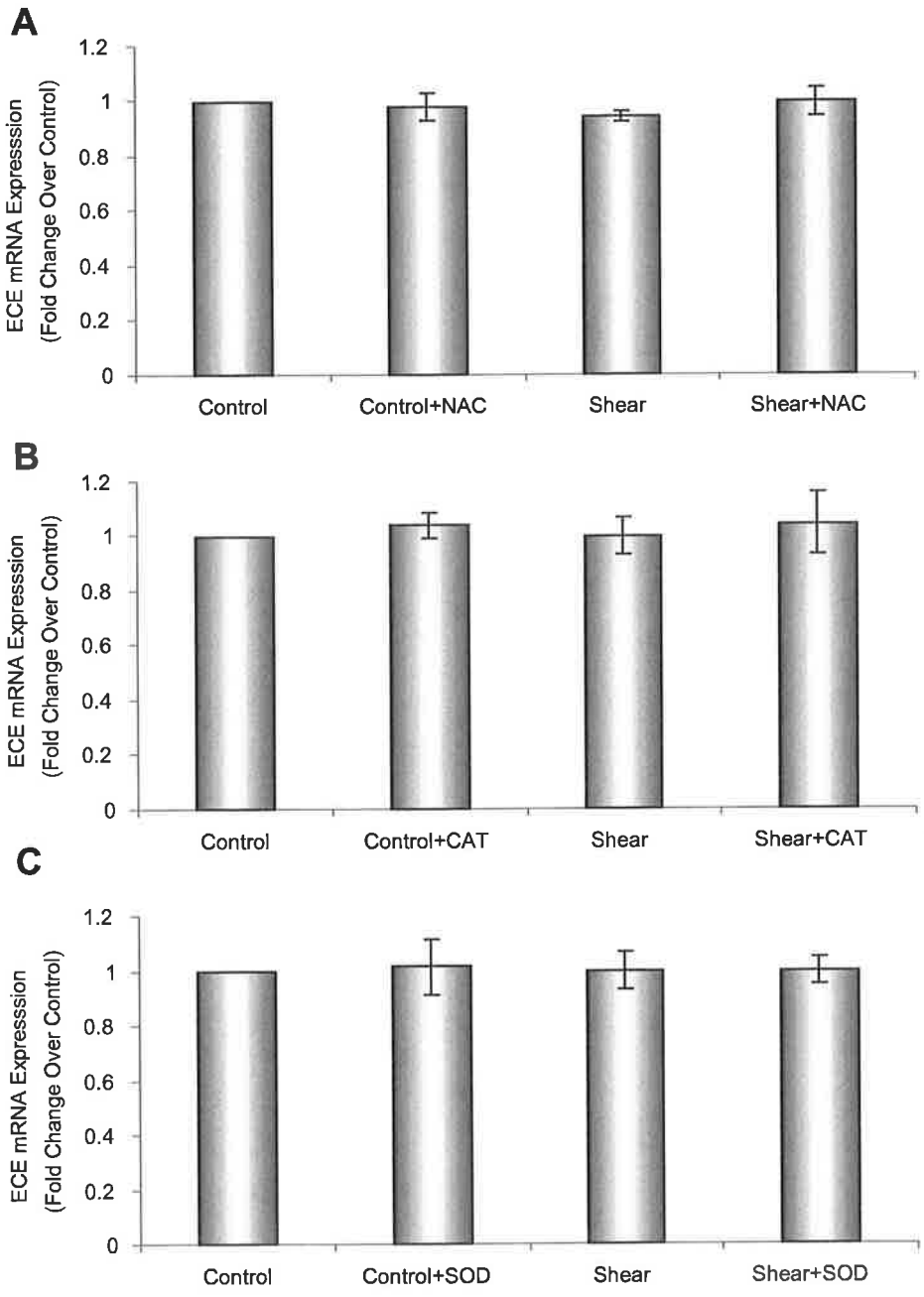


Fig 4.9 Effect of antioxidants on shear-dependent ECE expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence and presence of antioxidants (A) 5 mM NAC or (B) 1000 U/ml CAT or (C) 100 U/ml SOD, and monitored for ECE mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus static controls. dp=0.05 versus untreated shear.

4.2.4.5 Effect of antioxidants on shear stress-dependent regulation of NEP expression

The effect of NAC on the shear-induced regulation of NEP mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, NEP mRNA expression was attenuated to 0.39±0.04 fold of control. Levels of NEP mRNA were then raised to 1.42±0.18 fold of control in samples sheared in the presence of NAC (Fig 4.12 A).

The effect of CAT on the shear-induced regulation of NEP mRNA expression was then examined. Following exposure to shear stress, NEP mRNA expression was attenuated to 0.47±0.03 fold of control. Levels of NEP mRNA were then raised to 1.11±0.15 fold of static control in samples sheared in the presence of CAT (Fig 4.12 B).

Finally, the effect of SOD on shear-induced regulation of NEP mRNA expression was then examined. Following exposure to shear stress, NEP mRNA expression was attenuated to 0.58±0.04 fold of control. Levels of NEP mRNA were then raised to 1.06±0.05 fold of control in samples sheared in the presence of SOD (Fig 4.12 C).

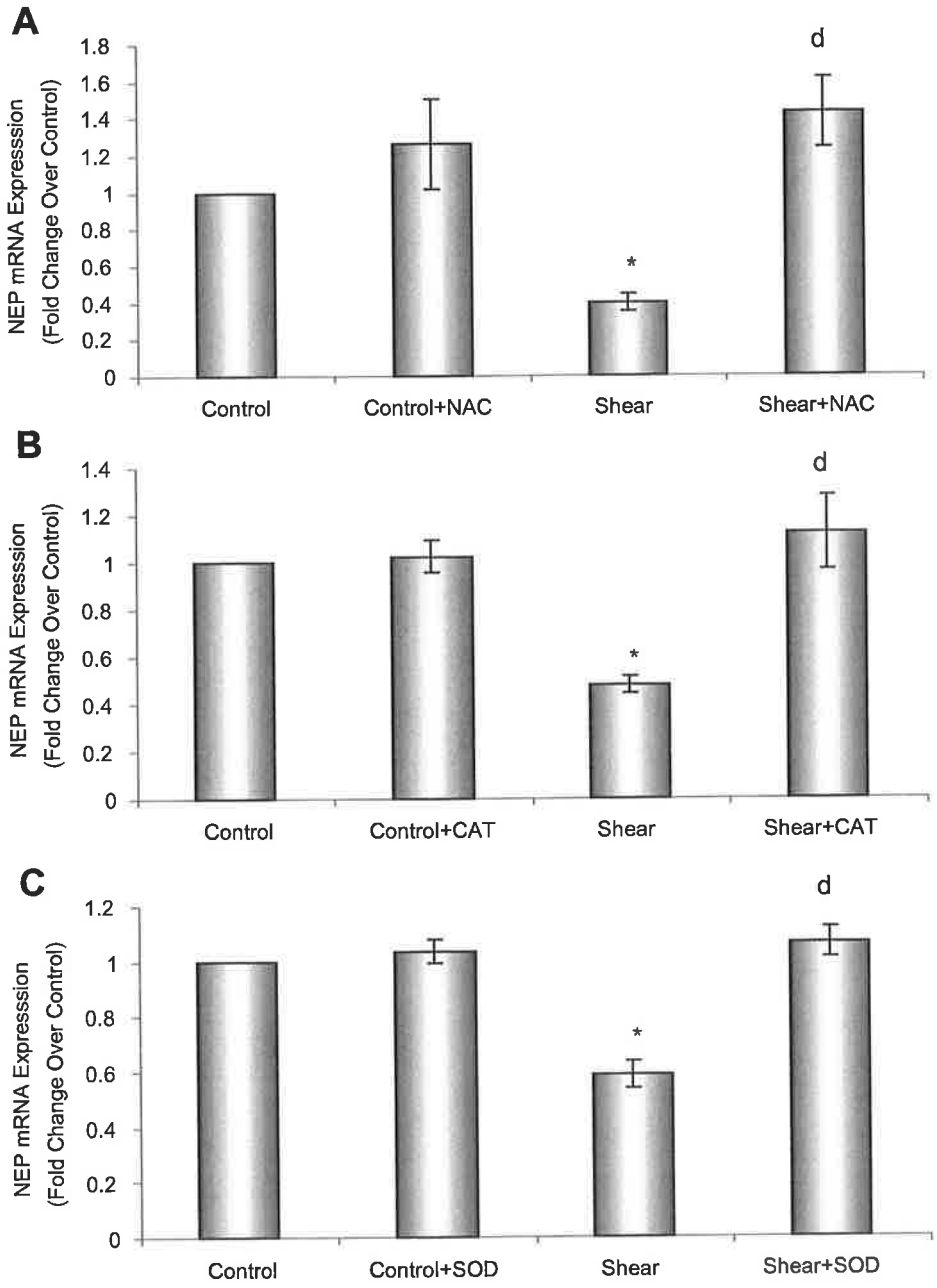


Fig 4.10 Effect of antioxidants on shear-dependent NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence and presence of antioxidants (A) 5 mM NAC or (B) 1000 U/ml CAT or (C) 100 U/ml SOD, and monitored for NEP mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus static controls. dp=0.05 versus untreated shear.

4.2.4.3 Effect of antioxidants on shear stress-dependent regulation of EP24.15 expression

The effect of NAC on the shear-induced regulation of EP24.15 mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, EP24.15 mRNA expression was increased to 1.43±0.07 fold of control. Levels of EP24.15 mRNA were then attenuated to 0.97±0.04 fold of control in samples sheared in the presence of NAC (Fig 4.10 A).

The effect of CAT on the shear-induced regulation of EP24.15 mRNA expression was then examined. Following exposure to shear stress, EP24.15 mRNA expression was increased to 1.4±0.04 fold of control. Levels of EP24.15 mRNA were attenuated to 1.03±0.02 fold of control in samples sheared in the presence of CAT (Fig 4.10 B).

Finally, the effect of SOD on shear-induced regulation of EP24.15 mRNA expression was then examined. Following exposure to shear stress, EP24.15 mRNA expression was increased to 1.44±0.12 fold of control. Levels of EP24.15 mRNA were then attenuated to 0.94±0.07 fold of control in samples sheared in the presence of SOD (Fig 4.10 C).

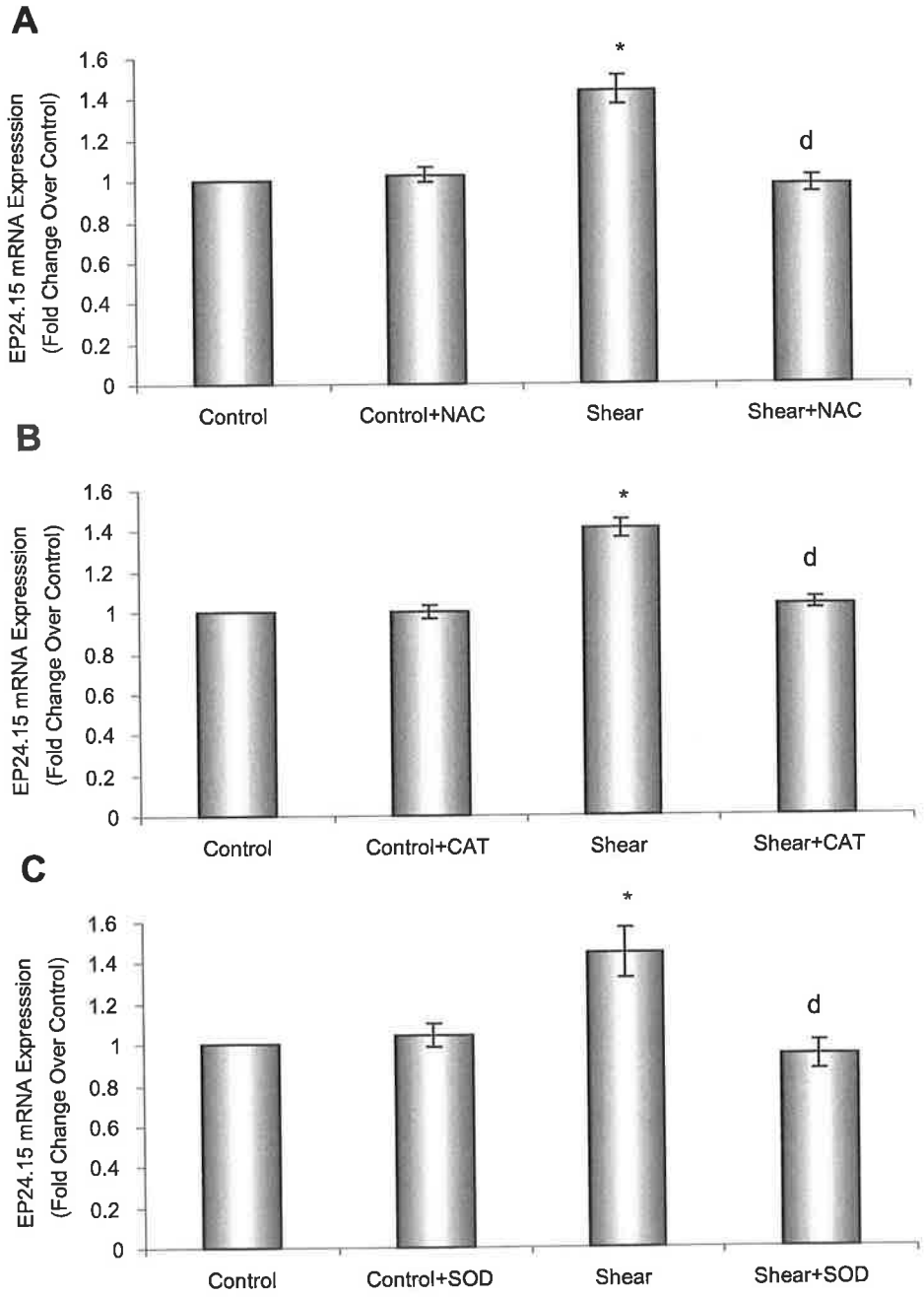


Fig 4.11 Effect of antioxidants on shear-dependent EP24.15 expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence and presence of antioxidants (A) 5mM NAC or (B) 1000 U/ml CAT or (C) 100 U/ml SOD, and monitored for EP24.15 mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus static controls. dp=0.05 versus untreated shear.

4.2.4.4 Effect of antioxidants on shear stress-dependent regulation of EP24.16 expression

The effect of NAC on the shear-induced regulation of EP24.16 mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, EP24.16 mRNA expression was attenuated to 0.67±0.05 fold of control. Levels of EP24.16 mRNA were then raised to 1.08±0.05 fold of control in samples sheared in the presence of NAC (Fig 4.11 A).

The effect of CAT on the shear-induced regulation of EP24.16 mRNA expression was then examined. Following exposure to shear stress, EP24.16 mRNA expression was attenuated to 0.71±0.01 fold of control. Levels of EP24.16 mRNA were then raised to 0.89±0.06 fold of control in samples sheared in the presence of CAT (Fig 4.11 B).

Finally, the effect of SOD on shear-induced regulation of EP24.16 mRNA expression was then examined. Following exposure to shear stress, EP24.16 mRNA expression was attenuated to 0.67±0.03 fold of control. Levels of EP24.16 mRNA were then raised to 0.95±0.04 fold of control in samples sheared in the presence of SOD (Fig 4.11 C).

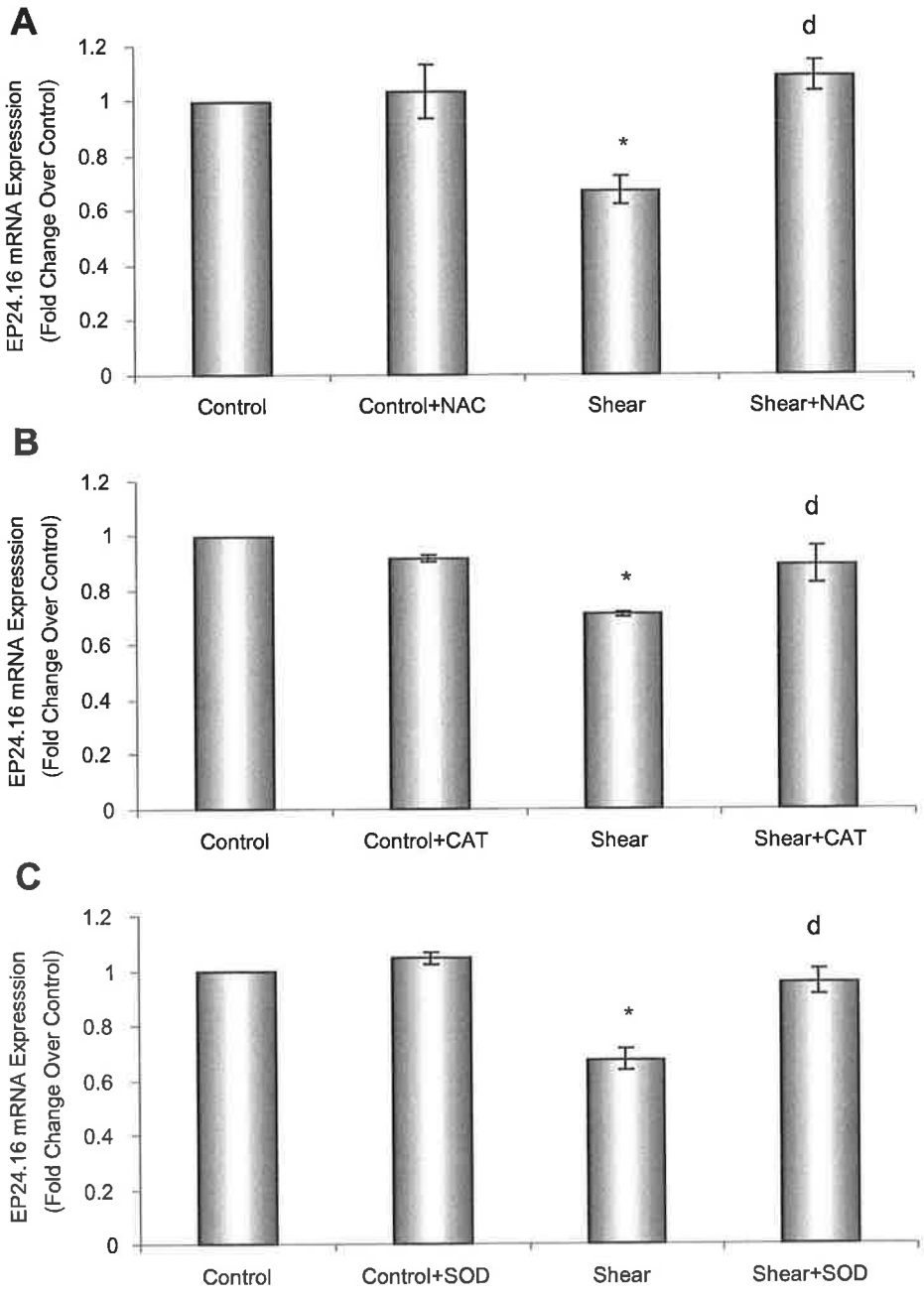


Fig 4.12 Effect of antioxidants on shear-dependent EP24.16 expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) in the absence and presence of antioxidants (A) 5 mM NAC or (B) 1000 U/ml CAT or (C) 100 U/ml SOD, and monitored for EP24.16 mRNA expression using Real-Time PCR. Histograms represent fold change in expression over untreated controls and are averaged from three independent experiments ±SEM; *p=0.05 versus static controls. dp=0.05 versus untreated shear.

4.3 Discussion

Having demonstrated in chapter 3 that expression of Thermolysin-like zinc metallopeptidases in vascular endothelial cells is sensitive to shear stress, our attention then turned to the possible mechanism by which the shear-induced responses were being mediated. Previous studies have shown that not only are a number of important endothelial cellular control mechanisms, such as cell fate via ASK-1 and Txnip, influenced by the oxidative state of the cell, in a shear-dependent or -independent manner (Harrison, 2005, Haendeler *et al.*, 2004) but also vasoactive molecules such as ECE and ET-1. This evidence prompted the hypothesis that the observed shear stress regulation of TLZM expression may similarly be influenced by the cellular oxidative state, and furthermore that the shear-induced responses may depend on the production of a transient oxidative burst as an integral part of its regulatory pathway.

In order to ascertain if shear stress mediated its effects on TLZM expression levels in a ROS-dependent manner, it was firstly needed to define the capability of the hemodynamic force to produce an oxidative state or ROS "burst". To that end, two separate assays were employed to measure the oxidative state of the cell under conditions of laminar shear stress. Levels of extracellular superoxide anion released by endothelial cells were measured under static and sheared conditions using of a spectrophotometric assay incorporating ferricytochrome C.

This assay has been used in numerous studies to adequately measure ROS production, so much so that a number of publications refer to this method as the "Gold Standard" in ROS measurement (Dikalov, Griendling *et al.* 2007). Ferricytochrome C is known to react with superoxide anion to form ferrocytochrome C, this reduction by superoxide anion results in a change in the extinction coefficient and thus absorbance of the substance. Thus a change in absorbance level is taken as a reading of superoxide anion production. Media samples of both static and sheared cells were measured at regular time points and the change in absorbance levels at 550 nm was monitored. Laminar shear stress produced a rapid increase of superoxide anion release, with levels increasing after 10 min of shear to a maximum of 8 fold relative to static between 60 and 90 min before once again beginning to decrease.

Whilst this assay provided evidence of the shear-induced transient ROS burst in endothelial cells, it is not without limitations. Several cellular reductants such as ascorbate and glutathione are capable of reducing ferricytochrome C in the absence of superoxide. Also reduced cytochrome C can be reoxidized by cytochrome oxidases, cellular peroxides and other oxidants including hydrogen peroxide. For this reason we employed an acetylated form of ferricytochrome C, a known modification that confers the greatest possible specificity for reaction solely with superoxide anion. However, in view of these drawbacks, it was decided that a second, more robust measurement of the shear-induced ROS burst was required.

