THE INFLUENCE OF MECHANICAL FORCES ON THE RENIN ANGIOTENSIN SYSTEM IN CULTURED HUMAN VASCULAR SMOOTH MUSCLE CELLS

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Abstract

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In cardiovascular disease, the deleterious effects of Angiotensin II (Ang II) on the vasculature are well recognised. This is not necessarily due to elevated levels of circulating Ang II. An alternative hypothesis implicates the role of vascular cell signalling in disease. Therefore this thesis examined the role of the Ang II type I (AT_1) receptor in a cell culture model of hypertension: cultured human vascular smooth muscle (VSM) cells were exposed to cyclical mechanical strain regimes (Flexcell®) designed to mimic the forces generated by hypertension in vivo.

Early experimentation demonstrated cyclical mechanical strain and Ang II induction of VSM cell gene expression and synthesis of extracellular matrix proteins. Importantly, AT_1 receptor antagonism inhibited the strain-induced fibrogenic activity of VSM cells suggesting a synergistic relationship between the renin-angiotensin system and mechanical strain.

The response of VSM cell AT_1 receptor to mechanical strain was analysed by three-colour flow cytometry. After accounting for non-specific binding, two distinct populations of human VSM cells were identified based on their level of AT_1 receptor expression. In the population of VSM cells with a high expression of AT_1 receptor, cyclical mechanical strain resulted in an increase in the expression of the AT_1 receptors.

This thesis has revealed for the first time, conclusive evidence that mechanical strain up-regulates cell-surface expression of AT_1 receptors on human VSM cells. This may highlight a mechanism whereby mechanical strain may lead to sensitisation of human VSM cells to Ang II in the early stages of vascular disease.

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Contents

Chapter 1

Introduction

1.1 Introduction

Our current knowledge of the biology of arterial blood vessels is hindered by the lack of investigations of the interactions between different environmental factors that shape the biological function of vessel walls. This knowledge is crucial to our understanding of the development of disease in the vasculature.

We know that several key factors namely the renin angiotensin system (RAS), the sympathetic nervous system (SNS), mechanical forces and nitric oxide exert an influence on the function of cells and proteins within the vessel wall. Although it is not clear what triggers the development of arteriosclerosis or atherosclerosis, there is compelling evidence that both the RAS and mechanical strain have a deleterious effect on vascular cell function.

There remain a number of unknowns. The mechanism by which mechanical forces exert an effect on cellular function in vascular smooth muscle (VSM) cells is not clear; controversy still surrounds the nature of Angiotensin (Ang) II synthesis in VSM cells and the reliance on an intact RAS within the cell. Importantly, the relationship between neuro-humoral factors and mechanical strain has not been elucidated in human cells nor has the contribution from each been determined.

Despite a lack of full understanding of the molecular and cellular pathophysiology, large-scale outcome clinical studies have highlighted the benefits in treating vascular disease with drugs that inhibit the RAS. Arguably and perhaps controversially, these therapies may have advantages over and above blood pressure (BP) reduction.

This thesis will explore the cellular basis of the relationship between the RAS and mechanical strain in human VSM cells by using a cell culture model mimicking the effects of pressure on VSM cells under varying conditions.

1.2 Blood Vessel Structure

1.2.1 Introduction

Arterial blood vessels comprise of large arteries, arterioles and capillaries. In essence, these vessels comprise of endothelial and vascular smooth muscle cells, matrix proteins and adipocytes. The vasculature is a complex structure that does not exists purely as a tube to facilitate transport of blood, but has a unique and dynamic biology with intricate intercellular, cell-matrix and matrix-matrix interactions and interactions with circulating agents such as neuro-humoral factors, cytokines and growth factors.

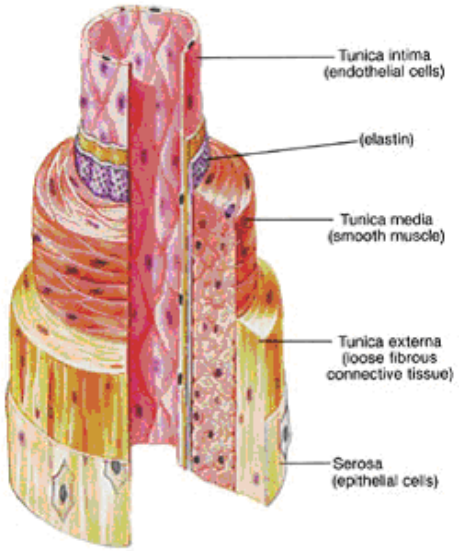
Hypertension is associated with changes to the resistance vessels (small arteries < 500 um) (Bohlen et al., 1986; Mulvany et al., 1990; Schiffrin, 1992). Vascular changes, particularly an increase in media:lumen ratio, are as a result of haemodynamic and hormonal factors. Endothelial function, assessed by response to acetylcholine or noradrenaline, has also been shown to be impaired in resistance arteries (Rizzoni et al., 1996; Tesfamariam et al., 1988); however in renovascular hypertension (increased RAS activity) and to a lesser extent in subjects with hyperaldosteronism, resistance arteries undergo greater vascular wall growth and less vascular wall remodeling than subjects with essential hypertensions (Rizzoni et al., 1996).

Increased matrix deposition within vessel walls and the heart are characteristic findings in hypertensive subjects (Feigl et al., 1963; Safar et al., 1984). This increase in vascular matrix renders the vasculature less compliant and profoundly influences the haemodynamic performance of large vessels. Characteristically, reduced vascular compliance in larger conduit arteries is associated with a widening of pulse pressure,

increased systolic pressure and an increase in cardiac after-load resulting in left ventricular hypertrophy (Feigl et al., 1963; Pannier et al., 1989; Safar et al., 1984).

The stimuli and mechanisms responsible for increased matrix deposition within the vessels of hypertensive subjects are poorly defined and less well understood. Nevertheless, the likely target for such stimuli is the VSM cell within the media of blood vessels as the proliferating VSM cell are one of the major sources of matrix production within the vasculature (Burke et al., 1977). High blood pressure has been found to be closely linked to increased vascular collagen synthesis (Lopez et al., 2004). Hypertension imposes a chronic increase in cyclical mechanical stress on blood vessels and this leads to chronic cyclical stretching of VSM cell within the vascular media (Ooshima et al., 1974; Wolinsky, 1982).

Figure 1.1 Cross-section of the arterial wall



1.2.2 Vascular Matrix

The extra cellular matrix (ECM) is the largest component of the blood vessel wall comprising of 60% of intimal volume. This comprises of collagens types I, III, IV, V,

VI; proteoglycans (chondroitin sulphate PG, heparan sulphate PG, dermatan sulphate PG & keratan sulphate PG); elastin, glycoproteins, fibronectin and laminin (Dolley et al., 1995, Stary et al., 1992, Wright et al., 1995). Collagen is a triple helical structure comprising of 3 polypeptide α chains, the majority of which is the interstitial collagens type I and III. Collagens type IV, V and VI account for only 0.5-1% of the ECM within blood vessel walls (Stary et al., 1992). ECM proteins are synthesised predominantly by VSM cells, but also endothelial cells; in particular the latter secrete basement membrane ECM proteins (Ruoslahti et al., 1997).

Basement membrane ECM proteins are specialised and comprise of Type IV collagen, laminin and proteoglycans. The basement membrane ECM lining endothelial cells have isoforms different to that supporting VSM cells. Elastin is found predominately in the media and musculo-elastic layers, while fibronectin, along with laminin is a major component of normal intima. Conversely, adventitia is rich in collagen type I & III, contributing to the stiffness of the blood vessel wall.

Integrins are heterodimeric membrane proteins that cement cell-matrix and matrix-matrix interactions. Although most ECM proteins are ligands for integrins, fibronectin has been investigated most extensively (Ruoslahti et al., 1997). These interactions are important in maintaining the integrity of the vessel wall and orientation of cells within it wall. Importantly they can respond to changes in blood flow. This is evident in diseases such as Marfan's and Ehlers-Danlos syndromes, where there is a genetic mutation for fibrillar collagens and elastin respectively leading to blood vessel structural changes (Curran et al., 1993; Dietz et al., 1995).

Endothelial cells have α6β1, α5β1, α2β1 and αVβ3 receptors for laminin, fibronectin, collagen and vitronectin (Conforti et al., 1992). VSM cells have a large complement of β1 and αV receptors of which αVβ3 is the most characterised (Liaw et al., 1995).

The majority of studies investigating the role of these ligands have involved *in vitro* studies with RGD motif antibodies that can inhibit ligand interactions. αVβ3 has been shown to be important for VSM cell migration (Liaw et al., 1995), fibronectin matrix assembly (Wu et al., 1996) and VSM cell proliferation (Assoian et al., 1996). The role of integrins in mechano-sensing will be discussed in detail in section 1.5.

The vascular matrix is a dynamic system of continuous deposition and degradation under the influence of matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases - TIMPs). These in turn are regulated by neuro-humoral factors and other cytokines. This appears to be key to the development of arteriosclerosis and reduction in blood vessel compliance. MMP activity will be discussed in more detail in section 1.2.3.

The exact regulation of the vascular matrix is not clear, but the most influential neuro-humoral factors appear to be Ang II, mechanical strain and noradrenaline stimulation of VSM cells, acting via a common pathway (i.e. transforming growth factor (TGFβ). $TGF\beta_1$ is a powerful fibrogenic cytokine in a variety of tissues. In renal fibrosis, $TGF\beta_1$ leads to increased production of ECM proteins, cell surface integrins and a reduction in MMP activity (Border et al., 1994). In studies with cultured mesangial cells, angiotensin II was shown increase type I collagen and fibronectin (Kagami et al., 1994; Wolf et al., 1992) synthesis through a $TGF\beta_1$- dependent mechanism (Kagami et al., 1994).

Ageing is also consistent with a shift to increasing matrix and as a result leads to less compliant vessels as well as changes to ECM biology and therefore endothelial cell and VSM cell function; for example changes to the ECM composition of basement membranes allows VSM cell migration to the lumen. This ultimately leads to atherosclerosis and arteriosclerosis, but the contribution from each of the many influences on vascular matrix biology is difficult to define.

For our experimentation, we chose to analyse collagen αI, collagen αIII and fibronectin mRNA as well as fibronectin protein synthesis as these matrix components are characteristically deposited in fibrogenic states and most readily investigated. Our group has previously published data revealing Ang II and strain induced collagen protein synthesis in VSM cell and therefore did not repeat this work. Ang II and mechanical strain are potent promoters of fibrogenesis in the human vasculature (O'Callaghan et al., 2000).

1.2.3 Matrix Metalloproteinases

MMPs are zinc/calcium dependent endopeptidases that regulate changes to the ECM in blood vessel walls. There are 4 main groups of enzymes namely the interstitial collagenases, gelatinases, stromeolysins and membrane-bound MMPs (Birkedal-Hansen et al., 1993; Murphy et al., 1992). A full list of the characterised MMPs, their substrates, actions and inhibitors can be found in the reviews by Cleutjens and Stetler-Stevenson (Cleutjens, 1996; Stetler-Stevenson, 1996). The resultant subtle changes in the balance of MMP activity can lead to significant pathological changes in vascular disease; for example, an increase in elastase activity leading to degradation of ECM proteins is

thought to be fundamental to the loss of vessel wall integrity and resultant aneurysmal development. However, the first series of MMP inhibitor novel drug compounds to be tested in clinical studies have been directed at a diverse range of disease states including arthritides, cancer (to inhibit metastatic spread) and periodontal disease (Parsons et al., 1997).

MMPs are secreted by a wide variety of cell types including endothelial cells, VSM cells and macrophages as latent pro-enzymes that require extra-cellular activation. Plasmin and uPA can lead to activation of the enzymes by cleavage of 10-80 amino acids from the N-terminus (Cleutjens, 1996; Dollery et al., 1995). TIMPs, tetracycline (by its zinc-chelating property) and α2 macroglobulin are inhibitors of MMPs. Four TIMPs have been characterised and are synthesised by macrophages (Dollery et al., 1995) to form a high affinity irreversible complex with its MMP to rendering it inactive.

Gene expression of MMPs is influenced by several factors at the transcriptional level. interleukin (IL)-1, platelet derived growth factor (PDGF) and tissue necrosis factor (TNF)α promote gene expression, while TGFβ, heparin and corticosteriods reduce gene expression (Dollery et al., 1995).

95 kDa MMP-9 and 72 kDa MMP-2 (formerly known as type IV collagenases / gelatinases) effectively degrade collagen type IV and elastin (Katsuda et al., 1994; Murphy et al., 1991; Senior et al., 1991). Stromeolysins1 and 2 and punctuated MMP (PUMP) are also able to degrade of type IV collagen, but are more potent proteoglycan-degrading enzymes (Murphy et al., 1991). The interstitial collagens (type I and III) are degraded by MMP-1 (interstitial collagenase) and MMP-8 initially forming gelatins, which are further degraded by MMP-2 and MMP-9, stromeolysins and elastases.

MMP activity has been investigated in numerous animal models and some human studies to determine its role in vascular tissue. Rat thoracic aorta VSM cell ability to migrate across the basement membrane depended on their phenotype. Proliferating cells had 20-fold MMP-2 activity compared to quiescent VSM cells. Only proliferating cells have the ability to migrate (Pauly et al., 1994). VEGF stimulated MMP-1, MMP-3 and MMP-9 synthesis in cultured human aortic VSM cells leads to accelerated cell migration through ECM-based *in vitro* gels (Wang et al., 1998). In contrast, nitric oxide inhibits VSM cell migration and reduces MMP-2 and MMP-9 activity in rat aortic transfected cells with eNOS (Gurjar et al., 1999). These studies highlight the influence of VSM cell phenotype in disease progression, which begs the question: does an altered VSM cell phenotype become more sensitive to the effects of other cytokines or growth factors?

The expression of matrix components in the aortas of older (30 months) was compared to younger Fisher 344XBN rats (6 months). The intimal thickness was greater and the expression of TGFβ ICAM 1 and fibronectin was increased in older rat aorta. Studies with early passaged VSM cells from young and old rats were stimulated with IL-1, TNFα and TGFβ revealing exaggerated MMP-2 production in older rat cells (Li Z et al., 1999), therefore further supporting a hypothesis that these cells are sensitive to the effects of pro-fibrotic cytokines.

In heart failure, MMP activity increases during progression of the disease (Spinale et al., 1998), however regulation of this may occur due to cytokine influences on MMP inhibitors. In neonatal rat ventricular cells, Li (Li Y et al., 1999) noted that TNFα and IL-1b altered the regulation of TIMPS 1-4.

In cultures of human saphenous veins, quiescent VSM cells secrete MMP-2 and TIMP 1 & 2, but stimulation with IL-1 and TNFα results in activation of MMP-1, MMP-3 (stromeolysins) and MMP-9 (Galis et al., 1994). MMP-2 synthesis is further enhanced by injury as evident in thrombin stimulation of cultured human saphenous VSM cells (Galis et al., 1997) or human saphenous veins harvested in preparation for vein grafts (George et al., 1997).

In a study of SHRs, chronic AT_1 receptor blockade with losartan resulted in left ventricular inhibition of TIMP-1 expression and stimulation of collagenase activity. This suggests a mechanism by which Ang II may cause myocardial fibrosis by depressing the collagenase-mediated EC degradation of collagen (Varo et al., 2000).

1.2.4 Vascular Smooth Muscle Cells

VSM cells are the predominant cell of the vessel and are the major source of matrix proteins. These cells are situated in the media, embedded in a layer of ECM lined by a thin line of basement membrane proteins. They are formed in a circular pattern at 45^{o} to the blood flow. Cellular function is aimed predominantly at vessel contraction, however other roles are vital and include maintaining the integrity of the matrix milieu and factors that lead to an interruption of the VSM cell biology ultimately lead to changes to the matrix structure and consequently disease.

There are numerous cellular-cellular and cellular-matrix interactions between vascular cells and their milieu; the most widely characterized being modulated via the integrin RGD receptor. Therefore, *in vitro* VSM cells behave differently to their normal *in vivo* biological function.

Chamley-Campbell et al conducted one of the earliest studies of VSM cell cytoskeleton. *In vivo*, a healthy vessel environment ensures that cells maintain a "contractile" quiescent phenotype. However, a series of studies revealed that there was increasing loss of smooth muscle (SM) myosin as the phenotype of the cell switched from "contractile" to "synthetic" in passaged cells. When present myosin was observed as striated "myosin aggregates" within the cytoplasm; SM and non-muscle (NM) actin was observed in early and late passaged cells as long fibrils densely positioned along the longitudinal axis (Chamley-Campbell et al., 1979). This is supported by studies of cultured VSM cells from adult rat aorta demonstrate low levels of SM myosin heavy chain (MHC) 1 & 2 and high levels of non-muscle myosin in proliferating cells, but high levels of SM MHC and NM MHC in growth arrested cells (Rovner et al., 1986). Cultured rat aorta VSM cells grown in 10% fetal calf serum for 3-5 days had significant reduction in their SM MHC mRNA with a greater reduction in SM2 (80%) compared to SM 1 (30%). In contrast NM myosin increased. There were no changes to SM myosin heavy chain expression in quiescent serum-deprived media. This may explain the change of phenotype from contractile to synthetic (Babij et al., 1992). Smooth muscle α-actin was studied in cultured rat aortic VSM cells and revealed down regulation in growing sub-confluent cells and a higher expression in serum-deprived growth arrested cells (Owens et al., 1986).

In cultured human VSM cells, growing cells undergo a phenotypic shift from "contractile" quiescent status to a proliferative secretary phase that resembles the phenotype seen on atherosclerosis (Hultgårdh-Nilsson et al., 1997; Thyberg J 1996). This is characterised by down regulation of the SM myosin isoforms and up regulation of non-muscle myosin (Kim et al., 1993; Zanellato et al., 1990). The finding that cells exposed to mechanical strains up-regulate SM myosin and grow suggests that the

phenotypic characteristics of VSM cells in culture can be influenced by the biomechanical environment.

In experimentally induced atherosclerosis in rabbit aorta, VSM cells exhibit an immature type of phenotype with SM MHC-1 expression without SM MHC-2 (Zanellato et al., 1990). In hypertensive animal models (rabbit model of renovascular hypertension (Pauletto et al., 1994) and stroke-prone hypertensive rats (Contard et al., 1993)), immature-type VSM cells have been identified in the hypertrophied media of large vessel walls. Interestingly the VSM cells in hypertensive rabbit resistance vessels do not exhibit the same phenotypic shift (Pauletto et al., 1995).

These phenotypic changes are also seen in endothelial cells; bovine endothelial cells, extracted from cardiac tissue, had a greater expression of fibronectin and collagen type I (suggesting a more proliferative phenotype) in cloned 'spout cell' cultures compared to a normal cobblestone monolayer morphology (Myers, 1993).

Interestingly, the interaction between vascular cells and the ECM in culture can influence cell biological activity. Early studies with rat arterial cells plated in primary culture on differing ECM substrates revealed that a laminin substrate delayed the modulation of cells from a contractile to a synthetic phenotype (Hedin et al., 1988). Collagen synthesis is greater in VSM cells cultured on a fibronectin and type I collagen substrate compared to plastic, but lower on Matrigel® and type IV collagen (Powell et al., 1997). There was no assessment of VSM phenotype in this study, but the latter would suggests that VSM cells co-cultured with a basement membrane-ECM were more likely to remain quiescent.

1.2.5 Endothelial cells

Endothelial cells line the lumen of the vessel and persist as a solitary cell layer that act as the barrier between blood and the vessel as a whole. Its function has become increasingly important, as greater characterisation has been explored. Most importantly are the role of nitric oxide (NO) and its effect on the vasculature. NO is one of several cytokines that are synthesised by endothelial cells; the others include prostacyclin, endothelium-derived hyperpolarising factor, endothelin and angiotensin II. NO is a potent vasodilator and its production is stimulated by NO synthase (NOS), which has two isoforms namely endothelial NOS and inducible NOS. Endothelial NOS is produced in small amounts, but in inflammatory states, inducible NOS is expressed in large amounts by leucocytes and VSM cells. Inducible NOS may also stimulate endothelial cells to produce NO. In contrast, high concentrations of TNFα can reduce the levels of endothelial NOS (Sprague and Khalil 2009).

Endothelial cells are adhered to matrix, particularly the basement membrane. It is hypothesised that disruption to the basement membrane leads to biological dysfunction of endothelial cells and subsequent disease. This basement membrane is therefore crucial to maintaining the integrity of the blood vessel wall. Interactions with the endothelial cells and blood constituents are increasingly characterised and importantly include lipids and Ang II. Endothelial cells are not the predominant synthesizers of matrix proteins, but there is evidence that some collagens are products of endothelial cells in vitro.

Early studies with co-cultures of bovine aortic endothelial cells and VSM cell, divided by a semi-permeable membrane, on varying substrates revealed differential VSM cell migration. Endothelial cells and endothelial cell conditioned media increased VSM cell

migration on plastic and fibronectin, but not on type I collagen. This highlighted an endothelial cell released pro-migratory factor that failed to stimulate VSM cells on type I collagen (Powell et al., 1996). Further experimentation highlighted endothelial cell induced down regulation of VSM cell type I collagen gene expression (Powell et al., 1997). Moreover, co-cultures revealed evidence of reduced TFGβ1 activation as a consequence of increased endothelial cell PAI-1 (Powell et al., 1998).

From human cardiac tissue, endothelial cells and fibroblasts have demonstrated serum-induced growth and MMP and TIMP expression at gene expression and protein levels. (Tyagi et al., 1995).

1.2.6 The blood vessel in disease

In atherosclerosis, the classical view regarding changes to matrix composition highlights the initial role of oxygen free radical activated macrophages crossing the endothelium. These macrophages secrete TNFα, IL-1, PDGF which promote increased gene expression of MMPs. Enhanced activation of pro-enzymes occur, in part stimulated by plasmin / uPA, shifting the balance between MMP-TIMP complexes and initially leading to degradation of the basement membrane-ECM. This allows proliferating VSM cells to migrate to the lumen and triggering the development of atherosclerosis.

The RAS is also implicated as a key factor in the development of cardiovascular disease, although the majority of studies have been conducted in animals and investigations have related to cardiac rather than blood vessel pathology. Activated macrophages, stimulated by inflammatory mediators at the time of a vascular event,

undergo a phenotypic shift and invade the intima of coronary vessels. This leads to increased macrophage secretion of Ang II, which in turn both stimulates further macrophage activation in an autocrine manner and stimulates macrophage synthesis of $TGF\beta_1$. Evidence is provided by cultured cardiac fibroblasts, co-incubated with $TGF\beta_1$, which undergo a phenotypic change to myofibroblasts; the latter results in further synthesis of Ang II and $TGF\beta_1$ and consequently production of collagens type I & III. (Sun et al., 1997; Weber et al., 1997).

In animal models of left ventricular hypertrophy, spontaneously hypertensive rats (SHR) had increased gene expression of type I and II collagens in the left ventricles and increased gene expression of fibronectin, type IV collagen and $TGF\beta_1$ in aortas compared to Wistar-Kyoto rats. Treatment of SHR animals with ACE inhibitors or AT_1 receptor antagonists reduced the gene expression of all ECM proteins suggesting a critical role for Ang II via the AT_1 receptor (Ohta et al., 1994). The role of cardiac fibroblasts was underscored with a study revealing cultured cells co-incubated with angiotensin II had increased expression of type III collagen and reduced activity of MMP-1 (interstitial collagenase) that was dependent on AT_1 receptor presence (Brilla et al., 1995).

In SHR animals AT_1 receptor antagonism suppressed the gene expression of cardiac TGFβ1 and matrix proteins (Collagen types I, III and IV, fibronectin and laminin). (Kim et al., 1995). Similarly in a post MI experimental model rat heart, AT_1 receptor antagonism attenuated cardiac fibrosis (Ju et al., 1997).

The relationship between matrix turnover and left ventricular hypertrophy (LVH) in hypertensive patients was examined after treatment with ACE inhibitors (Laviades et al., 1998). Although there were no differences in serum free MMP-1 and TIMP-1 levels

between normotensive and never treated hypertensive patients; those hypertensive patients with LVH had greater levels of TIMP-1 and lower values of MMP-1. ACE inhibitor treatment for one year resulted in increasing free MMP-1 and decreasing TIMP-1 compared to baseline values. Moreover serum carboxy-terminal telopeptide of collagen type 1 was increased suggesting greater collagen type 1 degradation.

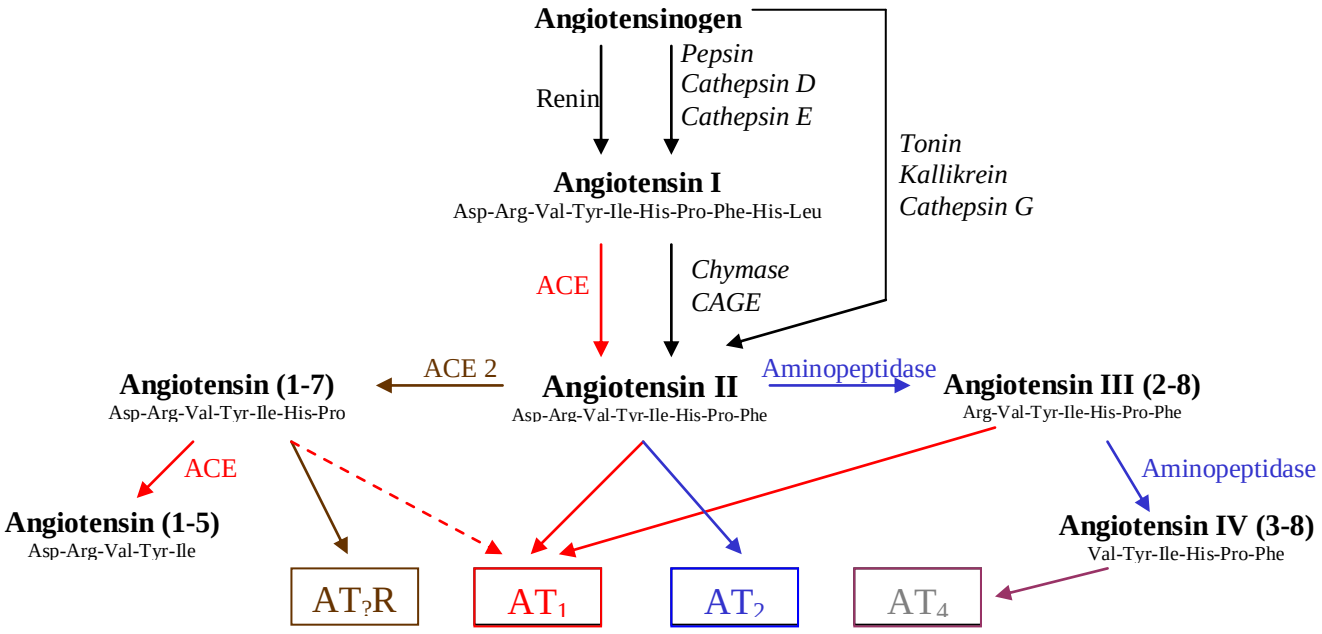
1.3 Renin Angiotensin System

1.3.1 Introduction

The RAS is crucial to the normal regulation of BP. It interacts with a variety of other cytokines and peptides and consequently plays a predominant role on blood vessel wall function. Importantly, there is mounting evidence from basic scientific and clinical studies that the RAS has deleterious effects on vascular function in disease.

The various components of the RAS have differing influences on vessel biology and these are discussed below. However, it is unclear what the relative contributions that each of these factors serve and exactly how these factors mediate their effect on vascular tissue.

Figure 1.2 Renin-Angiotensin System



CAGE: chymostatin-sensitive Ang II-generating enzyme.

Up regulation of the RAS activity and thus production of Ang II and aldosterone ultimately results in arterial vasoconstriction and salt and water retention in the renal tubules. In physiological terms, this prevents major organ ischemia when the BP falls. However, Ang II may compound the changes to the vasculature that occur in disease and therefore its action becomes deleterious.

1.3.2 Angiotensin II

1.3.2a Activity

Ang II is a potent vasoactive octapeptide with a wide biological profile with activity on VSM cells, endothelial cells (Wolf et al., 1996), platelets, renal fibroblasts (Ruiz-Ortega et al., 1997) and neurones causing diverse activities such as growth, oxidative stress (via nuclear factor κB (NF κB) – Braiser et al., 1996), fibrogenesis, arterial vasoconstriction, vasopressin secretion and hyper-coagubility. Via the AT_1 receptor, Ang II triggers a cascade of secondary messenger systems predominantly phospholipase C (see section 1.4.2) leading to Protein Kinase C (PKC) activation and calcium channel activation resulting in raised intracellular calcium (Dzau et al., 1994).

1.3.2b Actions on VSM cells

Ang II modulates VSM cell biology predominantly through its stimulation of the AT_1 receptor. Its documented properties in animal and human VSM cells, acting via the AT_1 receptor, include growth induction (Makita et al., 1995), activation and up regulation of $TGF\beta_1$ (Gibbons et al., 1992; Liu et al., 1997), basic fibroblast growth factor (bFGF)

and PDGF and increasing migratory potential of VSM cells (Liu et al., 1997). Interestingly Liu reported that high dose Angiotensin II (10^{-6} M) lowered the migratory potential while low dose (10^{-8} M) stimulated migration (Liu et al., 1997). Downstream in cell signaling terms, Ang II has also been shown to promote growth by stimulating the expression of proto-oncogenes namely c-fos c-myc, c-jun (Naftilan et al., 1989; Taubman et al., 1989). These factors are responsible for promoting hypertrophy and hyperplasia. In healthy vessels, the actions of Ang II are counteracted by endothelium derived substances such as nitric oxide and prostaglandin I_2 (prostacyclin).

Human VSM cells possess a functionally active gp91phox-containing neutrophil-like NAD(P)H oxidase. Ang II regulates the enzyme by inducing phosphorylation of p47phox, translocation of cytosolic subunits, and de novo protein synthesis (Touyz et al., 2002). Further studies (Zafari et al., 1998) indicate that AT_1 receptor-mediated production of super oxide generated by the NADH/NADPH oxidase is followed by an increase in intracellular H_2 O_2, suggesting a specific role for these oxygen species and scavenging systems in modifying the intracellular redox state in vascular growth. Ang II stimulates O_2^- generation by activating an NADH/NADPH oxidase. Importantly, inhibition of this enzymatic pathway by diphenylene iodonium (DPI) or by anti-sense transfection of p22phox, a critical component of the NADPH oxidase, inhibits Ang II-induced hypertrophy (Griendling et al., 1994; Ushio-Fukai et al., 1996).

In disease models, Ang II has a deleterious effect on the vasculature. Ang II induced fibronectin synthesis in animals via a BP-independent mechanism (Kim et al., 1996) and ACE inhibitors and AT_1 antagonism prevented accumulation of collagen in rat aortas independently of BP (Benetos et al., 1996). In balloon injury vessels, resulting in the removal of the endothelial cells, intimal hyperplasia occurs via platelet activation and

growth factor (bFGF/TGFβ) activity as well as loss of endothelium derived inhibitory factors (Madri et al., 1989; Powell et al., 1989). In cultured rat aortic VSM cells, Ang II increases FGF2 but not FGF1 mRNA (Peifley et al., 1998). Thus atherosclerosis can be viewed as an Ang II dependent disease: the key features of atherosclerosis include VSM cell growth and migration, inflammatory cell activation (monocyte /macrophages), platelet and plasminogen activator inhibitor 1 activation (Dzau et al., 1994).

1.3.2c Generation of Angiotensin II

Cultured aortic VSM cells from SHRs reveal a greater production of Ang II than cells cultured from Wistar Kyoto (WKY) rats. Angiotensinogen, cathepsin D and ACE mRNA are also increased (Fukuda et al., 1999). Unpublished data from Fukuda reveals a change from contractile to synthetic VSM cell phenotype in SHRs are associated with the generation of Ang II.

This observation is important to the discussion regarding a hypertensive VSM phenotype, but also relates to the controversial subject regarding the nature of Ang II synthesis in vascular (i.e. extra-renal) cells. The key question is whether there is a fully intact RAS in vascular cells?

There is conflicting evidence of the activation of angiotensinogen mRNA in cardiac tissue in rat models of myocardial infarction or volume overload (Iwai et al., 1995; Lindpainter et al., 1993; Pieruzzi et al., 1995). Angiotensinogen mRNA in WKY and Wistar rats was identified in aortic medial smooth muscle cells and peri-aortic fat by northern blot and in situ hybridization (Naftilan et al., 1991). Angiotensinogen mRNA was also identified by ribonuclease protection assay in human heart homogenates but

only in atria, muscles of the conducting system and sub-endocardial layer of the LV. These findings were supported by immunohistochemistry (Sawa et al., 1992). However, angiotensinogen mRNA expression in the heart was less than 0.1% of liver (Dzau et al., 1997) and angiotensinogen was not found in supernatant of serum-deprived rat neonatal cardiomyocytes (van Kesteren et al., 1999).