Intracellular production of ROS was monitored through the use of dihydroethidium, a superoxide anion-specific, cell-permeable fluorescent dye. Cells dyed with dihydroethidium and examined using standard fluorescence microscopy, will produce of a mildly fluorescent blue/purple cytoplasm. Upon the production of intracellular superoxide anion, the dye will react specifically with this ROS to result in the production of ethidium. Ethidium binds nucleic acid and hence results in the production of a fluorescent red/orange nucleus (Peshavariya, Dusting *et al.* 2007). Increases in ROS production are taken as an increase in the occurrence and intensity of nuclear staining. Cells exposed to shear stress (10 dynes/cm^2, 3h), showed a dramatic increase in ROS production was after 1 hr (Fig 4.2, iv) in sheared samples, rising to a maximum after 2 h (Fig 4.2, vi) and dropping once again after 3h (Fig 4.2, viii). This increase not only mirrored the ferricytochrome C results but also concurred with previously published studies that incorporated fluorescent dyes in ROS measurement procedures (Masatsugu *et al.*, 2003, (Hwang, Saha *et al.* 2003). Studies by Hwang *et al*, identified a 2.5 fold increase in superoxide anion production in sheared cells over control in response to 15 dynes/cm^2. This level of ROS increase was measured after only 1 h of shear and had returned to control level after 18 h (Hwang, Saha *et al.* 2003). We thus concluded that exposure of BAECs to laminar shear stress resulted in a rapid and transient increase in ROS production.

We next sought to ascertain the impact of oxidative state on metallopeptidase expression levels. To this end we incorporated two methods of artificially inducing an oxidative state in endothelial cells and measured mRNA response of the TLZMs. Ang II, a known mediator of superoxide anion production in endothelial cells, via activation of NAD(P)H oxidase (Wang *et al.*, 2001), was employed to induce production of superoxide anion. Cells were incubated with Ang II before harvesting for mRNA analysis. All members of the TLZM family responded to Ang II-induced superoxide in a similar manner as to shear stress, clearly indicating the possibility that shear stress mediates its effects on TLZM expression via oxidative state. We further sought to provide evidence of this through the use of hydrogen peroxide. Hydrogen peroxide is itself an oxygen species and hence can be applied directly to the cells.

As with Ang II superoxide induction, expression of TLZM mRNA levels was modulated in an almost identical manner to that of shear stress by hydrogen peroxide. This showed definitively that TLZM expression in BAECs is regulated by cellular oxidative state. These results also raise the possibility that this regulation is not specific to individual oxygen species, in that both superoxide anion and hydrogen peroxide produced the same effect on TLZM mRNA levels. Indeed with such a plethora of reactive and non-reactive oxygen species produced in the endothelium, both directly and as breakdown products of other oxygen species, it remains a possibility that the overall oxidative state is responsible for altered expression levels.

Finally we sought to conclusively evidence that the observed shear stress regulation of TLZM expression was mediated via oxidative state. To this end we employed a number of antioxidants in our shear stress paradigm in an attempt to attenuate the observed differences in mRNA expression. Through the use of specific and non-specific antioxidants, we sought to attenuate the shear-induced ROS burst and thus the ROS-associated changes in TLZM mRNA expression. Firstly we employed N-Acetyl-L-cysteine, a broad spectrum antioxidant which acts to increase cellular levels of glutathione. Glutathione is a known antioxidant and detoxification tripeptide, which will scavenge and, hence, decrease all present oxygen species. Shear stress in the presence of NAC did not induce the previously noted changes in any TLZM mRNA expression and thus strongly validated the proposed hypothesis for the role of shear-dependent ROS in modulation of TLZM expression. Secondly, we employed the oxygen species-specific superoxide dismutase which acts specifically on superoxide anion. SOD dismutates two molecules of superoxide anion to produce hydrogen peroxide which is in turn broken down to produce oxygen and water. As with NAC, shear stress in the presence of SOD did not induce the previously noted changes in any TLZM mRNA expression and thus further validated the proposed hypothesis. Lastly, catalase, which catalyzes the breakdown of hydrogen peroxide to oxygen and water, was finally employed to assess the impact of oxidative state on TLZM expression via shear stress. Results with catalase again mimicked the other antioxidant treatments, with shear stress in the presence of CAT failing to induce the previously noted changes in any TLZM mRNA expression and once again validating the proposed hypothesis.

4.4 Conclusion

Through use of the assays employed it was clearly evidenced that shear stress had the ability to produce a transient burst of ROS in the endothelium. It was also clearly demonstrated that an induced oxidative state, such as that by Ang II or hydrogen peroxide, could alter mRNA expression levels of the members of the TLZM family. Through these oxidative states it was also evidenced that the changes in expression of TLZM family members could be modulated via more than one specific oxygen species. Finally antioxidant studies provided conclusive evidence for the role played by ROS in the shear-induced changes in expression levels.

These antioxidant treatments not only definitively indicated that shear stress mediates its regulation of TLZM expression and consequently vessel tone via ROS, but also indicated again that it is an overall oxidative state that is responsible, rather than a specific oxygen species. This finding is based on the fact that SOD inhibited the shear-induced effects on TLZM expression by removing superoxide anion, but through its action would also have produced hydrogen peroxide. This would seem to indicate that superoxide anion was the responsible oxygen species, however, incubation with catalase also inhibited the shear-induced effects, indicating that neither oxygen species alone are responsible and again that the entire oxidative state of the cell, contributed to by a number of ROS, is responsible for shear-mediated regulation of TLZM expression in the vasculature. To our knowledge, this is the first study to fully evidence this pathway.

The next chapter will focus on the signal transduction mechanisms that enable vascular endothelial cells to sense and translate shear stress into a change in oxidative state and consequently endopeptidase expression. Particular emphasis will be placed on NEP, a member of the TLZM family for which no data has been published in relation to shear sensitivity.

Chapter 5 Results – Section 3

5.1 Introduction

We next decided to probe the signalling pathways mediating shear-induced changes in metallopeptidase expression levels in BAECs. For these studies, we focused exclusively on NEP, a prototypical member of the TLZM family for which no previous information on hemodynamic sensitivity has been published. It was sought firstly to ascertain the exact mechanisms and complexes through which shear stress modulated the oxidative state and hence NEP expression. Secondly, the regulatory pathways that allow the endothelial cell to sense the hemodynamic force of shear stress and translate it into a cellular signal were examined.

To this end, it was initially sought to identify the source of the shear stress-induced ROS shown to alter NEP expression. With such a plethora of reactive and non-reactive oxygen species within in the vascular endothelium (including $O_2^{-\bullet}$, $NO^{\bullet}$, $ONOO^{-\bullet}$,$OH^{\bullet}$ and H_2O_2), work focused on elucidating the role of the major source of non-toxic levels of ROS, NAD(P)H oxidase, which has been evidenced to produce $O_2^{-\bullet}$, a known precursor of most other oxygen species. Indeed previously discussed data (chapter 4) evidenced a clear role for $O_2^{-\bullet}$ and its immediate breakdown product H_2O_2, in modulating expression levels of NEP and other members of the TLZM family in vascular endothelial cells. These findings were, at least in part, in agreement with previously published studies that show a clear link between expression levels of ECE and oxidative state (Masatsugu *et al.*, 2003).

Previous studies have demonstrated the ability of shear stress to modulate activity of NAD(P)H oxidase in endothelial cells. Oscillatory shear has been shown to induce a prolonged and deleterious activation, with a consequent rise of the oxidative state of the cell to a toxic level. Laminar shear has, however, been shown to transiently activate NAD(P)H oxidase, resulting in a temporal production of ROS which dissipates over time due to endogenous antioxidant mechanisms (De Keulenaer *et* al., 1998). Through the use of the NAD(P)H oxidase-specific pharmacological inhibitor, Apocynin, we sought to elucidate the role played by this membrane bound source of ROS, in the previously evidenced shear-induced ROS burst. Apocynin functions by inhibiting the association of the cytosolic $p47^{phox}$ and $p67^{phox}$ subunits with the membrane bound $gp91^{phox}$ subunit and hence preventing the formation of an active complex. Without active complex formation, NAD(P)H oxidase in unable to produce superoxide anion in response to stimuli such as shear stress.

It was then sought to elucidate the mechanotransductive processes that enabled the endothelial cell to sense and respond to shear stress with activation of NAD(P)H oxidase, and consequent ROS production and attenuation of NEP expression. Mechanotransduction is the process by which a mechanical signal, such as the tractive force of shear stress, is converted into a chemical signal via an intermediate molecule or structure. A number of complexes present in the endothelial cell have been implicated in this transduction process, chief among them are, integrins, G-proteins, and tyrosine kinase receptors.

These predominantly membrane-bound complexes have been shown to participate in a number of shear-dependent-signaling cascades in the vasculature and have been shown to play a role in processes involving vessel tone and control thereof. Indeed a number of studies have shown a conclusive link between these transductive complexes and a number of vasoactive peptides and peptidases.

Integrin studies have, as previously discussed in section 1.6.2, been shown to play an important role in vasoactive pathways, including MAPK activation and integrin induced up-regulation of ET-1 mRNA expression (Lehoux *et al.*, 2003; Chen *et al.*, 2001). In order to specifically study the role of integrins, linear- and cyclic-RGD peptide inhibitors were used. The RGD motif (Arg-Gly-Asp) is an integrin-recognition motif found in many ligands and hence functions in ligand and receptor binding. Linear-RGD is a tripeptide consisting of a flexible structure which allows for binding to differing conformations. Cyclic-RGD peptides contain a rigid skeleton to closely bind with its receptor. These specifically designed RGD containing antagonists can bind and inactivate ligand receptors and hence ameliorate their effects.

In order to specifically examine the role of PTK, genistein, an ATP-competitive inhibitor was used. Kinases function by transferring a phosphate from ATP to another protein, resulting in a functional change in the protein. Genistein competes with ATP, resulting in a non-active enzyme-substrate complex and hence inhibits the activity of protein tyrosine kinases.

G-proteins have also been strongly implicated in similar vascular processes. In a similar manner to integrins, G-proteins have been shown to activate MAPK pathways (Schriffin and Touyz, 1998) in addition to regulation of the activity of vasoactive peptides, Ang 1-7 and ET (Ferreira and Santos, 2005). In addition, expression and activity of TLZM family members, EP24.15 and EP24.16 has been evidenced to be regulated via cyclic strain in a G-protein-dependent manner (Cotter *et al.*, 2004). With such evidence in place, it was hypothesized that the shear and ROS induced changes in NEP expression levels may involve similar mechanotransductive pathways. In order to ascertain the role played by G-proteins in the signal transduction of the shear stress attenuation of NEP expression, we firstly looked at the a subunit, specifically the G_ia subunit. PTX is a noncovalently linked heterohexameric protein that is structurally and functionally divided into subunits A and B, similarly to other bacterial toxins such as cholera toxin (CT) and *Escherichia coli* heat-labile toxin. The A subunit (A protomer) is composed of a single peptide (S1) with ADP-ribosyltransferase activity, that modifies GTP-binding regulatory proteins (G proteins), thus interfering with G protein-dependent signal transduction. PTX has been found to specifically inhibit the G_ia subunit.

To ascertain fully the role played by G-proteins in the signal transduction of the shear stress attenuation of NEP expression, we secondly looked at the Gβγ subunit and examined its role via transfection of the ß-Ark-ct plasmid. There are several biochemical mechanisms that contribute to ß-adrenergic receptor desensitization.

Most importantly is agonist-occupied active receptors becoming phosphorylated by ß-ARK, facilitating the subsequent binding of the inhibitor protein ß-arrestin to the receptors. Receptor function is consequently inhibited by up to 70%. $G_{\beta\gamma}$ subunits anchored to the membrane are available to interact with ß-ARK after G-protein activation and dissociation. Recently, peptides derived from the $G_{\beta\gamma}$-binding domain of ß-ARK have been shown to act as ß-ARK inhibitors by competing for $G_{\beta\gamma}$ and preventing translocation. The ß-ARK-ct is a nonkinase peptide derived from ß-ARK that competes for binding to the βγ-subunits of heterotrimeric G proteins, a process that is required for ß-ARK activation.

In order to explore the possible function played by small G-proteins, GTPases (those mainly found in the cytosol which act downstream of many cellular reactions) we used a Rac1 specific inhibitor to asses the role played by small G-proteins in shear stress attenuation of NEP expression. NSC23766 is a cell-permeable pyrimidine compound that specifically and reversibly inhibits Rac1 GDP/GTP exchange activity by interfering with Rac1 interaction with the Rac-specific GEF (guanine nucleotide exchange factor) Trio and Tiam1. Of the superfamily of GTPases, Rac1 was selected due to its role as an active subunit in the NAD(P)H oxidase complex.

5.2 Results

5.2.1 Effect of Apocynin on shear-dependent modulation of NEP Expression

In order to ascertain the role of NAD(P)H oxidase in the shear-induced attenuation of NEP, we employed a highly specific pharmacological inhibitor, Apocynin.

The effect of Apocynin (10 mM) on the shear-induced regulation of NEP mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, NEP mRNA expression was attenuated to 0.48±0.06 fold of control. In the presence of Apocynin, levels of NEP mRNA were then raised to 1.08±0.09 fold of control in sheared samples (Fig 5.1 A).

NEP protein expression levels were also measured using western blotting and visualized using immunocytochemistry. Following shear stress, NEP protein expression was attenuated to 0.4±0.02 fold of control. In the presence of Apocynin, levels of NEP protein were then raised to 0.99±0.005 fold of control in sheared samples (Fig 5.1 B). Measured changes in protein levels were then visualized using immunocytochemistry, with images further mimicking the previously measured changes (see Fig 5.2). Images clearly indicate a shear stress-induced attenuation in NEP protein levels whilst this attenuation is ameliorated in the presence of apocynin (Fig 5.2(ii) and (iv) respectively).

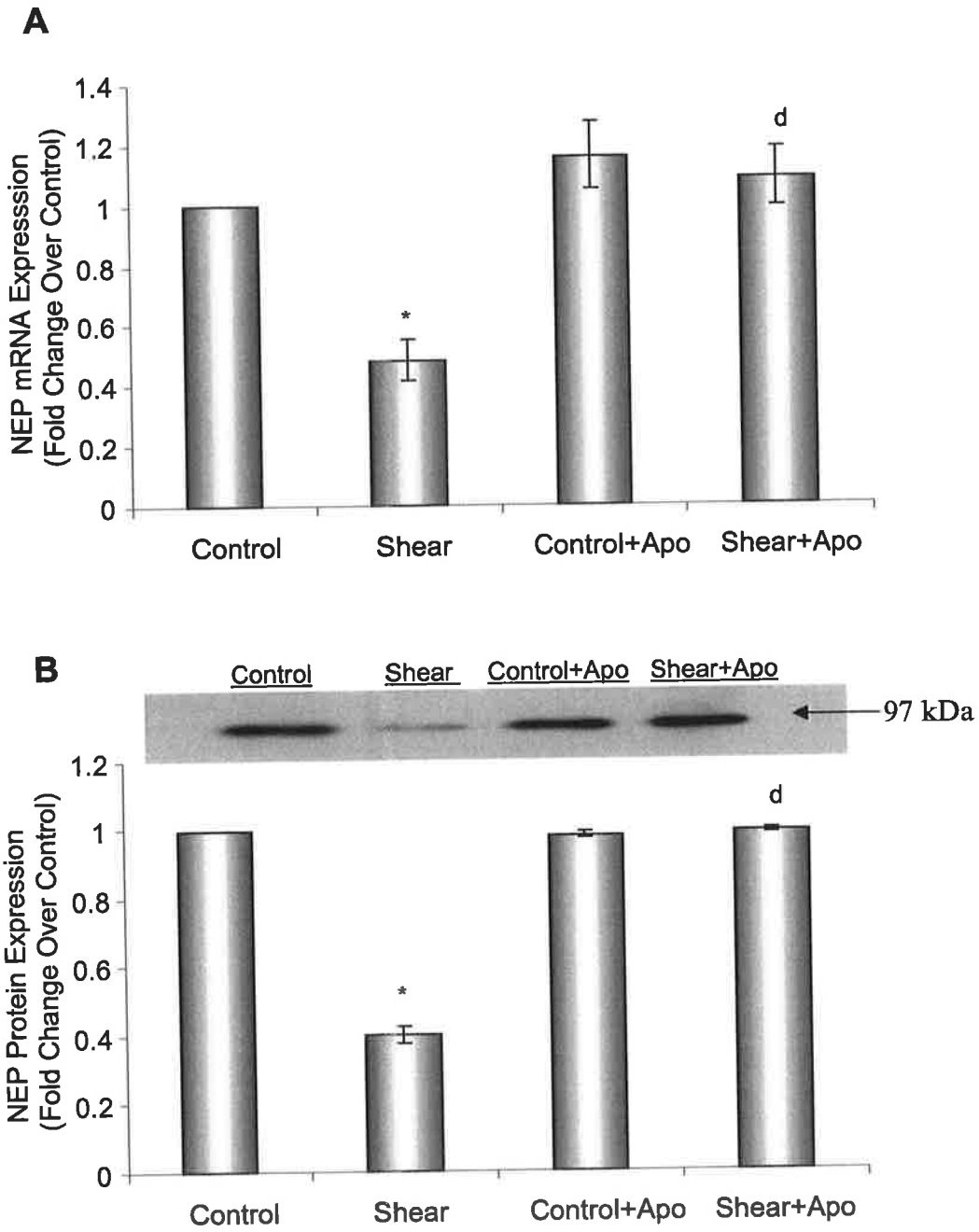


Fig 5.1 Effect of Apocynin on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 10 mM apocynin (Apo) and monitored for changes in NEP (A) mRNA expression and (B) protein expression.. Histogram in (A) represent fold change in mRNA levels over untreated controls and is averaged from three independent experiments ±SEM. Histogram in (B) represents fold change in band intensity over untreated control and is averaged from three independent experiments ±SEM. Representative blot is shown. *p=0.05 versus static controls. dp=0.05 versus untreated shear.

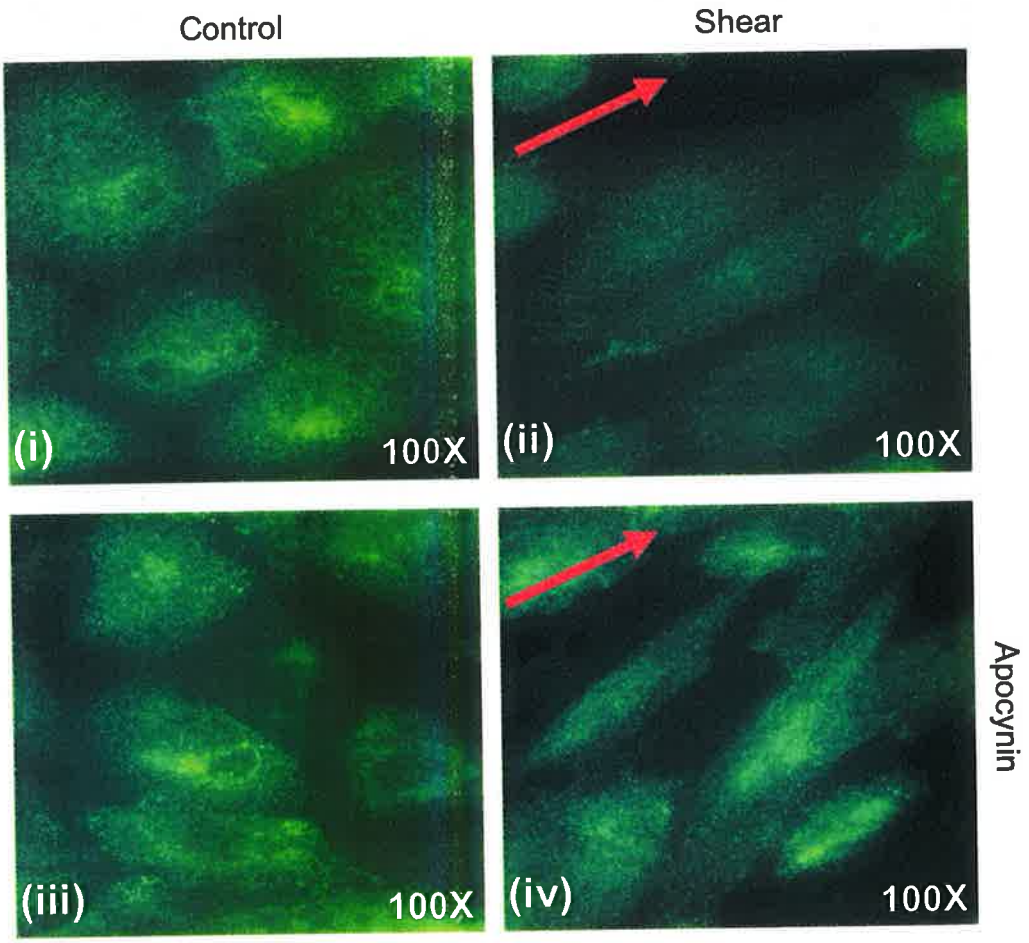


Fig 5.2 Effect of Apocynin on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 10 mM apocynin and visualised using immunocytochemiistry for changes in NEP expression. Untreated control (i), untreated shear (ii), apocynin-treated control (iii) and apocynin treated shear (iv), were monitored using standard fluorescence microscopy (100X). Red arrows indicate the direction of flow. Images are representative of three independent experiments.

5.2.2 Effect of Linear-RGD on shear-dependent modulation of NEP Expression

In order to ascertain the role of integrins in the shear-induced attenuation of NEP, we employed a specific pharmacological inhibitor, Linear-RGD (lRGD).

The effect of lRGD (0.5 mM) on the shear induced regulation of NEP mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, NEP mRNA expression was attenuated to 0.53±0.12 fold of control. In the presence of lRGD, NEP mRNA levels showed no statistically significant changes, with sheared samples in the presence of lRGD also showing an attenuation of 0.55±0.16 fold of control (Fig 5.3 A). NEP protein expression levels were also measured using western blotting and visualized using immunocytochemistry.

Following shear stress, NEP protein expression was attenuated to 0.47±0.01 fold of static control. In the presence of lRGD, NEP protein levels showed no statistically significant changes, with sheared samples in the presence of lRGD also showing an attenuation of 0.63±0.13 fold of control (Fig 5.3 B). Measured changes in protein levels were then visualized using immunocytochemistry, with images further mimicking the previously measured changes (Fig 5.4). Images clearly indicate a shear stress induced attenuation in NEP protein levels whilst this attenuation is not ameliorated in the presence of lRGD (Fig 5.4(ii) and (iv) respectively).

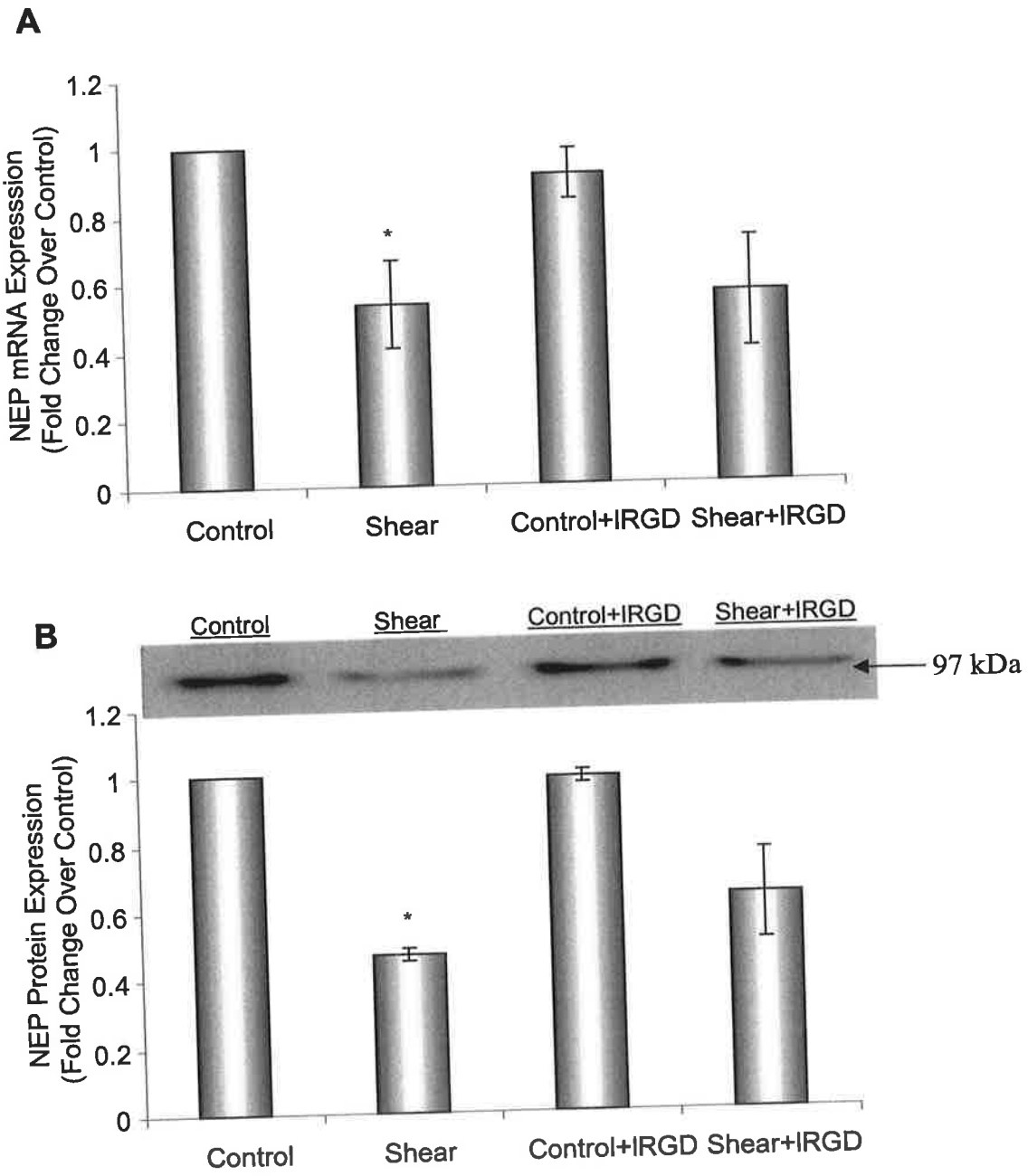


Fig 5.3 Effect of Linear RGD on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 0.5 mM lRGD and monitored for changes in NEP (A) mRNA expression and (B) protein expression.. Histogram in (A) represent fold change in mRNA levels over untreated controls and is averaged from three independent experiments ±SEM. Histogram in (B) represents fold change in band intensity over untreated control and is averaged from three independent experiments ±SEM. Representative blot is shown. *p=0.05 versus static controls. dp=0.05 versus untreated shear.

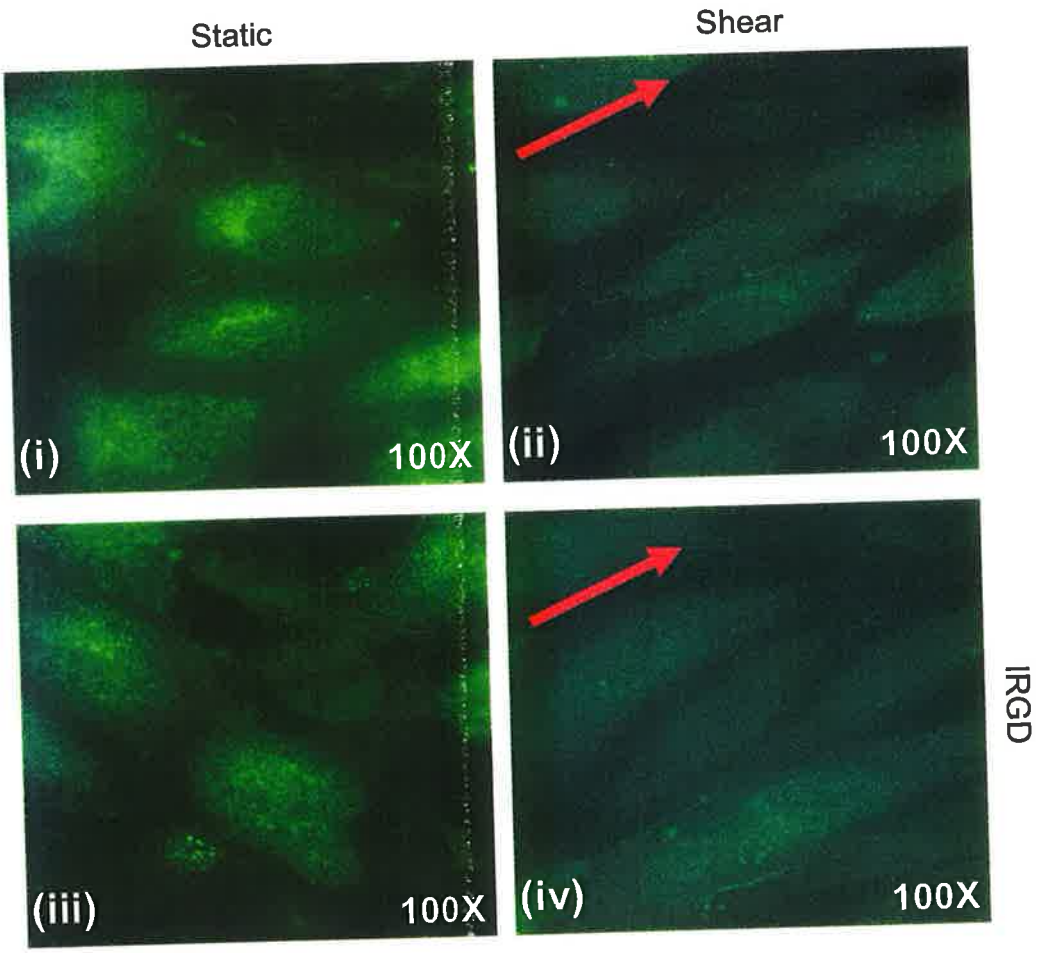


Fig 5.4 Effect of lRGD on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 0.5 mM lRGD and visualised using immunocytochemiistry for changes in NEP expression. Untreated control (i), untreated shear (ii), lRGD treated control (iii) and lRGD treated shear (iv) were monitored using standard fluorescence microscopy (100X). Red arrows indicate the direction of flow. Images are representative of three independent experiments.

5.2.3 Effect of Cyclic-RGD on shear-dependent modulation of NEP Expression

In order to fully ascertain the role of integrins in the shear-induced attenuation of NEP, we employed a highly specific pharmacological inhibitor, Cyclic-RGD (cRGD).

The effect of cRGD (100 μM) on the shear induced regulation of NEP mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, NEP mRNA expression was attenuated to 0.46±0.01 fold of control. In the presence of cRGD, NEP mRNA levels showed no statistically significant changes, with sheared samples in the presence of cRGD also showing an attenuation of 0.45±0.03 fold of control (Fig 5.5 A). NEP protein expression levels were also measured using Western blotting and visualized using immunocytochemistry.

Following shear stress, NEP protein expression was attenuated to 0.49±0.03 fold of control. In the presence of cRGD, NEP protein levels showed no statistically significant changes, with sheared samples in the presence of cRGD also showing an attenuation of 0.49±0.03 fold of control (Fig 5.5 B). Measured changes in protein levels were then visualized using immunocytochemistry, with images further mimicking the previously measured changes (Fig 5.6). Images clearly indicate a shear stress-induced attenuation in NEP protein levels whilst this attenuation is not ameliorated in the presence of cRGD (Fig 5.6(ii) and (iv), respectively).

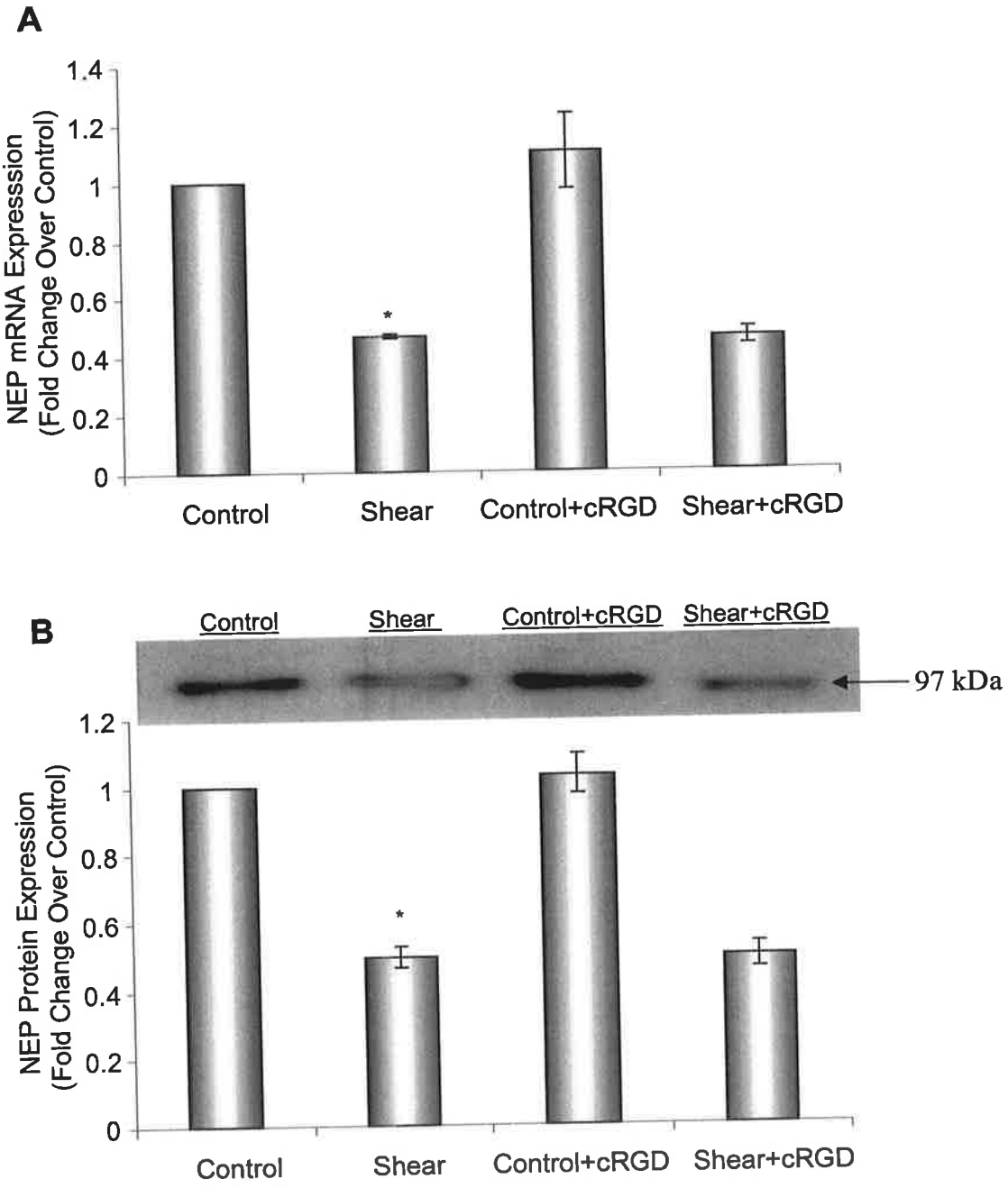


Fig 5.5 Effect of Cyclic RGD on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 100 μM cRGD and monitored for changes in NEP (A) mRNA expression and (B) protein expression.. Histogram in (A) represent fold change in mRNA levels over untreated controls and is averaged from three independent experiments ±SEM. Histogram in (B) represents fold change in band intensity over untreated control and is averaged from three independent experiments ±SEM. Representative blot is shown. *p=0.05 versus static controls. dp=0.05 versus untreated shear.

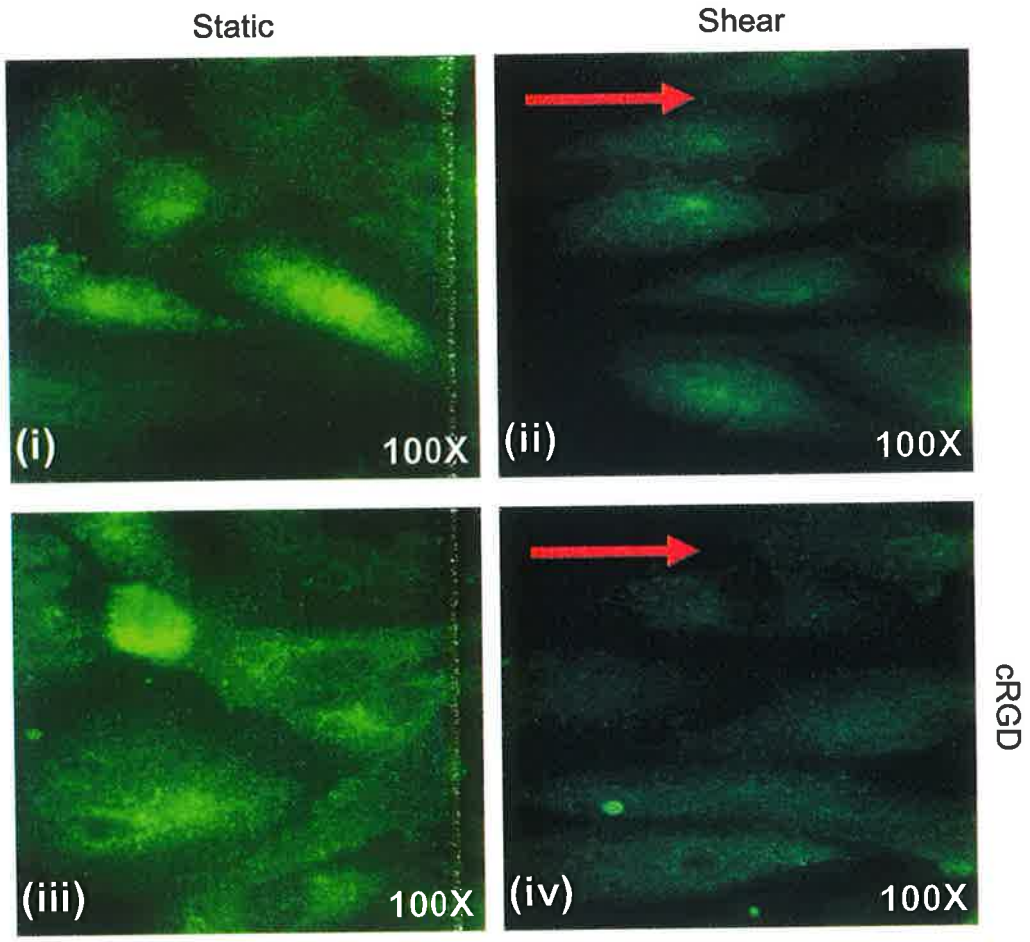


Fig 5.6 Effect of cRGD on shear-induced modulation of NEP Expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 100 μM cRGD and visualised using immunocytochemiistry for changes in NEP expression. Untreated control (i), untreated shear (ii), cRGD treated control (iii) and cRGD treated shear (iv) were monitored using standard fluorescence microscopy (100X). Red arrows indicate the direction of flow. Images are representative of three independent experiments.

5.2.4 Effect of Genistein on shear-dependent modulation of NEP Expression

In order to ascertain the role of PTK in the shear-induced attenuation of NEP, we employed a highly specific pharmacological inhibitor, genistein.

The effect of genistein (50 μM) on the shear-induced regulation of NEP mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, NEP mRNA expression was attenuated to 0.5±0.04 fold of control. In the presence of genistein, NEP mRNA levels showed no statistically significant changes, with sheared samples in the presence of genistein also showing an attenuation of 0.5±0.04 fold of control (Fig 5.7 A). NEP protein expression levels were also measured using western blotting and visualized using immunocytochemistry.

Following application of shear stress, NEP protein expression was attenuated to 0.46±0.03 fold of control. In the presence of genistein, NEP protein levels showed no statistically significant changes, with sheared samples in the presence of genistein also showing an attenuation of 0.47±0.03 fold of controls (see Fig 5.7 B). Measured changes in protein levels were then visualized using Immunocytochemistry, with images further mimicking the previously measured changes (see Fig 5.8). Images clearly indicate a shear stress-induced attenuation in NEP protein levels, whilst this attenuation is not ameliorated in the presence of genistein (Fig 5.8(ii) and (iv), respectively).

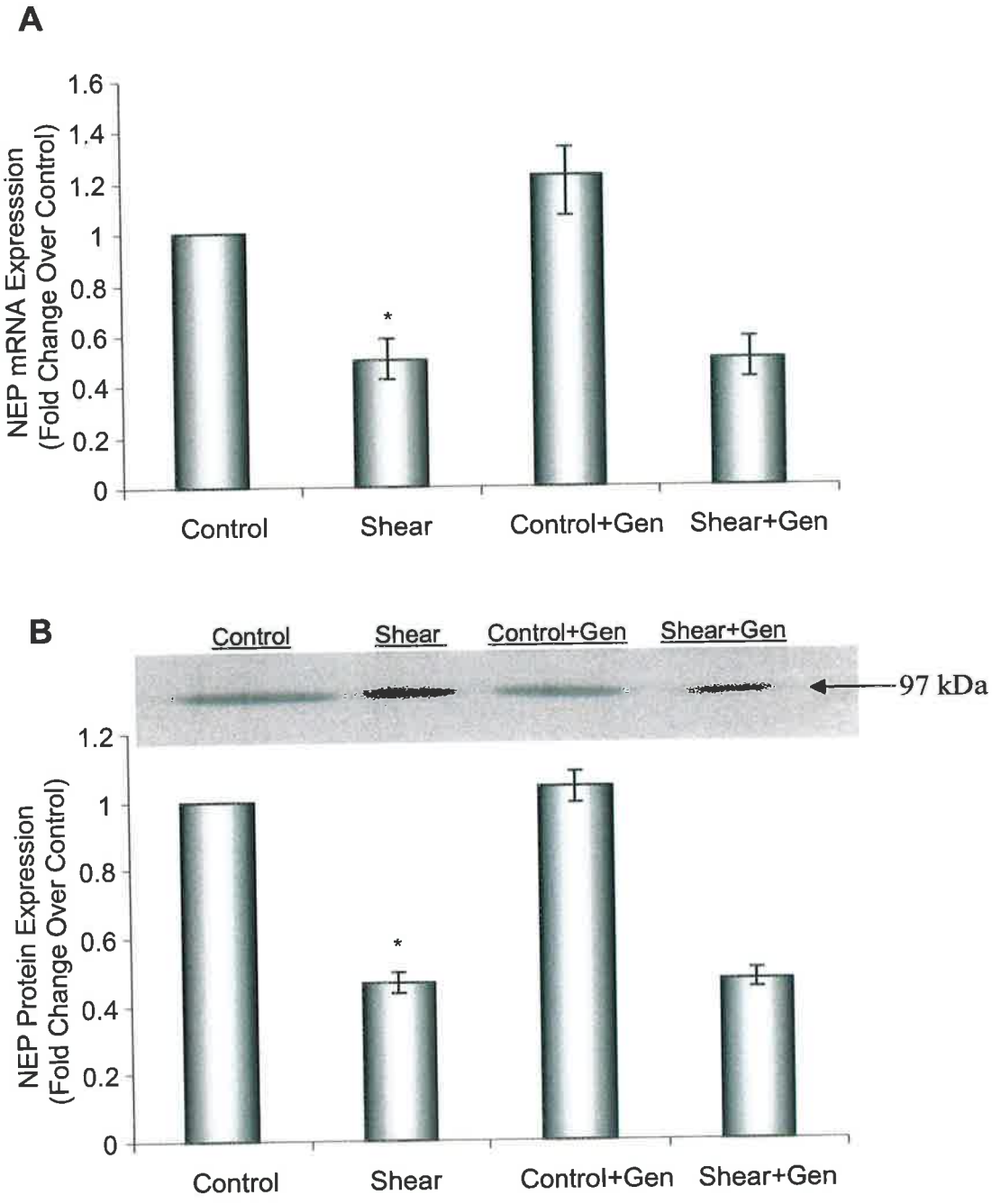


Fig 5.7 Effect of Genistein on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 50 μM Genistein (Gen) and monitored for changes in NEP (A) mRNA expression and (B) protein expression.. Histogram in (A) represent fold change in mRNA levels over untreated controls and is averaged from three independent experiments ±SEM. Histogram in (B) represents fold change in band intensity over untreated control and is averaged from three independent experiments ±SEM. Representative blot is shown. *p=0.05 versus static controls. dp=0.05 versus untreated shear.