Renin mRNA has been identified in human kidney, adrenal gland, placenta and saphenous vein by rtPCR (Paul et al., 1993). In a rat carotid artery balloon injury model, renin mRNA was identified by PCR and immunohistochemistry, but this does not conclusively prove that renin is synthesised de novo in VSM cells (Iwai et al., 1997). Earlier studies suggested that renin is taken up from circulation (Gohlke et al., 1992; Louden et al., 1983). Further evidence to support this has revealed a positive correlation between cardiac and plasma renin (Heller et al., 1998; Hirsch et al., 1999) with a high tissue-plasma concentration (Danser et al., 1997). Finally doubt has been cast on de novo vascular renin from three studies. Renin was not identified in isolates of rat heart (de Lannoy et al., 1998) or in the supernatant of serum-deprived rat neonatal cardiomyocytes (van Kesteren et al., 1999). After bilateral nephrectomy, renin was not synthesised by cardiac or extra-renal vascular tissue (von Lutterotti N 1994). Part of the discrepancies may relate to the sensitivity of radio-immunoassays (van Kesteren et al., 1999).

ACE has been demonstrated in VSM cells and macrophages of atherosclerotic plaques (Dzau et al., 1994). ACE mRNA was detected in rat hearts (Schunkert et al., 1990) and both cardiac ACE mRNA and protein increases in post myocardial infarction (MI) and volume overload models (Passier et al., 1995; Pieruzzi et al., 1995; Schunkert et al., 1990; Yamada et al., 1991). Although not specifically identifying ACE, Ang I

conversion to Ang II was observed in isolated perfused rat hearts (de Lannoy et al., 1998).

There was initially some doubt to the presence of extra-renal Ang II. Evidence of plasma Ang II in anephric patients was reported by some (Mizuno et al., 1990; Yu et al., 1972), but not others (Brown et al., 1971; Düsterdieck et al., 1971). More recently, Ang II was synthesised at cardiac tissue sites in the normal heart (De Mello et al., 2000), post MI and in response to volume overload (Danser et al., 1994).

Of particular importance to this thesis, mechanical strain has been proposed to increase Ang II release from intra-cellular granules (Dostal et al., 1992; Sadoshima et al., 1993), but this is not supported by later work (van Kesteren et al., 1999; Yamazaki et al., 1995), perhaps suggesting the Ang II in "granules" originated from receptor endocytosis?

On balance, vascular cells are likely to sequester renin and angiotensinogen from the circulating plasma to eventually produce Ang II de novo.

1.3.2d Species differences in RAS activity

There are species differences in the RAS involving Ang I and II and in particular renin, which have been documented for 30 years (Hollenberg, 2000). With the angiotensins, there is no suggestion that there is a functional element to this difference. Importantly in the rat, the AT_1 receptor has two isoforms namely AT_{1A} and AT_{1B} – both of which share a high degree of homology (Sasanura et al., 1992). Similarly, there is no suggestion that

this is functionally important, but in studies in this field, the use of a human model might potentially reduce the risk of erroneous results.

The major differences between species relate to the generation of Ang II. As an example in the rat, almost all the Ang II is produced via the ACE pathway (Bacells et al., 1997), but ACE only accounts for 70% of Ang II generation in humans (Hollenberg, 2000). In contrast to Bacells, in a study of human atrial appendages, TGFβ1 mRNA concentration correlated with ACE mRNA but not chymase mRNA (Ohmichi et al., 1997).

In cell culture studies, Ang II had a differential effect on rat aortic VSM cells compared to human saphenous vein VSM cells: the latter revealed less intense mitogen activated protein (MAP) kinase phosphorylation (Hollenberg, 2000). Alternatively, this may relate to differences in the vessel wall origins of the cells.

1.3.3 Other Angiotensins

Ang II is metabolised in the circulation (Semple et al., 1976) and cerebral ventricles (Harding et al., 1986) by Angiotensin-Converting Enzyme (ACE) 2 acting at the C-terminus to produce Ang (1-7) and aminopeptidase A acting at the N-terminus producing Ang III. Ang (1-7) can also be synthesised directly from Ang I by neutral endopeptidases (Mustafa et al., 2001).

Much less is known about the biology of ACE 2, but it is expressed in heart endothelial cells, renal tubular epithelial cells (Donoghue et al., 2002; Tipnis et al., 2000), intestine (Harmer et al., 2002) and lungs (Komatsu et al., 2002). ACE 2 also cleaves Ang I and the C-terminal residues from several unrelated peptides (apelin-13 or dynorphinA), suggesting that ACE 2 activity may not be limited to the RAS (Donoghue et al., 2002).

Ang (1-7) binds the AT_1 receptor weakly 100-1000 fold lower than Ang II (Pendleton et al., 1989). In addition, it may mediate its action through a non- AT_1non- AT_2 receptor that has yet to be characterised. Ang (1-7) produces vasorelaxation in canine coronary arteries possibly by a NO-dependent mechanism (Brosnihan et al., 1996) and has been shown to inhibit VSM cells proliferation (Strawn et al., 1999) effectively opposing the actions of Ang II. The role of ACE 2 as a cardio-protective balance to ACE in and outside the RAS is under investigation (Danilczyk et al., 2006; Ferrario CM 2006).

Ang (1-7) inhibits cultured rat aortic VSM cell growth as evidenced by ^{3}H-thymidine incorporation. However this effects was not inhibited by AT_1 and AT_2 receptor antagonism suggesting the activity of a third non- AT_1 non- AT_2 receptor (Freeman et al., 1996). Experimentally Ang (1-7) levels in rats increase after treatment with ACE inhibitors (Campbell et al., 1993). ACE hydrolyses Ang (1-7) by cleaving 2 amino acids from the molecule to form Ang (1-5) (Ferrario, 2006).

Ang III is a heptapeptide with a binding affinity to the AT_1 receptor about 10-fold lower than Ang II (Pendleton et al., 1989). It demonstrates biological activity similar to Ang II, but in addition may be the final mediator of Ang II activity in the central nervous system (Reaux et al., 1999; Zini et al., 1996).

Ang IV is the product of aminopeptidase N action at the N-terminus of Ang III. (Mustafa et al., 2001). It has similar affinity to the AT_1 receptor as Ang (1-7). However it exhibits high affinity for the AT_4 receptor – notably 10 times greater than Ang III (Swanson et al., 1992). Ang II has lower affinity and Losartan shows no binding activity for the AT_4 receptor (Miller-Wing et al., 1993). AT_4 receptor has been identified in the brain, heart, kidney, VSM cells and adrenal cortex (de Gasparo et al., 2000; Mustafa et al., 2001). Ang IV causes vasodilation via an NO-dependent mechanism (Haberl et al.,

1991), but at high doses causes vasoconstriction via the AT_1 receptor (Li et al., 1997). However, there is greater evidence of Ang IV actions in neural tissue, notably improving cognition (Delorenzi et al., 1997) and involvement in neuronal development (Moeller et al., 1996).

1.4 Angiotensin Receptors

1.4.1 Introduction

Ang II acts on cells via at least two angiotensin receptors (type 1 (AT_1) and type 2 (AT_2) that have been identified on vascular cells namely cardiomyocytes, VSM cells and endothelial cells as well as in the kidney, adrenal glands, sweat glands, gonads, pituitary and the central nervous system (Takeda et al., 2001). AT_1 receptors are highly expressed in foetal tissue suggesting a role in development (Shanmugam et al., 1996). In rat cardiomyocytes, receptors were localized to the sarcolemma, T-tubules and nuclei and on the transluminal side of endothelial cells and fibroblasts (Takeda et al., 2001).

The fibrogenic actions of Ang II are directed through the AT_1 receptor subtype. The role of AT_2 is less known, but activity at this receptor is thought to be in contrast to AT_1 activity in that it promotes nitric oxide production and thus vasodilation. There is some evidence to support AT_2 receptor activity promoting cellular senecesence, but the exact result of this is unclear.

1.4.2 Structure of the AT_1 receptor

The AT_1 receptor is a polypeptide of approximately 360 amino acids with seven transmembrane domains. It has a single gene located on chromosome 3, which encodes a 47 Kbp precursor mRNA strand with 5 exons that undergo alternative splicing to produce mature mRNA (Curnow, 1996). It has 30% homology with the AT_2 receptor, which is encoded on chromosome X.

The AT_1 receptor is coupled to a G-protein and activation leads to dissociation of its sub-units and in turn activating phospholipase C-β1 (PLC), phospholipase D and cytosolic phospholipase A_2 common with other G-protein linked receptors (Capponi, 1996).

Of particular note, human VSM cells derived from saphenous veins possess Ang II receptors almost exclusively of the AT_1 subtype in cultured cells at the third passage and these cells are therefore devoid of AT_1 receptors (personal communication: Dr ME Cooper (Melbourne) and Dr J McLay (Aberdeen)). Primary cultures of human umbilical arterial endothelial cells revealed ^{125}I-Ang II binding with kD of $1.98x10^{-9}$ with a maximum binding of $2.84x10^{-13}$ mol/mg protein. By the third passage this binding was no longer evident. (Ko et al., 1997). These examples are relevant to our model, however it is not possible to extrapolate this to arterial VSM cells *in vivo*. Notwithstanding, we can neglect any confounding bias from Ang II interactions with the AT_2 subtype in cultured cells.

1.4.3 Biology

1.4.3a Ligand Interactions

Ang II assumes a twisted U-shape when interacting with the AT_1 receptor as the C-terminus penetrates the interior of the space defined by the transmembrane domains. Mutagenesis studies have hypothesised that the C-terminus binds to lys199 on the 5th domain and the guanidine group of Arg2 binds to asp281 on the 7th domain. Agonists induce a conformation change leading to G protein activation (Inoue et al., 1997). This work was supported by later studies (Laporte et al., 1999) that used photosensitive

analogs of Ang II for binding studies; this revealed the val3 region of Ang II binding with Ile172 on the 2nd domain and the pocket for the C-terminus penetration being formed predominantly by the 3rd, 5th, 6th and 7th domains (Boucard et al., 2000).

Internalisation of the AT_1 receptor–Ang II complex occurs with a $t_{1/2}$ of less than 2 minutes (Anderson et al., 1993). Only AT_1 receptors are internalised (AT_2 lack the Ser-Thr-Leu motif (Hunyady et al., 1994, Smith et al., 1998) and importantly antagonists binding to the AT_1 receptor does not lead to internalisation. In addition, angiotensin receptors are de-sensitised after phosphorylation of the receptor leads to uncoupling of the receptor from the G-protein (Thomas et al., 1996) and prolonged Ang II stimulation (van Kats et al., 1997).

Ang II stimulation of AT_1 receptors has a variable effect on their expression. Cultured rat thoracic VSM cells AT_1 receptor mRNA was reduced by 30% at 4 hours and its $t_{1/2}$ reduced from 6 to 2 hours after co-incubation with Ang II (Nickenig G 1996). Similarly, Ang II-stimulated rat aortic VSM cell AT_1 receptor mRNA and protein was down regulated by 70% and 35% respectively (Lassengue et al., 1995). In contrast, Ang II increased AT_1 receptor expression in the kidney (Cheng et al., 1995). A high salt diet increased AT_1 mRNA in male Wistar aorta and mesenteric arteries, which was negated by a non-pressor infusion of Ang II. However a pressor dose of Ang II increases AT_1 mRNA over a 14-day period. Importantly, this could suggest that in hypertensive states, Ang II positively influences receptor density (Wang et al., 1998). Is this because of a shift in vascular cell phenotype?

1.4.3b Signalling

Activated PLC generates diacylglycerol and inositol triphosphate, the latter releasing calcium from intracellular stores (Griendling et al., 1986). In addition plasma membrane calcium channels are activated which promotes extra-cellular calcium entry into the cells. These events occur in seconds, but other signaling pathways are activated.

Calcium and diacylglycerol activate PKC leading to activation of ERK 1/2 (Abraham et al., 1998). Prolonged agonist activity results in addition STAT pathway up regulation. Nevertheless, the receptor exhibits de-sensitisation and internalises either via clathrin-coated pits or caveolae, once stimulated by an agonist (Thomas et al., 1996). Studies of rat VSM cells have reported internalisation within caveolae suggesting that this allows intracellular signal transduction. Subsequently, internalised receptors are either degraded in lysosomes or recycled courtesy of their Ser-Thr-Leu motif (Hunyady et al., 1994; Ullian et al., 1989). However, the complement of cell-surface receptors are maintained by de novo synthesised AT_1 receptors or recycled receptors.

1.4.4 Intracellular receptors

More recently, the presence of intracellular angiotensin receptors has been investigated. As described in section 1.3.2c, the presence of an intact intracellular renin-angiotensin system in vascular cells is controversial.

All components of the renin-angiotensin system have been identified in cardiac and VSM cells (Kato et al., 1993; Passier et al., 1996), however, this does not necessarily equate to de novo synthesis. Evidence for intracellular binding sites for Ang II exists in

cardiomyocytes and VSM cells (Robertson et al., 1971), and more recently in liver nuclei (Kiron et al., 1989). A7r5 cells (Filipeanu et al., 2001), which are devoid of functional surface AT_1 receptors, produce a biological effect to Ang II.

Although the origins of intracellular AT_1 receptors are not clear, the most likely source is internalised plasma membrane receptors. However, some argue reports of it may represent contaminants at the time of cell culture experimentation (De Mello et al., 2000). Whole kidneys from Ang II-infused Sprague-Dawley rats revealed (Zhuo et al., 2001) increased endosomal Ang II levels (inhibited by AT_1 receptor blocker) and increased AT_{1A} receptor binding in the endosomes, suggesting AT_1 receptor internalisation may be the mechanism of increased intracellular Ang II. This is speculative, but perhaps the role of intracellular Ang II is to compensate for the desensitisation of AT_1 plasma membrane receptors after prolonged stimulation?

Mutagenesis studies that eliminated the cell surface receptor in cultures rat hepatoma cells (Cook et al., 2001) revealed a potential intracellular action of losartan. Although antagonist-receptor complexes are not internalised, losartan passes freely into the cytoplasm because of its hydrophobic properties. Moreover Ang II production from these transformed cells remained intracellular via a renin dependent pathway, but nevertheless had a growth stimulatory effect via PDGF.

1.4.5 Regulation of Angiotensin Receptors

Proliferating rat aortic cultured VSM cells expressed 20% reduced AT_1 receptor mRNA and membrane associated protein compared to growth arrested cells (Nickenig and Sachinidis et al., 1996).

Nitric oxide down regulates AT_1 mRNA gene expression in rat VSM cells by 90% in a dose dependent manner (Ichiki et al., 1998). *In vivo*, the administration of nitric oxide synthase inhibitor (L-NAME) prevented the hypotensive effect of losartan in SHRs (Cachofeiro et al., 1996) suggesting a contributory role of nitric oxide in mediating the beneficial effects of AT_1 receptor antagonism or alternatively AT_2 agonism.

Other important factors reduce AT_1 receptor expression. Noradrenaline down regulates rat aorta AT_1 receptor mRNA via the α1 receptor in vivo and in cultured VSM cells (Du et al., 1997). Epidermal growth factor (EGF) reduces AT_1 receptor density in cultured aortic VSM cells by a mechanism that may involve a phenotypic shift (Ullian et al., 1989). Lead reduces AT_1 receptor density in cultured rat aortic VSM cells (Carsia et al., 1995). Glucose down regulates cultured rat VSM cells AT_1 receptor density without changing receptors affinity via glucose–stimulated PKC activation (Williams et al., 1992).

AT_1 receptor gene expression in myocardial biopsies taken from hearts following human transplantation was about 4-fold lower compared to donor hearts (Gullestad et al., 1998) suggesting that in heart failure receptor expression is lower. No account was taken of any treatment effect. In diseased rat models, AT_1 and AT_2 receptor gene expression in glomeruli and tubules was reduced in diabetic spontaneously hypertensive rats compared to non-diabetic SHRs (Bonnet et al., 2002). In contrast, hypercholesterolaemic rabbits have 2-fold AT_1 receptor expression and LDL up regulates AT_1 receptor gene expression in cultured rat aortic VSM cells (Nickenig and Sachinidis et al., 1997).

Importantly, uni-axial strain (10% cell elongation) increased AT_1 (predominantly due to AT_{1A}) and AT_2 receptor mRNA in rat neonatal cardiomyocytes at 12 hours by 2.8 and

3.3 fold respectively. Binding assays confirmed this increase was at the cell surface with 2.2 and 2.6 fold increases for AT_1 and AT_2 receptors, but no observed change in affinity (Kijima et al., 1996). However, the influence of mechanical strain on the renin-angiotensin system is not clear and this will be discussed in greater detail in Section 1.5.

1.4.6 Ang II (type II) Receptor

Key features of AT_2 receptors have been their role to antagonise the effects of AT_1 receptor actions of Ang II. AT_2 receptor stimulation leads to nitric oxide synthesis in the aorta, heart and kidney and stimulation of bradykinin leading to vasodilatation and natriuresis. (Horiuchi et al., 1999; Johren et al., 2004). The AT_1 and AT_2 receptor share 34% amino acid homology (Johren et al., 2004). The N-terminus and C-terminus of Ang II interact with the N-terminus and the inner half of the 3rd transmembrane domain of the AT_2 receptor respectively (Servant et al., 1997).

AT_2 receptor stimulation leads to activation of several pathways. Activation of phosphatases such as serine/ threonine phosphatase 2A and Src homology 2 domain phosphatase 1 results in inactivation of Extracellular Related Kinases (ERKs) leading to growth inhibition and apoptosis. Similarly, activation of phosphatases potentially inactivates the AT_1 receptor. Stimulation of phospholipase A2 activity and arachidonic acid formation results in potassium channel opening and hyperpolarisation.

The AT_2 receptor is the predominant receptor in the myometrium (Bouley et al., 1998) but also present in vascular tissue, brain, adrenal medulla and ovary. Foetal AT_2 receptor mRNA increases during embryonic development, but after birth AT_2 receptor

levels decrease substantially (Shanmugam et al., 1996). It is proposed that AT_2 receptors have a role is growth inhibition and differentiation during late gestation.

Cultured mesangial cells from Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats revealed differing expression of AT_1 and AT_2 receptors. Normotensive rats had evidence of both receptor types whereas hypertensive rats only the AT_1 sub-type. The association of a renal hypertrophy and lack of AT_2 receptor emphases their role in modulating the effects of Ang II via the AT_1 receptor (Goto et al., 1997). In cultured vascular cells, AT_2 receptor expression is low or absent and gene expression is down regulated by Ang II and noradrenaline in rat neonatal cardiomyocytes (Kijima et al., 1996).

In heart disease (cardiomyopathy, post MI, hypertensive hearts models), the expression of AT_2 receptors increases with an increased ratio of expression compared to AT_1 with the greatest density of AT_2 receptors in the infarcted regions (Wharton et al., 1998). Blood vessel injury results in re-expression of AT_2 receptors (Akishita et al., 2000).

In SHR thoracic aorta, mRNA expression for AT_2 is elevated, but down regulated by AT_2 antagonism. The physiological effect of the AT_2 antagonism reduced the medial cross-section of the SHR aorta (Otsuka et al., 1998).

In the rat mesenteric vasculature (Cao et al., 1999), Ang II infusion promoted growth and proliferation of VSM cells, which was attenuated by antagonists to both AT_1 and AT_2 receptors suggesting that the action of Ang II is modulated via both receptors. However in the same study only Ang II activity via the AT_1 receptor resulted in increase in BP and plasma renin activity. Other evidence suggests that Ang II promotes trophic changes without causing an elevation in BP (Li et al., 1998). Could this be through the

AT_2 receptor? Similar work (Sabri et al., 1997) in rat aorta revealed Ang II-induced hypertension caused medial wall hypertrophy and VSM cell content of increased NM myosin and fibronectin. AT_2 antagonism prevented the vascular hypertrophy but not the increase in BP or phenotypic change. AT_1 antagonism reversed the Ang II effects in all modalities. This suggests that the actions of Ang II via the AT_1 receptor lead to BP increases and phenotypic changes with some vascular hypertrophy. However the major role of AT_2 activity is trophic.

1.5 Mechanical Strain

1.5.1 Introduction

Most *in vitro* studies of human cellular activity whether it be the investigation of normal physiology or pathophysiology is undertaken in static conditions. However, in reality, a number of cells are continually under the influence of mechanical forces. This is most apparent in, but not limited to, the cardiovascular, respiratory and musculoskeletal systems. Arguably, to definitively study these systems, it is imperative to examine the effects of mechanical strain.

Mechanical forces on blood vessels include shear stress, pressure and tensile stress (Bishop, 1998). The effect of this force on the biology of vascular cells has only recently been explored and most studies have been conducted in animal tissue.

There is good evidence that mechanical strain induces cell proliferation, hypertrophy and ECM synthesis in some vascular cells. Importantly, mechanical forces act synergistically with other neuro-humoral factors to effect changes to vascular cells and it is this interaction with the RAS that will be explored in more detail.

1.5.2 Cellular mechano-sensing

Three key potential mechano-sensing mechanisms have been proposed:

1.5.2a Cytoskeleton

VSM cells are maintained in shape by actin filaments and microtubules, but adapt to differing environments such as adherence to matrix proteins in culture. Cells maintained

under mechanical stress are synthetically active and undergo replication, but once the tension is relieved the cells become non-proliferative (Grinnell, 1994).

The cytoskeletal role in sensing stretch was determined in studies using rat portal veins in organ baths (Zeidan et al., 2003). Inhibition of actin polymerization reduced the growth effect of pressure loading. However, other experimental evidence has revealed that disruption to the actin filaments by either cytochalasin D or colchine did not inhibit strain-induced protein synthesis or c-fos expression in cardiomyocytes (Sadoshima et al., 1992).

1.5.2b Integrins

Integrins are heterodimeric transmembrane proteins consisting of one α and one β chain binding specific integrin receptors. From knockout studies, fibronectin and the integrin α5β1 or αvβ3 were identified as fundamental to the development of the heart and vasculature. These integrins have been shown to be essential for mechanically induced pro-collagen synthesis (Reynolds et al., 1998) and downstream activation of MAP kinase, Extra-cellular Related Kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) (MacKenna et al., 1998). Blocking RGD motif peptides can prevent the strain-induced replicative response in aortic VSM cells (Wilson et al., 1995), but not in neonatal cardiomyocytes (Sadoshima et al., 1992).

1.5.2c Stretched activated ion channels

Specific stretch activated channels have been identified on most cells (Sachs et al., 1998, Sachs 2010). The initiator of channel opening is stretch, but its continued

activation is modulated by ion flux (Na^+, K^+ or Ca^{++}). Little is currently known about the activation and their physiological role, but stretch activated channels have been identified in VSM cells (Davies et al., 1992) and rat ventricular myocytes (Bett and Sachs 2000); in the latter, it is postulated that stretch may lead to fracture of the cytoskeleton, which leads to ion channel activation.

1.5.3 Intracellular Signaling in Mechanically Stretched cells

Several key mechanisms have been identified in modulating the effects of mechanical strain on cells. Mechanical strain-induced activation of phospholipases resulting in an increase in inositol tris-phosphate (IP3) and diacylglycerol (DAG) and leading to PKC activation and Ca^{++} influx in cardiomyocytes (Sadodshima et al., 1993) and endothelial cells (Rosales et al., 1992). MAP kinases (ERK1/2) are up regulated by PKC in cardiomyocytes (Sadodshima et al., 1993) and cardiac fibroblasts (Papakrivopoulou et al., 2004). The activation of MAP kinases has only in part been inhibited by PKC inhibitors suggesting that this pathway is not exclusively PKC- dependent.

Stretch-induced induction of MAP Kinase in cardiomyocytes has been inhibited by an AT_1 receptor blockade. This has been constant finding in the literature and is discussed in more detail below.

Strain-induced MAP kinase induction leads to pro-collagen synthesis. (Papakrivopoulou et al., 2004) and nuclear factors such as c-fos, c-Jun, c-myc and erg-1 are up regulated in stretched cardiomyocytes (Komuro et al., 1991; Sadodshima et al., 1992) suggesting a mechanism for cardiomyocyte hypertrophy. In endothelial cells, strain up regulates

NFκB, activator protein (AP)-1 and cAMP response element (CRE) binding proteins (Du et al., 1995).

1.5.4 Effects of Mechanical Strain on Vascular Smooth Muscles Cells

Cultured rat aortic VSM cells reveal a strain induced increase in SM MHC (both SM1 and SM2 isoforms) and a decrease in the NM isoform of myosin HC-A (Reusch et al., 1996). This phenotype is representative of the "contractile" quiescent VSM cell described by Chamley-Campbell in section 1.2.4. Interestingly, this effect was reduced when cells were plated on a fibronectin (compared to collagen type 1 and laminin) substrate suggesting an ECM role in determining cell differentiation. These changes in SM MHC were not evident in 10% serum suggesting GF inhibition of phenotype shift.

Growth of VSM cells, in response to stretch was in part dependent on the expression of key differentiation markers. Adult rat aortic VSM cells grown on pronectin capillaries that expressed differentiation markers such as myosin and calponin (i.e. a contractile function) revealed a much greater growth response to pulsatile flow (Cappadona et al., 1999) compared to those rat aortic VSM cells that weakly expressed the differentiation markers. However, these high myosin-expressing cells revealed no significant strain-induced increases in ERK1/2.

In human studies, cultured VSM cells (3-5 passages) from saphenous veins demonstrate increased cell proliferation in response to pulsatile pressure than cells from internal mammary vessels (Predel et al., 1992). Further work comparing arterial and venous VSM cells revealed a significant increase in growth in stretch-induced venous cells only

(Dethlefsen et al., 1996). VSM cells from veins are effectively mechanical strain-naïve and consequently may alter their phenotype under experimental stretch.

Cyclic stretch potentiated rabbit aortic VSM cell proliferation in serum-containing media only (Birukov et al., 1995). Further work conducted in our laboratory (Personal communication: Dr C Kemp) revealed strain-induced VSM growth was dependent on 5% serum-containing media. The use of high percentage serum lead to enhanced cell proliferation, but the use of 5% serum kept the cells maintained within the G_0 cell cycle phase.

DNA array technology, with access to 5000 genes, only identified 3 (Plasminogen activator 1, cyclo-oxygenase 1 and Tenascin C) transcripts in cultured human aortic VSM cells with a strain-induced increase greater than 2.5 fold and 10 transcripts with a decrease in expression at 12 or 24 hours. Notably MMP-1 was down regulated by 3.9-fold (Feng et al., 1999). This is inconsistent with other data and although the investigators used state-of-the-art technology, only 4% cell elongation was achieved and this is too little.

1.5.5 Effects of Mechanical Strain on the Vascular Matrix

Cyclical mechanical strain induces ECM synthesis in VSM cells. A 2 to 4 fold increase in collagen type I and III synthesis was identified in rabbit aortic medial cells (Leung et al., 1976), increased collagen synthesis was observed in porcine VSM cells under the influence of 10% cell elongation at 3 cycles per minutes (Sumpio et al., 1988) and a five-day exposure of human VSM cells to strain increased fibronectin and collagen protein concentrations and TGFβ1 mRNA expression (O'Callaghan et al., 2000). This

fibrogenic effect was inhibited when stretched cells were co-incubated with TGFβ1 antibodies (O'Callaghan et al., 2000).

Application of *in vitro* mechanical strain significantly increases collagen and / or pro-collagen expression. However, a cell-specific response was observed: notably VSM cells, fibroblasts and cardiomyocytes increase collagen expression to strain while the effect on endothelial cells leads to reduced collagen production (Sumpio et al., 1990).

Mitogenic response to mechanical forces (cyclical mechanical strain at 15-20 KPa) in rat VSM cells was dependent on the matrix on which the cells were plated (Wilson et al., 1995). VSM cell DNA synthesis was increased with collagen, fibronectin and vitronectin substrates, but not elastin or laminin. Importantly this effect was blocked by the integrin binding peptide GRGDTP and antibodies to β3 and αvβ5 integrins.

Mechanical strain (at very low levels up to 4% cell elongation) did not influence MMP-1 synthesis from VSM cells plated on fibronectin, but gene expression of ets-1 (transcriptional activator of MMP-1) was down regulated. However, PDGF- and TNFα-induced MMP-1 expression was suppressed (Yang et al., 1998).

In contrast, stationary (5%), but not cyclical (1 Hz), uniaxial strain significantly increased MMP-2 and MMP-9 expression in cultured human saphenous vein SMC (Asanuma et al., 2003); but these MMPs do not degrade interstitial collagens. MMP-1 degrades interstitial collagens and therefore the strain-induced increase in collagen synthesis may be a result of inhibition of its degradation as well as pro-active synthesis.

In vivo experimentation is rare, but pressure overload of rat cardiomyocytes by artificially inducing a significant increase in afterload by the application of an aortic constricting ring lead to an increase in TGFβ1 mRNA (Takahashi et al., 1994).

1.5.6 Effects of Mechanical Strain on Endothelial Cell Biology

Endothelial cells are exposed to all forms of mechanical forces and may transmit these forces to the cells deeper in the blood vessel wall, by the release of peptides such as PDGF and TGFβ1, by either pressure or increased permeability (Gray et al., 1993; Resnick et al., 1993). Strain-induced endothelin (ET)-1 has also been reported (Gray et al., 1993; Macarthur et al., 1994). However, stretch-induced secondary messenger (cAMP) activation is not consistent across endothelial cell type and origin; its activation has been observed in arterial and microvascular endothelial cells, but not endothelial cells derived from umbilical and saphenous veins (Manolopoulos et al., 1993) or cardiomyocytes (Sadodshima et al., 1993). At least 15% mechanical strain is required to induce endothelial cell replication (Banes et al., 1990).

There is evidence of a feedback loop protecting the vasculature. Bovine aortic endothelial cells, exposed to cyclical mechanical strain, revealed an increase in nitric oxide synthase (eNOS) gene expression (Awolesi et al., 1995). In contrast, reactive oxygen species are up regulated: cyclical strain induced H_20_2 release in cultured porcine aortic endothelial cells and exhibited greater activity of NADH/NADPH oxidase (Howard et al., 1997).

Mesangial cells, cardiomyocytes, lung and cardiac fibroblasts have also been investigated. Cyclical strain increased cultured rat mesangial cell TGFβ1 mRNA twofold at 36 hours followed by increased secretion of TGFβ1 at 48 to 72 hours (Riser et al., 1996). Further studies confirmed stretch-induced increases in mRNA expression for collagens types I and IV, fibronectin as well as TGFβ1 in mesangial cells and the ECM mRNA increase was inhibited by anti-TGFβ1 neutralising antibodies (Hirakata et al., 1997).

Mechanical stretch lead to a significant increase in collagen type 3 gene expression in isolated rat neonatal heart fibroblasts (Carver et al., 1991) and PDGF release from lung fibroblasts (Bishop et al., 1992). Cardiomyocyte response to mechanical forces (Sadoshima et al., 1993; Yamazaki et al., 1995) and in particular its interaction with the RAS will be discussed in the next section.

1.5.7 Synergy between Mechanical Strain and the Renin-Angiotensin-System

There have been a number of studies in predominantly animal cultures attempting to identify the relationship between mechanical strain and the RAS (Malhotra et al., 1999; Sadoshima et al., 1993; van Kesteren et al., 1999; Yamazaki et al., 1995). It is unclear whether mechanical strain and Ang II act synergistically to accelerate matrix synthesis by VSM cells. Of particular importance, there had been no studies preceding this thesis reporting the interaction between Ang II and mechanical strain in human VSM cells. This distinction is important because previous studies have generated species dependent differences in matrix protein synthesis in response to mechanical strain.

The evidence from these studies is part dependent on the concept of an intact RAS within VSM cells that synthesises Ang II de novo. This remains controversial as highlighted in section 1.3.2c.

Currently, most data has suggested that mechanical strain leads to an up regulation of Ang II production and in part exerts its effects on the vasculature through the RAS as well as directly from mechanical forces (Yamazaki et al., 1995; Sadoshima et al., 1993) However, Touyz has shown that stretched cells have an augmented response to the effects of Ang II in culture (Touyz et al., 1999). This has lead to the suggestion that post

receptor co-operativity could be relevant to this interaction, however the function of the AT_1 receptor under the influence of strain was unexplored prior to this thesis.