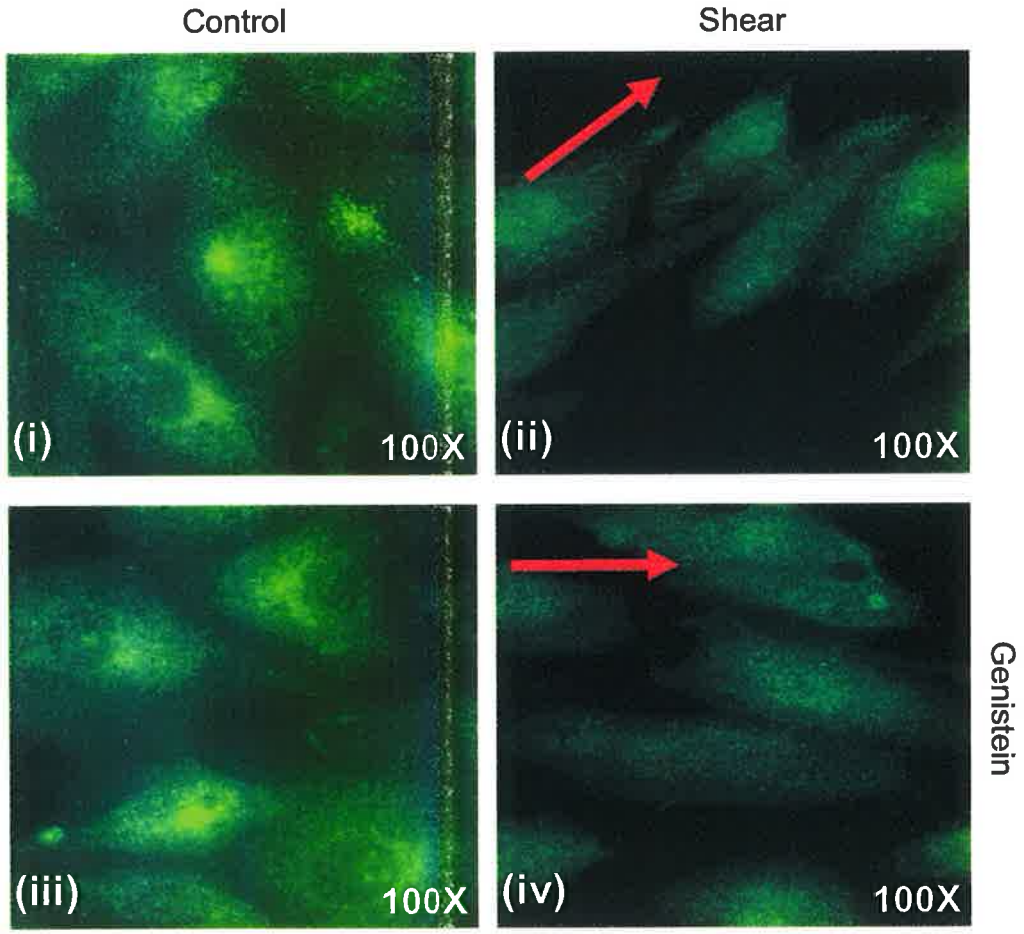


Fig 5.8 Effect of Genistein on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 50 μM Genistein and visualised using immunocytochemiistry for changes in NEP expression. Untreated control (i), untreated shear (ii), genistein treated control (iii) and genistein treated shear (iv) were monitored using standard fluorescence microscopy (100X). Red arrows indicate the direction of flow. Images are representative of three independent experiments.

5.2.5 Effect of PTX on shear-dependent modulation of NEP Expression

In order to ascertain the role of the $G_i a$ subunit in the shear-induced attenuation of NEP, we employed a highly specific pharmacological inhibitor, Pertussis toxin (PTX).

The effect of PTX (100 ng/ml) on the shear-induced regulation of NEP mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress NEP mRNA expression was attenuated to 0.51±0.07 fold of control. In the presence of PTX, levels of NEP mRNA were then raised to 1.25±0.13 fold of control in sheared samples. The static control in the presence of PTX, however, also exhibited an increase in NEP mRNA expression of 1.22±0.12 fold of untreated control, indicating a shear-independent pathway for NEP expression regulation (Fig 5.9 A).

NEP protein expression levels were also measured using Western blotting and visualized using immunocytochemistry. Following application of shear stress NEP protein expression was attenuated to 0.41±0.006 fold of control. In the presence of PTX, levels of NEP protein were then raised to 1.47±0.03 fold of control in sheared samples. Again, however the static control in the presence of PTX also exhibited an increase in NEP protein expression of 1.44±0.03 fold of untreated control, further indicating a shear-independent pathway for NEP expression regulation (Fig 5.9 B).

Measured changes in protein levels were then visualized using immunocytochemistry, results reflecting the transcriptional data as seen in (Fig 5.10). Images clearly indicate a shear stress-induced attenuation in NEP protein levels, whilst both the PTX-treated static and sheared samples show an increase in NEP protein levels (Fig 5.10 (ii) and (iii) and (iv), respectively).

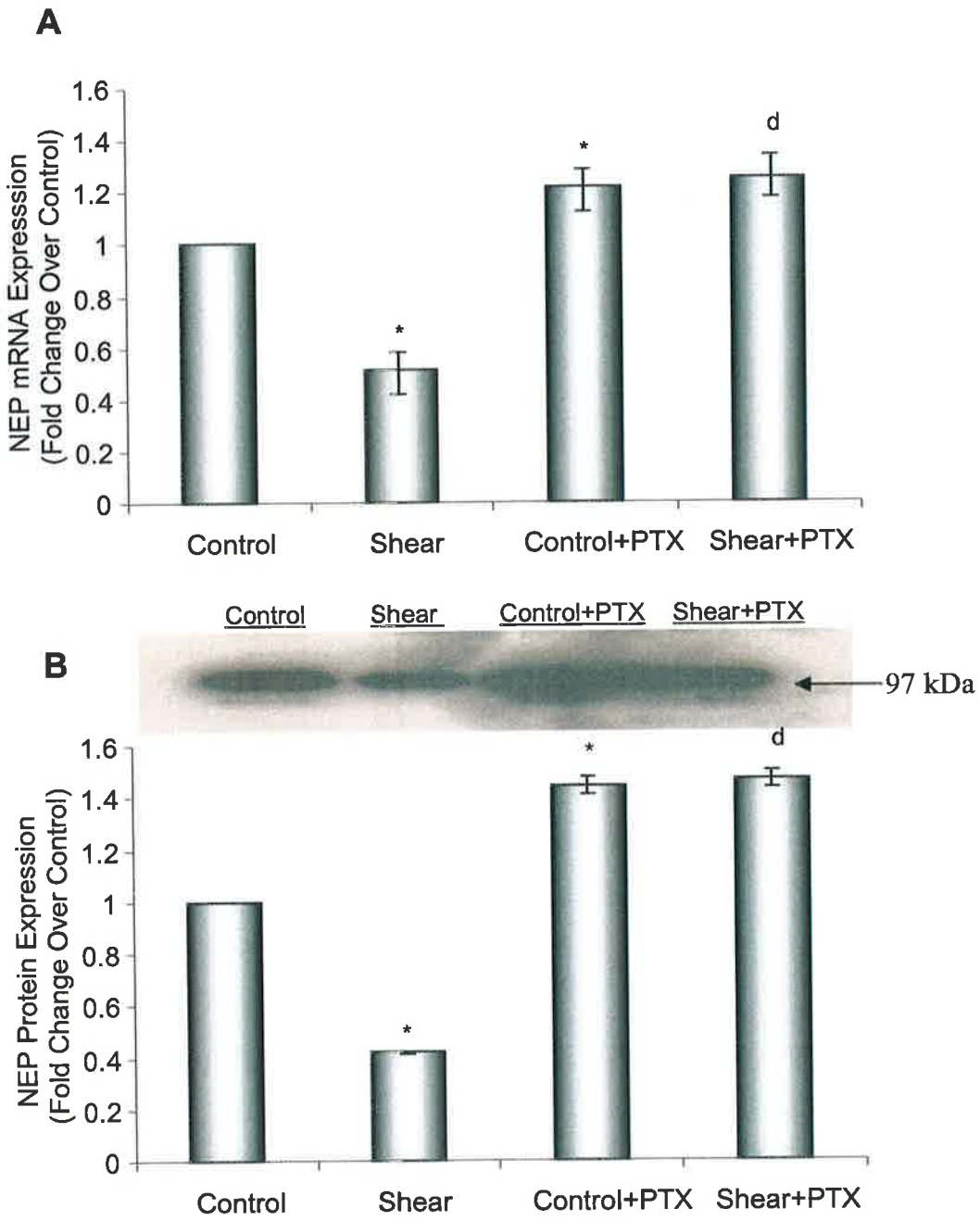


Fig 5.9 Effect of PTX on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 100 ng/ml PTX and monitored for changes in NEP (A) mRNA expression and (B) protein expression.. Histogram in (A) represent fold change in mRNA levels over untreated controls and is averaged from three independent experiments ±SEM. Histogram in (B) represents fold change in band intensity over untreated control and is averaged from three independent experiments ±SEM. Representative blot is shown. *p=0.05 versus static controls. dp=0.05 versus untreated shear.

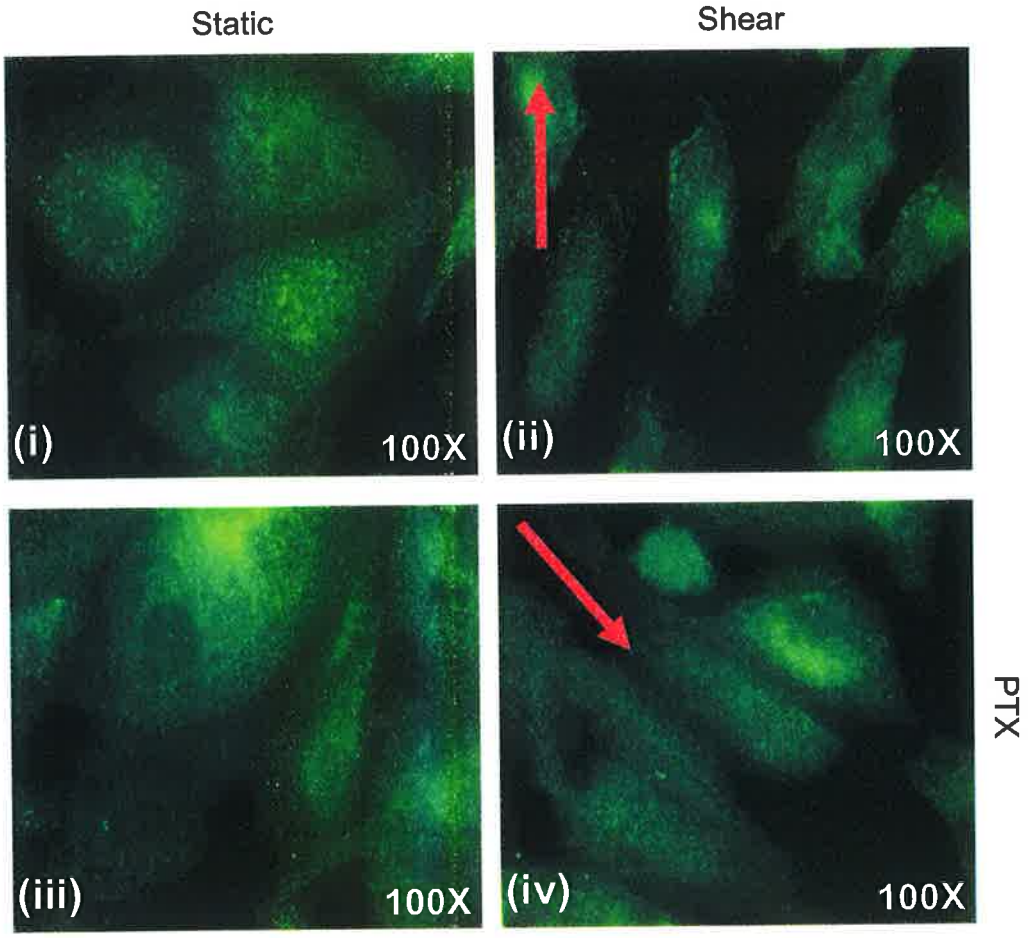


Fig 5.10 Effect of PTX on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 100 ng/ml PTX and visualised using immunocytochemiistry for changes in NEP expression. Untreated control (i), untreated shear (ii), PTX treated control (iii) and PTX treated shear (iv) were monitored using standard fluorescence microscopy (100X). Red arrows indicate the direction of flow. Images are representative of three independent experiments.

5.2.6 Effect of ß-Ark on shear-dependent modulation of NEP Expression

In order to ascertain fully the role played by G-proteins in the signal transduction of the shear stress attenuation of NEP expression, we secondly looked at the Gβγ subunit and examined its role via transfection of the ß-Ark-ct plasmid.

The effect of ß-Ark (1 μg DNA/10cm^2) on the shear-induced regulation of NEP mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress, NEP mRNA expression was attenuated to 0.44±0.01 fold of control. In the presence of ß-Ark, levels of NEP mRNA were then raised to 0.99±0.11 fold of control in sheared samples (Fig 5.11 A).

NEP protein expression levels were also measured using Western blotting and visualized using immunocytochemistry. Following application of shear stress NEP protein expression was attenuated to 0.53±0.03 fold of control. In the presence of ß-Ark, levels of NEP protein were then raised to 0.97±0.003 fold of control in sheared samples (Fig 5.11 B). Measured changes in protein levels were then visualized using immunocytochemistry, with images further mimicking the previously measured changes (Fig 5.12). Images clearly indicate a shear stress-induced attenuation in NEP protein levels, whilst this attenuation is ameliorated in the presence of ß-Ark (Fig 5.12(ii) and (iv) respectively).

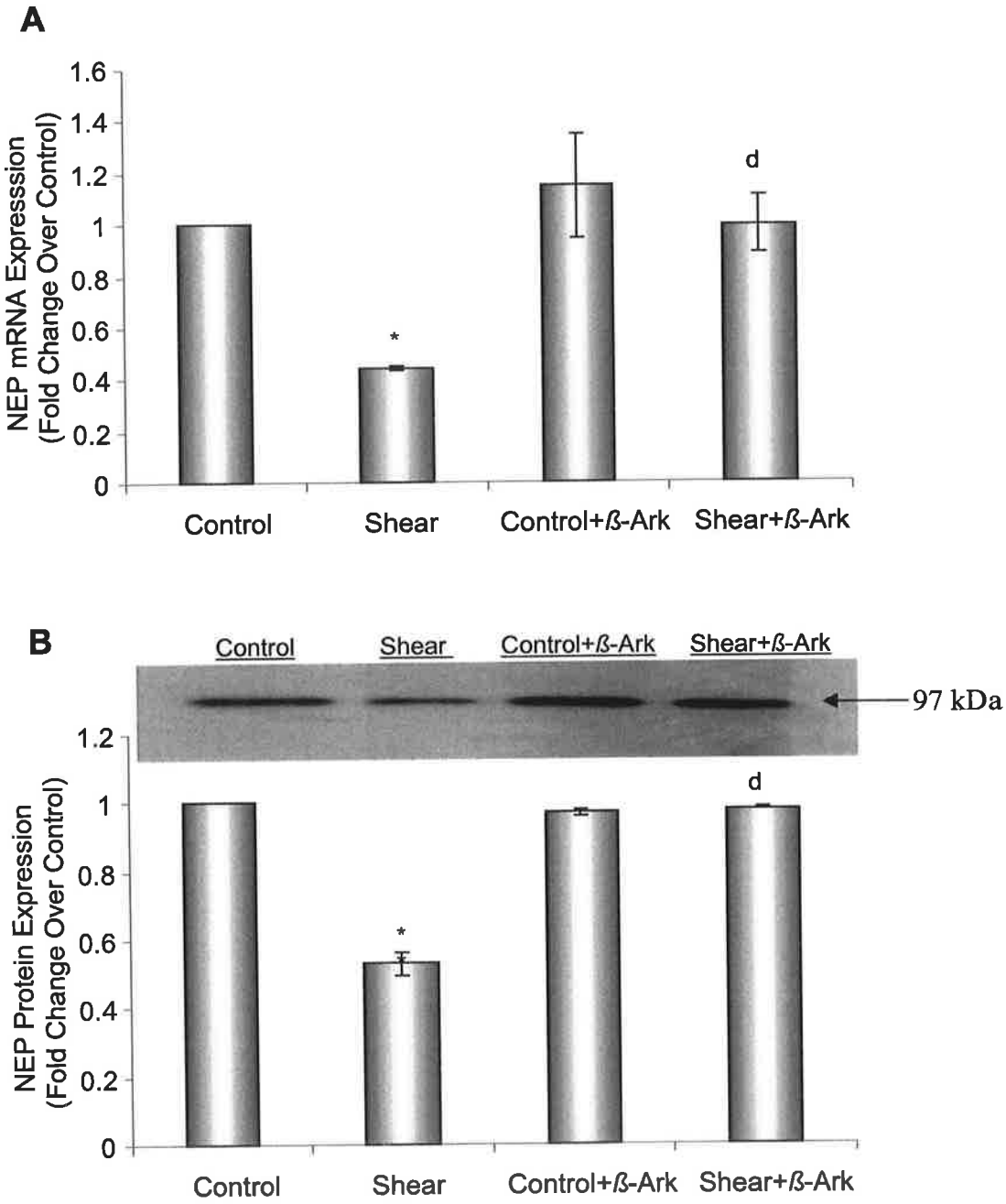


Fig 5.11 Effect of *ß*-Ark on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 1 μg DNA/10cm^2 ß-Ark and monitored for changes in NEP (A) mRNA expression and (B) protein expression.. Histogram in (A) represent fold change in mRNA levels over untreated controls and is averaged from three independent experiments ±SEM. Histogram in (B) represents fold change in band intensity over untreated control and is averaged from three independent experiments ±SEM. Representative blot is shown. *p=0.05 versus static controls. dp=0.05 versus untreated shear.

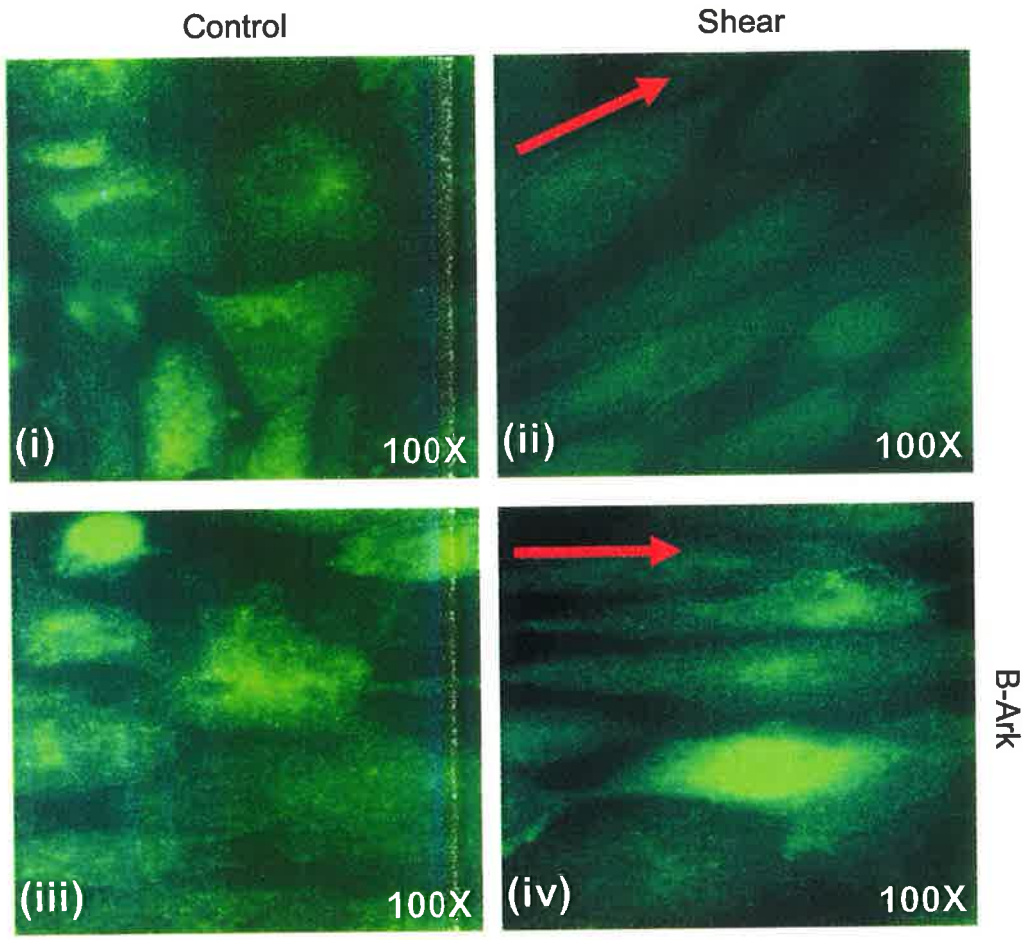


Fig 5.12 Effect of *β*-Ark on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 1 μg DNA/10cm^2 *β*-Ark and visualised using immunocytochemiistry for changes in NEP expression. Untreated control (i), untreated shear (ii), *β*-Ark treated control (iii) and *β*-Ark treated shear (iv) were monitored using standard fluorescence microscopy (100X). Red arrows indicate the direction of flow. Images are representative of three independent experiments.