Initially, strain-induced release of Ang II from granules within rat neonatal cardiomyocytes was proposed (Sadoshima et al., 1993). Further evidence in the same cell culture model demonstrated attenuation of strain-induced cell signalling (c-fos gene expression (Kojima et al., 1994), PKC (Komuro et al., 1995), Raf-1 kinase (Yamazaki et al., 1996; Komuro et al., 1995), MAPK kinase activation (Yamazaki et al., 1995) and MAP Kinase Kinase (Yamazaki et al., 1996; Komuro et al., 1995) with AT_1 receptor antagonism. These latter observations suggested that the effects of mechanical strain were at least in part, being mediated via the AT-1 receptor.

An organ culture of rabbit aorta *ex vivo* (Bardy et al., 1996) and cultured rabbit aortic VSM cells (Li et al., 1997) have been the models bearing most resemblance to the adult human to date. Both studies revealed attenuation of stretch-induced matrix protein synthesis by AT_1 receptor antagonism, but interestingly, the former identified an increase in cellular Ang II production.

Using conditioned media from stretched cardiomyocytes, endothelial cells, VSM cells and cardiac fibroblasts administered to static cardiomyocytes identified strain-induced synthesis of Ang II, ET-1 and TGFβ1 leading to induction of molecular markers of vascular hypertrophy (van Wamel et al., 2001).

But significantly, others (van Kesteren et al., 1997) failed to identify an increase in Ang II in strain-conditioned media. Cellular activation of the RAS and an increase in AT_1 receptors (but not receptor affinity) in neonatal cells has also been proposed (Kijima et

al., 1996). Ang II binding studies revealed greater density of AT_1 receptor in SHR cardiomyocytes compared to WKY (Touyz et al., 1996).

Further support for a stretch-induced increase in receptor number has been shown in cultured rat neonatal cardiomyocytes. Stretch up-regulated AT_{1A}, AT_{1B} and AT_2 receptor mRNA and AT_1 receptor density on cell membranes, whilst Ang II down regulated AT_{1A} and AT_{1B} receptor mRNA with no effect on AT_2 receptor mRNA (Malhotra et al., 1999).

Fundamentally, mechanical strain appears to sensitise vascular cells to the effects of Ang II, but the exact mechanism or mechanisms is still unclear.

The role of platelet-derived growth factor PDGF has also been highlighted. Rabbit aortic VSM cells were exposed to 10% elongation resulting in cell proliferation and increased gene expression for PDGF-B (Li et al., 1997). In addition to AT_1 receptor antagonism and ACE inhibition, anti-PDGF-AB neutralizing antibodies inhibited stretch-induced cell proliferation. AT_1 receptor antagonism inhibited the increased gene expression for PDGF-B. Results suggest that synergy between mechanical strain and the RAS and a subsequent up-regulation of PDGF-B mRNA. In a similar series of experiments, AT_1 receptor antagonism inhibited the stretch-induced increased gene expression for TGFβ1 gene expression and protein synthesis (Li et al., 1998).

The synergy between stretch, RAS and platelet-derived growth factor has also been explored in rat VSM cells. Compared to either sole Ang II or strain stimulation of cells, the application of strain in cell cultures co-incubated with Ang II significantly increases DNA synthesis (Sudhir et al., 1993), which in turn was completely eliminated by anti-

PDGF-AB neutralizing antibodies. Interestingly in their experiments there was no attenuation of sole Ang II –induced DNA synthesis by anti-PDGF-AB.

Ang II down regulates the gene expression and presence of α1B adrenoreceptor in confluent quiescent rat aortic VSM cells via its stimulation of the AT_1 receptor (Clements et al., 1997). However, this effect is attenuated by cyclical mechanical stretching suggesting that stretch may also sensitise the VSM cells to sympathetic nervous system stimulation.

1.6 Ang II and Mechanical Strain Activation of MAP Kinase

1.6.1 Introduction

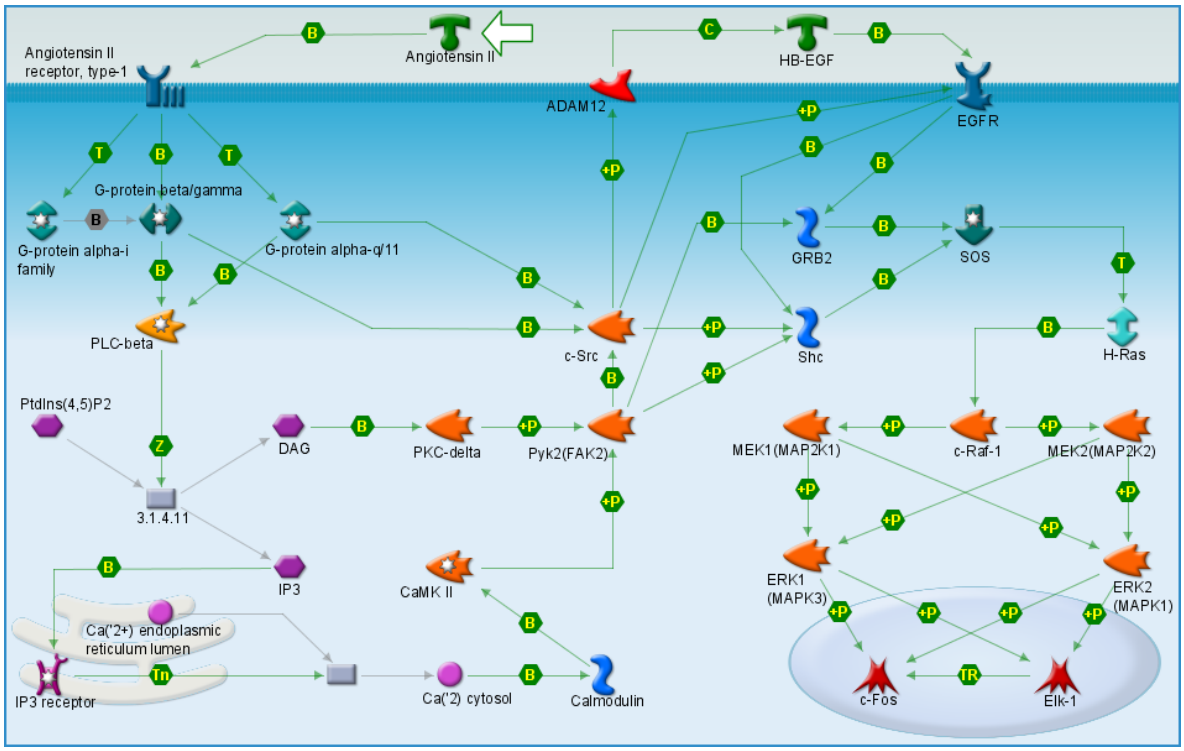
It is not clear how much each of the numerous intracellular signaling pathways contribute to the biological effects of Ang II and mechanical strain in VSM cells. A number of pathways have been studied including the Mitogen-Activated Protein Kinases (MAPKs) and more recently oxidative stress pathways. As we propose to investigate this pathway in our experimentation, a review of the evidence of intracellular signalling via MAP kinase is presented below.

1.6.2 Ang II and MAP Kinases

Evidence exists to show that Ang II activates the MAP kinase ERK 1/2 via the AT_1 receptor in cardiomyocytes, VSM cells, mesangial cells and renal proximal tubular cells (Hannken et al., 2001; Mondorf et al., 2000; Touyz et al., 2001; Zimpelmann et al., 2001). Moreover, Ang II activates ERK 1/2 via AT_2R in a neuronal cell line (PC12W cells) (Stroth et al., 2000). The majority of these studies have been conducted in animal or transformed cell-lines. Further studies, particularly in vascular cells, have revealed Ang II activation of p38 MAPK and JNK (Aoki et al., 2000; Touyz et al., 2001; Viedt et al., 2000).

With regard to VSM cells, several studies, in both human and animal cells, provide arguments to support the hypothesis that while activation of ERK 1/2 maintains a basal activity for growth, Ang II activation of p38 MAPK and JNK might be the key pathways for increased vascular growth or synthesis of matrix proteins.

Figure 1.3: Cartoon demonstrating the signally pathway after angiotensin II receptor activation (With permission map from: MetaCore by GeneGo, a Thomson Reuters Business).



In animal studies, increased Ang II-induced activation of ERK 1/2 and p38 MAPK was present in SHR compared to WKY rats. In addition, Ang II-induced matrix protein synthesis (as measured by ^{3}H-proline incorporation and pro-collagen I and III mRNA) was attenuated by SB212190, a selective p38 MAP kinase inhibitor in SHR rats only (Touyz et al., 2001). A further study also revealed a greater Ang II-induced activation of ERK 1/2 in SHR rats. Interestingly, a phosphatidylinositol-3 kinase inhibitor blocked angiotensin II-stimulated ERK1/2 activation in SHR but not in WKY revealing further differences in signal transduction (El Mabrouk et al., 2001). Furthermore, stroke-prone spontaneously hypertensive rats revealed similar ERK 1/2 and p38 MAPK activation, but increased JNK activation compared to normotensive rats (Izumi et al., 2000).

Only one study has compared the effects of Ang II to other stimuli. Using cultured neonatal rat cardiomyocytes, phenylephrine and Ang II activated ERK 1/2, but phenylephrine stimulation had a greater effect than Ang II on p38 MAPK and JNK activation (Aoki et al., 2000).

In one small human study, the effect of Ang II on cultured VSM cellular signaling was gauged by examining small peripheral resistance arteries from hypertensive and normotensive patients. Although the density of AT_1 receptors was determined to be identical, VSM cells from hypertensive subjects exhibited greater ERK 1/2 activation; p38 MAPK and JNK activation was not examined (Touyz et al., 2001).

1.6.3 Role of oxidative stress in mediating Ang II

There is some evidence, although conflicting, to support the hypothesis that Ang II activation of ERK 1/2 and other MAP Kinases is mediated by reactive oxygen species (ROS) in VSM cells. Importantly, one study has shown that H_2O_2 activates ERK 1/2 in VSM cells, highlighting a direct stimulus from oxidative stress (Frank et al., 2000). The activation of ERK 1/2 by Ang II was inhibited by N-acetylcysteine (NAC), a potent antioxidant, in rat aortic VSM cells (Ichiki et al., 2001). Furthermore, using diphenyleneiodonium (DPI) to inhibit NADH/NADPH oxidase resulted in attenuation of ERK 1/2 activation by Ang II (Frank et al., 2000). However, the data is not consistent. In rat VSM cells, Ang II was observed to increase the synthesis of reactive oxygen species (ROS) and activate ERK 1/2, p38 MAPK and JNK. Using anti-sense to $p22^{phox}$ (a subunit of the NAD(P)H oxidase), attenuation of Ang II-activation of ROS, p38 MAPK and JNK was evident, but not ERK 1/2 (Viedt et al., 2000). Furthermore,

although the authors have not suggested oxidative stress as the link, augmentation of Ang II–induced activation of janus-activated kinase (JAK) / signal transducers and activators of transcription (STAT) pathway in cultured VSM cells was demonstrated when co-incubated in a hyperglycemic environment (Amiri et al., 1999).

1.6.4 Mechanical Strain and Cell Signalling

Fewer studies have investigated the action of mechanical force on vascular cellular signaling and most used animal cells as the model. To date rabbit and rat aortic isolates, neonatal and adult rat VSM cells and neonatal rat cardiomyocytes, bovine and transformed (H441) pulmonary artery endothelial cells, skeletal VSM and mesangial cells have been investigated (Chess et al., 2000; Ingram et al., 1999; Kito et al., 2000; Lehoux et al., 2000; Martineau et al., 2001; Reusch et al., 1997; Yamazaki et al., 1996). This area of research is particular relevant as the cells exist in dynamic environments such as the vascular wall.

Experimental mechanical force can be applied to whole tissue isolates in an organ bath or cultured cells. Flexcell® provide the apparatus that permits the cyclical stretching of cultured cells up to 30% of their original length by plating cells on flexible elastomer wells that deform under the influence of a vacuum. Other techniques include the application of pressure with an organ bath (Lehoux et al., 2000) to whole tissue or the use of receptor-coated ferromagnetic beads that stretch cultured cells in a magnetic field (Goldschmidt et al., 2001).

In all of these studies, mechanical pressure results in cellular activation of ERK 1/2. Several deserve comment. Yamazaki also noted that transferring stretch conditioned

media to static neonatal rat cardiomyocytes activated ERK 1/2 and this effect was partially inhibited by a non-specific Ang II receptor (Yamazaki et al., 1996). Moreover, more recently, Kubo perfused a pressure load into rat aortic isolates both endothelial intact and denuded. The pressure-induced activation of ERK 1/2 was inhibited by losartan (AT_1 receptor antagonist), Pepstatin A (renin inhibitor) and Captopril (ACE inhibitor). These studies contribute to the controversial hypothesis that stretch activates the renin-angiotensin system in rat vascular cells. Other MAPKs have not been investigated in VSM cells, however cyclical strain has been shown to activate p38 MAPK and JUN in bovine pulmonary artery endothelial cells (Kito et al., 2000) and p38 MAPK in mesangial cells (Ingram et al., 1999).

1.7 Clinical Relevance of the Thesis

The importance of inhibiting the renin-angiotensin system in cardiovascular disease is well supported by key clinical trials. Blockade of the RAS has also been shown to be beneficial in post-myocardial infarction, diabetic nephropathy and non-diabetic renal failure, heart failure (AIRE investigators, 1993; CONSENSUS-1 investigators, 1987; Kober et al., 1995; Pfeffer et al., 1993; SOLVD investigators, 1992; Swedberg et al., 1992; ISIS-4, 1995) and in those at increased cardiovascular risk (Hope Investigators, 2000).

The work from this thesis examines a laboratory model of hypertensive disease in VSM cells and consequently cannot mimic clinical practice precisely. However, excessive mechanical strain and activation of the RAS often co-exist in hypertensive patients and this laboratory model aims to explore synergy between these key factors and provide data about the relative expression of VSM cell AT_1 receptors in pre-hypertension.

1.8 Summary

VSM cells are fundamental to the integrity of the blood vessel wall and play a pivotal role in its structure and function. These cells not only have a functional role in vessel contraction, but are the predominate source of ECM proteins. More importantly, VSM cell biology is influenced by numerous neuro-humoral and other factors including reactive oxygen species, but key influences include the RAS and mechanical forces.

VSM cell phenotype can adapt both *in vivo* and in experimental models. Proliferating cells are characterised by a "synthetic" phenotype whereas quiescent cells are maintained in "contractile" state. In cell culture, a phenotypic shift towards the synthetic model occurs as a consequence of growth media, but interestingly mechanical strain maintains the cell in a "contractile" like-phenotype with highly expressed differentiation markers.

The dominant effects of Ang II on most vascular cells are via the AT_1 receptor. These actions include arterial vasoconstriction, growth and fibrogenesis. After stimulation, the ligand-receptor complex is internalized and stimulation with Ang II ultimately leads to receptor down regulation. It is not clear if vascular cells are able to synthesise the RAS components de novo or whether studies, purporting to demonstrate de novo synthesis, are in fact identifying internalized ligand-receptor complexes.

Mechanical strain acts via a number of key mechano-sensors to promote its growth and fibrogenic potential. Inhibition of the RAS attenuates the effects of mechanical strain on vascular cells suggesting a synergistic relationship between mechanical strain and the RAS. It is not clear what underlying mechanism explains this phenomenon and most of the studies to date have involved animal cells. Although animal studies may not

accurately reflect the RAS activity in human cells, they suggest that strain sensitises the cells to the effects of Ang II.

This thesis will explore the interaction between mechanical strain and the RAS in human vascular smooth muscle cells using a cell culture model of VSM cells mimicking the effects of strain *in vivo*. Importantly, it will aim to answer a key question regarding the expression of AT_1 receptors on cells exposed to mechanical forces and thus provide further evidence of the pathogenesis of hypertension.

Hypothesis

The hypothesis of this thesis is that cell surface AT_1 receptor expression in cultured human vascular smooth muscle cells is up-regulated by mechanical strain.

Therefore the specific aims of the thesis are to:

1. Characterise cultured human vascular smooth muscle cells and assess cell viability of cells after mechanical stretch
2. Quantify vascular smooth muscle cell extracellular protein synthesis in response to Angiotensin II
3. Quantify vascular smooth muscle cell extracellular protein synthesis in response to mechanical strain
4. Quantify vascular smooth muscle cell ERK I/II synthesis in response to Angiotensin II and mechanical strain
5. Quantify vascular smooth muscle cell AT_1 receptor expression in response to mechanical strain

Chapter 2

Methods

2.1 Human Vascular Smooth Muscle Cell Culture

Cell culture work was carried out in a dedicated cell culture laboratory. All work was performed aseptically in a type II laminar flow hood. Cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

2.1.1 Primary Culture of Vascular Smooth Muscle Cells

Human vascular smooth muscle (VSM) cells were obtained from two sources. Venous VSM cells were obtained from human saphenous veins during surgical procedures and arterial VSM cells were obtained from discarded umbilical cords. Ethics committee approval and consent had been given appropriately. After removal of the endothelial layer by debridement, the VSM cell layer was diced into sections of less than 1 mm^3. The venous cells were suspended in media containing RPMI 1640 (Sigma), 0.5% chick embryo extract (Gibco, Life Technology), 2 mmol/L L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin supplemented with 20% fetal bovine serum (FBS) (Gibco, Life Technologies) and cultured in humidified air at 37^oC with 5% CO_2. The arterial cells were treated likewise but M199 media was used in place of RPMI 1640. (The use of M199 media which contains adenine, AMP and ATP might influence the vascular cell culture biology due to the potential effect of adenosine). Medium was changed after 24 hours to remove non-adherent cells and thereafter every 48-72 hours. The cells became confluent in 5-7 days.

2.1.2 Passage of cells

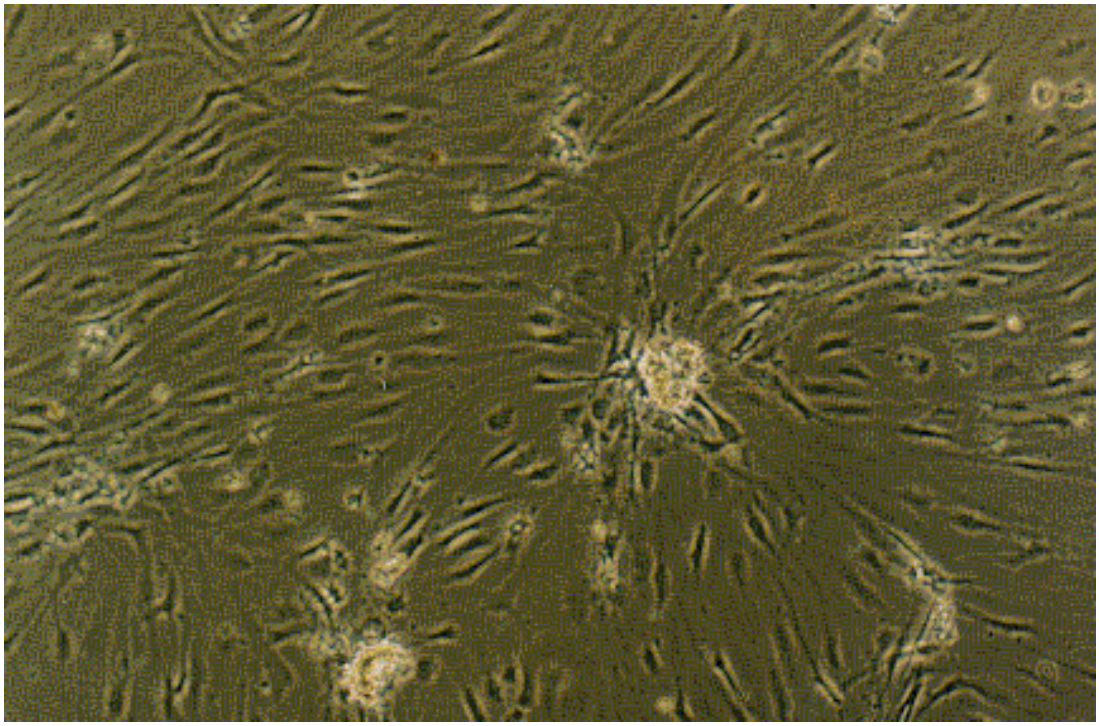
The cell monolayers were passaged every 5-7 days. Confluent cells were washed once with HBSS. Trypsin EDTA (0.1% trypsin, 0.02% EDTA) (Life Technologies) was added and incubated for 5 minutes at 37°C until all the cells had detached as assessed by microscopy. The trypsin was inactivated by adding 10ml of standard medium containing 10% FBS, and centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet re-suspended in complete medium. Cell concentration was measured using a modified Neubauer chamber and the cells seeded at approximately 10^5 cells per well. Human VSM cells from the second to the fourth passage, which had been serum-deprived for 48 hours, were used for experimentation. Cells not required for immediate experimentation were re-suspended in a small volume of FBS and stored in liquid nitrogen.

2.1.3 Cell Counting

Cells were counted using the Neubauer chamber. Volumes are listed for 10 cm diameter plates (35 mm diameter wells). The cells are washed once in warm Modified Eagles Media (MEM) or phosphate-buffered saline (PBS) containing 100 units/ml penicillin, 100 µg/ml streptomycin and 1% fungizone (250 µg/ml Amphotercin B - Gibco, Life Technologies) and aspirated. 2 ml (0.5 ml) of warm 0.1% Trypsin is added, the cells agitated and incubated for 2 to 5 minutes at 37^{o}C to promote separation. The cells are washed once in warm MEM (or PBS) and then re-suspended in 7 ml (2 ml) MEM. 20 µl is taken and placed on the Neubauer chamber to be counted using microscopy. All cells in each of 4 boxes of 16 squares both sides are counted. Multiplying the average value

of cells per box by 10^4 gave cells per ml, which multiplied by 7 (2) gave the total cell number in the sample. By the addition of 0.2% trypan blue, live cells could be distinguished from dead cells as the later have permeable cell membranes that take up the dye when viewed.

Figure 2.1 Human VSM cells (venous origin) in culture on plastic well (x40).



2.2 Cell Culture Experimentation

Individual experiments are detailed in the Results sections; however this section presents a broad outline of the cell culture methodology.

2.2.1 Ang II Experimentation

After preparation and serum deprivation, VSM cells were incubated in RPMI 1640 (1% FBS) as described above. Experimental cells were co-incubated with Ang II (usually at a concentration of 10^{-6}M) in the usual culture environment of humidified air at 37^{o}C with 5% CO_2 for up to 24 hours. Controls were treated identically except for the absence of Ang II. The AT_1 receptor blocker used in the experiments was Losartan donated by Merck, USA; this was added at a concentration of 10^{-5}M usually 30 minutes prior to the addition of Ang II.

2.2.2 Mechanical Stretch Experimentation

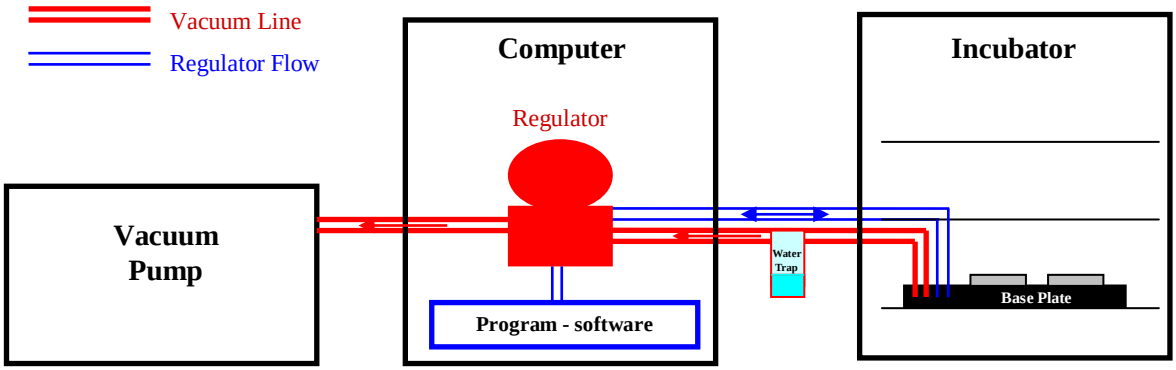
The basis for these series of experiments was to place the VSM cells in an *in vitro* environment that reflected the mechanical forces applied to these cells *in vivo*.

2.2.2a Flexcell Mechanical Strain Systems

The equipment and culture plates used for the application of mechanical strain were obtained from Flexcell Corp (Pennsylvania, USA). There were two main systems available for the application of mechanical strain of cells, namely the FX-2000 and FX-3000. The older of the systems (FX-2000) was used initially as it provided a greater

number of samples for the early experiments. However, in the main, the FX-3000 was utilised as it allowed more reproducible quantities of stretch, offered greater flexibility and provided concurrent, but varied, levels of mechanical stretch. A schematic outline of the mechanism is shown in figure 2.2.

Figure 2.2 Ouline Diagram of Flexcell Mechanical Strain System



Essentially, cells were seeded onto a flexible elastomer culture well contained within a 6-well culture plate. These plates were placed onto a specially designed base situated within a standard incubator that permitted a computer-generated vacuum to be applied beneath the plates and thus deform the membrane as shown in figure 2.4. Cells adhered to the membrane would therefore be stretched in the usual cell culture environment of humidified air at 37^{o}C with 5% CO_2. A mechanism housed within the computer regulated the flow of air from the base to the vacuum pump to generate the required level of stretch. Thus a stretch:relaxation cycle could be programmed into the computer, mimicking as close as possible the pattern and percentage cell elongation and relaxation experienced by VSM cells in large vessels during the cardiac cycle *in vivo*.

FX-2000 System

This system used the 6-well (25 mm diameter) Flex I plates. The Flex I plates (figure 2.3) allowed the flexible membranes to be easily pushed out of the plates following the experiment; this became particularly useful when conducting the immunofluorescence studies (see Results 3). However, it only provided one pattern of cyclical stretch and the amplitude was altered by twisting dials on the front of the computer to the desired level. To ensure that cells adhered to the base of the well, the plates were manufactured with one of four covering extracellular matrix substrates: collagen type I, collagen type IV, vitronectin or laminin.

Figure 2.3 – Flex I Culture Plate for FX-2000

When the vacuum was applied beneath the membranes, the base would deflect downwards forming a hemi-spherical cap. This resulted in a biaxial strain – radially and circumferentially. In the circular direction the strain would be equal due to the symmetry of the membrane. However, in the radial direction, cells were exposed to different levels of strain, thus cells towards the periphery would be stretched more than

the cells in the centre as highlighted in figure 2.4. This was the system's major disadvantage.

Figure 2.4 – Exaggerated schematic demonstrating cell elongation under pressure

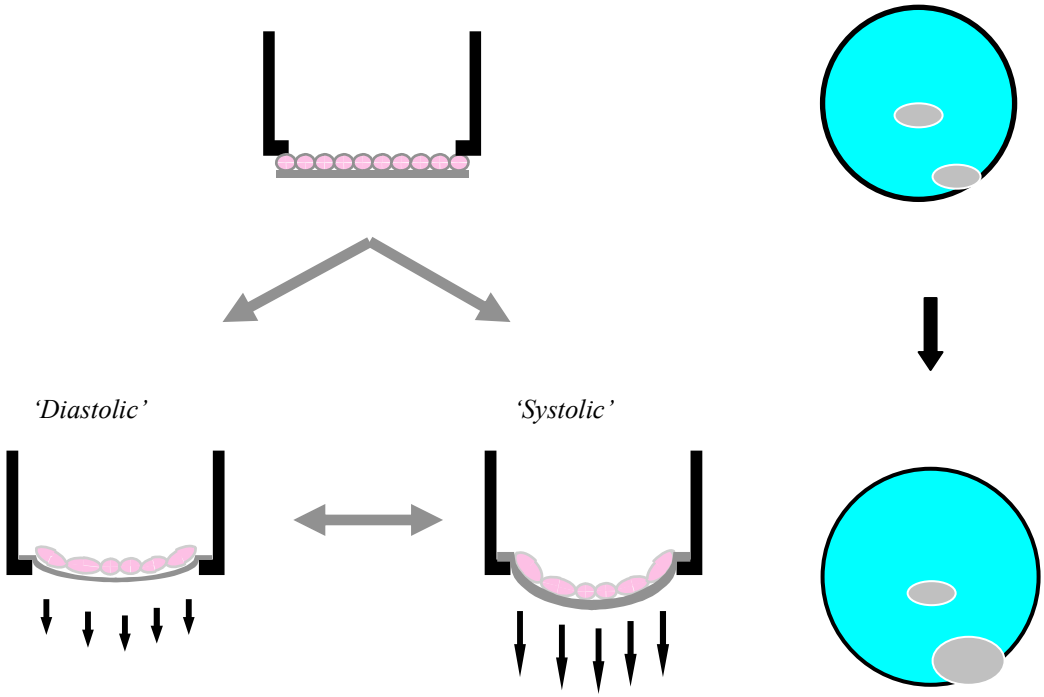
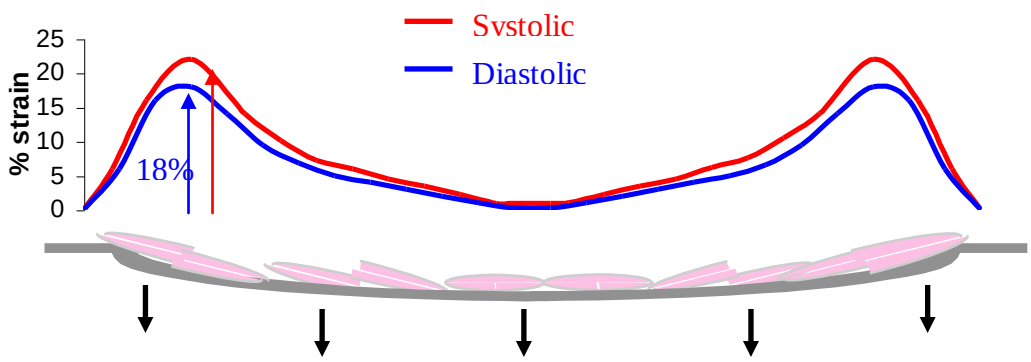


Figure 2.5: Diagrammatic representation of the varying ***radially*** stretch depending on the site of the cell on the membrane. This example demonstrates the maximum (systolic) and minimum (diastolic) cell strain pattern equivalent to 120/80 mmHg.



FX-3000 System

This system (Figures 2.6 and 2.7) was more versatile and importantly permitted varying patterns (sine / square / triangular waves) to be applied to culture cells. Consequently it was used for the majority of the stretch experimentation.

Figure 2.6: FX-3000 System (Vacuum pump not shown)

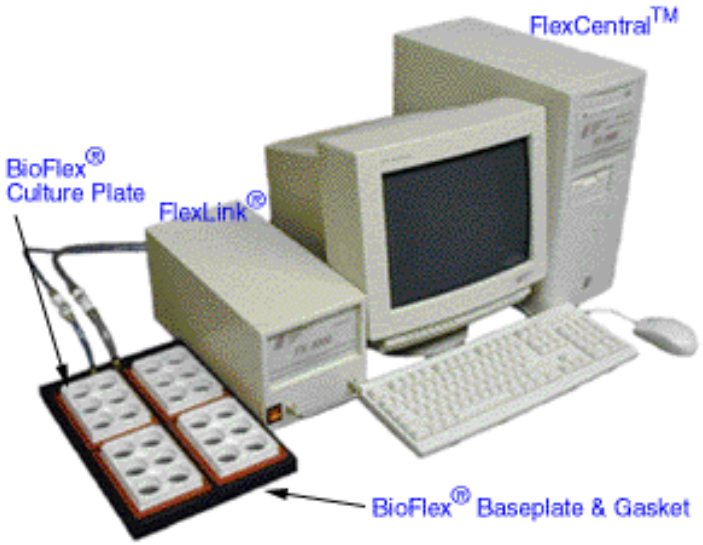


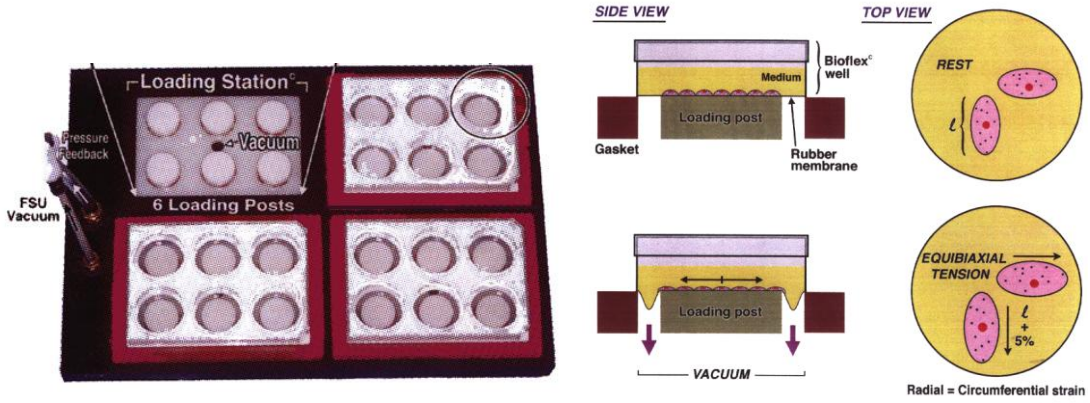
Figure 2.7: FX-3000 Setup in the Tissue Culture Laboratory

The system allowed up to four 6-well (35 mm diameter) Bioflex plates to be placed on a single base; however by using an additional regulator (FlexLink) gave the opportunity to deliver a different stretch regime to a further series of culture plates at the same time.

The Bioflex plates differed from the Flex I plates in a number of its properties. The wells were larger and membranes were fixed under tension to the bottom of the wells. If the membrane was excised from the plate, then the membrane would fold along with the cells adhered to it. The Bioflex plates were primarily designed to provide uniform biaxial radial and circumferential strain across the membrane surface by the use of 25 mm diameter loading posts. These loading posts would be situated directly beneath the

wells and lubricated with a silicone-based gel to reduce friction (see figure 2.8). There were clear advantages for using the Loading Posts, as it could be certain that a majority of the cells would be stretch according to the experimental protocol. Nevertheless, the use of loading posts limited the level of pressure that could be applied to the cells.

Figure 2.8: Bioflex Plates using loading posts to provide uniform biaxial strain



Similarly the same variety of extracellular matrix substrates was available.

2.2.2b Mimicking Blood Pressure levels using the Flexcell Systems

The experiments aimed to reproduce the mechanical forces on VSM cells *in vitro* as occur *in vivo*. Unfortunately, the Flexcell systems cannot simply translate in vivo patterns and levels of pressure into the laboratory. However, they can provide a workable model for the study of cultured cells that usually exist in a mechanical environment.

As highlighted above, the FX-3000 system allowed the use of central loading posts to give a uniform pattern of strain to the majority of cells. However, with loading posts, the cells overlying the central 25 mm post were exposed indirectly to the pressure generated by the vacuum pump. Therefore, although almost all experimental cells receive a similar level of strain, by the very nature of the system, the maximum level of cell elongation was only 8-9%.

FX-2000 Flexcell System

By varying the load and computer-enhanced application of vacuum, a variety of pressures could be applied to alter the peak (i.e. systolic) and trough (i.e. diastolic) pressures. However, it was not possible to easily set a level of pressure for both systolic and diastolic equivalents independently. Therefore with trial and error, table 2.1 highlights a number of the pressures that could be applied with the resulting cell elongation as determined at the periphery (data supplied by Flexcell).

Table 2.1

Pressure Applied (KPa)	**Equivalent mmHg**	**Approximate Cell Elongation**
- 15.9 / - 10.6 KPa	~120 / 80 mmHg	22 / 18 %
- 21.2 / - 13.3 KPa	~ 160 / 100 mmHg	26 / 20 %
- 26.6 / - 15.9 KPa	~ 200 / 120 mmHg	30 / 22 %

FX-3000 Flexcell System

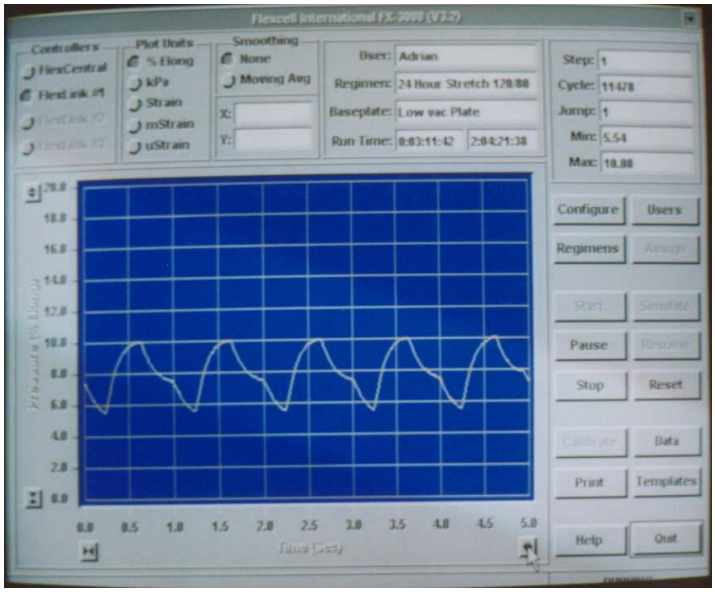
Without loading posts, the application of a vacuum pressure provided a similar level of cellular deformation to the FX-2000 Flexcell System as per table 2.2.

Table 2.2.

Pressure Applied (KPa)	**Equivalent mmHg**	**Approximate Cell Elongation**
- 18.4 / - 15.4 KPa	~138 / 116 mmHg	22 / 18 %
- 20.8 / - 16.9 KPa	~ 156 / 127 mmHg	26 / 20 %
- 23.3 / - 18.4 KPa	~ 175 / 138 mmHg	30 / 22 %

Using the loading posts, the maximum equilibiaxial strain resulted in 9.6 / 5.4 % cell elongation from a negative pressure of –7.8 / 3.6 KPa.

Figure 2.9: Representative Photograph of Waveform Pattern Generated by FX-3000



2.2.2c Cell culture

For all experiments, cells were seeded onto the stretch culture plates at a starting confluence of 30-40% and incubated in RPMI 1640 (15% FBS) to allow growth. Cells usually required one cycle of media change at 2 days to provide an approximate cell confluence of 80-90%. This period was appropriately adapted depending on the speed of cellular growth. Prior to experimentation, cells were serum deprived for 48 hours.

Studies conducted by my colleague, Dr. Christian Kemp during 1996-9, identified the optimum growth media for cells to be preserved in a mechanical environment. Using a series of varying growth media (1% FCS – 5% FCS – 10% FCS - 15% FCS) under the influence of mechanical strain, he identified that high percentage growth factor media lead to enhanced proliferation of the cells whilst low percentage growth factor media resulted in reduced cell numbers. In experiments exposing cultured human VSM cells to mechanical strain over 72 hours, the use of 5% FCS resulted in an increase in DNA

synthesis (^{3}H-thymidine incorporation), but no change in cell number nor protein synthesis (^{3}H-leucine incorporation); in contrast, the use of higher percentage growth media resulted in significant increases in all of the above indices and promoted entry into the G_2/M cell cycle phase, as identified by flow cytometry. It is likely that cellular activity in response to stretch is dependent of a minimum growth factor requirement. The use of 5% FCS kept the cells maintained within the G_0 cell cycle phase and consequently this percentage of growth factor were used for all stretch experiments.

Similarly, Ang II was co-incubated at a concentration of 10^{-6}M. Those experiments requiring an AT_1 receptor blocker used Losartan added at a concentration of 10^{-5}M, 30 minutes prior to the commencement of the mechanical strain or the addition of Ang II. If experimentation continued for more than 24 hours, media (including added agents) was changed daily.

All active and control experiments (including static controls) used identical culture plates to prevent any possible bias from different substrates influencing the outcome.

2.3 Cell Characterisation in standard culture and following mechanical strain

Confluent vascular smooth muscles (VSM) cells have a cobblestone appearance in culture. In this regard they can clearly be differentiated from cultured fibroblast or endothelial cells. However, as discussed in the Introduction, although contractile VSM cells express the structure protein α-actin, the synthetic and the intermediate VSM cell phenotype express a greater abundance of α-actin. Historically, our laboratory have a broad experience of using VSM cells, but a series of experiments were conducted to characterise the cells both prior to experimentation and following the influence of mechanical strain.

The elastomer membranes in the stretch plates auto-fluoresced effectively preventing use of standard microscopy in conducting these studies. A confocal microscope was therefore used. However, the confocal microscope could not image cells adhered to culture plates as the imaging stage could only accept cells attached to a flat plate up to 50mm wide. This limited the studies to using the Flex I plates as the elastomer membranes could be retrieved from the culture wells and positioned on the imaging stage without disrupting the integrity of the cells.

Following cell culture experimentation, the cells were washed carefully twice in *PBS*, before incubating with 4% Formaldehyde (in *PBS*) for 10 minutes at $4^{o}C$ to fix the cells. The cells were washed 3 times for 5 minutes each in *PBS* and this was typical of each wash step for this protocol. Cells were permeablised in *TBS/0.5% Triton* for 10 minutes at room temperature. The cells were washed in PBS. Non-specific binding was reduced by incubating with 2% BSA in PBS for 20 minutes at room temperature followed by washing in PBS.

The antibody steps are detailed below. Each incubation was followed by washing the cells in PBS, as described above. Primary antibodies for cell characterisation studies were a mouse monoclonal anti- α-actin antibody (Sigma) and mouse monoclonal anti-smooth muscle (SM) myosin against both heavy chains SM1 and SM2 (Sigma). Antibody concentrations were used according to initial experimentation and supplier's instructions:

1. Cells were incubated in the primary antibody (1:500) diluted in 2% BSA in PBS for 90 minutes at room temperature.
2. Cells were incubated in a rabbit anti-mouse antibody (1:100 - DAKO) acting as a link antibody diluted in 2% BSA in PBS for 60 minutes at room temperature.
3. Cells were incubated in the fluorochrome-conjugated antibody (anti-rabbit IgG – Alexafluor 568 (1:1000) – Molecular probes USA) for 60 minutes at room temperature in the dark.

Following immunostaining, the cells were maintained in a physiological buffer until viewed by confocal microscopy, usually within 2 hours. Control samples were treated identically, but BSA replaced either the primary antibody or link antibody.

2.3.1 Measurement of Cell Viability after Mechanical Strain

VSM cells (passage 5) were cultured as described above and seeded equally onto stretch plates (Flex I: $5x10^4$ cells / well; Bioflex: 10^5 cells / well). After the application of mechanical strain FX-2000 Flex I and FX-3000 Bioflex with Loading Posts, cells were analysed in the Neubauer chamber (see Section 2.1.3) with and without 0.2% trypan blue to determine cell viability.

2.4 Molecular Biology

Molecular biology experimentation was carried out in a nuclease-free environment. Plastic consumables or equipment was either purchased nuclease-free, autoclaved prior to use or baked at 120°C for at least 6 hours. Glassware was sterilised by baking at 160°C for 6 hours. All reagents and chemicals were of molecular biology grade. Any equipment not suitable for autoclaving (e.g. electrophoresis cells etc) was treated with 2% Hydrogen peroxide for at least 30 minutes and cleaned by rinsing in autoclaved DEPC-treated water.

2.4.1 cDNA probe preparation

A cDNA probe for Collagen I(α1) were kindly donated by Dr. Jill Norman (University College London) as plasmid vector (pBR322) constructs transformed into viable E.coli. The cDNA probe for AT_1 receptor was donated by Dr. David Lodwick (University of Leicester) as plasmid vector pBluescript. (The cDNA probe for fibronectin, ready for radiolabelling, was donated by Dr. James Metcalfe - University of Leicester). GAPDH cDNA was used as a control for RNA loading.

The E. Coli were stored at –70°C in Luria Broth (Millers LB broth base, 25g/L, Life Technologies) containing glycerol. This permitted storage for extended periods of time without loss of cell viability. All plasmids contained an ampicillin resistance gene to allow selection of E.Coli containing the cDNA of interest from wild-type E. Coli. The length of the cDNA for Collagen I(α1) was 1600 base pairs and for Collagen III(α1) was 1800 base pairs. Both cDNA inserts were contained within EcoR1 (G↓AATTC)

restriction sites. The length of the cDNA for the AT_1 receptor was 730 base pairs and inserted within PstI (CTGCA↓G) and EcoR1 restriction sites.

2.4.2 Extraction of cDNA from plasmids

2.4.2a Preparation from Culture

Initially, culture media for plasmid growth was prepared by inoculating 50ml Luria Broth (autoclaved at 131°C for 20 minutes) with Ampicillin at a concentration of 50µg/ml and pre-warmed to 37°C. E. Coli containing plasmid cDNA from storage was incubated with the growth media at 37°C for 16 hours (overnight) with vigorous agitation, producing a cloudy suspension. A second incubation step was undertaken with 1 ml of the overnight growth media incubated under identical conditions with fresh sterile 50 ml ampicillin-containing Luria Broth for 24 hours.

For Collagen I(α1) and Collagen III(α1), a chloramphenical-enhanced plasmid preparation was required as the yield from initial experimentation was low. In addition to the first incubation as described above, the second incubation was undertaken for 5 hours to allow adequate growth before the addition of Chloramphenical at a concentration of 180µg/ml and further incubation at 37°C for 24 hours with vigorous agitation.

2.4.2b Extraction and Purification of plasmid

Extraction of plasmid DNA from culture was performed using a commercial kit (QIAGEN Plasmid Purification Kit using the QIAGEN-tip 100 (midi) protocol)

following the manufacturer's instructions. The bacterial cells were harvested by centrifugation at 6000g for 30 minutes at 4^{o}C in a Sorvall RC 5B Plus high speed centrifuge. The bacterial pellet was re-suspended in *Re-suspension buffer* and incubated for 5 minutes at room temperature with *Lysis buffer* after gentle mixing. Chilled neutralizing buffer (3M potassium acetate pH 5.5) was added to precipitate the DNA and after gentle mixing, incubated for 20 minutes on ice. Supernatant containing DNA plasmid was recovered by double centrifugation: initially 20,000 g for 30 minutes at 4^{o}C and re-centrifugation for 15 minutes at 4^{o}C (Sorvall SS-34).

The resulting supernatant was filtered using QIAGEN filters (equilibrated with buffer: 750 mM NaCl; 50mM MOPS pH 7; 15% isopropanol; 0.15% Triton X-100). The QIAGEN filter was washed twice (1M NaCl; 50mM MOPS pH 7; 15% isopropanol) to remove contaminants. The DNA was recovered from the QIAGEN filter in *Elution buffer*.

The DNA was precipitated from the solution by the addition of 0.7 volume isopropanol at room temperature and mixing. The DNA was pelleted by centrifugation at 15,000 g for 30 minutes at 4^{o}C in a Sorvall SS-34 fixed angle rotor. The supernatant was carefully discarded and the pellet was washed in 70% ethanol and re-centrifuged at 15,000 g for 30 minutes at room temperature. The supernatant was discarded and the DNA re-suspended in 1M TRIS-HCl (pH 8). The DNA was precipitated for a second time overnight by the addition and mixing with ethanol. The sample was pelleted by centrifugation as described above and pellet air-dried for 15 minutes. The DNA was dissolved in 1M TRIS-HCl (pH 8) and stored at 4^{o}C. The DNA could be visualised by 0.8% agarose gel electrophoresis as described below.

2.4.2c Restriction Endonuclease Digest of plasmid DNA

Excision of the cDNA probe was performed using the appropriate restriction endonucleases (Invitrogen Ltd, Paisley) used at a concentration of 1 unit per μg DNA. These were incubated with 10x buffer (Invitrogen Ltd, Paisley) for 60 minutes at 37°C then warmed at 65°C for 10 minutes to deactivate the endonuclease.

2.4.2d DNA identification and quantification

The quantity of DNA was determined by spectrometry (Ultraspec III spectrophotometer – Pharmacia) measuring the absorbance at 260nm and 280nm. DNA measured at the 260nm absorbance giving a value of 1 is equivalent to 50 μg/ml DNA.

The integrity of the DNA was assessed by electrophoresis. A 0.8% agarose (Sigma) gel was prepared by dissolving agarose in 1x Tris-Acetate-EDTA (TAE, Sigma) by micro-waving. The gel was allowed to cool to approximately 50°C and further TAE was added to the appropriate volume. Ethidium bromide (50μg/ml) was added before pouring into a gel tray. The gel set with the addition of a comb to provide sample wells and placed in an electrophoresis tank containing TAE. Samples were prepared by the addition of loading buffer (5:1 ratio) and were resolved by the application of a constant voltage of 80V for two hours. By electrophoresing a standard DNA size ladder concurrently, DNA samples could be analysed for size and quantity; the latter supported by spectrometry.

Figure 2.10: Plasmid Digestion of Collagen α(I) cDNA

1 – Undigested Plasmid DNA

2 – Plasmid DNA digested by EcoR1

3 – 1 kB DNA ladder (4k, 3k, 2k, 1.6k, 1k)

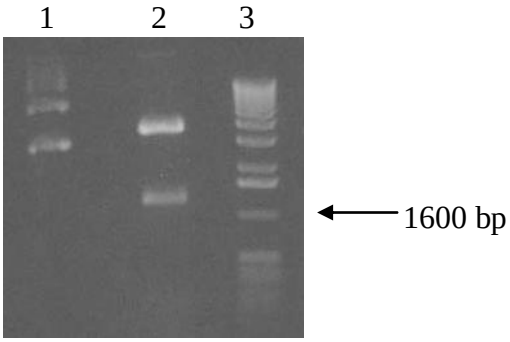
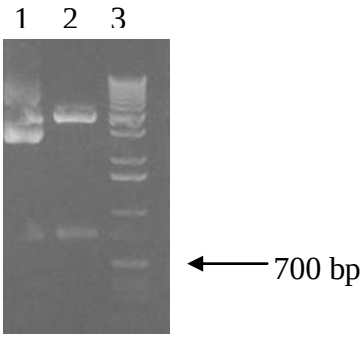


Figure 2.11: Plasmid Digestion of AT $_1$ Receptor cDNA

1 – Undigested Plasmid DNA

2 – Plasmid DNA digested by EcoR1

3 – 1 kB DNA ladder (4k, 3k, 2k, 1.6k, 1k)



2.4.3 Labeling of cDNA with ^{32}P

cDNA probes were radiolabelled using the RadPrime DNA labelling system (Gibco, Life Technologies) utilising random primers and the large Klenow Fragment of DNA Polymerase I. Briefly, samples were all prepared on ice. Approximately 25ng cDNA (dissolved in water) was denatured at 100^{o}C for 10 minutes. RadPrime buffer (including oligodeoxyribonucleotide primers), unlabelled dNTP (dATP, dGTP, dTTP in equal volumes) and radiolabelled [α-^{32}P]dCTP (3000 Ci/mmo/l, 10 mCi/ml – Amersham, UK) were added to the cDNA and mixed. Finally the Klenow Fragment

was added and briefly mixed before incubating at 37°C for 60 minutes. The final product was denatured in preparation for hybridization by boiling for 10 minutes at 100°C and then quenching on ice for 1 minute.

2.4.4 RNA extraction and isolation

Extraneous material is removed by a PBS wash. Solution A lyses cells and neutralises RNAses. DNA and other debris preferentially dissolve in chloroform whereas RNA dissolves in alcohol, finally RNA precipitates out of alcohol as it cools and the alcohol washes are used to remove any debris associated with the RNA pellet (Xie et al., 1991).

Cultured cells were washed twice with cold PBS then lysed by the addition of *Solution A* (water saturated phenol/TRIS saturated phenol). The cell lysate was transferred to a microfuge tube and vigorously mixed with chloroform/isoamyl alcohol. (24:1). The sample was incubated on ice for 30 minutes and then centrifuged at 12,000 rpm for 20 minutes at 4^0C. The colourless aqueous layer was decanted into a fresh microfuge tube containing 800 µl of 100% cold ethanol and store at -70°C for at least 2 hours to precipitate the RNA. The RNA pellet was recovered by centrifugation at 12000g for 30 minutes at 4°C and rinsed once in 75% ethanol. After further centrifugation (12,000 rpm for 10 minutes at 4°C), the pellet was left to air dry. The RNA was dissolved in nuclease-free water and incubated for 10 minute at 37°C to ensure complete dissolution. The samples were frozen at –70°C until required. Prior to electrophoresis, the RNA solution was incubated at 65°C for 15 minutes and quenched on ice for 1 minute (rapid cooling prevented renaturation).

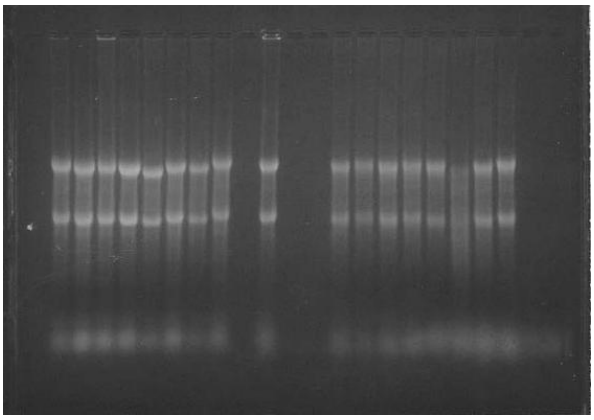
2.4.5 RNA identification and quantification

The total RNA was quantified by spectrometry (Ultraspec III spectrophotometer – Pharmacia) at the absorbances of A_{260} and A_{280}. RNA measured at the 260nm absorbance giving a value of 1 is equivalent to 40 μg/ml total RNA. The ratio of 260nm /280nm was determined to give an indication of the quality of the RNA preparation. The ratio was typically 1.6. This data was entered into an Excel spreadsheet that permitted automatic calculation of loading volumes for further experimentation.

2.4.6 RNA Formaldehyde Gel Electrophoresis

A 1.2% agarose gel was prepared by dissolving agarose in water by micro-waving. The gel was allowed to cool to approximately 50°C and in a fume hood, 1x MOPS and formaldehyde plus further water were added to the appropriate volume. Ethidium bromide (50μg/ml) was added before pouring into a gel tray. The gel set with the addition of a comb to provide sample wells and placed in an electrophoresis tank containing 1x MOPS buffer.

Figure 2.12: Example of Northern Blot RNA Formaldehyde Gel revealing ribosomal RNA signals



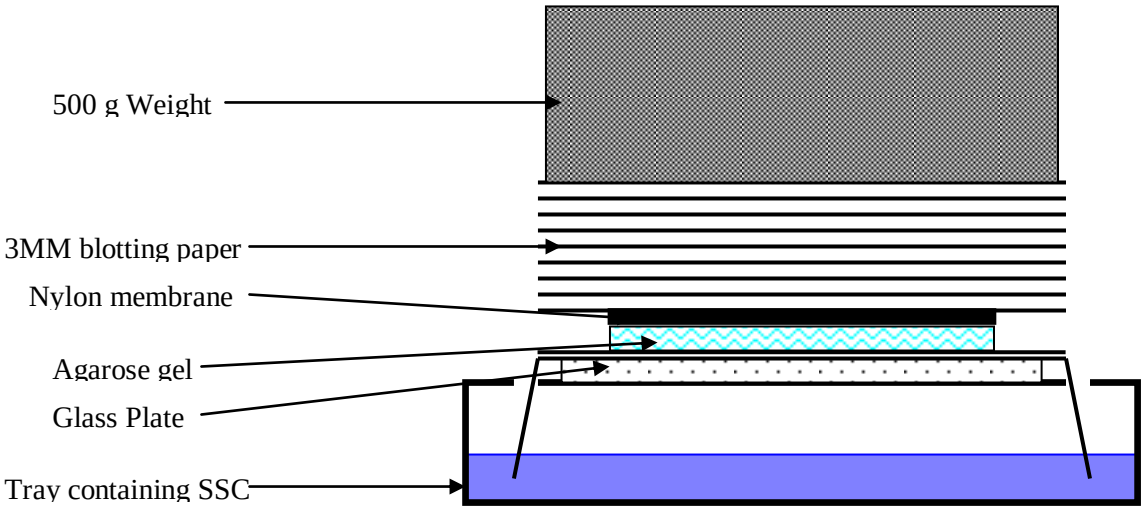
Samples were prepared by the addition of freshly prepared sample buffer (see appendix 1) as dictated by values calculated to give an even loading of total RNA. Samples were heated at 65^{o}C for 15 minutes and quenched on ice for 1 minute prior to loading. The samples and RNA ladder (Gibco, Life Technologies) were resolved by the application of a constant voltage of 75V for 4 hours.

2.4.7 Northern Blot Transfer

After electrophoresis, the gel was soaked in 20xSSC for 60 minutes to remove the formaldehyde. Figure 2.13 represents a cartoon of the northern blot apparatus, which facilitates RNA transfer from the gel to a nylon membrane (Hybond N - AmershamPharmaciaBiotech). The base of the apparatus comprised of a tray half full with 20xSSC. Two sheets of 3MM Whatmann chromatography paper formed wicks to soak up the 20xSSC and were supported by a glass plate positioned over the tray. The gel was placed (wells down) on the wick ensuring that air bubbles were excluded.

The perimeter of the tray was prepared to prevent SSC flowing around the gel. A gel-size piece of pre-soaked Hybond was placed on top, again ensuring that air bubbles were excluded. The tower was completed with two pieces of Whatmann paper soaked in 2x SSC, a further 10 sheets of Quick draw blotting paper (Sigma), one to two inches of paper towels and finally a 500g weight. Transfer was allowed to proceed for 16 to 48 hours.

Figure 2.13: Cross-section cartoon of the northern blot apparatus



On completion the nylon membrane and gel were placed on a UV transilluminator to check that transfer had occurred efficiently. The filter was rinsed in 6x SSC to remove any pieces of agarose and air-dried for 5-10 minutes. The RNA was fixed to the nylon membrane by cross-linking using a UV cross-linker (70,000µJ/cm^2, AmershamPharmaciaBiotech) pre-calibrated for use with Hybond-N membranes. The filter was stored, in Saran wrap, at 4^{o}C.

2.4.8 Hybridisation

The blotted membranes were initially incubated with SSC for 30 minutes at 42^{o}C. The membranes were then allowed to equilibrate in freshly prepared pre-hybridisation buffer (Appendix 1), with the addition of 200µl of denatured salmon sperm DNA, for 4 hours at 42^{o}C.

A ^{32}P-labelled cDNA probe (section 2.4.1) was prepared and then denatured at 95^{o}C for 5 minutes and quenched on ice. The radio-labelled cDNA and a further 200µl of denatured salmon sperm DNA was added to a hybridisation solution (Appendix 1). This hybridisation solution replaced the pre-hybridisation buffer and the membranes were incubated for at least a further 4 hours at 42^{o}C.

Following hybridisation, the filters were washed in increasingly stringent solutions to remove non-specific binding of the ^{32}P-labelled cDNA probe. Lower concentrations of SSPE and higher temperatures provided greater stringency. Following each wash, a hand-held radioactivity monitor provided a guide to the level of ^{32}P remaining bound to the membrane.

2.4.9 Autoradiography

Following stringency washing, the membrane was covered in Saran wrap and exposed to Kodak film using an intensifying screen at –70^{o}C. The exposure time was adjusted according to the activity of the probe on the membrane in order to give an image that could be analysed. The film was processed using an automated film processor. The signal intensity was quantified by scanning densitometry using a software package. The mean density of all signals were corrected for the house-keeping gene (GAPDH) and expressed in arbitrary units as percentage increase over control conditions.

2.4.10 Stripping membranes of the radio-labeled cDNA probe

The bound probe was stripped off the membrane to allow its re-use. The membrane was incubated in stripping solution (Appendix 1), initially at 95^{o}C, for at least 2 hours. (The solution was allowed to cool over this time). The membrane was then allowed to partially dry (until the sheen was lost) before storage at 4^{o}C.

2.5 Protein Chemistry

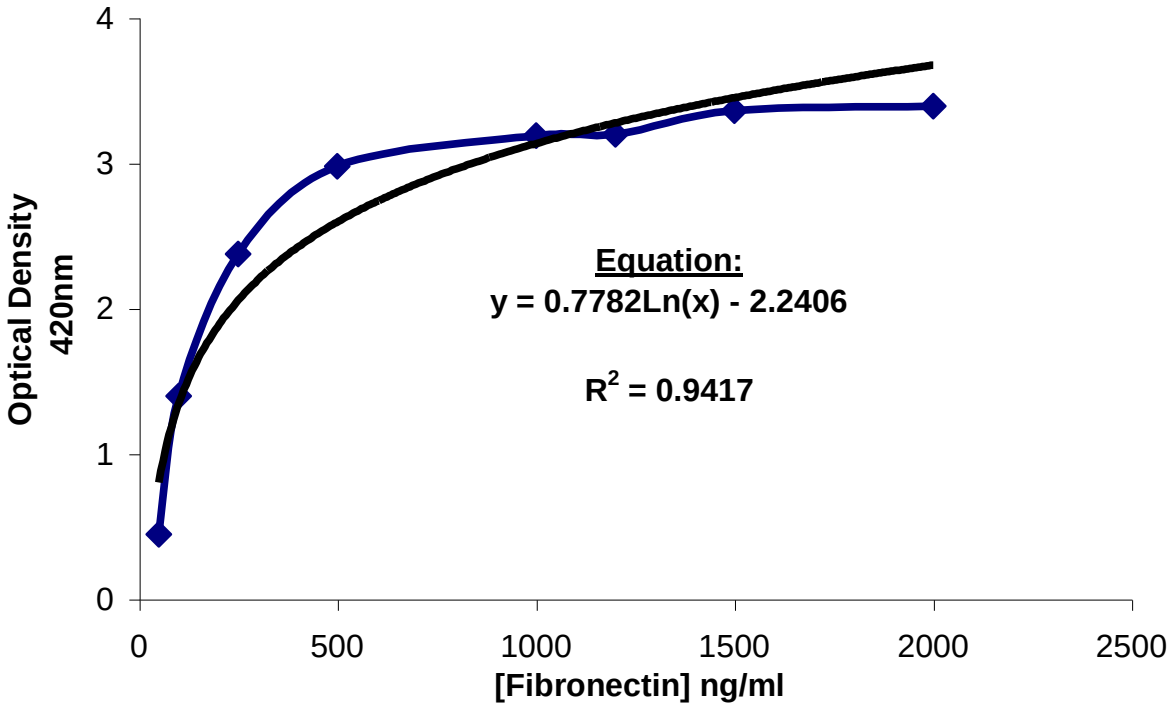
2.5.1 Assays for Cell Culture Supernatant Protein

Cell supernatants were examined for fibronectin synthesis by use of a sandwich ELISA developed in the laboratory by Dr. Chris O'Callaghan. Media was collected fresh at the completion of the cell culture experimentation. Care had been taken to ensure all wells had been incubated in identical volumes. The supernatant was briefly centrifuged at 1000g to exclude cellular debris and stored at –20^{o}C until analysed. Reagents for the ELISA are listed in Appendix 1.

Immunoplates (Sigma) were coated overnight at 4^{o}C with 1:1000 rabbit anti-human fibronectin antibody diluted in *Coating Buffer*. Excess antibody was removed by washing thrice with 1x PBS-Tween. Non-specific protein binding sites were blocked by incubating the plate with 2% Bovine Serum Albumin (BSA – Sigma) in *Washing Buffer* (WB) for 1 hour at room temperature. The plates were washed as above (PBS-Tween) and after every incubation step described below. Standards were made from serial dilutions of standard fibronectin (Sigma) to concentrations of 0-2,000ng/ml and analysed in duplicate. Samples or standards were added to the wells in duplicate / dilutions and incubated at room temperature for 2-3 hours or overnight at 4^{o}C. For the secondary antibody step, the plates were incubated with 1:500 mouse monoclonal anti-fibronectin (Sigma) diluted in WB for 2 hours at room temperature. Horseradish peroxidase (HRP) conjugated anti-mouse IgG 1:1000 in WB was added and incubated for 1-2 hours at room temperature. Peroxidase activity was detected by adding *Citrate phosphate buffer* (containing a chromogenic substrate) and incubated for 5-10 minutes at room temperature. The reaction was stopped using 1M sulphuric acid and the absorbance read at 492nm using a plate scanner (Titertek multiscan plus automated

microtitre plate reader) within 30 minutes. A standard curve was generated and a logarithmic relationship identified. Fibronectin concentrations that there not discriminated by optical density using the log-linear standard curve were repeated at greater dilution to ensure that there was an adequate range of optical density for a given range of fibronectin concentrations. Fibronectin concentrations were determined from the optical density (420nm) by use of an Excel spreadsheet incorporating the logarithmic equation. An example of a standard curve is shown in figure 2.14.

Figure 2.14: Representative Fibronectin ELISA Standard Curve



2.5.2 Assays for Cellular Protein

The isolation of cellular protein for western blotting was adapted for analysis of the ERK 1/2 and the AT_1 receptor proteins in whole cell homogenates. Reagents are listed in Appendix 1.

2.5.3 Isolation of total cellular protein

At the completion of cell culture experimentation, the media was aspirated and cells washed in PBS. This latter step was ignored in the ERK 1/2 experiments because of time pressure. Liquid nitrogen was added briefly to freeze the cells. The cells were incubated in ice-cold *Lysis Buffer* for a few minutes before scraping the cell homogenate into an eppendorf tube. Cellular debris was discarded after centrifugation at 14,000g (4,500 rpm) for 10 minutes at 4^oC.