5.2.7 Effect of Rac1 inhibitor NSC23766 on shear-dependent modulation of NEP Expression

In order to ascertain the role of Rac1, small G-protein, in the shear-induced attenuation of NEP, we employed a highly specific pharmacological inhibitor, NSC23766.

The effect of NSC23766 (50 μM) on the shear-induced regulation of NEP mRNA expression was monitored using Real-Time PCR and gene specific primers. Following exposure to shear stress NEP mRNA expression was attenuated to 0.59±0.13 fold of control. In the presence of NSC23766, levels of NEP mRNA were then raised to 1.03±0.09 fold of control in sheared samples (Fig 5.13 A).

NEP protein expression levels were also measured using Western blotting and visualized using immunocytochemistry. Following shear stress NEP protein expression was attenuated to 0.54±0.06 fold of control. In the presence of NSC23766, levels of NEP protein were then raised to 0.99±0.003 fold of control in sheared samples (Fig 5.13 B). Measured changes in protein levels were then visualized using immunocytochemistry, with images further mimicking the previously measured changes (Fig 5.14). Images clearly indicate a shear stress-induced attenuation in NEP protein levels whilst this attenuation is ameliorated in the presence of NSC23766 (Fig 5.14(ii) and (iv), respectively).

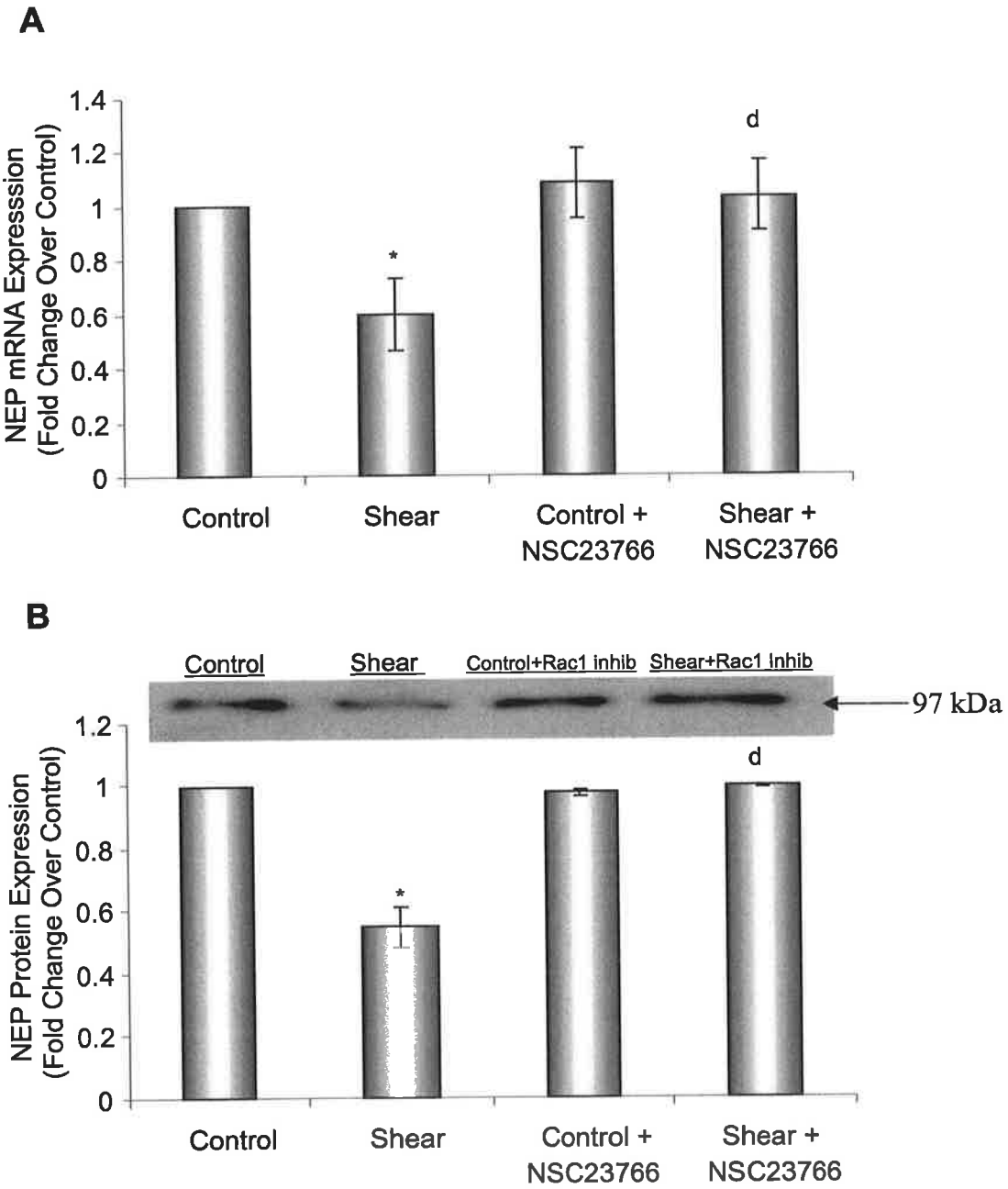


Fig 5.13 Effect of NSC23766 on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 50 μM NSC23766 and monitored for changes in NEP (A) mRNA expression and (B) protein expression.. Histogram in (A) represent fold change in mRNA levels over untreated controls and is averaged from three independent experiments ±SEM. Histogram in (B) represents fold change in band intensity over untreated control and is averaged from three independent experiments ±SEM. Representative blot is shown. *p=0.05 versus static controls. dp=0.05 versus untreated shear.

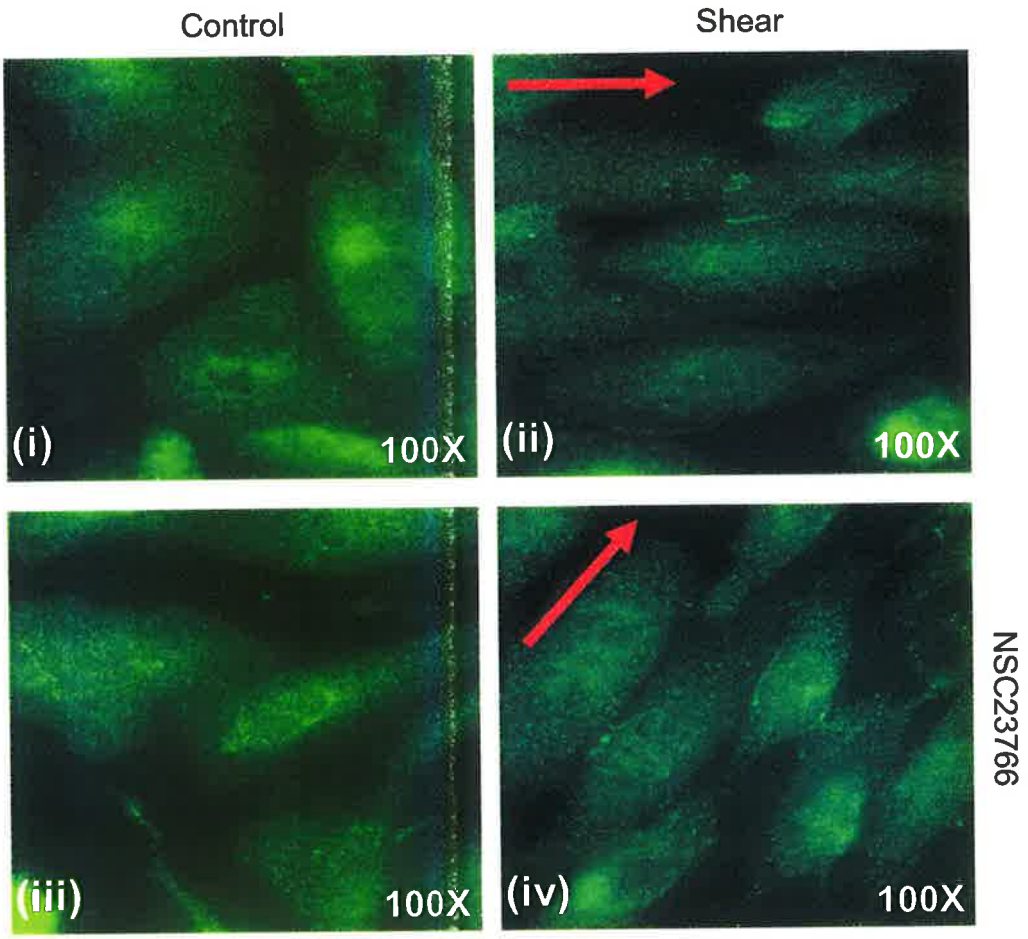


Fig 5.14 Effect of NSC23766 on shear-induced modulation of NEP expression. BAECs were exposed to shear stress (10 dynes/cm^2, 24 h) ± 50 μM NSC23766 and visualised using immunocytochemiistry for changes in NEP expression. Untreated control (i), untreated shear (ii), NSC23766 treated control (iii) and NSC23766 treated shear (iv) were monitored using standard fluorescence microscopy (100X). Red arrows indicate the direction of flow. Images are representative of three independent experiments.

5.3 Discussion

Blood vessels are constantly exposed to hemodynamic forces in the form of cyclic stretch and shear stress due to the pulsatile nature of blood pressure and flow. ECs, as the interface between vessel and blood, are subjected to these forces and possess the capacity to convert these mechanical stimuli into intracellular signals that affect cellular functions such as proliferation, apoptosis, migration, permeability, remodeling and gene expression. In order that the vessel and consequently the ECs that comprise that vessel adequately respond to changes in mechanical forces and hence maintain vessel tone and function, ECs use multiple sensing mechanisms to detect changes in the forces applied by blood flow, leading to the activation of signaling networks.

The purpose of this study was to identify the specific signaling pathway in endothelial cells mediating the shear-dependent reduction in NEP, a prototypical member of the thermolysin-like zinc metallopeptidases. Previous chapters have discussed in detail the ability of shear stress to modulate cellular oxidative state and in turn regulate metallopeptidase gene expression. Our first goal in this chapter was to ascertain the source of shear stress-induced ROS production. To this end, we employed a pharmacological inhibitor of NAD(P)H oxidase (Apocynin), an important ROS producing complex.

Many studies have previously demonstrated that the membrane-bound NAD(P)H oxidase complex is responsible for production of superoxide anion in vascular endothelial cells, the main precursor of other oxygen species, and is also responsive to hemodynamic stimulation.

NAD(P)H oxidase is a multicomponent enzyme, consisting of two main structures, cytosolic and membrane-bound. The cytosolic component comprises of a number of subunits, namely $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the small G-protein, Rac1. The membrane complex is made up of $gp91^{phox}$ and $p22^{phox}$ (collectively termed cytochrome b_{558}), with $gp91^{phox}$ as the major catalytic subunit (Parkos *et al.*, 1987). Upon activation of the enzyme, the GDP on the small G-protein Rac1 is exchanged for GTP and $p47^{phox}$ is phosphorylated by PKC. These two separate events trigger a conformational change in the cytosolic components facilitating firstly, association with each other and then membrane bound cytochrome b_{558}. Once all components have translocated to the membrane, the active complex is formed. NAD(P)H, the reduced substrate of the enzyme, binds to $gp91^{phox}$ subunit, with a subsequent transfer of two electrons to two molecules of oxygen to produce two molecules of $O_2^{-\bullet}$. Apocynin functions by inhibiting the association of the cytosolic $p47^{phox}$ and $p67^{phox}$ subunits with the membrane bound $gp91^{phox}$ subunit and hence preventing the formation of an active complex and $O_2^{-\bullet}$ production. Apocynin treatment clearly demonstrated a potential role for NAD(P)H oxidase-derived superoxide anion production in the shear stress-induced attenuation in NEP expression levels in BAECs.

Other main sources of ROS within the vascular endothelial cell include the mitochondrial electron transport chain, xanthine oxidase, and uncoupled NO synthases. We cannot rule out the possibility that these additional ROS sources may also have contributed to the observed temporal shear-induced ROS burst and consequent attenuation in NEP expression. In particular, xanthine oxidase, which has been shown to be sensitive to activation by oscillatory shear stress (McNally, Davis *et al.* 2003). However, whilst NAD(P)H oxidase is not the only source of ROS in the endothelium, these results indicate that its activation is required for shear-dependent regulation of NEP gene expression. Further study incorporating pharmacological and molecular inhibitors to specifically target other ROS sources is required in order to fully establish the role, if any, played by these other ROS sources in the observed shear stress-attenuation of NEP expression in the vascular endothelium.

Many receptors and protein complexes present on the endothelium surface allow vessels to detect subtle changes in the physical environment, from which different mechanotransduction cascades can be initiated according to the nature of the mechanical stimulus. Within vascular endothelial cells, cytoskeletal proteins transmit and modulate the tension between focal adhesion sites, integrins and the extracellular matrix. In addition to the structural modifications induced by mechanical forces, they may lead to changes in the ionic composition of the cells via ion channels, stimulate various membrane receptors and induce complex biochemical cascades.

Many intracellular pathways, such as MAPK cascades are activated by mechanical forces such as shear and initiate via sequential phosphorylations, the activation of transcription factors leading to subsequent gene expression changes (Lehoux *et al.*, 2003). As evidenced by ourselves and others, mechanical forces can also alter cellular oxidative state, with downstream effects on transcription factor activation and gene expression. We subsequently endeavored to investigate the signal pathway components upstream of NAD(P)H oxidase in the regulation of shear-dependent NEP reduction in BAECs.

Integrins have been shown by numerous studies to be active in transmission of mechanical forces such as shear stress. Studies have shown integrin clustering with adapter proteins Shc and FAK in response to shear stress, both of which are linked to downstream activation of MAPK pathways (Chen *et al.*, 2002, Tzima *et al.*, 2001). Whilst no studies have been published that evidence a role for integrins in shear stress regulation of expression of TLZM family members, studies have shown Ang II to induce integrin subunit expression, thus indicating a possible role downstream of their expression (Kawano *et al.*, 2000, Eero *et al.*, 1999). To study the possible role played by integrins in the shear stress modulation of NEP expression, RGD-binding inhibitory peptides were employed. Our findings indicated no significant effect of RGD peptides on shear-dependent NEP regulation, appearing to rule out a role for integrins in these events.

However, these studies do not rule out the possibility of the involvement of RGD-independent integrins. RGD-independent binding motifs such as those found on aVß3 have been shown to play a role in the mechanisms of vascular endothelial cell adhesion (Pedchenko, Zent *et al.* 2004). The employment of RGD-independent inhibitors such as cardiotoxin A5 (Wu, Lee *et al.* 2006) are required to fully explore the possible role of integrins in NEP attenuation

Attention then turned to examination of the role played by PTK in the shear stress attenuation of NEP expression. PTKs play roles in many signaling pathways that lead to activation of MAPK. Indeed, genistein, a PTK inhibitor, has been shown to attenuate shear stress activation of ERK and JNK (Li *et al.*, 1997, Jo *et al.*, 1997, Takahasi *et al.*, 1996). PTKs have also been shown to play a role in the early phase of flow-dependent NO production (Corson *et al.*, 1996). These studies raise the possibility that responses seen in NEP in our shear stress model may be mediated via PTK activation. In order to elucidate the possible role of PTKs, genistein an ATP-competitive inhibitor of PTK activity was employed. As with integrin inhibition via RGD-peptides genistein treatment had no effect on shear-dependent NEP attenuation, appearing to rule out a role for PTK activation in these events. In order to fully elucidate the possible role of PTKs in shear-dependent NEP attenuation it is necessary to repeat these experiments with a variety of molecular and pharmacological inhibitors capable of inhibiting non-receptor as well as receptor TKs.

The role of G-proteins in the shear stress attenuation of NEP was then examined. These proteins represent the largest group of cell surface receptors encoded by the mammalian genome (>1% of human genes) and, in the cardiovascular system G-protein coupled receptors (GPCRs) are implicated in more or less every regulatory event. We firstly sought to examine the role played by the Ga_i subunit through the use of a specific pharmacological inhibitor, PTX. Previous studies by Cotter *et al* in 2004, clearly demonstrated that cyclic strain induced changes in EP24.15 and EP24.16 were Ga_i subunit-dependent, with PTX entirely attenuating the strain induced changes. In our shear stress model, PTX was shown to clearly affect the expression levels of the related TLZM, NEP. A 25% increase in NEP expression in both static and sheared samples treated with PTX was measured, indicating that this regulation of NEP expression was independent of shear stress, as both static and sheared samples showed similar increases in NEP mRNA and protein levels. This shear stress-independent pathway can be considered a regulatory mechanism, by which the endothelium can control a number of cell processes. The Ga_i subunit has been shown to inhibit action of adenylate cyclase (AC). AC is known to increase levels of both cAMP and cGMP with downstream effects on barrier function, adhesion molecule expression and cell proliferation (Sadhu *et al.*, 1999). Indeed analogues of cAMP have been shown to induce an increase in expression of ET-1 in human endothelial cells (Stewart *et al.*, 1994). Concomitantly, ANP, the substrate for NEP, has been shown to regulate levels of cAMP and cGMP, whilst NEP has been evidenced to act on and degrade circulating levels of ET-1.

Furthermore, AC activation by Ga_i subunit inhibition and forskolin treatment has been shown to induce increases in NEP expression and activity. This data taken in conjunction with the evidenced increased NEP expression levels via PTX clearly indicate regulatory pathways by which NEP via ANP can regulate a number of cellular processes in a Ga_i subunit dependent manner.

We next turned to the $G_{\beta\gamma}$ subunit and its possible function in shear-induced attenuation of NEP expression. ß-ARK-ct is a non-kinase peptide derived from ß-ARK that competes for binding to the βγ-subunits of heterotrimeric G-proteins and thus acts as a sequestering agent of βγ-subunits and inhibitor of their function. In our study we observed that transfection with the ß-ARK-ct plasmid produced a complete attenuation of the shear induced decrease in NEP mRNA and protein expression, thus strongly indicating a possible role for the βγ-subunit in this pathway. Previous studies have shown that the βγ-subunit can regulate activity of Phosphoinositide-3 kinase (PI3K), an important signal transduction kinase involved in a number of cellular signaling pathways (Stephens *et al.*, 1997). Furthermore, the phosphorylated phosphatidylinositol (Ptdlns) products, Ptdlns-3-P, Ptdlns-3,4-P, produced by the actions of PI3K, have been shown to play a role in NAD(P)H oxidase activation. Whilst little work has been carried out to fully elucidate the interaction between PI3K and its products and NAD(P)H oxidase, studies have shown that Ptdlns-3,4-P has the ability to directly bind to the oxidase subunits, $p47^{phox}$ and $p40^{phox}$ (Hawkins *et al.*, 2007).

Indeed a study by Ellson *et al* in 2006 clearly demonstrated a role for Ptdlns-3,4-P in NAD(P)H oxidase activity through mutation of the Ptdlns-3,4-P binding domain on the $p40^{phox}$ subunit, which resulted in an inability of Ptdlns-3,4-P to bind the subunit with a marked decrease in cellular oxidative responses.

The βγ-subunit has also been implicated in signaling through small G-proteins such as Rac1. Rac1 is itself a component required for assembly and activation of a functioning NAD(P)H oxidase complex. We further sought to examine the role played by the small G-protein, Rac1, in the demonstrated shear-induced attenuation of NEP through the use of a specific pharmacological inhibitor.

Inhibition of Rac1 produced a similar response to that of βγ-subunit sequestration, with the shear induced NEP attenuation being entirely reversed in the presence of the inhibitor. As Rac1 is a known component of NAD(P)H oxidase and required for its function, this result was not entirely unexpected in that NAD(P)H oxidase-derived ROS were previously shown to regulate NEP expression and concomitantly that shear stress regulated NAD(P)H oxidase activation. Indeed, previous studies have evidenced a link between Gβγ-subunits and Rac1 activation via GEFs. The novel GEF, p114RhoGEF, has been shown to regulate Rac1 activity and consequently NAD(P)H oxidase production of ROS in a Gβγ-subunit dependent manner (Niu *et al.*, 2003).

5.4 Conclusion

Through the use of specific pharmacological and molecular inhibitors of known signal pathway components, we explored the signaling pathway mediating shear stress-induced attenuation of NEP expression. Our data suggests that shear stress attenuates NEP expression in a Gβγ-subunit/Rac1/NAD(P)H oxidase-dependent and an integrin, PTK and Ga^{i} independent manner.

Chapter 6 – Final Summary

6.1 Final Summary

The endothelium, a thin monocellular layer, is a dynamic cellular interface between the vessel wall and the bloodstream. The unique position of the endothelium confers on it the ability to sense and respond to a variety of blood borne stimuli. Among these stimuli are included, humoral and mechanical factors. These include neurotransmitters, peptide hormones, growth factors and their associated peptidases, as well as mechanical forces such as shear stress and cyclic strain. These stimuli result in both biophysical and biochemical changes in the endothelium which are, in turn, translated to a pronounced response in the vessel wall (Cummins *et al.*, 2004).

As mentioned, blood flow itself imparts a strong influence on the vessel wall. Of particular importance are the mechanical forces of cyclic strain and shear stress. Cyclic strain is a circumferential force that acts outwardly on the vessel wall as a result of pulsatile blood flow, resulting in a stretching of the cells of the endothelium, whilst shear stress is a frictional or tractive force which drags against the endothelium. Both forces have been implicated in a number of endothelial cell responses and have been shown to influence a number of vasoactive processes. Stretch has been shown to influence NOS, and ET-1 expression (Awolesi *et al.*, 1995, Cheng *et al.*, 1996), to regulate of p53 and Akt phosphorylation (Mayr *et al.*, 2002, Persoon-Rothert *et al.*, 2002, Haga *et al.*, 2003), and to confer increased sensitivity of the endothelium to shear stress.