The supernatant was retained for:

1. Determination of protein concentration: Total cell protein was measured using a commercial assay (BioRad protein assay) based on a modified Lowry technique following the manufacturer's instructions. A standard curve was plotted for BSA from 1µg to 20µg. 4 volumes of either diluted sample or standard were mixed with a one volume of Biorad Dye Reagent Concentrate and applied in triplicates to the wells of a 96-well microtitre plate. The plate was incubated for ~15 minutes at room temperature before absorbances were read at 595nm. A linear standard curve was plotted from the absorbance versus standard protein quantities and protein sample quantities were calculated by linear regression analysis.

2. Storage for future western blotting: an equal volume of *2x Reducing buffer* was added to the remaining supernatant and boiled for 2-3 minutes before storing at -20^{o}C.

2.5.4 Western Blot Analysis

The Biorad mini gel apparatus was used for these experiments. Reagents are listed in Appendix 1.

2.5.5 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

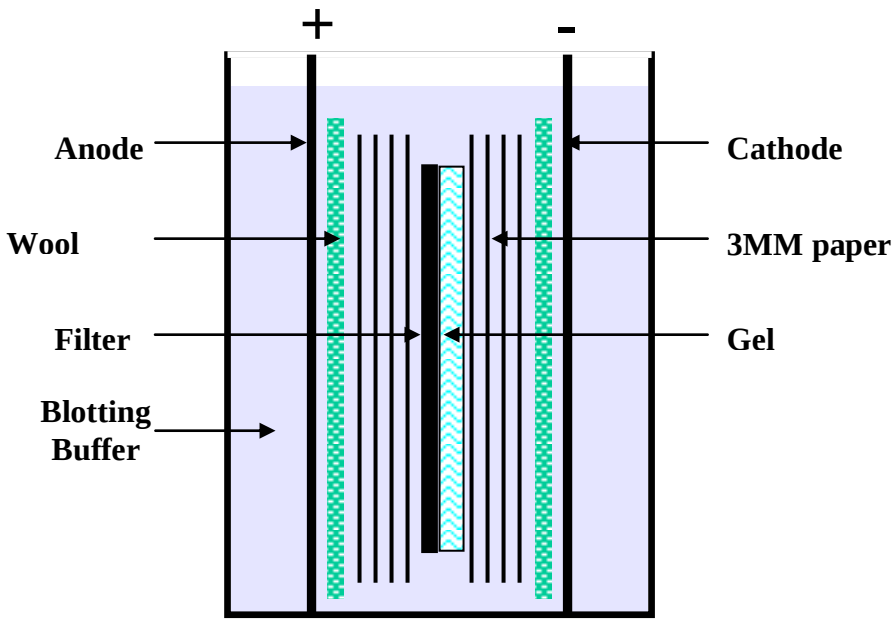
Ornstein (1964) and Davis (1964) originally devised PAGE to permits the analysis of protein defined by molecular weight. Initially proteins, denatured prior to electrophoresis, are bound to SDS and migrate through the acrylamide gel based on their SDS-polypeptide size. Proteins can later be transferred to nitrocellulose filters for more definite analysis by specific antibodies (western blotting).

The electrophoresis apparatus was constructed with a 10 % *Polyacrylamide Running Gel* and 5% *Polyacrylamide Stacking Gel*. 1x *Upper Buffer* and 1x *Lower Buffer* were added to the appropriate volume. Protein samples were aliquoted to allow equal amounts of protein to be loaded on the gel. Samples and molecular weight (Sigma High M.W. Standards SDS 6H) were boiled for 5 minutes and 30 seconds respectively prior to loading. Electrophoresis was conducted at constant current: 30 mA while the samples migrated through the stacking gel, 32-34 mA thereafter.

2.5.7 Western Blotting Transfer

The polyacrylamide gel was removed from the electrophoresis apparatus and soaked in blotting buffer for 10 minutes. A nitrocellulose filter (0.45μm Protein nitrocellulose, Cat. No. 401196, Schleicher & Schnell) and 8 sheets of Whatman 3MM paper were cut to the exact size of the gel and, with 2 wool pads, soaked in blotting buffer. Figure 2.15 represents a cartoon of the western blot apparatus, but briefly it was constructed carefully in layers to prevent air bubbles forming which would inhibit efficient transfer of the proteins. The filter was situated on the anode side of the gel as SDS-polypeptide complexes are negatively charged. Blotting buffer was used to aid transfer and a constant voltage of 100 V (0.75 amps) was applied for 1 hour. To prevent over-heating, the apparatus was placed in the cold room (4^{o}C). Following transfer, the blot was dismantled and the filter stained with Ponceau S to indicate whether the transfer has been successful. The blot was destained with distilled water and either stored at 4^{o}C or prepared for immunological probing.

Figure 2.15: Cartoon of Western Blot Apparatus



2.5.8 Antibody probing

Antibodies for ERK 1/2 (p44/42 Mitogen Activated Kinase) and the AT_1 receptor were purchased from New England Biolabs (Catalogue number 9100) and Santa Cruz (sc-570) respectively. Both were raised in rabbits. The ERK 1/2 antibody detected ERK 1/2 activation at both 42 kDa and 44 kDa sites. The AT_1 receptor antibody was a polyclonal antibody specific to the last 12 amino acids at the N-terminal.

The filter was initially incubated with 10% *Marvel/PBS-T* overnight at 4^{o}C to block non-specific binding. The filter was washed 5 times for 5 minutes each with *PBS-T* and this wash was repeated after each stage. The primary antibody for the specific protein and secondary antibody (Amersham anti-Rabbit Horseradish Peroxidase labeled) was diluted in 5% *Marvel/PBS-T* according to initial experimentation and supplier's instructions. The filter was incubated in the primary antibody for 2-3 hours at room temperature with gentle agitation. Following washes, the filter was incubated with the secondary antibody for 1 hour under the same conditions. The filter was washed. The detection of the proteins was obtained using the Amersham ECL detection system. Equal amounts of the ECL reagents A and B were mixed, such that 500μl could be evenly applied to each filter. After 1 minute, the excess was blotted off and the membranes covered with Saran wrap. The filter was then exposed to film at room temperature for between 5-30 minutes.

2.5.9 Stripping the Filters

The filters were initially washed for 5 minutes with *PBS-T*. To remove antibodies, the filter incubated in *Strip solution* for 30 minutes at 50^{o}C. Finally, the filters were washed 4 times for 5 minutes each with *PBS-T* before storing at 4^{o}C.

2.6 Radioligand Binding Studies

These series of studies aimed to assess the expression of AT_1 receptors on cultured human vascular smooth muscle cells. AT_2 receptors are not present on passaged cells, therefore inhibitory studies were not necessary (Personal communication from Dr ME Cooper (Melbourne) and Dr J McKay (Aberdeen)).

In one arm of the study, following cell culture experimentation, cells were washed once in cold PBS and then lysed by the addition of ice-cold *Lysis Buffer*. The lysate was collected and centrifuged at 4,000g for 5 minutes at 4^oC. The supernatant was retained for determination of protein concentration (section 2.5.3).

In the second arm of the study, cells were initially rinsed twice with ice-cold *Binding Buffer*. The cells were incubated for 90 minutes at 4^oC in radioligand buffer containing a fixed quantity of ^{125} I-Ang II Sar1, Ile8 (Quantity: 50 fmol – Activity: 2000 Ci/mmol (Amersham)) and varying concentrations of unlabelled Ang II (10^{-6} to 10^{-10} M). In addition, cells were incubated with unlabelled Ang II (10^{-5}M) to measure non-specific binding. The incubation was concluded with fast multiple rinses and aspiration using ice-cold buffer. All the incubations were conducted in the cold room to ensure a constant temperature of 4^oC. The cells were collected in *Harvest Solution* and the contents placed in LP3 tubes, suitable for counting.

The studies were conducted in triplicate to provide Hill plots and Scatchard analysis.

2.7 AT_1 receptor Immunocytochemistry

In addition to the cell characterisation studies, immunocytochemistry was used to identify the expression of AT_1 receptors on VSM cells under the influence of mechanical forces. Similarly, Flex I stretch plates and confocal microscopy was required.

Following cell culture experimentation, the cells were washed, fixed and permeablised as described in section 2.3, except all experimentation was conducted at 4^oC to prevent receptor internalisation. In an attempt to limit the immunostaining to the cell membrane, a series of experiments were conducted without permeablising the cells. Unfortunately, even when those cells were incubated in *Tyrodes Buffer*, the cells did not survive long enough to complete the experiment.

The antibody steps are detailed below. Each incubation was followed by washing the cells in PBS, as described previously. Antibody concentrations were used according to initial experimentation and supplier's instructions:

1. Cells were incubated in a rabbit polyclonal anti-AT_1 receptor (N10) antibody (Santa Cruz - 1:200) diluted in 2% BSA in PBS for 90 minutes at room temperature.
2. Cells were incubated in the fluorochrome-conjugated antibody (anti-rabbit IgG – Alexafluor 568 (1:1000) – Molecular probes USA) for 60 minutes at room temperature in the dark.

Following immunostaining, the cells were maintained in a physiological buffer until viewed by confocal microscopy, usually within 2 hours. Control samples were treated identically, but BSA replaced the anti-AT_1 receptor (N10) antibody.

2.8 Flow Cytometry

Conventional flow cytometry was performed to measure human AT_1 receptor expression on cells with or without the influence of mechanical forces. Mechanical strain was conducted using the *Bioflex®* plates and loading posts with an application of stretch for 48 hours.

All flow cytometry experiments were carried out at 4°C and in the presence of 0.05% sodium azide to prevent receptor recycling. Incubations were carried out in the dark to prevent bleaching of the fluorochrome and non-specific binding was prevented by performing all incubations and washes in the presence of 0.1% BSA.

Following cell culture experimentation, culture media was removed and cells were incubated in Enzyme-free Cell Dissociation Buffer (Gibco) for 15-20 minutes at 4°C with agitation to detach cells. Approximately $5x10^5$ cells were used per binding assay. All cells were pre-cooled on ice (5 min) before being pelleted by centrifugation at 300 g for 5 minutes at 4°C.

The supernatant was discarded and pellet re-suspended in *P/B/A* (control) or rabbit polyclonal anti-AT_1 receptor (N10) antibody (Santa Cruz) in excess. All assays were performed in triplicate and samples were left to equilibrate for 30 minutes at 4°C. After one wash in ice-cold P/B/A (centrifuged cells at 300 g for 5 minutes at 4°C then resuspended), cells were incubated in goat fluoroscein isothiocyanate (FITC)-conjugated $F(ab')_2$ anti-rabbit IgG (Sigma) for 30 minutes on at 4°C. The cells were washed as described above and incubated for 2 min in P/B/A containing propidium iodide (PI; 5 μg/ml) to allow a later assessment of cell viability. After a final wash in ice

cold P/B/A cells were re-suspended in 250 µl of *P/B/A* and immediately assayed for cell-associated fluorescence.

To correct for non-specific binding, VSM cells were incubated with the fluorochrome in the absence of anti-AT_1 receptor antibody. The non-specific binding was subtracted from the total binding to give the specific binding. As a positive control for AT_1 receptor surface staining, an anti-HLA Class I antigen (IgG1κ) monoclonal antibody was run with each study.

2.8.1 Data acquisition

Cell associated fluorescence intensity was evaluated by two colour flow cytometry with a FACScan flow cytometer equipped with an air-cooled 15 mW argon-ion laser coupled to LYSIS II software (Becton Dickinson). Excitation was at 488 nm and the fluorescence emission was collected between 515 nm and 550 nm. Fluorescence intensity was analysed with log amplifier output and light scatter data acquired in the linear mode. Dead cells were first live-gated out by PI (FL-2) staining before green fluorescence (FL-1), forward scatter (FSc; mean cell size) and side scatter (SSc; cellular complexity) were measured for 10^4 cells and data stored in list mode. The median channel fluorescence (MCF) was used as the measure of cell fluorescence intensity because this parameter has been shown to most accurately reflect the average fluorescence of cells in a cytometric sample (Sharrow, 1991).

In all cases the data presented have been corrected for non-specific binding by subtraction of auto-fluorescence and background staining.

The cytometer was regularly calibrated for fluorescence and light scatter with microbead standards (Dako FluoroSpheres, Dako Ltd). During the time course of the study no significant change in calibration was seen.

2.9 Statistics

Graph Pad and prism Statistical packages were used to analyse the data. For comparative studies, ANOVA, Mann Whitney and Student t-test were used as appropriate.

Chapter 3

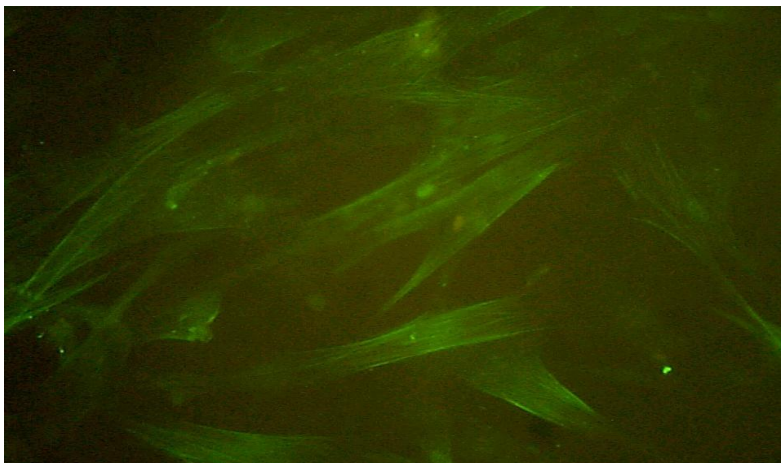
Cell Phenotype Characterisation and Cell Viability

3.1 Introduction

3.1.1 Cell Phenotype Characterisation

Experimentation depended on the successful isolation of human VSM cells from saphenous veins or umbilical cords. Laboratory colleagues had developed an expertise for the preparation of human VSM cells prior to the commencement of this thesis, however it had been important to demonstrate that endothelial cells or fibroblasts did not contaminate these cultures. Immunocytochemistry had been undertaken by H. Patel to characterise cultured cells (plastic wells) prepared as described in section 2.3; this clearly proved that cells prepared as human VSM cells were appropriately stained for α-actin (figure 3.1).

Figure 3.1: Venous human VSM cells plated on plastic dishes and stained for α-actin



I was confident that these studies did not need to be repeated. However, for all studies involving mechanical strain (including controls), cells were plated onto membranes coated with a variety of substrates. The experiments that define this thesis used membranes coated with type I collagen. The environment (namely the matrix substrate)

has an important influence on the cell phenotype and consequently the immunocytochemistry studies were repeated with antibodies for α-actin and smooth muscle myosin. Contractile cells will stain for both, but synthetic cells lose myosin smooth muscle. In addition, conducting these studies in freshly stretched cells would identify if these cells had undergone a phenotypic shift.

3.1.2 Cell viability

A further fundamental concern is the effect of mechanical strain on human VSM cell viability. As highlighted in section 2.2.2c, Dr. Kemp had demonstrated that cells survived intact under mechanical strain without excessive growth for at least 72 hours in 5% FCS. He only used the FX-2000 Strain Unit for his experimentation. Not wishing to repeat his thesis, basic studies of standard cell counting were undertaken for cells stretched by both the FX-2000 and FX-3000 strain units.

3.2 Experimental Design

3.2.1 Cell Phenotype Characterisation

Human VSM cells in early passages (2-3) were cultured on Flex I plates with collagen type I matrix substrate. When sub-confluent to 70-80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours. Cells were incubated in 5% FBS and exposed to cyclical mechanical strain by the FX-2000 system (Vacuum: -17/-10 kPa equivalent to Strain: 23/19% cell elongation). Controls were incubated identically except for the effect of mechanical strain. After 48 hours of continuous strain, cells were fixed and immuno-stained as described in section 2.3. Cell characterisation studies were performed in triplicate.

3.2.2 Cell Viability

Human VSM cells from passages 3 and 5 were cultured on Flex I and Bioflex plates respectively with collagen type I matrix substrates. Approximately 10^5 cells and 2.5x 10^5 cells were plated on each Flex I well and Bioflex well respectively and incubated in 15% FBS. After 2-3 days, cells were serum-deprived for 48 hours when ~ 70-80% sub-confluent and then exposed to mechanical strain incubated in 5% FBS. In the FX-2000 / Flex I arm, cells were stretched for 48 hours (Vacuum: -17/-10 kPa equivalent to Strain: 23/19% cell elongation). In the FX-3000 / Bioflex arm, cells were exposed to equibiaxial strain for 68 hours (Vacuum: -8/-4 kPa equivalent to Strain: 8/3.5% cell elongation). Controls were incubated identically except for the effect of mechanical strain. Cell counting (Section 2.1.3) using trypan blue provided estimates of cell number and viability.

3.3 Results

3.3.1 Cell Phenotype Characterisation

Immunocytochemistry of human VSM cells cultured on glass cover-slips revealed a distribution of α-actin filaments similar to those cultured on plastic (figure 3.1); however there was no evidence of smooth muscle myosin heavy chains SM1 & SM2.

Light microscopy of human VSM cells cultured on type I collagen (figure 3.2) revealed a similar appearance to those cells cultured in plastic wells. Cells are characteristically spindle-shaped and distributed in a random direction. However, after the application of mechanical strain, cells aligned at an approximate angle of 70^{o} to the direction of strain (figure 3.3). In contrast to cells cultured on glass cover-slips, immunocytochemistry of cells attached to type I collagen revealed the presence of both α-actin (figure 3.4) and myosin smooth muscle HC 1 & 2 (figure 3.5). Although it was not possible to undertake quantitative calculations due to potential bias, confocal microscopy revealed that those cells exposed to mechanical strain demonstrated an increased intensity of staining for both microfilaments (figure 3.6 and 3.7).

Figure 3.2: Human venous VSM cells in culture on Flex I plates

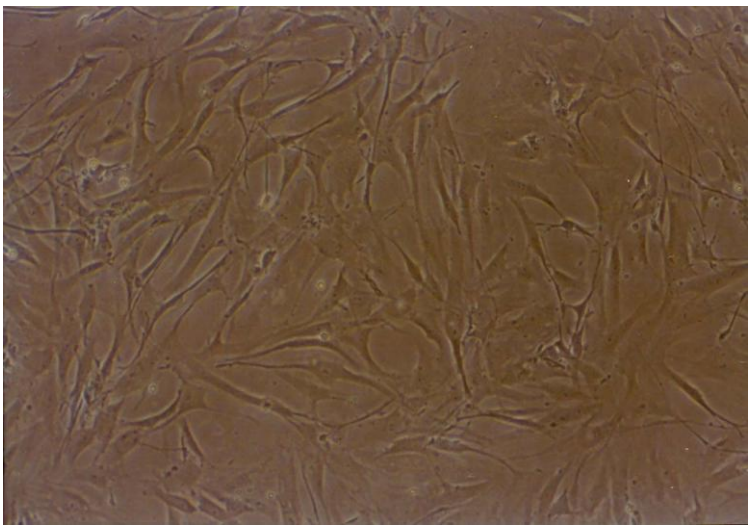
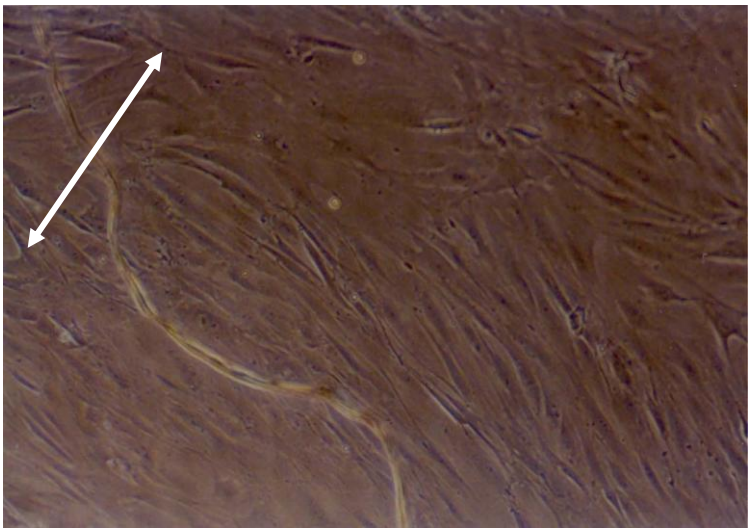


Figure 3.3: Human venous VSM cells in culture on Flex I plates after 48 mechanical strain



Arrow indicates direction of FX-2000-generated radial unidirectional mechanical strain.

Human VSM cells align at an approximate angle of 70^{o} to the direction of strain.

Figure 3.4: α-actin immunofluorescence study of non-stretch human venous VSM cells in culture (Flex I)

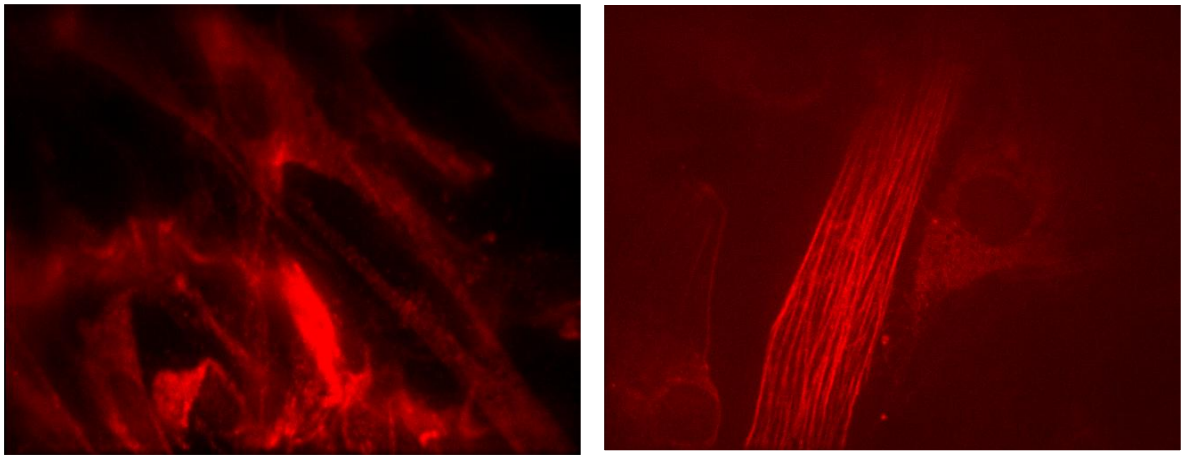


Figure 3.5: Smooth muscle myosin HC SM1 and SM2 immunofluorescence study of non-stretch human venous VSM cells in culture (Flex I)

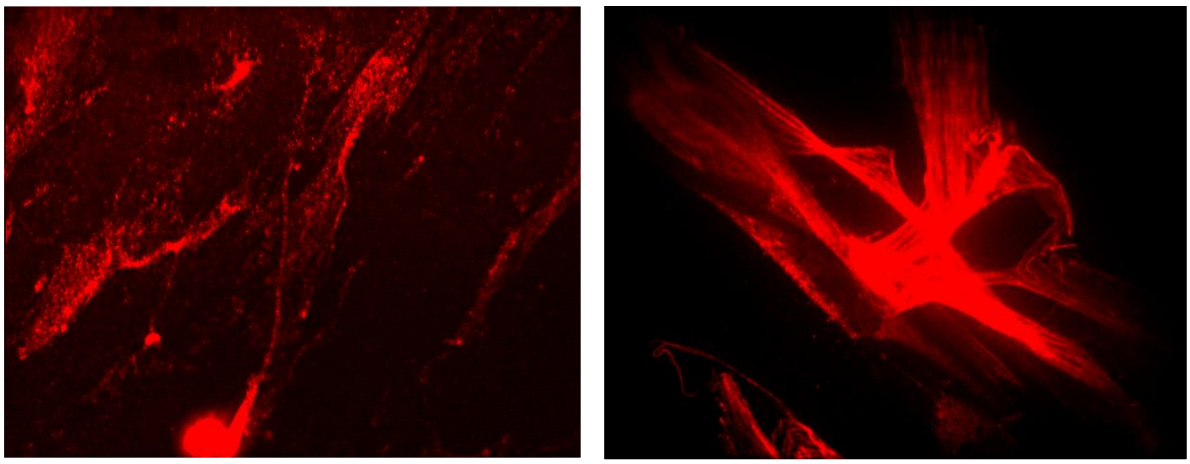


Figure 3.6: α-actin immunofluorescence study of human venous VSM cells in culture (Flex I) after 48 hours of mechanical strain

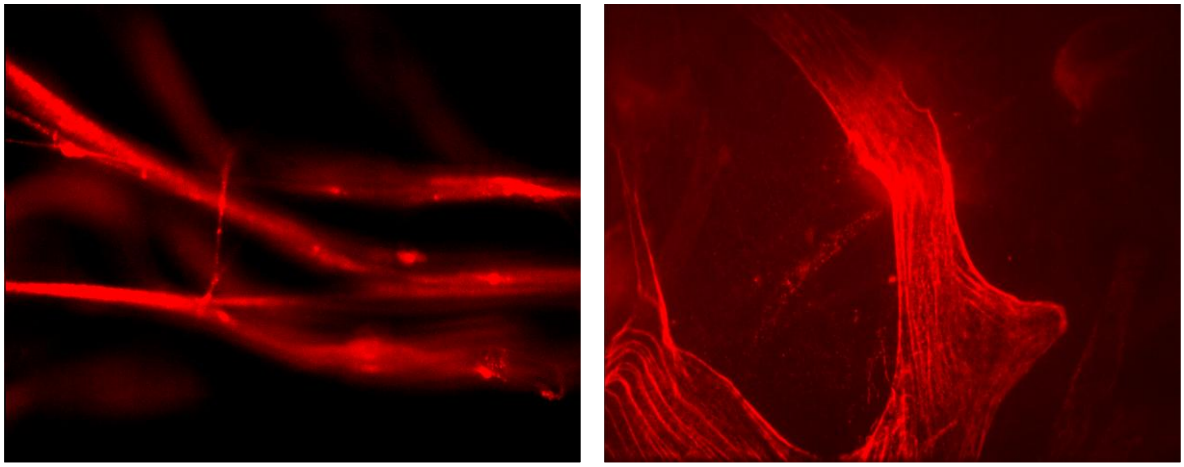
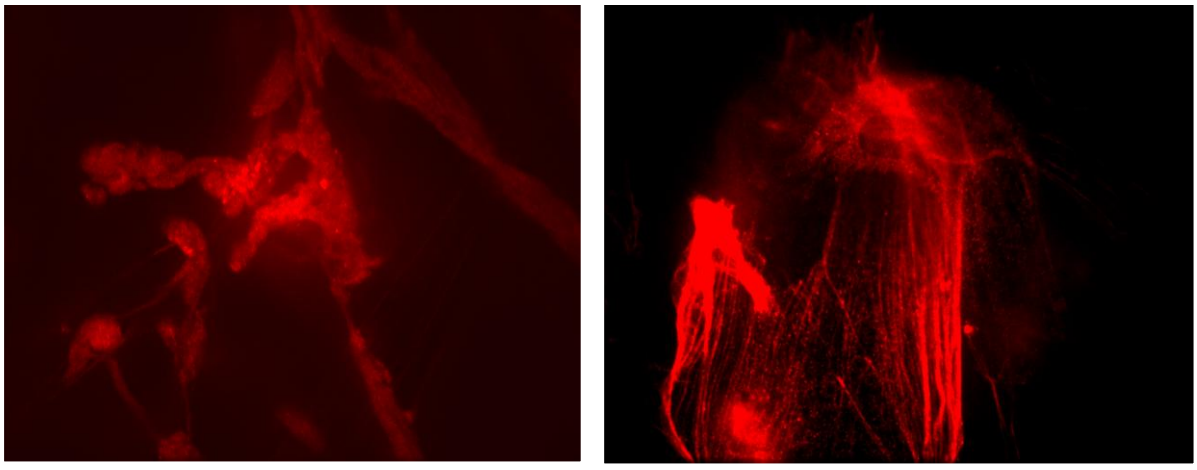


Figure 3.7: Smooth muscle myosin HC SM1 and SM2 immunofluorescence study of human venous VSM cells in culture (Flex I) after 48 hours of mechanical strain



3.3.2 Cell Viability

FX-2000 system (figure 3.8): Mechanical strain did not lead to an increase in human VSM cell number (Stretch - mean cells/well: 133333 +/- 10541 v. Static - mean cells/well: 135000 +/- 10878; n=6, p=0.91) and similarly there was no evidence of an increase in cell loss (Cell staining with trypan blue: both Stretch and Static - mean cells/well: 5000 +/- 2236; n=6, p>0.99).

FX-3000 system (figure 3.9): As above, mechanical strain did not lead to an increase in human VSM cell number (Stretch - mean cells/well: 310000 +/- 25430 v. Static - mean cells/well: 298333 +/- 17591; n=6, p=0.78), nor was there evidence of any increase in cell loss (Cell staining with trypan blue: Stretch - mean cells/well: 8333 +/- 3070 cf. Static - mean cells/well: 6667 +/- 4944; n=6, p=0.79).

Figure 3.8: Cell count after 48 hours mechanical strain generated by FX-2000

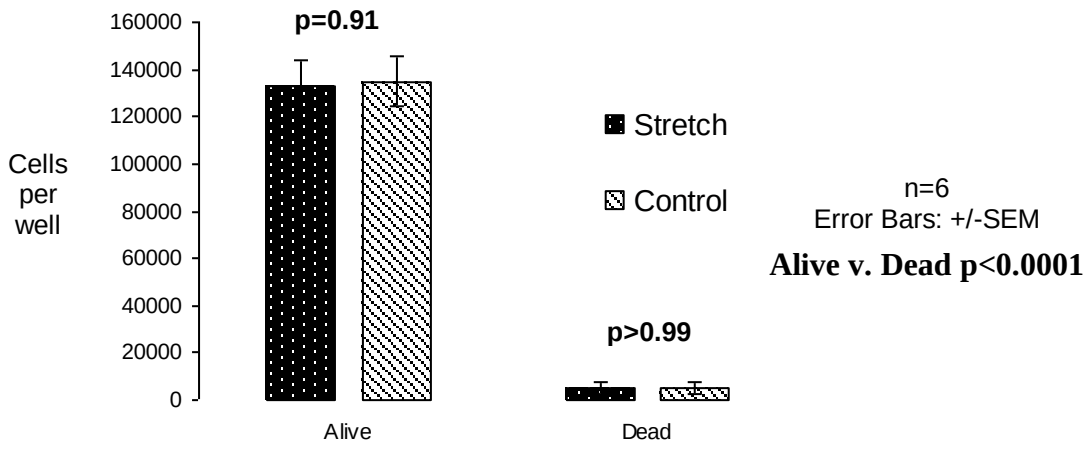


Figure 3.9: Cell count after 68 hours mechanical strain generated by FX-3000

3.4 Discussion

Reassuringly, we can be confident that our experimental cultured cells are a true population of human VSM cells with little or no evidence of endothelial cell or fibroblast contamination. For all definitive experiments, cells younger than the 5^{th} passage were used and very early passaged cells (split 2) were used in those studies seeking to characterise the expression of AT_1 receptors. It is recognised that cells passaged over multiple occasions (greater than 10), are likely to undergo significant phenotypic change to a cell type similar to fibroblasts. In addition, cultured human VSM cells lose AT_1 and AT_2 receptors in primary culture and early successive passages.

Although human VSM cells cultured on plastic and glass are microscopically similar to those cells cultured on a Type I collagen matrix, immunocytochemistry demonstrated a fundamental difference. Historical studies of human VSM cells have documented the existence of at least two phenotypic populations. Contractile (or quiescent) human VSM cells exist in the normal physiological environment of a blood vessel and have abundant evidence of both α-actin and smooth muscle myosin structural proteins. In contrast, synthetic (or proliferative) human VSM cells are more likely to be located close to areas of atherosclerosis and potentially play a pivotal role in plaque development. These cells have less contractile proteins and can be differentiated experimentally from contractile human VSM cells by their lack of smooth muscle myosin.

The lack of smooth muscle myosin immunofluorescence for cells cultured on plastic or glass cover slips was surprising. One potential confounder could be laboratory technique; the negative finding is just more difficult to prove. However, the immunofluorescence studies of cells attached to glass were conducted at the same time

as positive staining for smooth muscle myosin was observed on cells attached to the collagen type I substrate (Flex I). This raises the possibility that traditionally cultured human VSM cells on plastic or glass may be predominantly synthetic. Consequently, this suggests that *in vitro* studies examining the biological activity of cultured cells may be influenced by the substrate to which they are attached. In contrast, human VSM cells on Flex I stretch plates have a preserved contractile phenotype. However, the influence of mechanical strain results in an increase for both contractile microfilaments. Rather than mechanical strain leading to a more proliferative synthetic phenotype, these observations highlight the development of a more pronounced contractile phenotype. This might therefore represent another phenotypic isoform – namely a hypertensive phenotype. The purpose of this thesis is to examine this phenotype in more detail, particularly the response to Angiotensin II and the expression of AT_1 receptors.