Shear stress acts directly on the cellular membrane, and thus allows for the rapid transfer of extracellular signals into the cellular space and the induction of appropriate cellular responses. Of primary importance among these responses is the ability of the endothelium to alter expression of vasoactive molecules which regulate vessel tone.

Vascular tone, referred to as the level of constriction versus dilation that a vessel experiences, is regulated by a number of competing vasoconstrictor and vasodilatory influences, namely ET-1 and NO, respectively. The circulating levels of these and other, vasoactive molecules have all been evidenced to be modulated by shear stress (Cooke *et al.*, 1997, Morawietz *et al.*, 2000), indicating the ability of mechanical forces to regulate vessel tone, likely via alteration in peptide expression and associated peptidase function. Concomitantly, disruption of these processes and consequently the ability of the endothelium to mediate vessel tone, can lead to vascular dysfunction and CVD. Thus, the forces that regulate expression of these peptidases are implicated as possible emergent targets for clinical treatment of such dysfunction. Recently a number of physiological systems such as the renin-angiotensin and endothelin systems have become targets of clinical interest. The principal regulatory peptidases involved, ACE and ECE respectively, have been shown to be sensitive to regulation by shear stress.

Currently a number of pharmacological inhibitors of these peptidases are used clinically in the treatment of endothelial dysfunction and CVD. The orally active ACE inhibitors, captopril and enalapril, have been used with much success in the treatment of hypertension and CHF (Kelly and O'Malley 1990). Selective ECE inhibitors have also entered clinical trials. The ECE-1-selective compounds CGS-35066 and SM-19712, have been shown to effectively reduce hypertension and the risk of acute myocardial infarction, respectively (Jeng 2003). In conjunction with this, a related ectoenzyme, namely NEP, the principal enzymatic regulator of the natuiretic peptide system, has recently joined ACE and ECE as a possible clinical target, with dual ACE/NEP, ECE/NEP and ACE/ECE,NEP inhibitors currently under investigation. To date, the most widely studied is omapatrilat, an ACE/NEP inhibitor that exerts prolonged antihypertensive effects and has been shown in one study of 5770 patients with CHF to reduce cardiovascular death by 9% (Jandeleit-Dahm 2006). However, very little work has focussed on the regulation of these peptidases by blood flow associated mechanical forces, a major determinant of vascular diseases. We, therefore, examined the role of shear stress in regulating expression of TLZMs in vascular endothelial cells.

Previous studies have demonstrated shear stress-responsiveness in members of this family, namely ECE and ACE. Indeed, the promoter region of the ECE gene contains at least four classic SSRE sequences, whilst the ACE promoter region contains two novel SSRE sequences.

Shear studies carried out by Morawietz *et al.*, and Masatsugu *et al.*, have shown a clear down-regulation in ECE expression due to shear stress (Morawietz *et al.*, 2000; Masatsugu *et al.*, 2003), with a similar study by Rieder and co-workers demonstrating a clear shear-induced attenuation of ACE mRNA and protein expression levels (Rieder *et al.*, 1997). These studies, in parallel with work previously carried out in our laboratory on the effects of cyclic strain on EP24.15 and EP25.16 expression levels (Cotter *et al.*, 2003), clearly indicate the role for hemodynamic regulation of vasoactive peptidases of the TLZM family within the vascular endothelium.

Subsequently we examined mRNA and protein expression of ACE, ECE, NEP, EP24.15 and EP24.16 in response to chronic shear stress. We clearly demonstrated the ability of shear stress to regulate expression levels of these important endopeptidases. Levels of ACE, ECE, NEP and EP24.16 mRNA and protein were all attenuated in response to shear stress, whilst mRNA and protein levels of EP24.15 were increased. These results were in line with previously mentioned studies showing attenuation of ACE and ECE mRNA and protein expression in response to shear stress (Rieder *et al.*, 1997; Masatsugu *et al.*, 2003). This modulation in expression levels of these peptidases is a logical response to shear stress by the vasculature, in that, as previously discussed (Section 1.5), these peptidases and their associated peptides play an important role in controlling vessel tone.

Through modulation of their expression, the endothelium can alter the balance between vasoconstrictor and vasodilatory influences in response to hemodynamic forces such as laminar shear stress.

We next focused on elucidating the mechanisms through which shear stress altered metallopeptidase expression. Previous studies have shown that expression of vasoactive molecules such as ECE and ET-1 are influenced by shear stress via modulation of the cellular oxidative state (Masatsugu *et al.*, 2003). We therefore hypothesised that the shear stress-dependent regulation of TLZM expression may be influenced by the cellular oxidative state, and furthermore that shear-induced responses may depend on the production of a transient oxidative burst as an integral part of its regulatory pathway in vascular endothelial cells. To explore this hypothesis, we first examined the ability of shear stress to modulate the cellular oxidative state. Differing fluid shear patterns, laminar vs. oscillatory for example, have been evidenced previously to induce alteration in the cellular oxidative state. Whilst oscillatory shear stress has been shown to induce an incremental increase in cellular ROS levels, eventually leading to disruption of cellular mechanisms, laminar shear has been shown to induce a transient burst of ROS that act in a non-toxic signaling capacity (De Keulenaer *et al.*, 1998).

Through the use of appropriate assays, we observed a shear-induced ROS burst in BAECs that dissipated with time (1-4 h).

Induction of an artificial oxidative state by Ang II or H_2O_2 treatment yielded changes in TLZM expression identical to those induced by shear stress. Moreover, inclusion of antioxidants (NAC, SOD, CAT) prevented shear-dependent changes in metallopeptidase expression for all enzymes studied. We could therefore conclude that shear stress mediates its regulation of TLZM expression, at least in part, via modulation of cellular oxidative state (Fig 7.1).

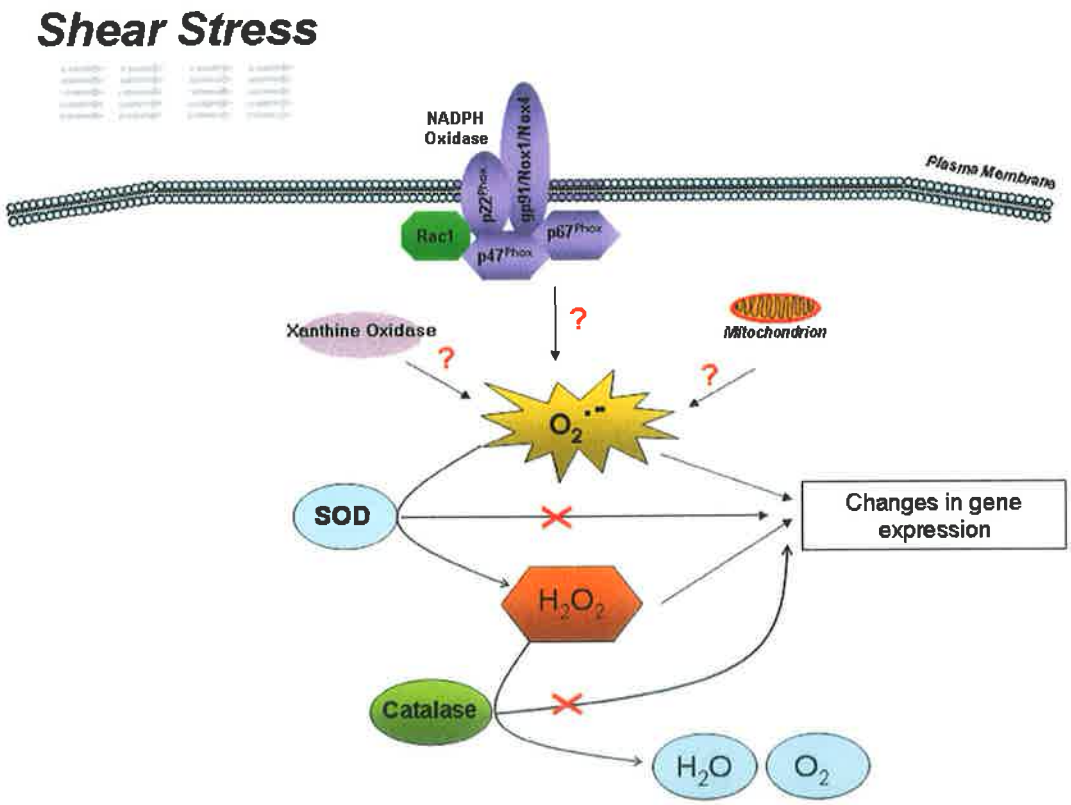


Fig 7.1 Diagrammatic representation of shear stress/ROS regulation of TLZM expression in BAECs

This data raises the possibility that overall oxidative state is responsible, rather than a specific oxygen species. This finding is based on the fact that whilst SOD inhibited the shear-induced effects on TLZM expression by removing superoxide anion, and thus implicating it as the oxygen species responsible, it would also through its action have produced hydrogen peroxide, which we have shown to alter expression levels in a manner similar to shear stress. Further to this, incubation with catalase also inhibited the shear-induced effects, indicating the possibility that neither oxygen species alone are responsible and, that the entire oxidative state of the cell, contributed to by a number of ROS, is responsible for the shear-mediated regulation of TLZM expression in the vasculature.

ROS are known to control a number of cellular processes by regulation of specific gene expression. Indeed transcription factors were the first signalling molecules to be identified as redox-sensitive (Poli *et al.*, 2004). Whilst ROS lack the accepted characteristics of traditional signalling molecules, they are small, highly reactive, short-lived and lack structural and conformational complexity and they have been evidenced to act as signalling molecules in a number of pathways. Among the primary targets for ROS are sulphydryl groups on cysteine residues. These residues can be oxidized to form disulphide bonds resulting in formation of intra- or inter-molecular disulphide bonds. These disulphide bonds alter protein conformation and hence influence DNA binding and enzymatic activity. In this way ROS levels within the cell can modulate both enzymatic activity and gene transcription (Cooper *et al.*, 2002).

One of the most widely studied redox sensitive transcription factors is that of NF-κB. The predominant and inactive form of NF-κB exists as a trimer of p65, p50 and I-κB subunits. NF-κB is activated by phosphorylation of two serine residues in I-κB, the inhibitory subunit. Once phosphorylated, I-κB is polyubiquitinylated and degraded, thus allowing NF-κB to translocated to the nucleus and activate transcription (Ghosh and Karin, 2002). Additional support for the involvement of ROS in regulation of gene transcription is the activation of NF-κB by many ROS elevating agents such as TNF-a, IL-1ß, phorbol 12-myristate 13-acetate (PMA), UV light, γ-rays, and lipid hydroperoxides. Concomitantly, studies incorporating antioxidants have shown their ability to block both ROS production and resultant NF-κB activation by these agents. As TLZM gene expression has been evidenced to be shear- and oxidative state-sensitive, it is possible to hypothesize that this sensitivity is mediated via alteration in activity of transcription factors, such as NF-κB. Indeed, NOS expression and NO production have been evidenced *in vivo* to be ROS sensitive in an NF-κB dependent manner and, as such, to regulate vessel tone (D'Acquisto *et al.*, 1999).

Once the roles of both shear stress and the oxidative state of the cell in the regulation of TLZM expression had been clearly evidenced, it was decided that future work would focus on a single prototypical member of the TLZM family, namely NEP. NEP has recently emerged as a clinical target in the treatment of CVD, with dual ACE/NEP and ECE/NEP inhibitors currently under study.

Despite the identification of NEP as a clinically important metallopeptidase, to our knowledge there have been no studies published which examine the effect of either blood flow and its associated mechanical forces or oxidative state on its expression in the vasculature. In order to address this, we sought firstly to ascertain the exact mechanisms through which shear stress modulated the oxidative state and hence NEP expression.

Initially my work focused on identifying the source of the shear stress-induced ROS shown to alter NEP expression. The cellular environment is home to a variety of reactive and non-reactive oxygen species, including $O_2^{-\bullet}$, $NO^{\bullet}$, $ONOO^{-\bullet}$,$OH^{\bullet}$ and H_2O_2, hence work focused on elucidating the role of the major source of non-toxic levels of ROS, NAD(P)H oxidase, which has been evidenced to produce $O_2^{-\bullet}$, a known precursor of most other oxygen species. De Keulenaer *et al*, evidenced a role for NAD(P)H oxidase in shear stress regulation of the cellular oxidative state. This study clearly defined differing roles for oscillatory versus laminar shear in the production of ROS. Oscillatory shear was shown to induce a steady and continuous rise in the levels of ROS present, eventually pushing the cell towards a toxic oxidative stress beyond the capacity of endogenous antioxidant systems. Laminar shear, however, was shown to initiate a transient ROS burst which dissipated with time, and thus provided a non-toxic signaling stimuli to the cell (De Keulenaer *et al.*, 1998). We evidenced this transient ROS burst and, through the use of an NAD(P)H oxidase specific pharmacological inhibitor (apocynin), clearly showed that the measured attenuation in NEP expression was NAD(P)H oxidase-dependent.

Future should focus on further elucidating the role of NAD(P)H oxidase in this pathway through the use of molecular inhibitors of the individual NAD(P)H oxidase subunits and through this possibly identify further and more pathway specific therapeutic targets.

Signal transduction components upstream of NAD(P)H oxidase activation were then examined for a putative role in these events. Integrins were initially examined due to previous studies evidencing them as shear stress-sensitive and active in transmission of mechanical forces. It was initially hypothesized that integrin activation may play a role in shear stress attenuation of NEP expression, in that previous studies have shown a role for integrins in shear stress regulation of the ET-1 expression, the main component of the endothelin system (Chen *et al.*, 2001). In order to explore this possibility, RGD-binding inhibitory peptides were employed. However, under the conditions studied, treatment with RGD peptides had no significant effect on shear-induced attenuation of NEP expression in BAECs, seemingly indicating a non-integrin dependent signaling pathway. The use of RGD peptides however, does not rule out the possibility of the involvement of non-RGD dependent integrins. Further study is required to rule out this possibility. Future studies should seek to employ non-RGD dependent integrin inhibitors and more specific molecular inhibitors. The role of greater levels of shear stress should also be examined. A novel study, and a possible model for future studies, by Chen and co-workers, employed magnetic twisting cytometry, to physically "twist" integrins

into an active conformation. Through this method they could apply directly to the integrins, shear stress of up to 20 dynes/cm^2 (Chen *et al.*, 2001).

We then examined of the possible role played by PTK in the shear stress attenuation of NEP expression. PTKs play roles in many signaling pathways including MAPK activation and have also been shown to play a role in the early phase of flow dependent NO production (Corson *et al.*, 1996). It was therefore possible to conclude that PTKs may play a similar role in the shear stress attenuation of NEP, another important vasoactive molecule. However, treatment with genistein, a PTK inhibitor, yielded no statistically significant effect on shear-induced changes in NEP expression. This indicates that in our model of shear stress, transmission of the shear stimuli is independent of PTK pathways. However, genistein is a receptor tyrosine kinase specific inhibitor, thus these results do not rule out a possible role for non-receptor tyrosine kinases. Further study incorporating inhibitors of non-receptor tyrosine kinases is required to fully explore this possibility.

The role of G-proteins was then examined. We sought through the use of a specific pharmacological inhibitor PTX, to examine the role played by the Ga_i subunit. Previous studies by Cotter *et al* in 2004, clearly demonstrated that cyclic strain induced changes in EP24.15 and EP24.16 were Ga_i subunit-dependent with PTX entirely attenuating the strain induced changes. In our shear stress model, PTX was shown to positively affect the expression levels, with increases in NEP mRNA and protein expression under both static and shear conditions treated with PTX.

These findings were interesting in that, previous work in our laboratory had shown cyclic strain-induced changes in expression of metallopeptidases EP24.15 and EP24.16 to be PTX-sensitive. However other previous studies have shown AC activation by PTX-induced Ga_i subunit inhibition and forskolin treatment to induce increases in NEP expression and activity, thus highlighting a possible shear-independent pathway for regulation of NEP expression.

Interest then turned to the $G_{ß}\gamma$ subunit and its possible function in the evidenced shear-induced attenuation of NEP expression. Transfection of the ß-ARK-ct encoding plasmid, a sequestering agent of βγ-subunits, produced a complete attenuation of the shear-induced attenuation in NEP mRNA and protein expression, thus strongly evidencing a role for the βγ-subunit in this pathway. Previous studies have shown the βγ-subunit can regulate activity of Phosphoinositide-3 kinase (PI3K), an important signal transduction kinase (Stephens *et al.*, 1997) and activator of NAD(P)H oxidase via Ptdlns-3-P, Ptdlns-3,4-P. Whilst little work has focused on elucidating the exact mechanisms of these interactions, studies have shown that Ptdlns-3,4-P has the ability to directly bind to the oxidase subunits, $p47^{phox}$ and $p40^{phox}$ (Hawkins *et al.*, 2007), thus indicating that further study, possibly involving a PI3K-specific inhibitor such as LY 294002 (Vlahos *et al.*, 1994) is required to fully elucidate the exact mechanism involved. The βγ-subunit has also been implicated in signaling through small G proteins, such as Rac1. Rac1 is itself a component required for assembly and activation of a functioning NAD(P)H oxidase complex and hence ROS production.

We sought to examine the role played by the small G-protein, Rac1, in shear-induced attenuation of NEP through the use of a specific pharmacological inhibitor. Inhibition of Rac1 produced an identical response to that of βγ-subunit sequestration, with shear-induced NEP attenuation being entirely reversed in the presence of the inhibitor. This was a logical response in that NAD(P)H oxidase derived ROS were previously shown to regulate NEP expression and concomitantly that shear stress regulated NAD(P)H oxidase activation. Indeed previous studies have linked Gβγ-subunits and Rac1 activation to NAD(P)H oxidase activity via the novel GEF, p114RhoGEF (Niu *et al.*, 2003). Future studies should focus on examining the extent of the possible role played by p114RhoGEF in these events.

This data taken in its entirety allows us to propose that shear stress attenuates NEP expression in a Gβγ-subunit/Rac1/NAD(P)H oxidase-dependent and an integrin, PTK and Ga^{i} independent manner (see Fig 7.2). This proposed pathway not only clearly delineates the mechanisms by which the endothelium senses and subsequently alters NEP expression and consequently, at least in part, vessel tone, but it also identifies for the first time, future clinical targets, inhibitors of which could form a battery of possible treatments to be used in conjunction with current enzymatic inhibitors. Also through the examination of NEP, a prototypical member of the TLZM family, it is possible to propose that regulation of expression of other members of this family, such as the clinically important ACE and ECE, may have similar regulatory pathways. Future work should focus on elucidating the possible similarities in expression pathways for other members of this family.

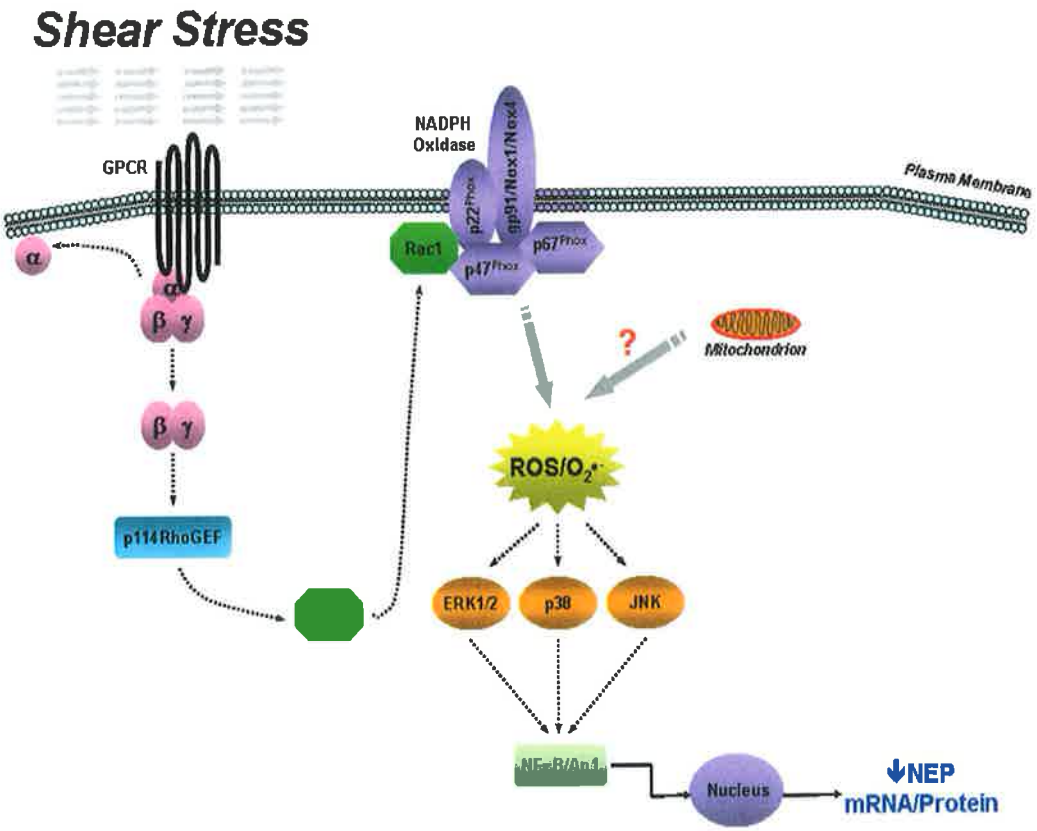


Fig 7.2 Signaling pathway model for shear stress attenuation of NEP expression via oxidative state in the endothelial cell.

Logical future studies should involve examination of alternate shear patterns, such as disturbed and oscillatory shear in relation to this pathway and consequently regulation of expression of NEP and other TLZMs within the vascular endothelium. Differing shear patterns, associated with vessel damage and lesion prone areas such as bifurcations, have been shown to induce higher levels of oxidative stress and endothelial dysfunction.