The cell viability studies confirm Dr. Kemp's work using the FX-2000 strain unit. Incubating cells in 5% FBS under the influence of mechanical strain did not result in an increase in cell number and importantly did not lead to higher cell attrition. Although it was not possible to account for cells that became detached from the flexible membrane, the similarity in live and dead cell numbers between stretch wells and their static controls is convincing. The use of FX-3000 strain unit, with use of the loading posts, provided a comparison between cells exposed to its equibiaxial strain and the uni-directional strain of the FX-2000 system. Similarly, the results revealed no difference in cell number or viability. It is therefore likely that Dr. Kemp's findings could be translated to cells exposed to equibiaxial strain.

In conclusion, these studies have determined that cultured human VSM cells were the predominant cell type under investigation and not adversely influenced by mechanical strain. This therefore provided a reassuringly robust workable *in vitro* model for further study. In addition, there is now clear proof from immunofluorescence studies that human VSM cells, under the influence of mechanical strain, do not switch phenotype to a synthetic proliferative cell type, but adapt with evidence of increased contractile proteins to an alternative hypertensive phenotype. It is the latter that form the basis of the thesis hypothesis.

Chapter 4

Angiotensin II treatment of Cultured

Human Vascular Smooth Muscle Cells

4.1 Introduction

Ang II is a potent vasoconstrictor and is vital in a normal human physiological response to low BP of any cause. Other properties of this octapeptide include its action on the kidney tubules to promote sodium and water retention and its stimulation of the adrenal cortex to enhance aldosterone synthesis. There is also overwhelming laboratory evidence of its pro-fibrotic nature. Arguably, it is a promoter of cardiovascular disease in susceptible individuals and therapies that inhibit its actions have proven clinical benefits in these subjects.

This chapter examines the effect of Ang II on extra-cellular matrix protein production in human VSM cells. There is prior published evidence of matrix protein gene expression and protein synthesis in predominantly animal and some human VSM cells as well as other cell types in tissues that develop fibrosis in disease or in response to noxious stimuli (e.g. cardiomyocytes and mesangial cells).

As described above in chapter 1, type I(αI) collagen and type III(αI) collagen are the matrix proteins that are predominantly synthesised in fibrosis. In addition other matrix proteins such as fibronectin are produced. It was important to determine whether our cultured human VSM cells would be influenced by Ang II before embarking on the mechanical strain experimentation. Therefore to gauge any response to Ang II, we undertook a series of experiments to examine type I(αI) collagen and fibronectin gene expression and fibronectin protein synthesis.

4.2 Experimental Design

Human venous VSM cells (approximately 5 x 10^5 cells/well) in passages (3-4) were cultured on 35 mm 6-well plastic culture plates. When sub-confluent to 80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours. Cells were treated with Ang II (10^6 M) in 1% FBS. 1% FBS was used as higher concentrations would cloud the effect of Ang II and interfere with the fibronectin ELISA. After incubation for 6 hours, cells were harvested and total RNA was isolated according to protocol (Chapter 2.4). Gene expression for type I(αI) collagen and fibronectin was analysed by northern blotting and standardised against the house-keeping gene GAPDH. After 12 hours, supernatant was collected, centrifuged at 1000g for 5 minutes at 4^{o}C to remove cellular debris and then stored at -20^{o}C until analysed for fibronectin protein synthesis by ELISA (Chapter 2.5.1).

For those experiments examining the effect of Ang II inhibition by use of an AT_1 receptor blocker, cells were pre-incubated with Losartan (10^5 M) approximately 30-60 minutes prior to the addition of Ang II to allow receptors to be appropriately inhibited.

All experiments were conducted in triplicate and controls were treated identically except for the addition of the relevant peptides or drugs. Drug and peptide concentrations and time courses were chosen on the basis of preliminary experimentation. Concentrations are obviously greater than *in vivo* physiological values and this reflects the down-regulation of Ang receptors in cultured human VSM cells.

4.3 Results

Ang II induced type I(αI) collagen and fibronectin gene expression in human venous VSM cells at 6 hours (191+/-93% and 103+/-19% increase over control; n=7) standardised to GAPDH (figure 4.1). This effect on matrix gene expression was partially inhibited by co-incubation with an AT_1 receptor blocker (Losartan) by limiting the increase to 53+/-35% and 10+/-54% for type I(αI) collagen and fibronectin respectively (n=4; ANOVA p=0.017 and p=0.051 respectively).

Ang II induced fibronectin protein synthesis by human venous VSM cells at 12 hours (10+/-2.5% increase over control; n=3 (figure 4.2)). This effect on matrix protein synthesis was reduced by co-incubation with Losartan by 23+/-9.5 % (n=3; ANOVA p= 0.028).

Blockade of the AT_2 receptor did not influence the activity of Ang II.

Figure 4.1: Collagen type I and Fibronectin mRNA expression in treated human venous VSM cells at 6 hours

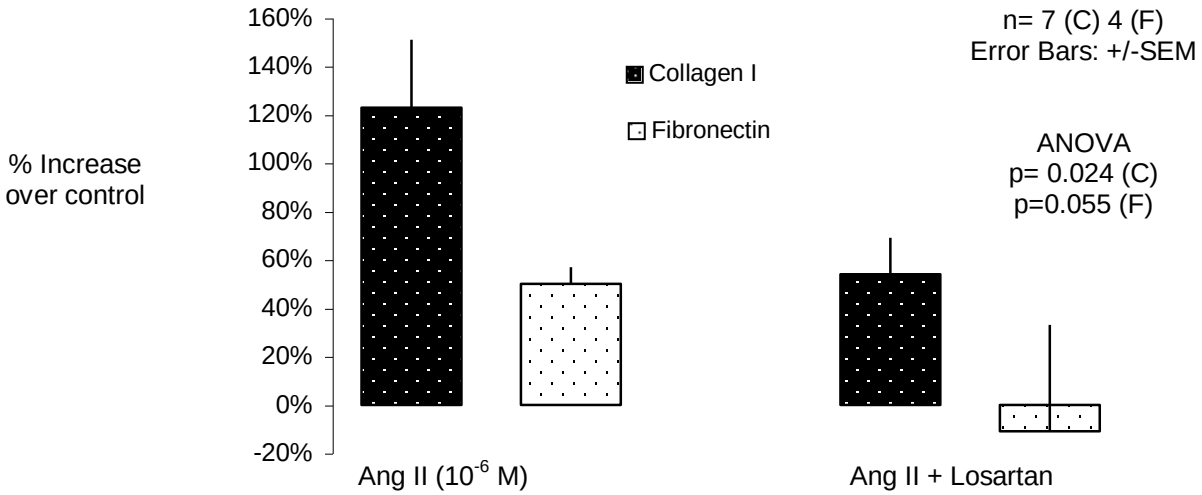


Figure 4.2: Fibronectin protein synthesis in treated human venous VSM cells at 12 hours

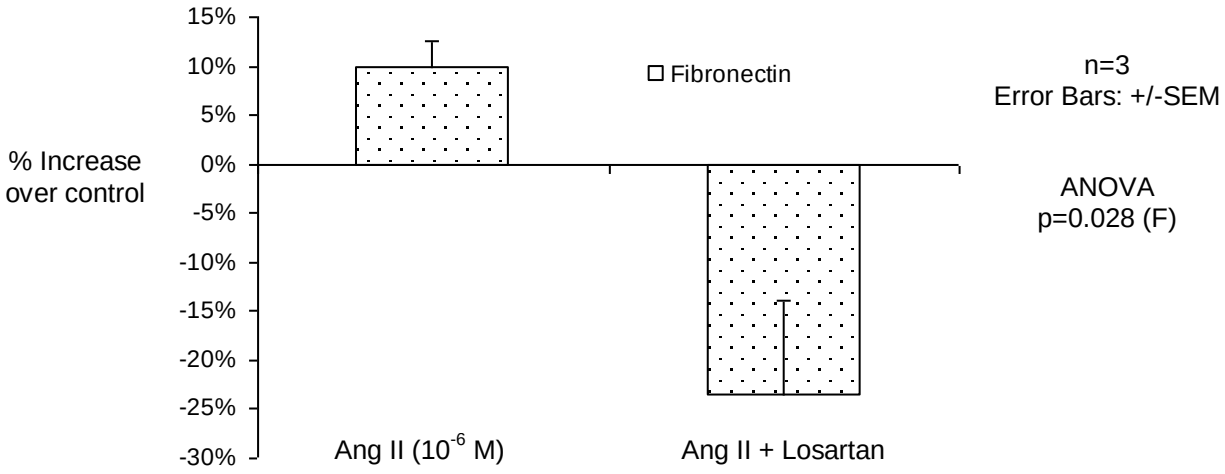


Table 4.1 Blot signal raw data for collagen type I and fibronectin mRNA expression in treated human venous VSM cells at 6 hours (standardised to GAPDH*)

Expt.	Untreated Control (CI)	Untreated Control (F)	Ang II		Ang II + Losartan	
			Collagen I	Fibronectin	Collagen I	Fibronectin
1	0.46	0.61	0.70	1.02	0.90	1.18
2	0.57	0.47	1.04	1.14	0.64	0.34
3	0.44	0.80	0.53	1.00	0.31	0.02
4	0.56	0.71	0.80	0.80	1.07	0.75
5	0.54	-	0.88	-	0.70	-
6	0.34	-	2.84	-	0.47	-
7	0.52	-	0.84	-	1.04	-
Mean	**0.49**	**0.65**	**1.09**	**0.99**	**0.73**	**0.57**
SEM	0.08	0.07	0.30	0.07	0.11	0.25

Table 4.2 Absolute values for fibronectin (ng) protein synthesis in treated human venous VSM cells at 12 hours

Expt.	Untreated Control	Ang II	Ang II + Los
1	744.00	794.00	605.00
2	1890.00	2177.00	1096.00
3	983.40	1057.20	887.30
Mean	**1205.80**	**1342.73**	**862.77**
SEM	349.01	424.00	142.27

*GAPDH mRNA expression did not appear to change significantly under any conditions tested in this thesis

4.4 Discussion

These series of experiments demonstrate that cultured human VSM cells increase matrix protein gene expression and synthesis after stimulation with Ang II and this effect is inhibited by an AT_1 receptor blocker. These studies confirm earlier work in other species as well as human cells. Studies by Dr. C. O'Callaghan (from our laboratory) revealed collagen synthesis in a similar population of cultured human VSM cells by a ^{3}H-proline incorporation method, thus underlining the validity of these results.

These series of experiments were conducted on cells plated on plastic culture wells. We have shown in Chapter 3 that cells plated on plastic may undergo a phenotypic shift to the "synthetic" variant. This phenotype is more likely to be present in vessels close to atherosclerosis and has a greater fibrogenic potential than normal 'contractile' human VSM cells. Consequently, plastic wells provide the best *in vitro* environment to reveal the effects of Ang II on human VSM cells if any exist. No formal studies examined this outcome using ECM substrates such as collagen type I; however later studies (often static controls) suggested that Ang II was similarly biologically active via the AT_1 receptor in cells incubated on a collagen type I substrate.

It was important to determine that Ang II mediated its biological activity via the AT_1 receptor in our population of cultured human VSM cells. This provided a secure basis for later studies involving cultured human VSM cells, particularly as this work progressed to examining the effect of mechanical forces.

Chapter 5

Cultured Human Vascular Smooth Muscle Cells under the influence of Mechanical Strain

5.1 Introduction

The effects of mechanical forces, whether it is shear stress or cyclical pressure, have rarely been examined in an *in vitro* cell culture system. However, it is arguably remiss of investigators to ignore such an important modality in the study of human VSM cells as these and other vascular cells are, forever and a day, exposed to cyclical strain from an early embryonic stage.

The background to the use of mechanical strain and its application has been discussed in section 2.2.2. This chapter details the initial mechanical transduction experimentation. Firstly, a qualitative study of human VSM cell survival on a variety of ECM substrates (collagen type I / elastin / pronectin / laminin) was investigated by determining both the proliferation of cells co-incubated with growth media and the response of these cells to cyclical mechanical strain. In a second series of experiments, the effect of cyclical strain on collagen I(αI) gene expression and fibronectin protein synthesis was determined.

5.2 Experimental Design

In the first series of experiments, human VSM cells (approximately 5 x 10^5 cells/well) in passages (3-4) were cultured on Bioflex® culture plates and exposed to growth media (15% FBS). When sub-confluent to 80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours and then exposed to 8.5% / 4.5% cyclical mechanical strain for 24 hours in 5% FBS. Cells were visualised by plain microscopy prior to and following mechanical strain.

In the second series, human VSM cells (approximately 2 x 10^5 cells/well) in passages (3-4) were cultured on Flex I culture plates. When sub-confluent to 80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours and then exposed to 22% / 18% cyclical mechanical strain for 24 hours in 5% FBS. Following mechanical strain, cells were harvested and total RNA was isolated according to protocol (Chapter 2.4.4). Gene expression for collagen I(αI) was analysed by northern blotting and standardised against the house-keeping gene GAPDH. Similarly following mechanical strain, supernatant was collected, centrifuged at 1000g for 5 minutes at 4^{o}C to remove cellular debris and then stored at -20^{o}C until analysed for fibronectin protein synthesis by ELISA (Chapter 2.5.1).

For those experiments examining the effect of Ang II inhibition by use of an AT_1 receptor blocker, cells were pre-incubated with Losartan (10^{-5} M) approximately 30-60 minutes prior to the application of mechanical strain to allow receptors to be appropriately inhibited.

All experiments were conducted in triplicate and controls were treated identically except for the addition of the relevant peptides or drugs. Drug and peptide

concentrations, magnitude of strain and time courses were chosen on the basis of preliminary experimentation. Concentrations are obviously greater than *in vivo* physiological values and this reflects the down-regulation of Ang II receptors in cultured human VSM cells.

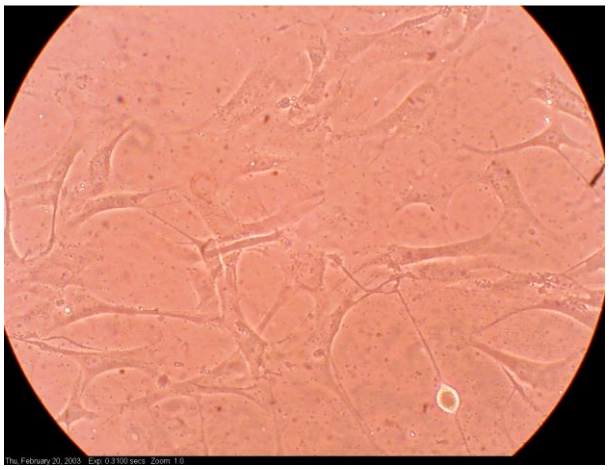
5.3 Results

5.3.1 Qualitative Study

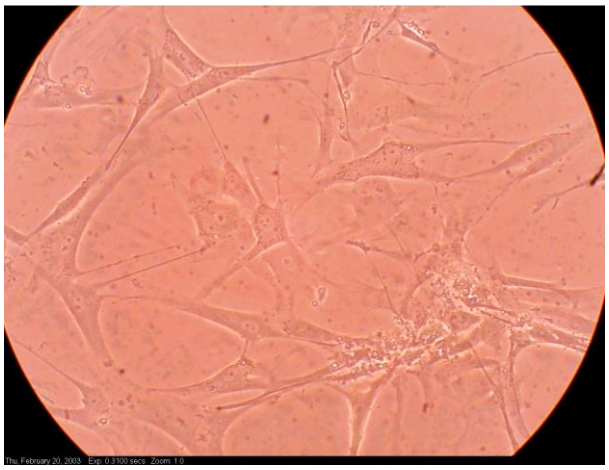
Human venous VSM cells only grow well on plastic, glass and type I collagen. The cells failed to proliferate on laminin, pronectin and elastin (figure 5.1). After the application of mechanical strain, cells on laminin, pronectin and elastin remained sparse thus limiting their usefulness for detailed investigation (figure 5.2). Cells adherent to collagen type I maintain their integrity as visualised by light microscopy permitting continued use of this substrate for all further investigations.

In addition, arterial cells derived from umbilical veins were investigated in a parallel study. Similarly only cells attached to collagen type I remained viable during initial cells growth and following cyclical mechanical strain. As demonstrated in figures 5.3 and 5.4, cells plated on other ECM substrates did not maintain their integrity and failed to remained adherent to the substrate after stretching.

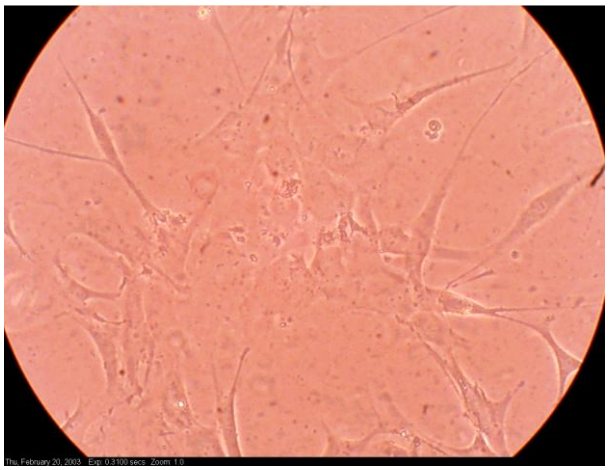
Figure 5.1: Human venous VSM cells incubated in high growth media on various ECM substrates for 5 days



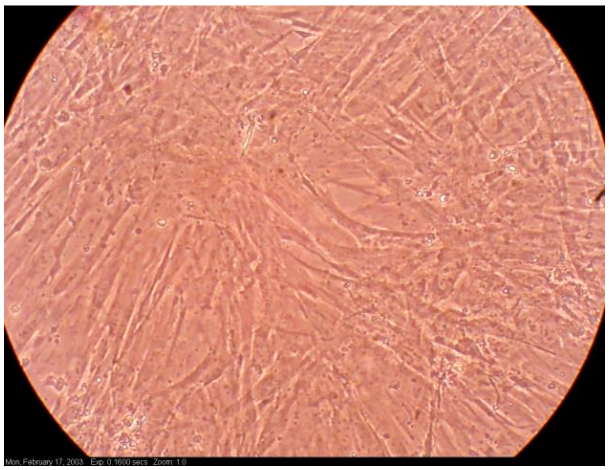
Laminin



Elastin

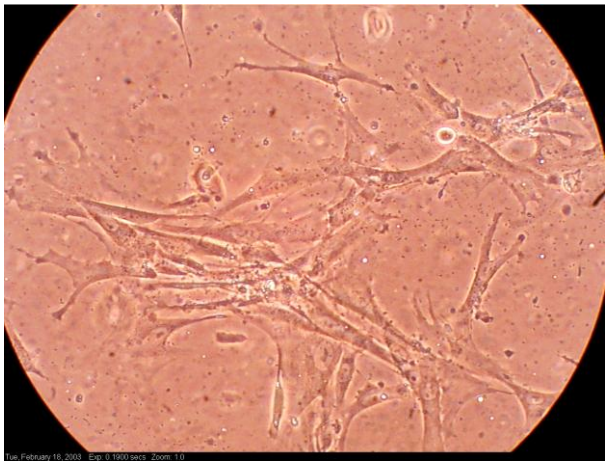


Pronectin

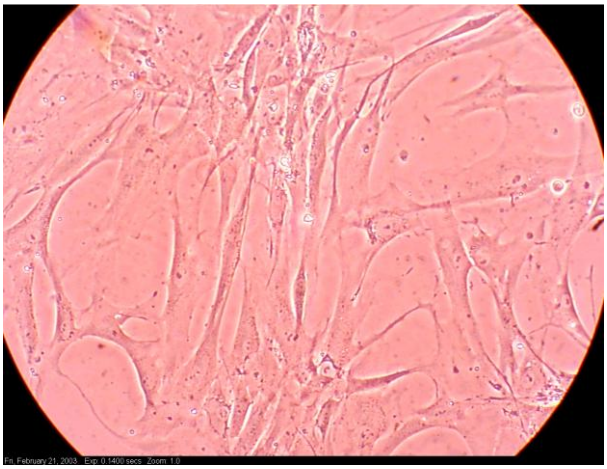


Collagen type I

Figure 5.2: Human venous VSM cells incubated on various ECM substrates then exposed to mechanical strain (8.5% / 3.5% cell elongation) for 24 hours



Laminin



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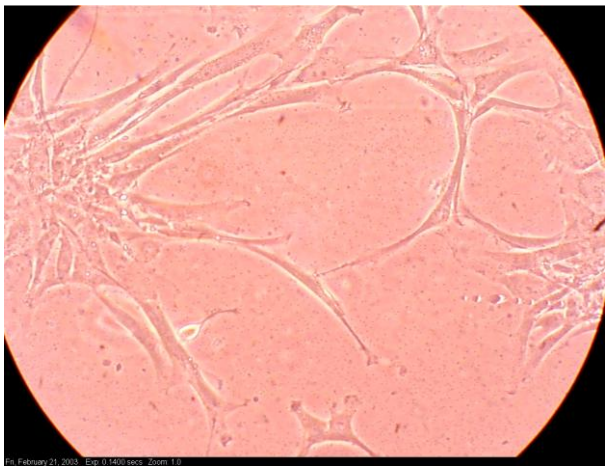
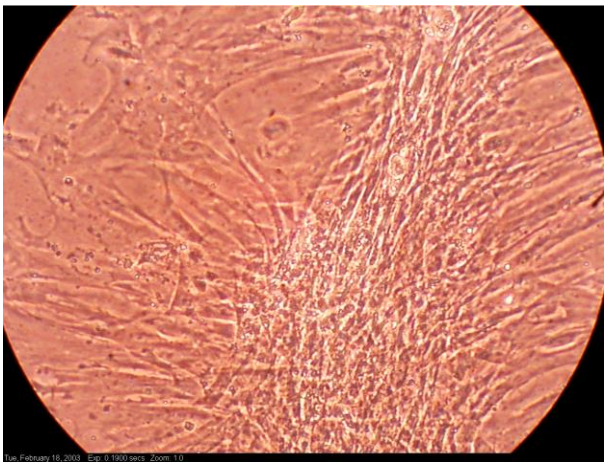
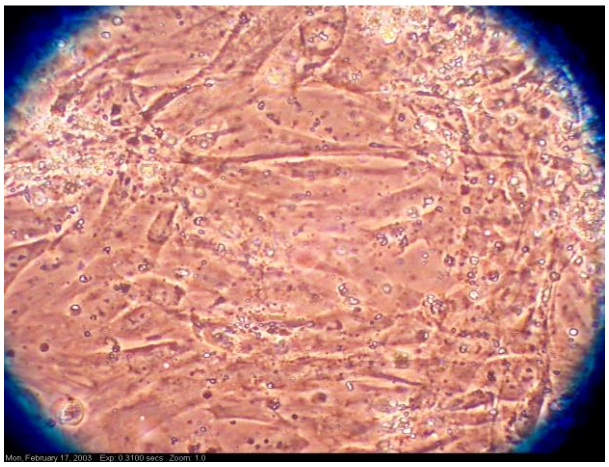


Pronectin

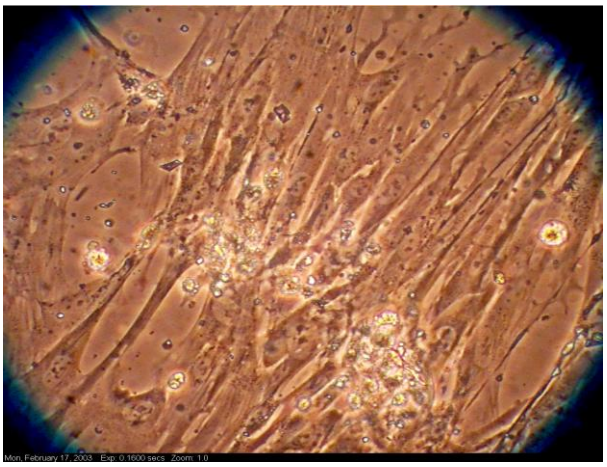


Collagen type I

Figure 5.3: Human arterial VSM cells incubated in high growth media on various ECM substrates for 4 days



Laminin



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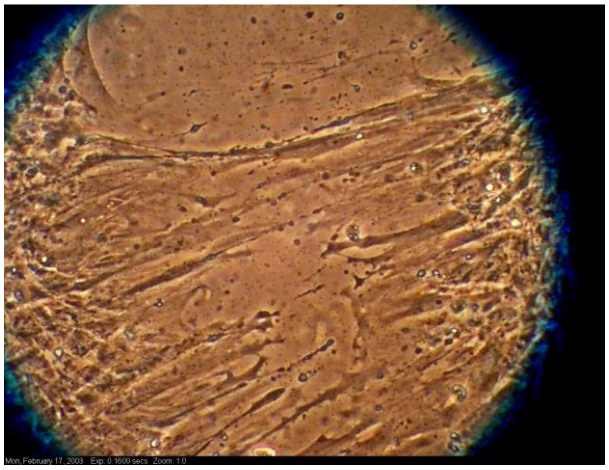
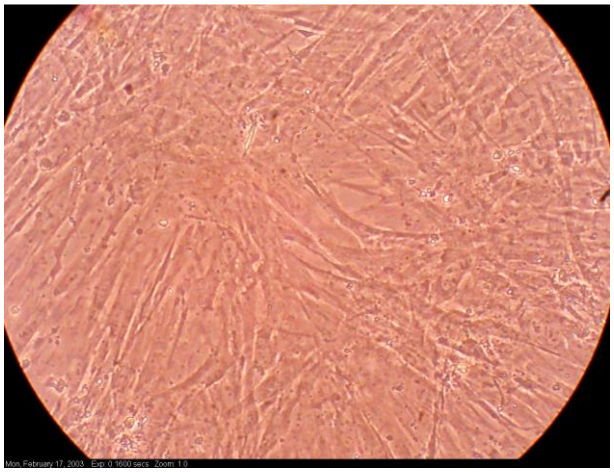
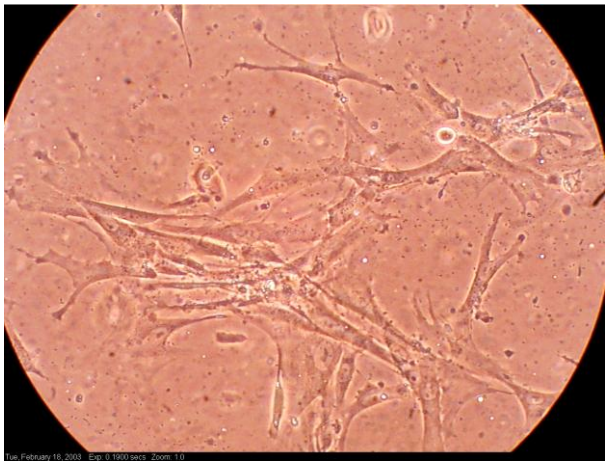


Pronectin

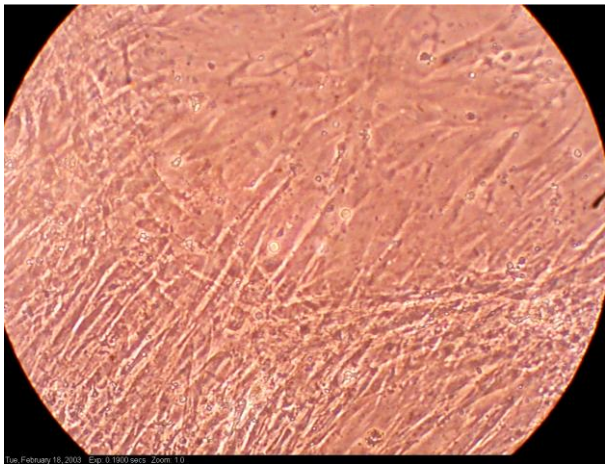


Collagen type I

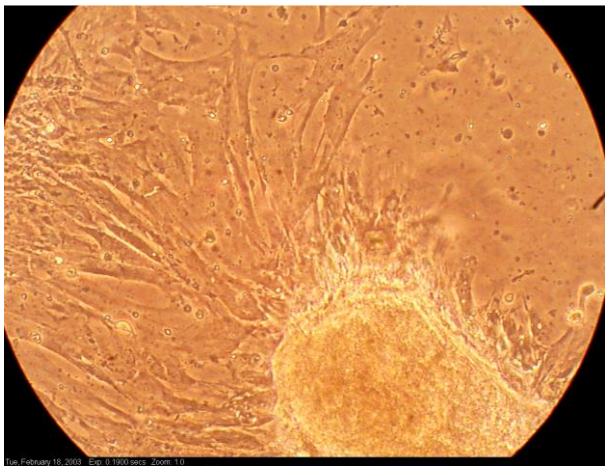
Figure 5.4: Human arterial VSM cells incubated on various ECM substrates then exposed to mechanical strain (9% / 9% cell elongation) for 16 hours



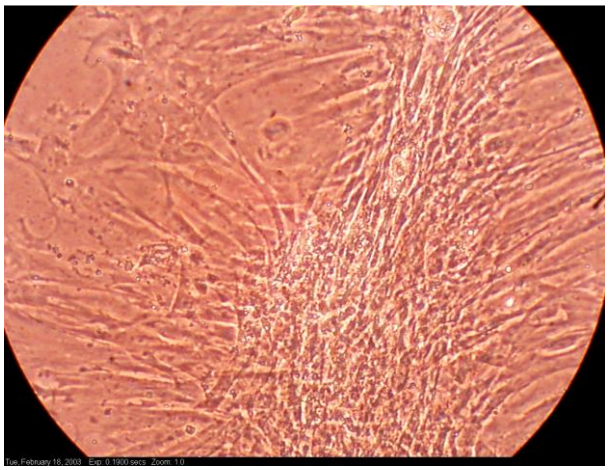
Laminin



Elastin



Pronectin



Collagen type I

5.3.2 ECM Synthesis

Cyclical mechanical strain induced collagen I(αI) gene expression in human venous VSM cells at 12 hours (100+/-39% increase over control; n=4) standardised to GAPDH (figure 5.5). Intriguingly, this effect on matrix gene expression was significantly inhibited by co-incubation with an AT_1 receptor blocker (Losartan) by limiting the increase in collagen I(αI) to 37+/-13% (n=4; AVOVA p=0.0046).

Cyclical mechanical strain induced fibronectin protein synthesis by human venous VSM cells at 24 hours (28.5+/-12% increase over control; n= 4; figure 5.6). Similarly, this effect on matrix protein synthesis was again significantly inhibited by co-incubation with Losartan by reducing the fibronectin synthesis by 14+/-4% (n=4; AVOVA p=0.0046).