In addition to this the functional impact of changes in expression should be assed through NEP activity assays, possibly measuring substrates such as ANP, ET-1 or bombosen levels. In this manner, it would be possible to fully exploit the potential therapeutic value of this pathway.

The potential therapeutic value of this pathway could also be explored through the repetition of this study in an *in vivo* model with the incorporation of an ApoE-/- mouse model. This would provide a greater understanding of the physiological impact and hence possible therapeutic value of the pathway components examined in disease states such as atherosclerosis and hypercholesterolemia. Indeed previous studies have shown that treatments incorporating physiologically relevant antioxidants such as vitamin C, have yielded somewhat positive results. Animal and human studies have shown that vitamin C can improve endothelial function in a number of cellular oxidative stresses such as those caused by coronary artery disease, diabetes, hypercholesterolemia, hypertension and heart failure (Schulz *et al.*, 2004). However, there are a number of downsides to vitamin C treatments, with higher concentrations leading to vitamin induced-toxicity and further ROS production. Other treatments involve direct action by pharmacological inhibitors of vasoactive peptidases such as ACE and peptide receptors such as AT-1 receptor. Whilst these treatment methods have been well-characterized and shown to improve endothelial function in a number of vascular disease states, they also are not without limitations.

Recently ACE inhibitors, used in the treatment of pre-eclampsia, pregnancy related hypertension, have been shown to cause serious teratogenic effects, such as fetal malformation and even fetal death (Quan, 2006, Atallah, 2006). These limitations and negative side effects of current treatment models further highlight the relevance of this study and the importance of future work in the search for potential, safer CVD treatment paradigms.

Chapter 7 References

Abe, J. & Berk, B. C. (1999) Fyn and JAK2 mediate Ras activation by reactive oxygen species. *J Biol Chem,* 274, 21003-10.

Alevriadou, B. R., Eskin, S. G., Mcintire, L. V. & Schilling, W. P. (1993) Effect of shear stress on 86Rb+ efflux from calf pulmonary artery endothelial cells. *Ann Biomed Eng,* 21, 1-7.

Atallah, A. N. (2006) Angiotensin-converting enzyme inhibitors during the first trimester of pregnancy increase the incidence of fetal malformation, whereas calcium intake (1.0 to 2.0 g/day) prevents preeclampsia. *Sao Paulo Med J,* 124, 243-4.

Ausubel, F. M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Struhl, K., (1991) *Current Protocols in Molecular Biology,* New York, Wiley Interscience.

Awolesi, M. A., Sessa, W. C. & Sumpio, B. E. (1995) Cyclic strain upregulates nitric oxide synthase in cultured bovine aortic endothelial cells. *J Clin Invest,* 96, 1449-54.

Ayajiki, K., Kindermann, M., Hecker, M., Fleming, I. & Busse, R. (1996) Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. *Circ Res,* 78, 750-8.

Azevedo, E. R., Newton, G. E., Parker, A. B., Floras, J. S. & Parker, J. D. (2000) Sympathetic responses to atrial natriuretic peptide in patients with congestive heart failure. *J Cardiovasc Pharmacol,* 35, 129-35.

Babior, B. M., Lambeth, J. D. & Nauseef, W. (2002) The neutrophil NADPH oxidase. *Arch Biochem Biophys,* 397, 342-4.

Banai, S., Shweiki, D., Pinson, A., Chandra, M., Lazarovici, G. & Keshet, E. (1994) Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis. *Cardiovasc Res,* 28, 1176-9.

Bartling, B., Tostlebe, H., Darmer, D., Holtz, J., Silber, R. E. & Morawietz, H. (2000) Shear stress-dependent expression of apoptosis-regulating genes in endothelial cells. *Biochem Biophys Res Commun,* 278, 740-6.

Bassenge, E. (1996) Endothelial function in different organs. *Prog Cardiovasc Dis,* 39, 209-28.

Beckman, K. B. & Ames, B. N. (1998) The free radical theory of aging matures. *Physiol Rev,* 78, 547-81.

Birnboim, H. C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol,* 100, 243-55.

Bohlen, H. G., Gore, R. W. & Hutchins, P. M. (1977) Comparison of microvascular pressures in normal and spontaneously hypertensive rats. *Microvasc Res,* 13, 125-30.

Burnett, J. C., Jr., Granger, J. P. & Opgenorth, T. J. (1984) Effects of synthetic atrial natriuretic factor on renal function and renin release. *Am J Physiol,* 247, F863-6.

Burnett, J. C., Jr., Kao, P. C., Hu, D. C., Heser, D. W., Heublein, D., Granger, J. P., Opgenorth, T. J. & Reeder, G. S. (1986) Atrial natriuretic peptide elevation in congestive heart failure in the human. *Science,* 231, 1145-7.

Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R. & Hamm, H. E. (2003) Insights into G protein structure, function, and regulation. *Endocr Rev,* 24, 765-81.

Chen, J., Fabry, B., Schiffrin, E. L. & Wang, N. (2001) Twisting integrin receptors increases endothelin-1 gene expression in endothelial cells. *Am J Physiol Cell Physiol,* 280, C1475-84.

Chen, K. D., Li, Y. S., Kim, M., Li, S., Yuan, S., Chien, S. & Shyy, J. Y. (1999) Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. *J Biol Chem,* 274, 18393-400.

Cheng, J. J., Chao, Y. J. & Wang, D. L. (2002) Cyclic strain activates redox-sensitive proline-rich tyrosine kinase 2 (PYK2) in endothelial cells. *J Biol Chem,* 277, 48152-7.

Cheng, J. J., Chao, Y. J., Wung, B. S. & Wang, D. L. (1996a) Cyclic strain-induced plasminogen activator inhibitor-1 (PAI-1) release from endothelial cells involves reactive oxygen species. *Biochem Biophys Res Commun,* 225, 100-5.

Cheng, J. J., Wung, B. S., Chao, Y. J. & Wang, D. L. (1996b) Cyclic strain enhances adhesion of monocytes to endothelial cells by increasing intercellular adhesion molecule-1 expression. *Hypertension,* 28, 386-91.

Chien, S., Li, S. & Shyy, Y. J. (1998) Effects of mechanical forces on signal transduction and gene expression in endothelial cells. *Hypertension,* 31, 162-9.

Chilian, W. M., Eastham, C. L. & Marcus, M. L. (1986) Microvascular distribution of coronary vascular resistance in beating left ventricle. *Am J Physiol,* 251, H779-88.

Chiu, J. J., Chen, L. J., Chen, C. N., Lee, P. L. & Lee, C. I. (2004) A model for studying the effect of shear stress on interactions between vascular endothelial cells and smooth muscle cells. *J Biomech,* 37, 531-9.

Chun, M., Liyanage, U. K., Lisanti, M. P. & Lodish, H. F. (1994) Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci U S A,* 91, 11728-32.

Cooke, J. P. & Dzau, V. J. (1997) Nitric oxide synthase: role in the genesis of vascular disease. *Annu Rev Med,* 48, 489-509.

Cooke, J. P., Rossitch, E., Jr., Andon, N. A., Loscalzo, J. & Dzau, V. J. (1991) Flow activates an endothelial potassium channel to release an endogenous nitrovasodilator. *J Clin Invest,* 88, 1663-71.

Cooper, C. E., Patel, R. P., Brookes, P. S. & Darley-Usmar, V. M. (2002) Nanotransducers in cellular redox signaling: modification of thiols by reactive oxygen and nitrogen species. *Trends Biochem Sci,* 27, 489-92.

Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C. & Harrison, D. G. (1996) Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res,* 79, 984-91.

Corvol, P.; Williams, TA. (1998) Peptidyl-dipeptidase A/angiotensin 1-converting enzyme. *Handbook of Proteolytic Enzymes.* San Diego: Academic Press. pp. 1066–1076.

Corti, R., Burnett, J. C., Jr., Rouleau, J. L., Ruschitzka, F. & Luscher, T. F. (2001) Vasopeptidase inhibitors: a new therapeutic concept in cardiovascular disease? *Circulation,* 104, 1856-62.

Cotter, E. J., Von Offenberg Sweeney, N., Coen, P. M., Birney, Y. A., Glucksman, M. J., Cahill, P. A. & Cummins, P. M. (2004) Regulation of endopeptidases EC3.4.24.15 and EC3.4.24.16 in vascular endothelial cells by cyclic strain: role of Gi protein signaling. *Arterioscler Thromb Vasc Biol,* 24, 457-63.

Couet, J., Li, S., Okamoto, T., Ikezu, T. & Lisanti, M. P. (1997) Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. *J Biol Chem,* 272, 6525-33.

Crack, P. J., Wu, T. J., Cummins, P. M., Ferro, E. S., Tullai, J. W., Glucksman, M. J. & Roberts, J. L. (1999) The association of metalloendopeptidase EC 3.4.24.15 at the extracellular surface of the AtT-20 cell plasma membrane. *Brain Res,* 835, 113-24.

Cummins, P. M., Cotter, E. J. & Cahill, P. A. (2004) Hemodynamic regulation of metallopeptidases within the vasculature. *Protein Pept Lett,* 11, 433-42.

Cunningham, K. S. & Gotlieb, A. I. (2005) The role of shear stress in the pathogenesis of atherosclerosis. *Lab Invest,* 85, 9-23.

Cushman, D. W. & Cheung, H. S. (1971) Concentrations of angiotensin-converting enzyme in tissues of the rat. *Biochim Biophys Acta,* 250, 261-5.

D'acquisto, F., Ialenti, A., Iuvone, T., Di Rosa, M. & Carnuccio, R. (1999) Inhibition of nuclear factor-kappaB prevents the loss of vascular tone in lipopolysaccharide-treated rats. *Eur J Pharmacol,* 365, 253-7.

Davies, P. F. (1995) Flow-mediated endothelial mechanotransduction. *Physiol Rev,* 75, 519-60.

Davies, P. F. & Tripathi, S. C. (1993) Mechanical stress mechanisms and the cell. An endothelial paradigm. *Circ Res,* 72, 239-45.

De Gasparo, M. (2002) [AT(1) and AT(2) angiotensin II receptors: key features]. *Drugs,* 62 Spec No 1, 1-10.

De Jonge, H. W., Dekkers, D. H., Tilly, B. C. & Lamers, J. M. (2002) Cyclic stretch and endothelin-1 mediated activation of chloride channels in cultured neonatal rat ventricular myocytes. *Clin Sci (Lond),* 103 Suppl 48, 148S-151S.

De Keulenaer, G. W., Chappell, D. C., Ishizaka, N., Nerem, R. M., Alexander, R. W. & Griendling, K. K. (1998) Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res,* 82, 1094-101.

Diamond, S. L., Sharefkin, J. B., Dieffenbach, C., Frasier-Scott, K., Mcintire, L. V. & Eskin, S. G. (1990) Tissue plasminogen activator messenger RNA levels increase in cultured human endothelial cells exposed to laminar shear stress. *J Cell Physiol,* 143, 364-71.

Ellson, C., Davidson, K., Anderson, K., Stephens, L. R. & Hawkins, P. T. (2006) PtdIns3P binding to the PX domain of p40phox is a physiological signal in NADPH oxidase activation. *Embo J,* 25, 4468-78.

Esposito, F., Chirico, G., Montesano Gesualdi, N., Posadas, I., Ammendola, R., Russo, T., Cirino, G. & Cimino, F. (2003) Protein kinase B activation by reactive oxygen species

is independent of tyrosine kinase receptor phosphorylation and requires SRC activity. *J Biol Chem,* 278, 20828-34.

Exton, J. H. (1996) Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to G proteins. *Annu Rev Pharmacol Toxicol,* 36, 481-509.

Ferreira, A. J. & Santos, R. A. (2005) Cardiovascular actions of angiotensin-(1-7). *Braz J Med Biol Res,* 38, 499-507.

Frohlich, E. D. (2001) Local hemodynamic changes in hypertension: insights for therapeutic preservation of target organs. *Hypertension,* 38, 1388-94.

Fryer, B. H. & Field, J. (2005) Rho, Rac, Pak and angiogenesis: old roles and newly identified responsibilities in endothelial cells. *Cancer Lett,* 229, 13-23.

Galley, H. F. & Webster, N. R. (2004) Physiology of the endothelium. *Br J Anaesth,* 93, 105-13.

Garcia-Cardena, G., Comander, J., Anderson, K. R., Blackman, B. R. & Gimbrone, M. A., Jr. (2001) Biomechanical activation of vascular endothelium as a determinant of its functional phenotype. *Proc Natl Acad Sci U S A,* 98, 4478-85.

Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J. E. & Sessa, W. C. (1996) Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc Natl Acad Sci U S A,* 93, 6448-53.

Ghosh, S. & Karin, M. (2002) Missing pieces in the NF-kappaB puzzle. *Cell,* 109 Suppl, S81-96.

Griendling, K. K. & Alexander, R. W. (1996) Endothelial control of the cardiovascular system: recent advances. *Faseb J,* 10, 283-92.

Guardiola, F., Garcia-Cruset, S., Bou, R. & Codony, R. (2004) Artifactual oxidation of cholesterol during the analysis of cholesterol oxidation products: protective effect of antioxidants. *J AOAC Int,* 87, 493-8.

Gudi, S. R., Clark, C. B. & Frangos, J. A. (1996) Fluid flow rapidly activates G proteins in human endothelial cells. Involvement of G proteins in mechanochemical signal transduction. *Circ Res,* 79, 834-9.

Haendeler, J., Tischler, V., Hoffmann, J., Zeiher, A. M. & Dimmeler, S. (2004) Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. *FEBS Lett,* 577, 427-33.

Haga, M., Chen, A., Gortler, D., Dardik, A. & Sumpio, B. E. (2003) Shear stress and cyclic strain may suppress apoptosis in endothelial cells by different pathways. *Endothelium,* 10, 149-57.

Hawkins, P. T., Davidson, K. & Stephens, L. R. (2007) The role of PI3Ks in the regulation of the neutrophil NADPH oxidase. *Biochem Soc Symp*, 59-67.

Hendrickson, R.J., Cappadona, C., Yankah, E.N., Sitzmann, J.V., Cahill, P.A and Redmond, E.M. (1999) Sustained pulsatile flow regulates endothelial nitric oxide synthase and cyclooxygenase expression in co-cultured vascular endothelial and smooth muscle cells. *J Mol Cell Cardiol,* 31(3), 619-29.

Honing, M. L., Smits, P., Morrison, P. J., Burnett, J. C., Jr. & Rabelink, T. J. (2001) C-type natriuretic peptide-induced vasodilation is dependent on hyperpolarization in human forearm resistance vessels. *Hypertension,* 37, 1179-83.

Hwang, J., Ing, M. H., Salazar, A., Lassegue, B., Griendling, K., Navab, M., Sevanian, A. & Hsiai, T. K. (2003) Pulsatile versus oscillatory shear stress regulates NADPH oxidase subunit expression: implication for native LDL oxidation. *Circ Res,* 93, 1225-32.

Iba, T., Maitz, S., Furbert, T., Rosales, O., Widmann, M. D., Spillane, B., Shin, T., Sonoda, T. & Sumpio, B. E. (1991) Effect of cyclic stretch on endothelial cells from different vascular beds. *Circ Shock,* 35, 193-8.

Iba, T. & Sumpio, B. E. (1991) Morphological response of human endothelial cells subjected to cyclic strain in vitro. *Microvasc Res,* 42, 245-54.

Isenovic, E. R., Jacobs, D. B., Kedees, M. H., Sha, Q., Milivojevic, N., Kawakami, K., Gick, G. & Sowers, J. R. (2004) Angiotensin II regulation of the Na+ pump involves the phosphatidylinositol-3 kinase and p42/44 mitogen-activated protein kinase signaling pathways in vascular smooth muscle cells. *Endocrinology,* 145, 1151-60.

Ishida, T., Peterson, T. E., Kovach, N. L. & Berk, B. C. (1996) MAP kinase activation by flow in endothelial cells. Role of beta 1 integrins and tyrosine kinases. *Circ Res,* 79, 310-6.

Jalali, S., Del Pozo, M. A., Chen, K., Miao, H., Li, Y., Schwartz, M. A., Shyy, J. Y. & Chien, S. (2001) Integrin-mediated mechanotransduction requires its dynamic interaction with specific extracellular matrix (ECM) ligands. *Proc Natl Acad Sci U S A,* 98, 1042-6.

Jeske, N. A., Glucksman, M. J. & Roberts, J. L. (2003) EP24.15 is associated with lipid rafts. *J Neurosci Res,* 74, 468-73.

Jo, H., Sipos, K., Go, Y. M., Law, R., Rong, J. & Mcdonald, J. M. (1997) Differential effect of shear stress on extracellular signal-regulated kinase and N-terminal Jun kinase in endothelial cells. Gi2- and Gbeta/gamma-dependent signaling pathways. *J Biol Chem,* 272, 1395-401.

Kaschina, E. & Unger, T. (2003) Angiotensin AT1/AT2 receptors: regulation, signalling and function. *Blood Press,* 12, 70-88.

Kawano, H., Cody, R. J., Graf, K., Goetze, S., Kawano, Y., Schnee, J., Law, R. E. & Hsueh, W. A. (2000) Angiotensin II enhances integrin and alpha-actinin expression in adult rat cardiac fibroblasts. *Hypertension,* 35, 273-9.

Kim, W., Moon, S. O., Sung, M. J., Kim, S. H., Lee, S., So, J. N. & Park, S. K. (2003) Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. *Faseb J,* 17, 1937-9.

Kraemer, R., Pomerantz, K. B., Joseph-Silverstein, J. & Hajjar, D. P. (1993) Induction of basic fibroblast growth factor mRNA and protein synthesis in smooth muscle cells by cholesteryl ester enrichment and 25-hydroxycholesterol. *J Biol Chem,* 268, 8040-5.

Kuchan, M. J. & Frangos, J. A. (1993) Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. *Am J Physiol,* 264, H150-6.

Labrador, V., Chen, K. D., Li, Y. S., Muller, S., Stoltz, J. F. & Chien, S. (2003) Interactions of mechanotransduction pathways. *Biorheology,* 40, 47-52.

Landmesser, U., Dikalov, S., Price, S. R., Mccann, L., Fukai, T., Holland, S. M., Mitch, W. E. & Harrison, D. G. (2003) Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest,* 111, 1201-9.

Leal, J., Luengo-Fernandez, R., Gray, A., Petersen, S. & Rayner, M. (2006) Economic burden of cardiovascular diseases in the enlarged European Union. *Eur Heart J,* 27, 1610-9.

Lehoux, S. & Tedgui, A. (2003) Cellular mechanics and gene expression in blood vessels. *J Biomech,* 36, 631-43.

Lemaire, S., Lizard, G., Monier, S., Miguet, C., Gueldry, S., Volot, F., Gambert, P. & Neel, D. (1998) Different patterns of IL-1beta secretion, adhesion molecule expression

and apoptosis induction in human endothelial cells treated with 7alpha-, 7beta-hydroxycholesterol, or 7-ketocholesterol. *FEBS Lett,* 440, 434-9.

Leslie, S. J., Spratt, J. C., Mckee, S. P., Strachan, F. E., Newby, D. E., Northridge, D. B., Denvir, M. A. & Webb, D. J. (2005) Direct comparison of selective endothelin A and non-selective endothelin A/B receptor blockade in chronic heart failure. *Heart,* 91, 914-9.

Leung, D. Y., Glagov, S. & Mathews, M. B. (1977) Elastin and collagen accumulation in rabbit ascending aorta and pulmonary trunk during postnatal growth. Correlation of cellular synthetic response with medial tension. *Circ Res,* 41, 316-23.

Li, C., Chen, G., Gerard, N. P., Gerard, C., Bozic, C. R. & Hersh, L. B. (1995) Comparison of the structure and expression of the human and rat neprilysin (endopeptidase 24.11)-encoding genes. *Gene,* 164, 363-6.

Li, C. & Xu, Q. (2000) Mechanical stress-initiated signal transductions in vascular smooth muscle cells. *Cell Signal,* 12, 435-45.

Li, S., Kim, M., Hu, Y. L., Jalali, S., Schlaepfer, D. D., Hunter, T., Chien, S. & Shyy, J. Y. (1997) Fluid shear stress activation of focal adhesion kinase. Linking to mitogen-activated protein kinases. *J Biol Chem,* 272, 30455-62.

Lin, M. C., Almus-Jacobs, F., Chen, H. H., Parry, G. C., Mackman, N., Shyy, J. Y. & Chien, S. (1997) Shear stress induction of the tissue factor gene. *J Clin Invest,* 99, 737-44.

Lopez-Ilasaca, M. (1998) Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. *Biochem Pharmacol,* 56, 269-77.

Lotersztajn, S., Pavoine, C., Deterre, P., Capeau, J., Mallat, A., Lenguyen, D., Dufour, M., Rouot, B., Bataille, D. & Pecker, F. (1992) Role of G protein beta gamma subunits in the regulation of the plasma membrane Ca2+ pump. *J Biol Chem,* 267, 2375-9.

Luckhoff, A. & Busse, R. (1990) Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. *Pflugers Arch,* 416, 305-11.

Luscher, T. F. & Barton, M. (2000) Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. *Circulation,* 102, 2434-40.

Luscher, T. F., Yang, Z., Tschudi, M., Von Segesser, L., Stulz, P., Boulanger, C., Siebenmann, R., Turina, M. & Buhler, F. R. (1990) Interaction between endothelin-1 and endothelium-derived relaxing factor in human arteries and veins. *Circ Res,* 66, 1088-94.

Mackay, D. J. & Hall, A. (1998) Rho GTPases. *J Biol Chem,* 273, 20685-8.

Makino, T., Hattori, Y., Matsuda, N., Onozuka, H., Sakuma, I. & Kitabatake, A. (2003) Effects of angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade on beta-adrenoceptor signaling in heart failure produced by myocardial Infarction in rabbits: reversal of altered expression of beta-adrenoceptor kinase and G i alpha. *J Pharmacol Exp Ther,* 304, 370-9.