Figure 5.5: Collagen type I mRNA expression in treated human venous VSM cells at 12 hours

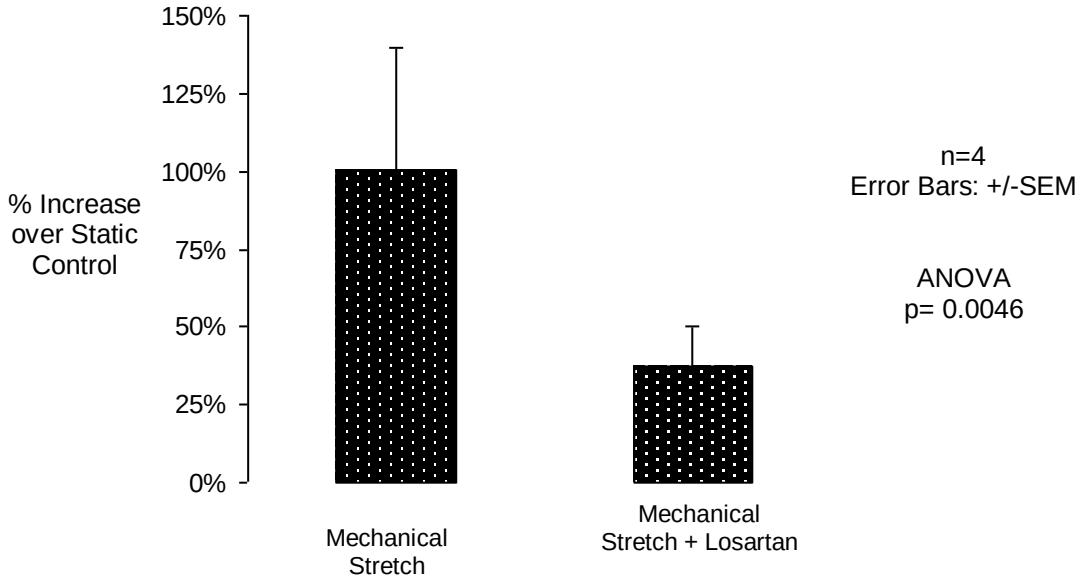


Figure 5.6: Fibronectin protein synthesis in treated human venous VSM cells at12 hours

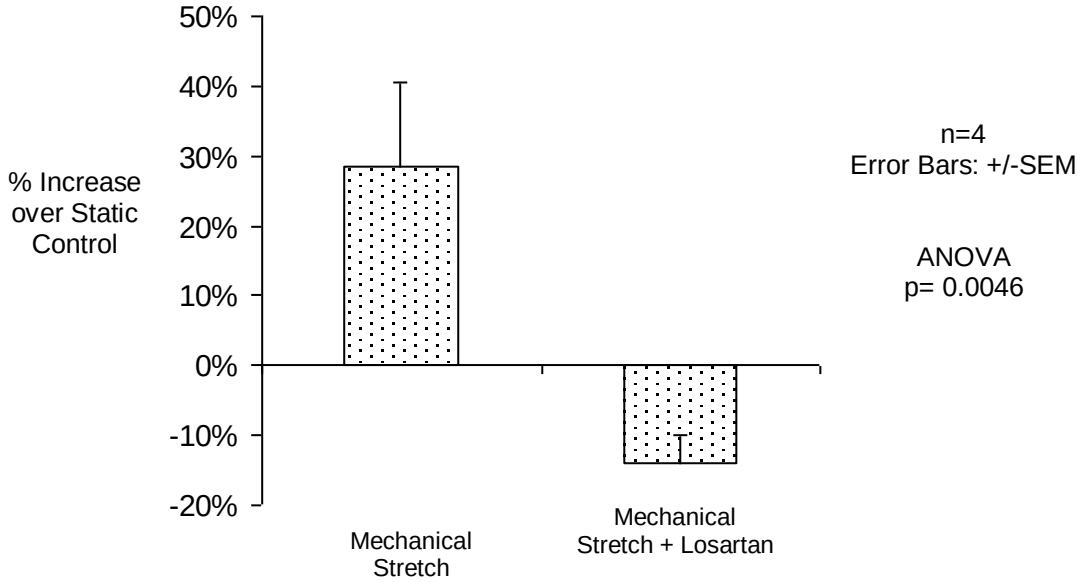


Table 5.1 Blot signal raw data for collagen type I mRNA expression in treated human venous VSM cells at 12 hours (standardised to GAPDH)

Expt.	Static	Stretch	Stretch + Los
1	0.276	0.393	0.37
2	0.276	0.355	0.34
3	0.37	1.1	0.64
4	0.53	1.218	0.616
Mean	**0.36**	**0.77**	**0.49**
SEM	0.06	0.23	0.08

Table 5.2 Absolute values for fibronectin (ng) protein synthesis in treated human venous VSM cells at 24 hours

Expt.	Static	Stretch	Stretch + Los
1	1475	1528	1415
2	919	1467	807
3	3106	4030	2299
4	348	418	311
Mean	**1462**	**1861**	**1208**
SEM	594	767	428

5.4 Discussion

The only consistent substrate that permitted our population of human venous VSM cells to proliferate was either plastic or collagen type I. Human VSM cells were rarely cultured on glass successfully. The literature supports the successful culture of VSM cells on type I collagen, fibronectin and elastin during the application of mechanical strain. Similarly, we can report that our population of venous VSM cells kept their integrity after the application of strain, but only those cells cultured on collagen type I were suitable for further investigation as cells remained sparsely populated on the alternative substrates. Although the evidence provided is based on light microscopy, it was unnecessary to proceed with more detailed immunofluorescence studies to discern this.

Adult arterial human VSM cells were not available for these studies. However, part of the interest in this work is to examine any environmental (ECM) influence on VSM cell phenotype and its function. Umbilical arterial VSM cells were therefore acquired. Unfortunately, these cells did not proliferate on the substrates except plastic and collagen type I. More detailed studies are outside the remit of this thesis, but it is hypothesised that the complex cell-matrix interactions are less well developed in these very immature cells. Consequently our studies investigating the effects of mechanical strain were conducted using human VSM cells from saphenous vein harvest on a collagen type I substrate.

Mechanical strain induced an increase in gene expression and protein synthesis of collagen type I and fibronectin respectively. This is well recognised, however intriguingly, the use of an AT_1 receptor antagonist attenuated this effect. Importantly no exogenous Ang II was added to the culture media. Previous studies in animals have

suggested that mechanical strain may increase ECM protein production via an interaction with the RAS, but uniquely, these studies have shown that AT_1 antagonism attenuates the effects of strain on human VSM cells.

Several theories for this exist, which is discussed in detail in section 1.5.7. Briefly, Sadoshima (Sadoshima et al., 1992 and 1993) proposed that strain-induced release of Ang II from granules within rat neonatal cardiomyocytes with Kojima (Komija et al., 1994) providing further evidence in the same cell culture model by demonstrating attenuation of strain-induced cell signalling with AT_1 receptor antagonism. But significantly, this later study and others failed to identify an increase in Ang II in strain-conditioned media. Finally, Kijima (Kimija et al.,1996) proposes not only cellular activation of the RAS, but also an increase in AT_1 receptors (but not receptor affinity) in neonatal cells.

Although no exogenous Ang II was added to the culture media of stretched cells, this hormone is present in FBS and even in very low concentrations, it might conceivably exhibit a physiological response without the need for cellular Ang II synthesis. We speculate that the fibrogenic response to mechanical strain is enhanced by up regulation of the AT_1 receptor, which is responsive to small quantities of Ang II in the culture media.

Chapter 6

Analysis of a Vascular Smooth Muscle Cell intracellular signalling protein (Extra-cellular Signal-Regulated Kinase 1/2) in response to Angiotensin II and Mechanical Strain

6.1 Introduction

The Extracellular Signal-Regulated Kinases (ERK) 1/2 (previously known as Mitogen Activated Protein (MAP) Kinases p42 / p44) are secondary messenger proteins modulating the response of cells to stimuli such as Ang II and mechanical strain. Activation via this pathway leads to downstream up regulation of other MAP Kinases resulting in both growth of cells and fibrogenesis and thus would be present in VSM cells.

Previous studies in the department by Dr. O'Callaghan had used a MAP Kinase assay to show that mechanical strain induced MAP kinase activity in human VSM cells (personal communication). This is supported by other studies for both strain and humoral factors such as Ang II (Touyz et al, 2001). Therefore, this regulatory pathway provided a useful target to examine the hypothesis and was conducted concurrently with the studies highlighted in the previous results chapters.

Therefore these experiments examined a number of issues. Fundamentally, if ERK 1/2 was inducible in the study population of human VSM cells by both Ang II and mechanical strain, this would allow the use of ERK 1/2 induction as a surrogate marker for human VSM cellular biological activity. Consequently, the assessment of the relative merits of Ang II and mechanical strain in promoting human VSM cellular activity could be assessed. In addition, by adapting the design of experiments, the interaction between mechanical forces and the RAS could be examined. These experiments measured phosphorylated ERK 1/2 as a marker of its activation.

6.2 Experimental Design

6.2.1 Ang II induction of ERK 1/2

Human venous VSM cells (approximately 5 x 10^5 cells/well) in passages (3-4) were cultured on 35mm 6-well plastic culture plates and exposed to growth media (15% FBS). When sub-confluent to 80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours. Cells were treated with Ang II (10^{-6} M) in 1% FBS. A series of time experiments had determined that peak ERK 1/2 activity occurred at 10 minutes for Ang II, therefore after incubation for 10 minutes, culture supernatant was removed and cells were snap-frozen with liquid nitrogen (speed was of essence as ERK 1/2 activity fell with time). Human VSM cell protein was harvested according to protocol and analysed by western blotting (section 2.5.3 to 2.5.9).

For those experiments examining the effect of Ang II inhibition by use of an AT_1 receptor blocker, cells were pre-incubated with Losartan (10^{-5} M) approximately 30 minutes prior to the addition of Ang II to allow receptors to be appropriately inhibited.

6.2.2 Mechanical Strain induction of ERK 1/2

Human venous VSM cells (approximately 5 x 10^5 cells/well) in passages (3-4) were cultured on Bioflex® (type I collagen) culture plates and exposed to growth media (15% FBS). When sub-confluent to 80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours and then exposed to 22% / 18% cyclical mechanical strain for 10 minutes in 1% FBS. It had previously been determined by a series of time experiments that peak ERK 1/2 activity occurred at 7½ to 10 minutes

(Personal communication Dr C. O'Callaghan). Therefore, after incubation for 10 minutes, cells were treated as described above and in Chapter 2.5.

6.2.3 Examining synergy between Ang II and Mechanical Strain

Two separate methods were used to examine this relationship. In the first study, human VSM cells were treated identically and then exposed to both Ang II and strain independently and in combination. In the second study, human VSM cells were first stretched for 24 hours and then immediately exposed to Ang II.

6.2.3a Experiment 1

Human venous VSM cells (approximately 5 x 10^5 cells/well) in passages (3-4) were cultured on Bioflex® culture plates as described above and rendered quiescent by serum-deprivation (1% FBS) for 48 hours. Cells were exposed to either 22% / 18% cyclical mechanical strain, Ang II (10^{-6}M) or both in 1% FBS for 10 minutes. After incubation, human VSM cell protein was harvested as described above.

6.2.3b Experiment 2

Human venous VSM cells were cultured on Bioflex® culture plates as per Experiment 1. Cyclical mechanical strain providing 22% / 18% cell elongation was applied to one cohort of human VSM cells incubated in 5% FBS for 24 hours. (An identical cohort of cells were incubated in 5% FBS at rest). Immediately following the end of the strain,

culture media was removed and cells incubated in 1% FBS for 10 minutes with or without Ang II (10^{-6}M). Human VSM cell protein was harvested as described above.

All experiments were conducted in triplicate and controls were treated identically except for the addition of the relevant peptides, drugs or strain regime.

6.3 Results

6.3.1 Ang II induction of ERK 1/2

Ang II induced ERK 1/2 protein activation in human VSM cells plated on plastic at 10 minutes (111+/-71% increase over control; n=3; figure 6.1). This effect on ERK 1/2 protein activation was partially inhibited by co-incubation with an AT_1 receptor blocker (Losartan) by limiting the increase to 53+/-41% (n=3; ANOVA p=0.53). Although this result was suggestive of Ang II activity via the AT_1 receptor, it has not been conclusively proven due to the wide distribution of results.

6.3.2 Mechanical Strain induction of ERK 1/2

Cyclical mechanical strain induced ERK 1/2 protein activation in human VSM cells at 10 minutes (87+/-43% increase over control; n= 4; Mann Whitney p=0.0286; figure 6.2).

6.3.3 Examining synergy between Ang II and Mechanical Strain

6.3.3a Experiment 1

Independently, Ang II did not influence (-7+/-8% increase over control; n=3), but cyclical mechanical strain (70+/-56% increase over control; n=3) induced ERK 1/2 protein activation in human VSM cells plated on type I collagen at 10 minutes (Figure 6.3). Synergistically, Ang II and mechanical strain induced an 84+/- 65% increase over control (n=3). The positive control related to cells co-incubated in 15% FBS.

6.3.3b Experiment 2

After the human VSM cells had been ‘primed’ by 24 hours of mechanical strain, there was a baseline increase in response to control 1% FBS (69+/-30% increase over static; n=3). When exposed to Ang II for 10 minutes, stretched cells augmented this response compared to unstretched cells (43+/-10% increase over static; n= 3; figure 6.4).

Figure 6.1: ERK 1/2 activation in treated human venous VSM cells plated on plastic at 10 minutes. Results represent % increase over control standardised to total protein content.

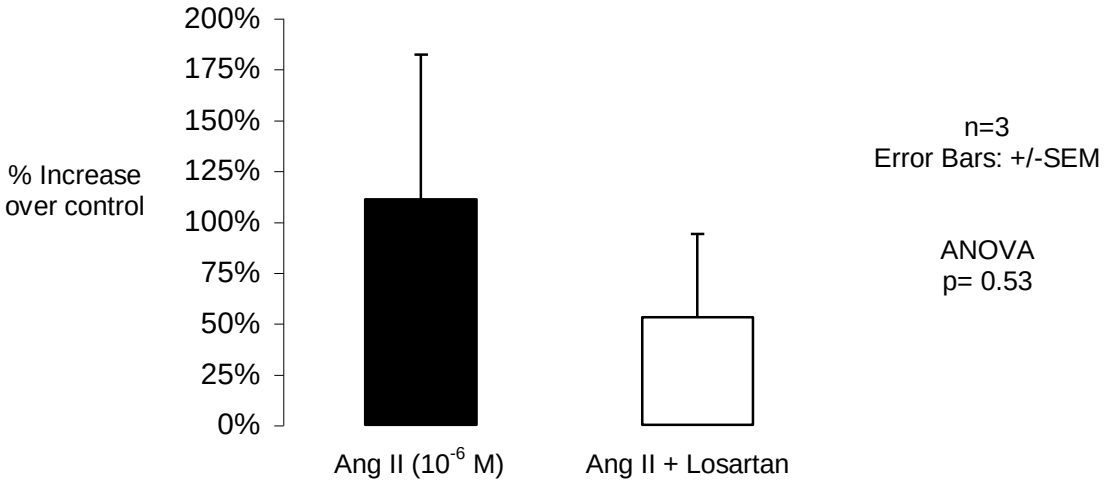


Figure 6.2: ERK 1/2 activation in human venous VSM cells after 10 minutes of mechanical strain. Results represent % increase over control standardised to total protein content.

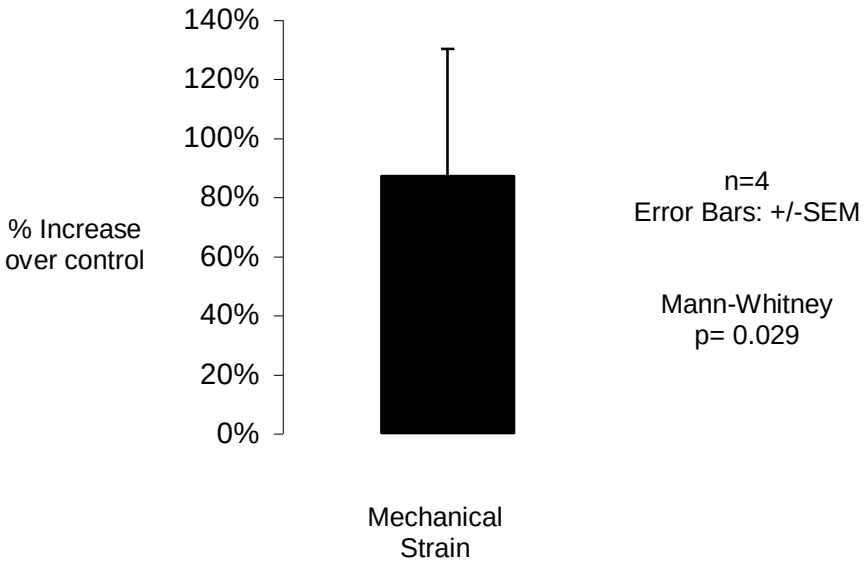


Figure 6.3: ERK 1/2 activation in human venous VSM cells after 10 minutes of Ang II, mechanical strain or both. Results represent % increase over control standardised to total protein content.

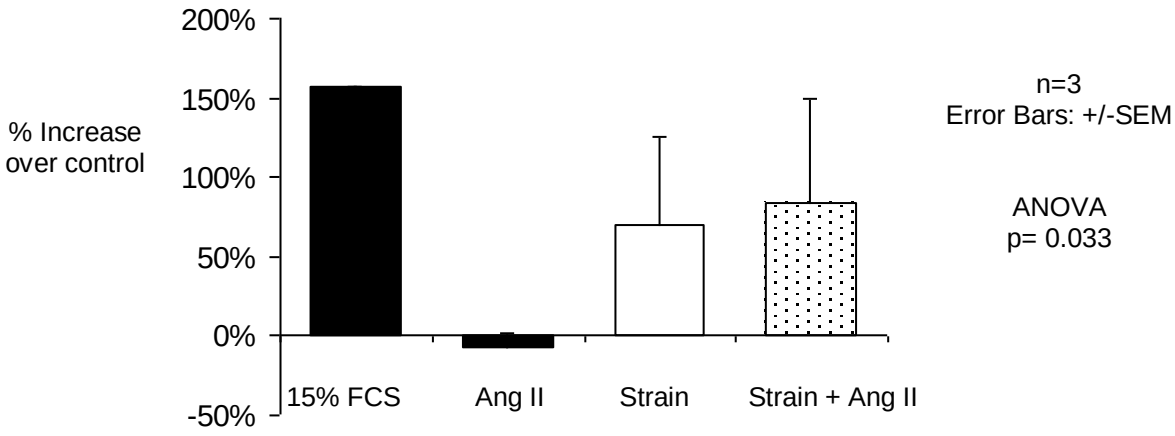


Figure 6.4: ERK 1/2 activation after 10 minutes of Ang II stimulation a cohort of pre-stretched human venous VSM cells and its unstretched control. Results represent raw data standardised to total protein content. The western blot for this experiment reveals each individual signal with positive controls (15% FCS) at the extreme.

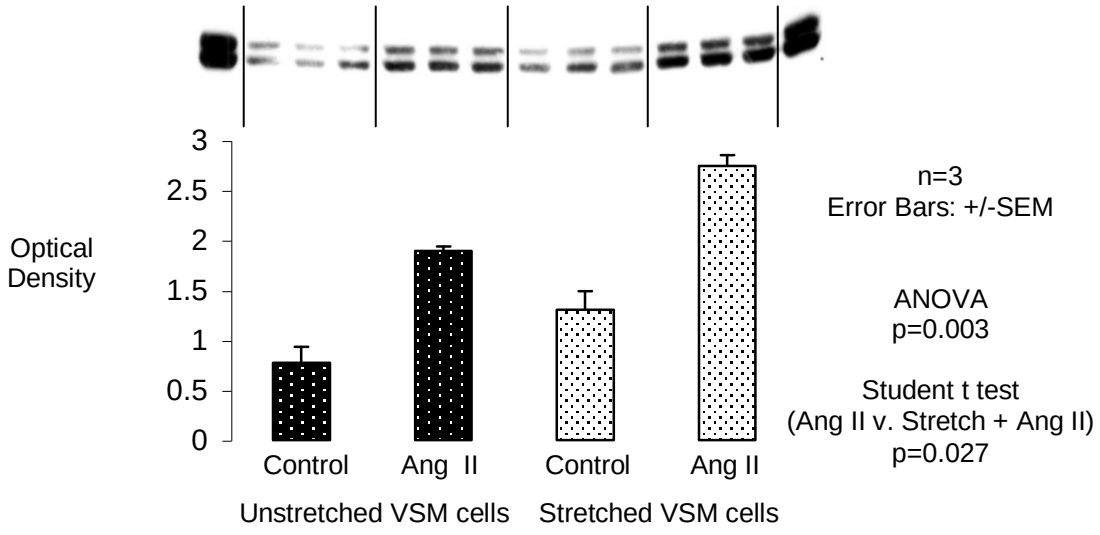


Table 6.1 Raw Optical Density values for ERK 1/2 protein activation in treated human venous VSM cells at 10 minutes standardised to total protein content

Expt.	Control	Ang II	Ang II + Los
1	0.719	1.711	1.525
2	2.81	2.172	2.105
3	0.42	1.34	0.72
Mean	**1.32**	**1.74**	**1.45**
SEM	0.75	0.24	0.40

Table 6.2 Raw Optical Density values for ERK 1/2 protein activation in human venous VSM cells after 10 minutes of mechanical strain standardised to total protein content

Expt.	Static	Stretch
1	1.066	3.37
2	2.687	3.294
3	2.493	3.932
4	2.169	3.315
Mean	**2.10**	**3.48**
SEM	0.36	0.15

Table 6.3 Raw Optical Density values for ERK 1/2 protein activation in human venous VSM cells after 10 minutes of stimuli standardised to total protein content

Expt.	Static	Ang II	Stretch	Stretch + Ang II
1	1.066	1.190	3.294	3.700
2	2.687	2.240	3.932	3.982
3	2.493	2.390	3.370	3.835
Mean	**2.08**	**1.94**	**3.53**	**3.84**
SEM	0.51	0.65	0.35	0.14

Table 6.4 Raw Optical Density values for ERK 1/2 protein activation in human venous VSM cells after 10 minutes of Ang II standardised to total protein content

Expt.	Unstretched Cells		Stretched Cells	
	Control	Ang II	Control	Ang II
1	0.848	1.996	1.542	2.825
2	0.467	1.841	0.938	2.527
3	1.005	1.834	1.43	2.877
Mean	**0.77**	**1.89**	**1.30**	**2.70**
SEM	0.16	0.05	0.19	0.11

6.4 Discussion

These experiments have shown that both mechanical strain and Ang II induces ERK 1/2 in our population of human VSM cells. This secondary messenger has been previously well characterised and acts to translate neuro-humoral stimuli into fibrogenic cellular activity. Thus this provides further evidence that our cell population is a true cohort of VSM cells and our culture model for the application of mechanical strain is robust.

In the comparator experiments, mechanical strain promoted greater ERK 1/2 activation than Ang II. In addition, the combination of strain and Ang II was not significantly greater than strain alone. Strain acts via integrin receptors (section 1.5.2) on human VSM cells to activate ERK 1/2. It is not known whether these receptors suffer the same attrition that Ang II receptors suffer in serial cultures, but one explanation for this observation may be the relative abundance of integrin receptors over Angiotensin receptors. The effect of strain may therefore account for near maximal ERK 1/2 activation. It is unlikely in such a short time-period (10 minutes) that integrin receptor activation would modulate a negative effect on AT_1 receptor function.

Importantly, the defining experiment demonstrated significantly increased activation of human VSM cell ERK 1/2 to Ang II in cells primed by 24 hours of mechanical strain. In these experiments, culture media was removed just following the application of strain and fresh 1% FBS containing Ang II was added. This effectively excludes the possibility that stretched human VSM cells synthesise Ang II de novo to explain these results. If the observation is purely a result of Ang II de novo synthesis, then static and stretched cells would have an identical response to Ang II. Other explanations could be either up-regulation of the MAP kinase pathway or the AT_1 receptor function; the latter forms our hypothesis and is examined in greater detail in the following chapter. The

observation of greater non-Ang II stimulated ERK 1/2 activation is likely to reflect a lag effect of the mechanical strain (cells were harvested just 15 minutes after the cessation of mechanical strain). Although the peak time for strain-induced ERK 1/2 activation was identified as 7½ to 10 minutes, ERK 1/2 never fell to baseline in timeline experiments up to 2 hours. Nevertheless, the magnitude of the response of stretched cells to Ang II compared to static cells is so much greater that this excludes the possibility that the same lag effect is responsible.

Chapter 7

Evidence for the Angiotensin II (type 1) receptor in cultured human vascular smooth muscle cells under the influence of mechanical strain

7.1 Introduction

From the experimentation detailed in the chapter above, this thesis has revealed evidence of a synergistic relationship between mechanical forces and the renin-angiotensin-system in human vascular smooth muscle cells. There are key observations that point to AT_1 receptor up-regulation and therefore this chapter will focus on the presence of the AT_1 receptor in stretched cells.

The receptor is a G-protein coupled seven-domain protein that activates a number of secondary messenger pathways. Ang II binding promotes receptor down-regulation and receptor-ligand complexes are reversibly internalised into lysozomes following agonist binding.

The evidence for changes to the quantity of AT_1 receptor in a *hypertensive* VSM cell phenotype is unclear. Cell culture experimentation with human VSM cells has been limited by the loss of Angiotensin receptors over relatively short periods of time. AT_2 receptors have been rarely investigated, but their presence is negligible after a few days in primary culture. AT_1 receptors are also lost during cell culture; a study of cultured primary human VSM cells demonstrated a significant loss of AT_1 receptor over a seven-day study (personal communication – Dr J McLay and Dr ME Cooper). Not only does this explain the need for supra-physiological concentrations of Ang II for cell culture studies, it also belies the reason for a surprising lack of published evidence in this field.

This chapter sets out to determine in a logical manner whether mechanical strain up-regulated the AT_1 receptor by initially investigating receptor gene expression and cell protein, before specifically identifying cell surface receptor kinetics by means of radio-ligand binding. Nevertheless, a definite answer was eventually dependent on

immunofluorescence studies. Importantly, the cell culture requirements demanded the use of human VSM cells from as low passage as possible, but at times this was hindered by a limited resource.

7.2 Total Cellular Gene Expression and Protein for AT_1 receptor

7.2.1 Introduction

The first set of studies asked a basic question regarding the expression of the AT_1 receptor mRNA and protein expression in whole human VSM cell extracts after exposure to mechanical strain.

7.2.2. Experimental Design

Human venous VSM cells (approximately 5 x 10^5 cells/well) in passages 2-3 were cultured on Bioflex® (type I collagen) culture plates and exposed to growth media (15% FBS). When sub-confluent to 80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours and then exposed to 22% / 18% cyclical mechanical strain for up to 24 hours in 5% FBS. For protein analysis, an additional strain regime of 10% /5% cell elongation was employed. Cells, treated identically except for the application of strain, served as controls.

At the completion of the experiment, cells were harvested for either RNA or total cellular protein as described in Chapter 2.4

In the first arm, RNA was prepared and then separated by gel electrophoresis before transfer onto a nylon membrane by northern blotting. A ^{32}P radio-labelled 1.7 Kb AT_1 receptor cDNA was used as the probe.

In the second arm of the study, total cellular protein was resolved by SDS-PAGE and transferred onto a membrane by western blotting. The AT_1 receptor antibody was a

polyclonal antibody specific to the last 12 amino acids at the N-terminal. After the membrane was incubation with the AT_1 receptor antibody and secondary Horseradish peroxidase conjugated antibodies (see section 2.5.8), it was exposed to autoradiograph film for 5-10 minutes at room temperature.

7.2.3 Results

Cyclical mechanical strain increased human VSM cell AT_1 receptor mRNA (130% increase over static control at 90 minutes and 260% increase at 3 hours). Despite hybridisation washing conditions not being very stringent, the signal following exposure to the radiolabelled cDNA probe was very weak. Therefore further experimentation was limited (see section 7.2.4).

Cyclical mechanical strain increased total human VSM cellular AT_1 receptor protein. At lower stretch (10%/6%), receptor protein increased by 43+/-18% (n=3) and at higher stretch (22%/18%) an increase of 32+/-15% (n=3) over static control was observed (figure 7.1).

Figure 7.1 Total human venous VSM cellular AT_1 receptor protein identified by western blotting after 24 hours of mechanical strain standardised as a % increase over control to total cell protein.

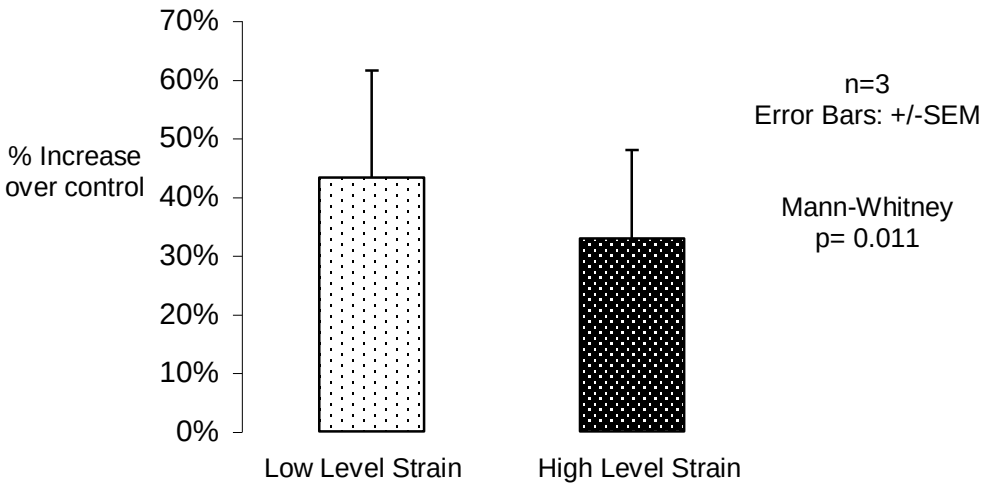


Table 7.1 Raw densitometry data standardised to total cell protein.

Expt.	Static	Low stretch	High Stretch
1	1.749	2.446	2.632
2	1.651	2.911	2.398
3	2.681	3.043	2.754
Mean	**2.03**	**2.80**	**2.59**
SEM	0.33	0.18	0.10

7.2.4 Discussion

These baseline studies provided enough evidence to embark on more detailed investigations. Analysis of total cellular AT_1 receptor protein was vigorously tested in a series of experiments and the results found to be robust in comparison to the mRNA assay. Nevertheless, it was unclear if this increase related specifically to the cell surface receptor as an increase in the cytoplasmic component of the receptor could similarly account for the observations. Consequently a detailed protocol for cell plasma membrane protein isolation was prepared. However, concerns over the validity of the technique, particularly with regard to the multiple sucrose gradient fractions and the recognised paucity of human VSM cell surface receptors resulted in a change of direction towards the use of radioligand binding techniques.

Although the cDNA probe for AT_1 receptor was prepared and purified without difficulty, it proved to be very weak. The result documented relates to only one experiment, as this could not be reproduced successfully. The expected band on the autoradiograph for the AT_1 receptor was sized correctly, but it was low in intensity and there was significant background interference. Even with low stringency washes following hybridisation, the use of fresh radiolabelled ^{32}P and adaptations to the timing of the hybridisations failed to improve the quality of the assay. Further experimentation was not justified.

7.3 Radioligand Binding Studies

7.3.1 Introduction

This technique has several clear advantages over other techniques in providing an answer to the specific question posed by this thesis. It can exclusively quantify the presence of receptor on the cell surface. In addition, it can determine receptor kinetics; this would provide not only a useful benchmark against the known dissociation constant of ~$1x10^{-8}$, but may highlight any change to the dissociation constant as a result of VSM cell exposure to mechanical strain.

In a second study, this technique was used to quantify the expression of AT_1 receptors in a series of control passaged cells cultured in plastic dishes to establish if AT_1 receptor expression reduces over time.

7.3.2 Experimental Design

7.3.2a Mechanical Strain

Human arterial VSM cells (approximately 5 x 10^5 cells/well) in their third passage were cultured on Bioflex® culture plates as described above and rendered quiescent by serum-deprivation (1% FBS) for 48 hours. Cells were exposed to 22% / 18% cyclical mechanical strain in 5% FBS for 24 hours and then assayed as described in Chapter 2.6. All experiments were conducted in triplicate and controls were treated identically except for the application of the strain regime. Hill and Scatchard plots were drawn to determine receptor density (B_{MAX}) and the dissociation constant (K_D).

7.3.2b Static Human VSM cells

In the second study, human arterial VSM cells (approximately 5 x 10^5 cells/well) in passages (1,2 & 4) were cultured on 35 mm 6-well plastic culture plates and exposed to growth media (15% FBS). When sub-confluent to 80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours. All experiments were conducted in triplicate and additional wells provided for protein quantification. Radioligand Binding studies were conducted as described in Chapter 2.6 to allow assessment of receptor density and the dissociation constant.

7.3.3 Results

7.3.3a Mechanical Strain

Only 4 points were credible for analysis namely the mean of three values from the results of adding cold Ang II at concentrations of 10^{-11} M, 10^{-9} M, 10^{-8} M, 10^{-7} M; non-specific binding was determined by the addition of cold ligand concentration of 10^{-6} M. All values were mean of three samples. The saturation binding curve has been plotted in figure 7.2.

The Hill plots for this study revealed strain and static cell assays had Hill coefficients of 0.9938 and 1.000 respectively (figure 7.3).

Scatchard analysis of the static human VSM cells (figure 7.4) reports the best-fit linear line of the 4 points plotted from the above data. Static cells reveal an AT_1 receptor dissociation constant (K_D) of $3.3x10^{-8}$ and AT_1 receptor density (B_{MAX}) of 125 fmol/ mg protein.

In comparison, Scatchard analysis of human VSM cells following mechanical strain (figure 7.4) also reports the best-fit linear line of 4 points. Importantly strain cells revealed an AT_1 receptor dissociation constant (K_D) of $5x10^{-8}$ and AT_1 receptor density (B_{MAX}) of 496 fmol/ mg protein.