Masaki, T. (2004) Historical review: Endothelin. *Trends Pharmacol Sci,* 25, 219-24.

Masatsugu, K., Itoh, H., Chun, T. H., Saito, T., Yamashita, J., Doi, K., Inoue, M., Sawada, N., Fukunaga, Y., Sakaguchi, S., Sone, M., Yamahara, K., Yurugi, T. & Nakao, K. (2003) Shear stress attenuates endothelin and endothelin-converting enzyme expression through oxidative stress. *Regul Pept,* 111, 13-9.

Massarelli, E. E., Casatti, C. A., Kato, A., Camargo, A. C., Bauer, J. A., Glucksman, M. J., Roberts, J. L., Hirose, S. & Ferro, E. S. (1999) Differential subcellular distribution of neurolysin (EC 3.4.24.16) and thimet oligopeptidase (EC 3.4.24.15) in the rat brain. *Brain Res,* 851, 261-5.

Masumoto, H., Kissner, R., Koppenol, W. H. & Sies, H. (1996) Kinetic study of the reaction of ebselen with peroxynitrite. *FEBS Lett,* 398, 179-82.

Matozaki, T., Nakanishi, H. & Takai, Y. (2000) Small G-protein networks: their crosstalk and signal cascades. *Cell Signal,* 12, 515-24.

Mayr, M., Hu, Y., Hainaut, H. & Xu, Q. (2002) Mechanical stress-induced DNA damage and rac-p38MAPK signal pathways mediate p53-dependent apoptosis in vascular smooth muscle cells. *Faseb J,* 16, 1423-5.

Mccormick, S. M., Eskin, S. G., Mcintire, L. V., Teng, C. L., Lu, C. M., Russell, C. G. & Chittur, K. K. (2001) DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. *Proc Natl Acad Sci U S A,* 98, 8955-60.

Meaney, S., Bodin, K., Diczfalusy, U. & Bjorkhem, I. (2002) On the rate of translocation in vitro and kinetics in vivo of the major oxysterols in human circulation: critical importance of the position of the oxygen function. *J Lipid Res,* 43, 2130-5.

Meininger, G. A. (1987) Responses of sequentially branching macro- and microvessels during reactive hyperemia in skeletal muscle. *Microvasc Res,* 34, 29-45.

Mervaala, E. M., Muller, D. N., Park, J. K., Schmidt, F., Lohn, M., Breu, V., Dragun, D., Ganten, D., Haller, H. & Luft, F. C. (1999) Monocyte infiltration and adhesion molecules in a rat model of high human renin hypertension. *Hypertension,* 33, 389-95.

Milligan, G. & Kostenis, E. (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol,* 147 Suppl 1, S46-55.

Milstien, S. & Katusic, Z. (1999) Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun,* 263, 681-4.

Miyakawa, A. A., De Lourdes Junqueira, M. & Krieger, J. E. (2004) Identification of two novel shear stress responsive elements in rat angiotensin I converting enzyme promoter. *Physiol Genomics,* 17, 107-13.

Morawietz, H., Talanow, R., Szibor, M., Rueckschloss, U., Schubert, A., Bartling, B., Darmer, D. & Holtz, J. (2000) Regulation of the endothelin system by shear stress in human endothelial cells. *J Physiol,* 525 Pt 3, 761-70.

Nakache, M. & Gaub, H. E. (1988) Hydrodynamic hyperpolarization of endothelial cells. *Proc Natl Acad Sci U S A,* 85, 1841-3.

Nakayama, T. (2005) The genetic contribution of the natriuretic peptide system to cardiovascular diseases. *Endocr J,* 52, 11-21.

Negishi, M., Lu, D., Zhang, Y. Q., Sawada, Y., Sasaki, T., Kayo, T., Ando, J., Izumi, T., Kurabayashi, M., Kojima, I., Masuda, H. & Takeuchi, T. (2001) Upregulatory expression of furin and transforming growth factor-beta by fluid shear stress in vascular endothelial cells. *Arterioscler Thromb Vasc Biol,* 21, 785-90.

Niu, J., Profirovic, J., Pan, H., Vaiskunaite, R. & Voyno-Yasenetskaya, T. (2003) G Protein betagamma subunits stimulate p114RhoGEF, a guanine nucleotide exchange factor for RhoA and Rac1: regulation of cell shape and reactive oxygen species production. *Circ Res,* 93, 848-56.

Noria, S., Cowan, D. B., Gotlieb, A. I. & Langille, B. L. (1999) Transient and steady-state effects of shear stress on endothelial cell adherens junctions. *Circ Res,* 85, 504-14.

Noria, S., Xu, F., Mccue, S., Jones, M., Gotlieb, A. I. & Langille, B. L. (2004) Assembly and reorientation of stress fibers drives morphological changes to endothelial cells exposed to shear stress. *Am J Pathol,* 164, 1211-23.

Norman, M. U., Reeve, S. B., Dive, V., Smith, A. I. & Lew, R. A. (2003) Endopeptidases 3.4.24.15 and 24.16 in endothelial cells: potential role in vasoactive peptide metabolism. *Am J Physiol Heart Circ Physiol,* 284, H1978-84.

Ogita, H. & Liao, J. (2004) Endothelial function and oxidative stress. *Endothelium,* 11, 123-32.

Ohno, M., Cooke, J. P., Dzau, V. J. & Gibbons, G. H. (1995) Fluid shear stress induces endothelial transforming growth factor beta-1 transcription and production. Modulation by potassium channel blockade. *J Clin Invest,* 95, 1363-9.

Olesen, S. P., Clapham, D. E. & Davies, P. F. (1988) Haemodynamic shear stress activates a K+ current in vascular endothelial cells. *Nature,* 331, 168-70.

Oliveira, V., Araujo, M. C., Rioli, V., De Camargo, A. C., Tersariol, I. L., Juliano, M. A., Juliano, L. & Ferro, E. S. (2003) A structure-based site-directed mutagenesis study on the neurolysin (EC 3.4.24.16) and thimet oligopeptidase (EC 3.4.24.15) catalysis. *FEBS Lett,* 541, 89-92.

Orlowski, M., Reznik, S., Ayala, J. & Pierotti, A. R. (1989) Endopeptidase 24.15 from rat testes. Isolation of the enzyme and its specificity toward synthetic and natural peptides, including enkephalin-containing peptides. *Biochem J,* 261, 951-8.

Orzechowski, H. D., Richter, C. M., Funke-Kaiser, H., Kroger, B., Schmidt, M., Menzel, S., Bohnemeier, H. & Paul, M. (1997) Evidence of alternative promoters directing isoform-specific expression of human endothelin-converting enzyme-1 mRNA in cultured endothelial cells. *J Mol Med,* 75, 512-21.

Pandey, S. K. & Anand-Srivastava, M. B. (1996) Modulation of G-protein expression by the angiotensin converting enzyme inhibitor captopril in hearts from spontaneously hypertensive rats. Relationship with adenylyl cyclase. *Am J Hypertens,* 9, 833-7.

Papadaki, M. & Eskin, S. G. (1997) Effects of fluid shear stress on gene regulation of vascular cells. *Biotechnol Prog,* 13, 209-21.

Parkos, C. A., Allen, R. A., Cochrane, C. G. & Jesaitis, A. J. (1987) Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. *J Clin Invest,* 80, 732-42.

Persoon-Rothert, M., Van Der Wees, K. G. & Van Der Laarse, A. (2002) Mechanical overload-induced apoptosis: a study in cultured neonatal ventricular myocytes and fibroblasts. *Mol Cell Biochem,* 241, 115-24.

Poli, G., Leonarduzzi, G., Biasi, F. & Chiarpotto, E. (2004) Oxidative stress and cell signalling. *Curr Med Chem,* 11, 1163-82.

Poston, L. (2002) Flow mediated responses in the circulation. *Introduction to vascular biology.* Second ed., Cambridge University Press.

Pueyo, M. E., Arnal, J. F., Rami, J. & Michel, J. B. (1998) Angiotensin II stimulates the production of NO and peroxynitrite in endothelial cells. *Am J Physiol,* 274, C214-20.

Quan, A. (2006) Fetopathy associated with exposure to angiotensin converting enzyme inhibitors and angiotensin receptor antagonists. *Early Hum Dev,* 82, 23-8.

Ramon De Berrazueta, J. (1999) [The role of calcium in the regulation of normal vascular tone and in arterial hypertension]. *Rev Esp Cardiol,* 52 Suppl 3, 25-33.

Ravichandran, K. S. (2001) Signaling via Shc family adapter proteins. *Oncogene,* 20, 6322-30.

Ray, R. & Shah, A. M. (2005) NADPH oxidase and endothelial cell function. *Clin Sci (Lond),* 109, 217-26.

Resnick, N., Collins, T., Atkinson, W., Bonthron, D. T., Dewey, C. F., Jr. & Gimbron, M. A., Jr. (1993) Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. *Proc Natl Acad Sci U S A,* 90, 7908.

Rieder, M. J., Carmona, R., Krieger, J. E., Pritchard, K. A., Jr. & Greene, A. S. (1997) Suppression of angiotensin-converting enzyme expression and activity by shear stress. *Circ Res,* 80, 312-9.

Rioli, V., Kato, A., Portaro, F. C., Cury, G. K., Te Kaat, K., Vincent, B., Checler, F., Camargo, A. C., Glucksman, M. J., Roberts, J. L., Hirose, S. & Ferro, E. S. (1998) Neuropeptide specificity and inhibition of recombinant isoforms of the endopeptidase 3.4.24.16 family: comparison with the related recombinant endopeptidase 3.4.24.15. *Biochem Biophys Res Commun,* 250, 5-11.

Rivilis, I., Milkiewicz, M., Boyd, P., Goldstein, J., Brown, M. D., Egginton, S., Hansen, F. M., Hudlicka, O. & Haas, T. L. (2002) Differential involvement of MMP-2 and VEGF during muscle stretch- versus shear stress-induced angiogenesis. *Am J Physiol Heart Circ Physiol,* 283, H1430-8.

Rosano, L., Spinella, F., Di Castro, V., Dedhar, S., Nicotra, M. R., Natali, P. G. & Bagnato, A. (2006) Integrin-linked kinase functions as a downstream mediator of endothelin-1 to promote invasive behavior in ovarian carcinoma. *Mol Cancer Ther,* 5, 833-42.

Roth, M. J., Tanese, N. & Goff, S. P. (1985) Purification and characterization of murine retroviral reverse transcriptase expressed in Escherichia coli. *J Biol Chem,* 260, 9326-35.

Ryan, J. W., Ryan, U. S., Schultz, D. R. & Day, A. R. (1976) Further evidence on the subcellular sites of kininase II (angiotensin converting enzyme). *Adv Exp Med Biol,* 70, 235-43.

Sadhu, K., Hensley, K., Florio, V. A. & Wolda, S. L. (1999) Differential expression of the cyclic GMP-stimulated phosphodiesterase PDE2A in human venous and capillary endothelial cells. *J Histochem Cytochem,* 47, 895-906.

Sadoshima, J. & Izumo, S. (1997) The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol,* 59, 551-71.

Sambrook, J., Fritsch, E.F., Maniatis, T., (1989) *Molecular Cloning: A Laboratory Manual,* New York.

Schaller, M. D. & Parsons, J. T. (1994) Focal adhesion kinase and associated proteins. *Curr Opin Cell Biol,* 6, 705-10.

Schaper, W. (1967) Tangential wall stress as a molding force in the development of collateral vessels in the canine heart. *Experientia,* 23, 595-6.

Schiffrin, E. L. & Touyz, R. M. (1998) Vascular biology of endothelin. *J Cardiovasc Pharmacol,* 32 Suppl 3, S2-13.

Schnitzer, J. E., Liu, J. & Oh, P. (1995) Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, annexins, and GTPases. *J Biol Chem,* 270, 14399-404.

Schriefer, J. A., Broudy, E. P. & Hassen, A. H. (2001) Inhibitors of bradykinin-inactivating enzymes decrease myocardial ischemia/reperfusion injury following 3 and 7 days of reperfusion. *J Pharmacol Exp Ther,* 298, 970-5.

Schulz, E., Anter, E. & Keaney, J. F., Jr. (2004) Oxidative stress, antioxidants, and endothelial function. *Curr Med Chem,* 11, 1093-104.

Schwarz, G., Callewaert, G., Droogmans, G. & Nilius, B. (1992) Shear stress-induced calcium transients in endothelial cells from human umbilical cord veins. *J Physiol,* 458, 527-38.

Searles, C. D. (2006) Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. *Am J Physiol Cell Physiol,* 291, C803-16.

Seymour, A. A., Asaad, M. M., Sheldon, J. H., Smith, P. L. & Rogers, W. L. (1995) Atrial natriuretic peptide in chronically hypertensive dogs. *Hypertension,* 26, 634-41.

Shen, J., Luscinskas, F. W., Connolly, A., Dewey, C. F., Jr. & Gimbrone, M. A., Jr. (1992) Fluid shear stress modulates cytosolic free calcium in vascular endothelial cells. *Am J Physiol,* 262, C384-90.

Shirotani, K., Tsubuki, S., Iwata, N., Takaki, Y., Harigaya, W., Maruyama, K., Kiryu-Seo, S., Kiyama, H., Iwata, H., Tomita, T., Iwatsubo, T. & Saido, T. C. (2001) Neprilysin degrades both amyloid beta peptides 1-40 and 1-42 most rapidly and efficiently among thiorphan- and phosphoramidon-sensitive endopeptidases. *J Biol Chem,* 276, 21895-901.

Shrimpton, C. N., Smith, A. I. & Lew, R. A. (2002) Soluble metalloendopeptidases and neuroendocrine signaling. *Endocr Rev,* 23, 647-64.

Shyy, J. Y., Lin, M. C., Han, J., Lu, Y., Petrime, M. & Chien, S. (1995) The cis-acting phorbol ester "12-O-tetradecanoylphorbol 13-acetate"-responsive element is involved in shear stress-induced monocyte chemotactic protein 1 gene expression. *Proc Natl Acad Sci U S A,* 92, 8069-73.

Sitbon, O., Badesch, D. B., Channick, R. N., Frost, A., Robbins, I. M., Simonneau, G., Tapson, V. F. & Rubin, L. J. (2003) Effects of the dual endothelin receptor antagonist bosentan in patients with pulmonary arterial hypertension: a 1-year follow-up study. *Chest,* 124, 247-54.

Skeggs, L. T., Jr., Kahn, J. R. & Shumway, N. P. (1956) The preparation and function of the hypertensin-converting enzyme. *J Exp Med,* 103, 295-9.

Skeggs, L. T., Jr., Marsh, W. H., Kahn, J. R. & Shumway, N. P. (1954) The existence of two forms of hypertension. *J Exp Med,* 99, 275-82.

Smaje, L., Zweifach, B. W. & Intaglietta, M. (1970) Micropressures and capillary filtration coefficients in single vessels of the cremaster muscle of the rat. *Microvasc Res,* 2, 96-110.

Smith, A. I., Lew, R. A., Shrimpton, C. N., Evans, R. G. & Abbenante, G. (2000) A novel stable inhibitor of endopeptidases EC 3.4.24.15 and 3.4.24.16 potentiates bradykinin-induced hypotension. *Hypertension,* 35, 626-30.

Smondyrev, A. M. & Berkowitz, M. L. (2001) Effects of oxygenated sterol on phospholipid bilayer properties: a molecular dynamics simulation. *Chem Phys Lipids,* 112, 31-9.

Stephens, L. R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A. S., Thelen, M., Cadwallader, K., Tempst, P. & Hawkins, P. T. (1997) The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell,* 89, 105-14.

Stewart, D. J., Cernacek, P., Mohamed, F., Blais, D., Cianflone, K. & Monge, J. C. (1994) Role of cyclic nucleotides in the regulation of endothelin-1 production by human endothelial cells. *Am J Physiol,* 266, H944-51.

Stone, D. K. (1998) Receptors: structure and function. *Am J Med,* 105, 244-50.

Sugden, P. H. & Clerk, A. (1997) Regulation of the ERK subgroup of MAP kinase cascades through G protein-coupled receptors. *Cell Signal,* 9, 337-51.

Sumner, M. J., Cannon, T. R., Mundin, J. W., White, D. G. & Watts, I. S. (1992) Endothelin ETA and ETB receptors mediate vascular smooth muscle contraction. *Br J Pharmacol,* 107, 858-60.

Sun, Y. & Oberley, L. W. (1996) Redox regulation of transcriptional activators. *Free Radic Biol Med,* 21, 335-48.

Takahashi, M. & Berk, B. C. (1996) Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. *J Clin Invest,* 98, 2623-31.

Takaishi, K., Matozaki, T., Nakano, K. & Takai, Y. (2000) Multiple downstream signalling pathways from ROCK, a target molecule of Rho small G protein, in reorganization of the actin cytoskeleton in Madin-Darby canine kidney cells. *Genes Cells,* 5, 929-936.

Tarpey, M. M., Beckman, J. S., Ischiropoulos, H., Gore, J. Z. & Brock, T. A. (1995) Peroxynitrite stimulates vascular smooth muscle cell cyclic GMP synthesis. *FEBS Lett,* 364, 314-8.

Tilakaratne, N. & Sexton, P. M. (2005) G-Protein-coupled receptor-protein interactions: basis for new concepts on receptor structure and function. *Clin Exp Pharmacol Physiol,* 32, 979-87.

Traub, O. & Berk, B. C. (1998) Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. *Arterioscler Thromb Vasc Biol,* 18, 677-85.

Tseng, H., Peterson, T. E. & Berk, B. C. (1995) Fluid shear stress stimulates mitogen-activated protein kinase in endothelial cells. *Circ Res,* 77, 869-78.

Tulis, D. A. & Prewitt, R. L. (1998) Medial and endothelial platelet-derived growth factor A chain expression is regulated by in vivo exposure to elevated flow. *J Vasc Res,* 35, 413-20.

Turner, A. J., Isaac, R. E. & Coates, D. (2001) The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. *Bioessays,* 23, 261-9.

Turner, A. J. & Tanzawa, K. (1997) Mammalian membrane metallopeptidases: NEP, ECE, KELL, and PEX. *Faseb J,* 11, 355-64.

Tzima, E. (2006) Role of small GTPases in endothelial cytoskeletal dynamics and the shear stress response. *Circ Res,* 98, 176-85.

Tzima, E., Del Pozo, M. A., Shattil, S. J., Chien, S. & Schwartz, M. A. (2001) Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment. *Embo J,* 20, 4639-47.

Vailhe, B. & Tranqui, L. (1996) [The role of the transforming growth factor beta 1 (TGF-beta 1) and of vascular endothelial growth factor (VEGF) on the in vitro angiogenesis process]. *C R Acad Sci III,* 319, 1003-10.

Valdenaire, O., Rohrbacher, E. & Mattei, M. G. (1995) Organization of the gene encoding the human endothelin-converting enzyme (ECE-1). *J Biol Chem,* 270, 29794-8.

Verhaar, M. C., Strachan, F. E., Newby, D. E., Cruden, N. L., Koomans, H. A., Rabelink, T. J. & Webb, D. J. (1998) Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation,* 97, 752-6.

Wang, B. W., Chang, H., Lin, S., Kuan, P. & Shyu, K. G. (2003) Induction of matrix metalloproteinases-14 and -2 by cyclical mechanical stretch is mediated by tumor necrosis factor-alpha in cultured human umbilical vein endothelial cells. *Cardiovasc Res,* 59, 460-9.

Wang, H. D., Xu, S., Johns, D. G., Du, Y., Quinn, M. T., Cayatte, A. J. & Cohen, R. A. (2001) Role of NADPH oxidase in the vascular hypertrophic and oxidative stress response to angiotensin II in mice. *Circ Res,* 88, 947-53.

Wettschureck, N. & Offermanns, S. (2005) Mammalian G proteins and their cell type specific functions. *Physiol Rev,* 85, 1159-204.

Wheeler-Jones, C. P. (2005) Cell signalling in the cardiovascular system: an overview. *Heart,* 91, 1366-74.

Wilson, E., Sudhir, K. & Ives, H. E. (1995) Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrin interactions. *J Clin Invest,* 96, 2364-72.

Wung, B. S., Cheng, J. J., Shyue, S. K. & Wang, D. L. (2001) NO modulates monocyte chemotactic protein-1 expression in endothelial cells under cyclic strain. *Arterioscler Thromb Vasc Biol,* 21, 1941-7.

Yamada, M., Inanobe, A. & Kurachi, Y. (1998) G protein regulation of potassium ion channels. *Pharmacol Rev,* 50, 723-60.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature,* 332, 411-5.

Yang, X., Rojanasakul, Y., Wang, L., Ma, J. Y. & Ma, J. K. (1998) Enzymatic degradation of luteinizing hormone releasing hormone (LHRH)/[D-Ala6]-LHRH in lung pneumocytes. *Pharm Res,* 15, 1480-4.

Zhao, S., Suciu, A., Ziegler, T., Moore, J. E., Jr., Burki, E., Meister, J. J. & Brunner, H. R. (1995) Synergistic effects of fluid shear stress and cyclic circumferential stretch on vascular endothelial cell morphology and cytoskeleton. *Arterioscler Thromb Vasc Biol,* 15, 1781-6.

Zheng, W., Brown, M. D., Brock, T. A., Bjercke, R. J. & Tomanek, R. J. (1999) Bradycardia-induced coronary angiogenesis is dependent on vascular endothelial growth factor. *Circ Res,* 85, 192-8.

Zou, M. H., Shi, C. & Cohen, R. A. (2002) High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. *Diabetes,* 51, 198-203.

Zou, M. H. & Ullrich, V. (1996) Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. *FEBS Lett,* 382, 101-4.

Zou, Y., Hu, Y., Metzler, B. & Xu, Q. (1998) Signal transduction in arteriosclerosis: mechanical stress-activated MAP kinases in vascular smooth muscle cells (review). *Int J Mol Med,* 1, 827-34.