7.3.3b Static Human Arterial VSM cells

Only the mean of three values (10^{-9} M, 10^{-8} M, 10^{-7} M) from the results of adding cold Ang II at concentrations of 10^{-11} M, 10^{-9} M, 10^{-8} M, 10^{-7} M were robust enough for evaluation; non-specific binding was determined by the addition of cold ligand concentration of 10^{-6} M. All values were mean of three samples, but wide confidence intervals were calculated. The results are tabulated in Table 7.1.

Hill plot numbers for all splits were poor and although the mean K_D values were all similar, these values were two orders of magnitude greater than the Scatchard analysis reported for both static and stretched cells.

The AT_1 receptor density (B_{MAX}) value was much greater in earlier split of cells. B_{MAX} values of 100, 40 and 25 corresponded to *Split 1* human VSM cells, *Split 3* human VSM cells and *split 4* human VSM cells.

Figure 7.2: Saturation binding curve for AT1 receptor radio-ligand binding to cultured human arterial VSM cells before and after 24 hours of cyclical mechanical strain.

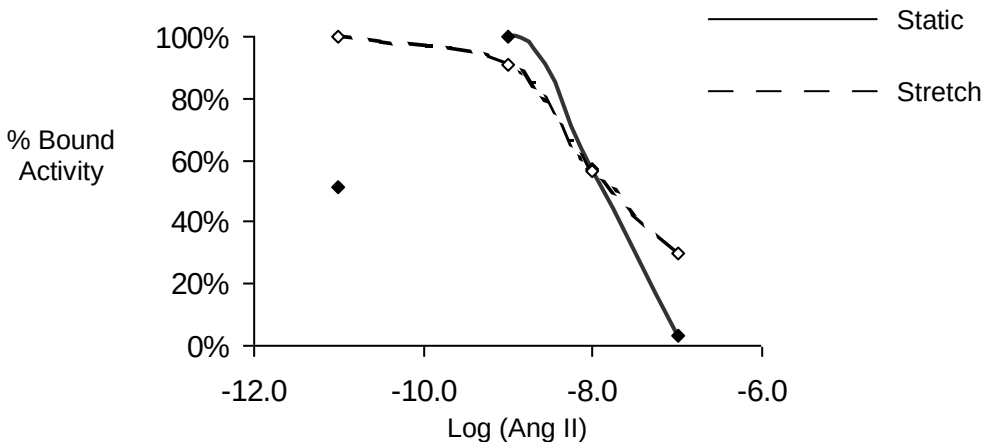


Figure 7.3: Hill Plot for AT1 receptor radio-ligand binding to cultured human arterial VSM cells before and after 24 hours of cyclical mechanical strain.

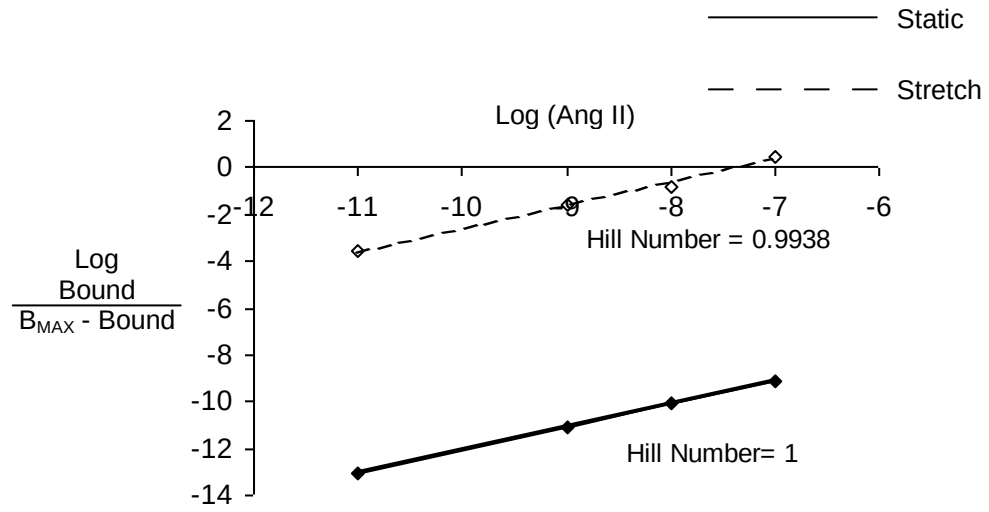


Figure 7.4: Scatchard analysis for AT1 receptor radio-ligand binding to cultured human arterial VSM cells before and after 24 hours of cyclical mechanical strain (n=3).

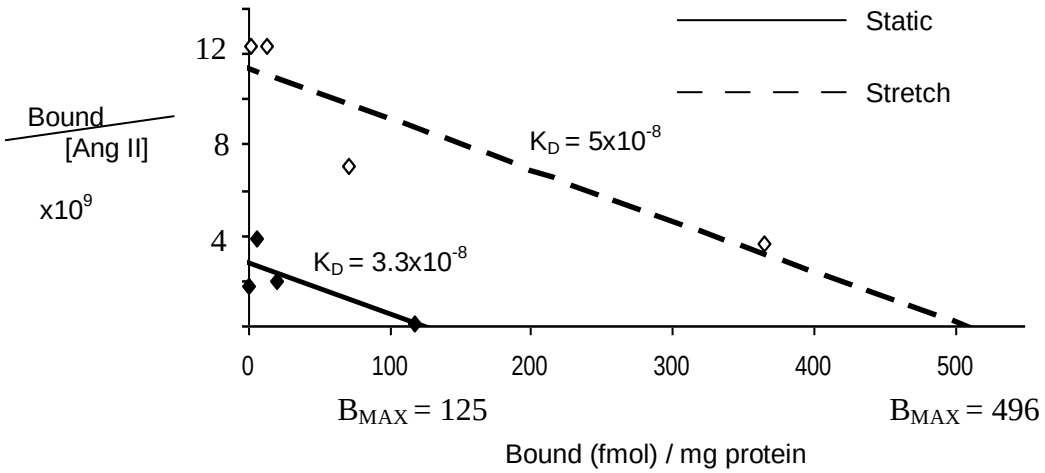


Table 7.2: AT_1 receptor-Ang II radio-ligand binding studies in early passaged human arterial VSM cells (n=3); S denotes passage number.

	Hill No.	K_D (M)	B_{MAX} (fmol/mg protein)
S1	0.77	2×10^{-6}	100
S2	0.73	2×10^{-6}	40
S4	0.66	1.25×10^{-6}	25

7.3.4 Discussion

The mechanical strain study demonstrated a 4-fold difference increase in stretched human VSM cell receptor density as determined by B_{MAX}. There is no published data available (even in static cultures / tissues) for comparisons to be made. Regrettably, the data exhibited a wide variance around the mean and the saturation binding curves were not complete. Nevertheless, the Hill Plot factor was 1 and the dissociation constant was similar to that reported by studies (Schachter et al., 1995). In addition, the dissociation constant was almost identical in the stretched and static human VSM cells. A tentative proposition can therefore be made that human VSM cells, influenced by cyclical mechanical strain, increase the number of cell-surface AT_1 receptors, but not their receptor affinity to Ang II.

It is important to acknowledge that these reported studies were conducted after a number of protocol adaptations and proved to be the most reproducible of all radio-ligand binding experiments undertaken for the thesis. Despite this, these results were not robust enough to draw this work to a firm conclusion. However, none of the other 10 studies gave a conflicting answer; the results were simply uninterpretable as the counts were so low, saturation binding and Hill plots could not be drawn.

However, it was encouraging that the study comparing cultured human VSM at different passages supported the concept that cultured human VSM cells lose AT_1 receptors with time. In addition, the data from *Split 1* human VSM cell triplicate samples revealed the lowest variance, suggesting that the experimental technique was reasonable and might be reproducible if only the target receptors were present on these cells. But there were discrepancies. Firstly, the Hill Plot factor was low probably as a consequence of not enough reproducible data at the extremes of the cold ligand sample

concentration. Alternative causes (i.e. the presence of other AT sub-types) are less likely, primarily because the stretch experiment reported a Hill Plot factor of 1. In addition, the Hill Plot value would be expected to be decreasing as the cells are passaged rather than increasing as demonstrated. Secondly, the dissociation constant was a factor of 100 greater than that reported in the stretch experiments and published work.

As highlighted above, the predominant factor limiting the value of the radio-ligand binding studies was the very low ^{125}-Iodine isotope count of the cell lysate at the completion of the experiment. This reflected low levels of sustained Ang II isotope binding after the cells were washed to remove non-specific bound ligand (even when competitive against cold Ang II at a concentration of 10^{-11}M). The ligand protein structure 'sar1,8' had been used successfully previously and is appropriate for the AT1 receptor in human cells. Through a series of studies, the methodology was adapted to maximise the experimental conditions and therefore improve the final cell lysate radio-activity count. The experiment was conducted at 4^{o}C (and in the cold room) to prevent receptor endocytosis. As the radio-isotope was batch manufactured, orders were timed to acquire fresh isotope and thus maximise its activity. In addition, the concentrations of cold Ang II were varied, particularly increasing the number of different concentrations across the range 10^{-8}M to 10^{-10}M. Moreover, to determine if cell origin was a factor, arterial Human VSM cells (previously acquired from vessels at the time of renal transplant harvest and stored in liquid N_2) were used in place of venous cells. Nevertheless, none of these adaptations significantly improved the activity of the final cell lysate.

Experimental technique could potentially have been verified by examining AT_1 receptors in homogenised animal heart tissue, where a high density of AT_1 receptor would be expected. However, whatever the outcome of this experiment, this work would not translate to human tissue and at best would only support the findings that have been observed above. Thus the sacrifice of the animal and the expense of the experiment could not be justified.

Consequently, one qualitative (immunofluorescence) study and one quantitative (flow cytometry) study were sought to resolve the issue AT_1 receptor existence on human VSM cellular plasma membranes.

7.4 AT_1 Receptor Immunofluorescence Studies

7.4.1 Introduction

The techniques employed to identify human VSM cell structural proteins (Chapter 2.3) were developed to examine AT_1 receptor changes on human venous VSM cells after the application of mechanical strain. This approach was important, as visual confirmation of receptor presence would support the conclusions from other experimental data that could only be viewed abstractly as graphs and values. Nevertheless, these series of studies were qualitative, as it would prove impossible to accurately quantify the immunofluorescence signal.

7.4.2 Experimental Design

Human venous VSM cells in early passages (2) were cultured on Flex I plates with collagen type I matrix substrate. When sub-confluent to 70-80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours. Cells were incubated in 5% FBS and exposed to cyclical mechanical strain by the FX-2000 system (Vacuum: -17/-10 kPa equivalent to Strain: 23/19%). Control static human VSM cells were incubated identically except for the application of mechanical strain. After 24 hours of continuous cyclical strain, cells were fixed and immuno-stained as described in section 2.7. Immunofluorescence signal was viewed under confocal microscopy. Multiple static control cell sample wells were investigated to obtain the optimum conditions for these immunofluorescence studies and it was determined that the lowest concentrations of primary rabbit polyclonal anti-AT_1 receptor (N10) antibody was 1:200

and secondary fluorochrome-conjugated antibody (anti-rabbit IgG – Alexafluor 568) was 1:1000.

In a second series of studies, human venous VSM cells were examined specifically for plasma membrane receptor signal. The protocol was adapted so that the cells were not permeablised following the cell culture experimentation for static control cells plated on type I collagen substrate. This would prevent antibody ligands penetrating the cellular cytoplasm and thus any binding would occur exclusively on the cell surface (the anti-AT_1 receptor antibody specifically targets the external N-terminal amino acids). Consequently, this would allow quantitative assessment of receptor immunofluorescence signal. Regrettably the cells did not survive the antibody incubations steps. Changing the incubation buffer from phosphate-buffered saline to a more physiological buffer (Tyrodes Buffer) delayed, but ultimately did not prevent osmotic fluid shifts across the cell membrane and destruction of the cells.

7.4.3 Results

Figure 7.6 illustrates the immunofluorescence binding of AT_1 receptors to human venous VSM cells in culture both at rest and following 48 hours of cyclical mechanical strain. Signal could be identified in the plasma membrane and throughout the cellular cytoplasm. The nucleus remained a shadow with no evidence of binding. The negative control samples substituted the primary anti-AT_1 receptor antibody for BSA and did not to reveal any immunofluorescence signal.

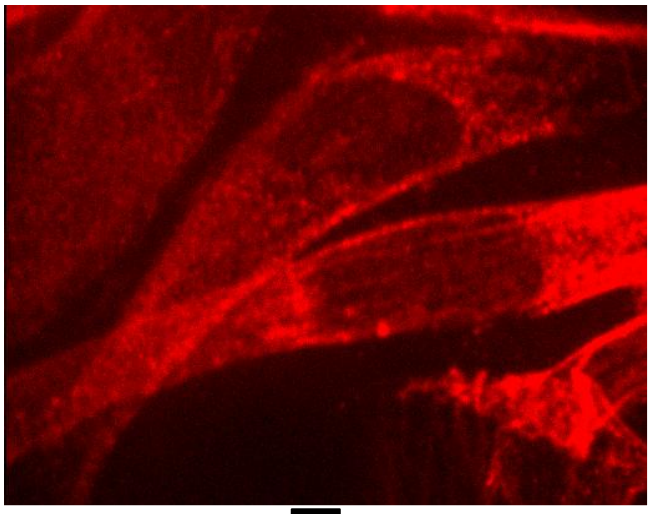
The objective of the study was to identify any differences in cell surface immunofluorescence between static and stretched cells. By using the Flex I membranes

and FX2000 strain unit, it was possible to view a homogenous cohort of cells at the centre and at the periphery of the well representing respectively the extremes of very low (almost static) and high pressures on cells.

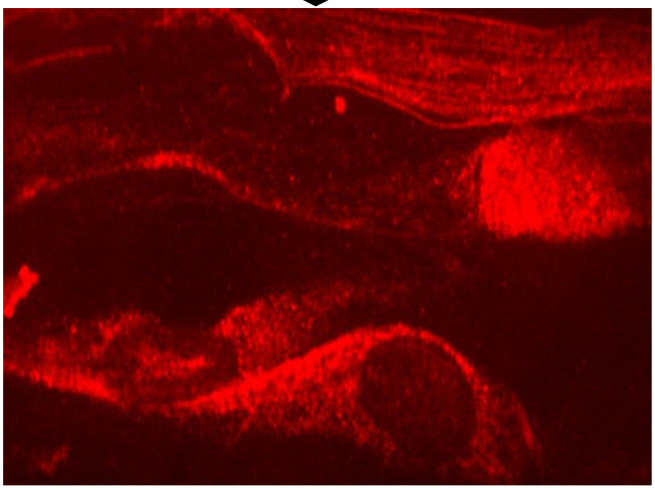
The cells were viewed by an independent observer (Dr Diane Hudman) and me. Human VSM cells exposed to stretch revealed increased signal at the cell surface membrane compare to static cells or those cells at the centre of the well exposed to very low pressures. Unfortunately it was not possible to quantify this signal.

Figure 7.6: Immunocytochemistry – revealing AT_1 receptor staining in human venous VSM cells plated on collagen type I in static cultures and following 48-hour cyclical mechanical strain (x100).

Static:



Post 48-hour strain:



Negative Control BSA and FITC 2^o:

7.4.4 Discussion

The studies revealed an increase in human VSM cell surface immunofluorescence for AT_1 receptors after a 48-hour period of cyclical strain. There are very limited studies available to compare and contrast results of these experiments with. Takeda (2001) published work revealing robust immunostaining of the AT1 receptors in human sweat glands with signal evident in the plasma membrane.

Signal could be identified in the plasma membrane of our cells, which represents cell surface receptors and also throughout the cellular cytoplasm, which was likely to represent internalised receptors or receptors synthesised de novo. As expected, the nucleus did not reveal any evidence of binding. Unfortunately, attempts to examine the external cell surface receptors failed. Several additional key experiments would be (1) dual antibody immunofluorescence for structural proteins and AT_1 receptors to determine whether a shift in cell phenotype is associated with changes to cell surface receptor density and (2) dual antibody immunofluorescence for a specific cell-surface membrane marker and AT_1 receptors.

In summary, these studies are not robust enough to stand alone, but may add to the data acquired from other studies reported in thesis and provide some useful pictographic support to the hypothesis.

7.5 Flow Cytometry Studies of the AT_1 receptor

7.5.1 Introduction

The implementation of flow cytometry studies was a natural progression following the microscopic immunofluorescence studies. Flow cytometers provide high-speed automated immunofluorescence on a cell-by-cell basis – aiming to measure populations of approximately 10,000 cells for each experimental sample. Although it does not provide pictorial observations, it is a validated quantitative analysis of single or multiple simultaneous measurements of immunofluorescence signal and can report cell-surface ligand binding as a mean value per cell. This provided an opportunity to resolve the hypothesis.

Therefore the aim of this work was to simply compare the expression of AT_1 receptors on stretched human VSM cell plasma membranes compared to a population of static control cells.

7.5.2 Experimental Design

Human venous VSM cells (approximately 5 x 10^5 cells/well) in their second passage were cultured on Bioflex® (type I collagen) culture plates and exposed to growth media (15% FBS). When sub-confluent to 80% cell coverage, cells were rendered quiescent by serum-deprivation (1% FBS) for 48 hours. Cell culture plates were positioned over a series of loading posts which afforded equibiaxial cyclical mechanical strain at 8% / 4.5% in 5% FBS. Static control cells were treated identically except for the application of strain.

Following cell culture experimentation, culture media was removed and cells were harvested by washing in Enzyme-free Cell Dissociation Buffer. The antibody labelling incubations were conducted as described in Chapter 2.8. Importantly, all flow cytometry incubations were carried out strictly at 4^{o}C and in the presence of 0.05% sodium azide to prevent receptor recycling. Furthermore, non-specific binding was prevented by performing all incubations and washes in the presence of 0.1% BSA.

The primary antibody was the same rabbit polyclonal anti-AT_1 receptor antibody to the N-terminal domain as this had been successful in other assays. Goat FITC $F(ab')_2$ anti-rabbit IgG (Sigma) served as the fluorochrome as it offered the best specific ligand binding.

The definite experiments were conducted on three separate occasions providing a sample number of 9. Each experiment was paired to static controlled cells. To correct for non-specific binding, human VSM cells were incubated with the fluorochrome in the absence of anti-AT_1 receptor antibody. An anti-HLA Class I antigen (IgG1κ) monoclonal antibody was examined with each study as a positive control for AT_1 receptor surface staining.

It was determined that the immunofluorescence signal would be analysed by comparing two key measurements between static and stretched human VSM cells. Therefore, at the outset a cohort of human VSM cells with significant quantities of immunofluorescence signal representing AT_1 receptors was identified for each sample. The number of cells identified in these cohorts was expressed as a percentage of the whole sample cell count. In addition, the median value of immunofluorescence signal for these cohorts was calculated.

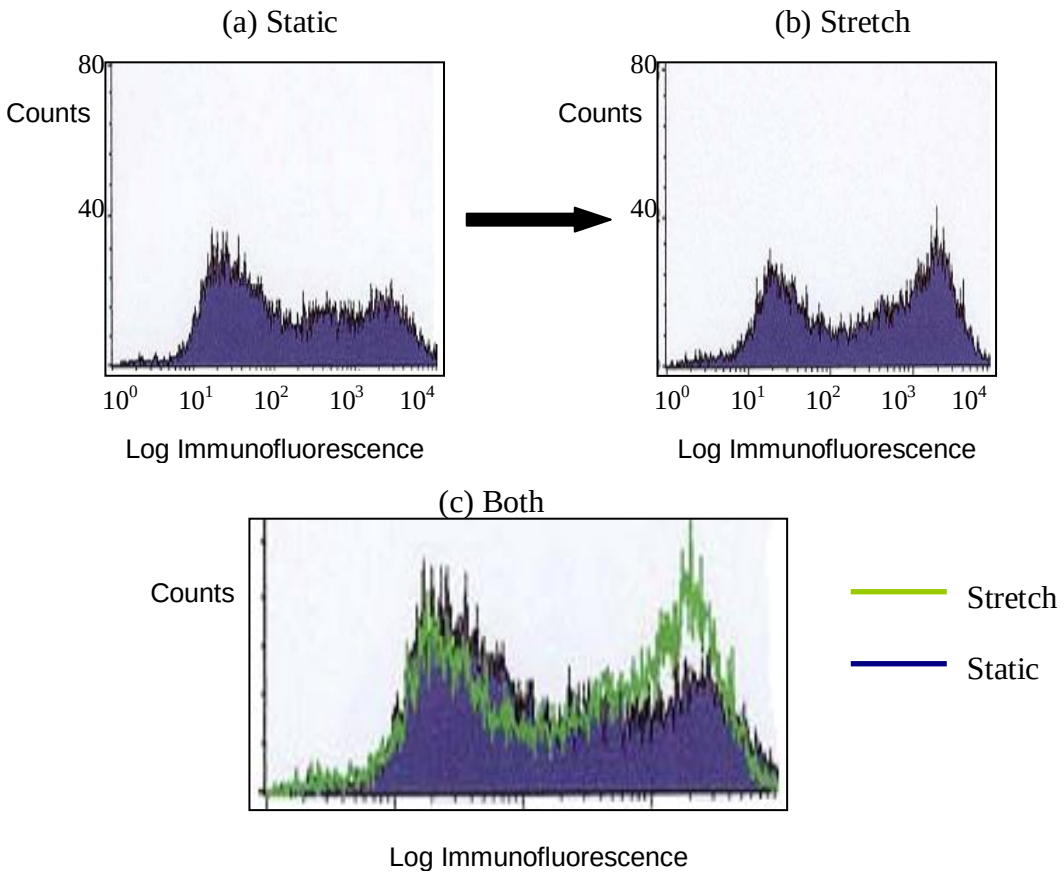
7.5.3 Results

7.5.3a Cell Population and Size

Repeated assessment of the cell population revealed consistency between samples. In addition, there was no change in cellular size after the application of mechanical strain, thus avoiding the need to adjust the results post analysis.

After accounting for non-specific binding, two distinct populations of human VSM cells with differing immunofluorescence intensity were observed (figure 7.7). The median value of the immunofluorescence intensity between the populations was separated by a magnitude of 10. This phenomenon remained consistent throughout all the experiments whether the human VSM cells were fresh from culture or after exposure to mechanical strain.

Figure 7.7: In both static and stretch cultures, two populations of human venous VSM cells (based on their expression of AT_1 receptors) were observed. Graph (c) is a composite of graph revealing the increased intensity of receptor expression at the higher values after stretch.



7.5.3b Effect of Mechanical Strain

The second population of human venous VSM cells were analysed to quantify the expression of AT_1 receptors. The data was computed from an arbitrary value set at 200. This value was chosen for two main reasons. Firstly it was the point at which non-specific binding was no longer evident and secondly it coincided closely with the second population of cells (figure 7.8). Cyclical mechanical strain increased both the median value of immunofluorescence signal (Static: 938.00 +/- 79.95; Stretch: 1124.85 +/- 133.43; 19.8% increase over static control; n=9; p= 0.0154 Students paired t-test – figure 7.9) and the percentage of cells with the higher intensity signal (Static: 38.2 +/- 2.1%; Stretch: 44.5+/-2.5%; 15.9% increase over static control; n=9; p=0.0141 Students paired t-test – figure 7.10). The value of signal intensity is proportional to AT_1 receptor quantity.

Figure 7.8: Analysis of results

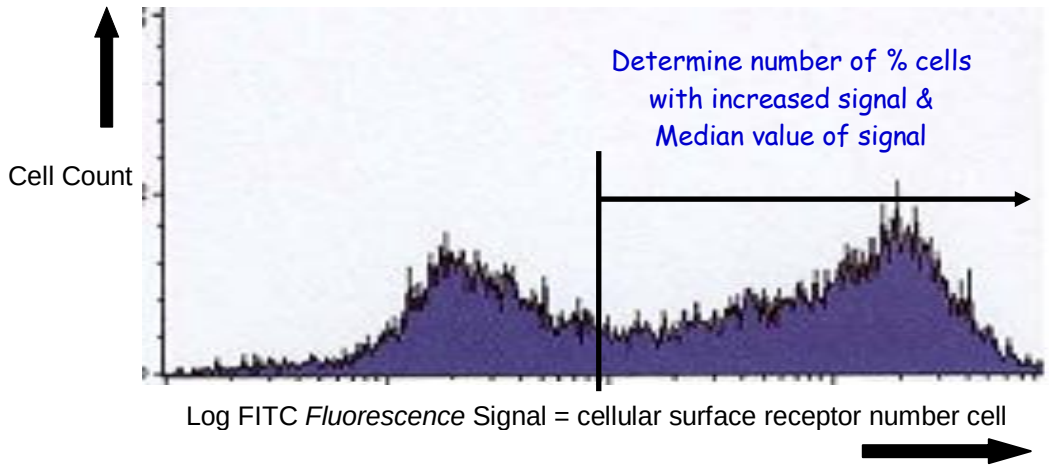


Figure 7.9: Median AT_1 receptor immunofluorescence in static human venous VSM cell cultures and after 48 hour cyclical mechanical strain

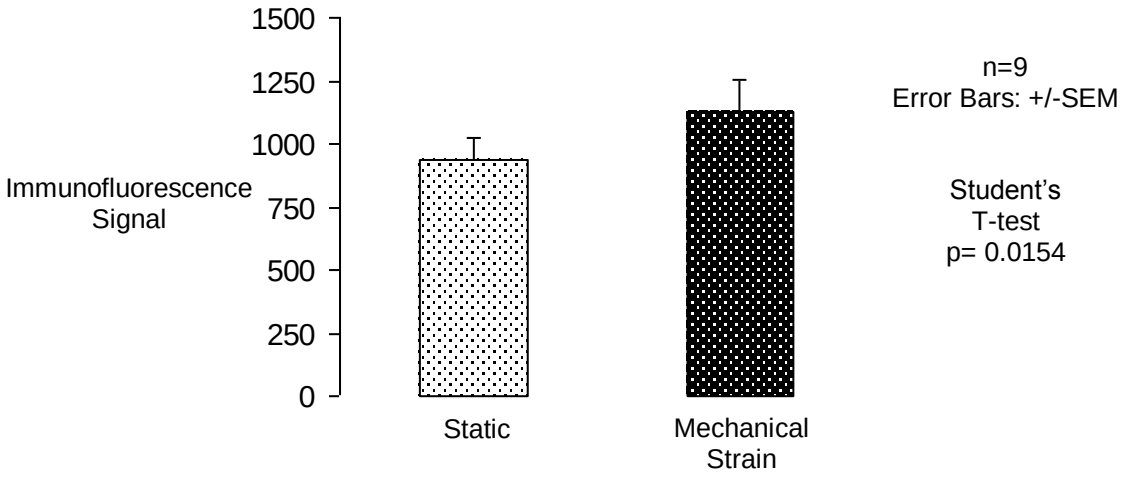


Figure 7.10: Percentage of cells with higher AT_1 receptor immunofluorescence in static human venous VSM cell cultures and after 48 hour cyclical mechanical strain

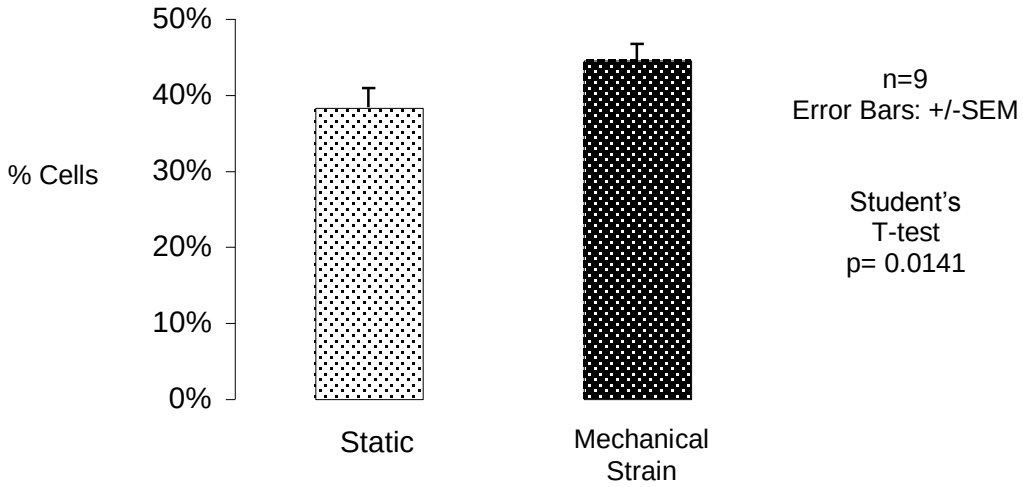


Table 7.2: Raw data for % cells AT_1 receptor immunofluorescence

Expt.	Static	Stretch
1	36.3%	43.9%
2	37.8%	37.9%
3	39.0%	36.4%
4	26.0%	43.8%
5	35.2%	41.6%
6	50.0%	55.4%
7	36.9%	49.3%
8	40.7%	36.5%
9	42.1%	55.9%
Mean	**38.2%**	**44.5%**
SEM	2.1%	2.5%

Table 7.3: Raw data for median AT_1 receptor immunofluorescence

Expt.	Static	Stretch
1	1154.78	1286.4
2	716.92	820.47
3	673.17	629.26
4	710.5	704.14
5	716.92	770.4
6	1197.09	1498.93
7	1050.71	1309.75
8	1274.88	1746.58
9	956	1357.73
Mean	**939.00**	**1124.85**
SEM	133.43	79.95

7.5.3c Quantification of human venous VSM cell expression of Epidermal Growth Factor receptor

There is no obvious explanation for approximately 50% of the cell population with a significantly higher expression of AT_1 receptors, but it may reflect a differing rate of attrition of AT_1 receptors in the cell culture environment. An alternative explanation would be non-VSM cell contamination, but this is unlikely from evidence provided by immunocytochemistry studies.

This occurrence was investigated by determining the expression of EGF receptors in human VSM cells with and without exposure to mechanical strain. The EGF receptor was chosen as it is primarily involved in modulating growth and animal studies have suggested its up-regulation in stretched VSM cells. In addition, it was possible to compare expression of both AT_1 and EGF receptors on the same population of cells by use of different immunofluorescence probes. The experimental design was identical except for the primary and secondary antibody incubation steps for immunofluorescence. In the primary incubation, a mouse monoclonal anti-EGF receptor antibody was co-incubated with the rabbit polyclonal anti-AT_1 receptor (N10) antibody; in the secondary incubation, a anti-mouse fluorescence tag was co-incubated with the goat FITC $F(ab')_2$ anti-rabbit IgG.

Mechanical strain did not influence the median value of immunofluorescence signal representing EGF receptor expression (Static: 14.2/- 1.3%; Stretch: 11.9% +/- 4.8%; n=3; p=0.715. In the comparative study, the majority of cells had low AT_1 receptor and EGF receptor signal (56.0%). Interestingly, there were very few cells that demonstrated

high intensity signal solely for the EGF receptor (1.1 %). However, only 13.3% cells that had increased AT_1 receptor signal also had EGF receptor signal.

7.5.4 Discussion

The flow cytometry experiments reveal for the first time the conclusive evidence that mechanical strain up regulates cell surface AT_1 receptors. Circumstantial evidence was first provided by the observation of synergy between the RAS and mechanical strain, later supported by evidence of increased AT_1 receptor protein and possibly immunocytochemistry. The radioligand binding studies were not conclusive and difficult to interpret; but never provided evidence to the contrary.

The advantages of flow cytometry and radioligand binding studies over the other experimental techniques are their ability to specifically identify AT_1 receptors on the cell surface. This allows the hypothesis to be examined successfully without any doubts of bias from internalised or unexpressed receptors. In addition, these studies have highlighted that fluorescent antibody-based experimental techniques appear to have advantages over radio-labelled ligand-based experiments when the target receptor is expressed in low numbers.

One potential bias of the flow cytometry was cell size. Previous studies have shown that cells proliferate after the application of mechanical strain in high concentrations of growth media and/or long periods of time (days). Similarly cells might undergo hypertrophy; there is no published data to neither refute nor confirm this. However, these studies revealed that there was no change in cellular size between stretched and static human VSM cells (data not shown).

It was intriguing to consistently identify two populations of human VSM cells based on their AT_1 receptor status. This observation was common to both static and stretched cells. As discussed above, it could reflect non-VSM cellular contamination. However, this would be unlikely: firstly there was no evidence of non-VSM cell contamination on light microscopy observed during the immunofluorescence studies. Secondly, non-VSM cells such as fibroblasts are not known to possess Angiotensin receptors. In addition, this is unlikely to reflect non-specific binding as the control sample peaked at significantly lower levels of immunofluorescence. Although cells for all individual experiments were acquired from a single source, it may be possible that there are subtle differences within the cell population that results in a differential expression of AT_1 receptors.

All VSM cells were initially grown on a plastic before transferring to a collagen type I matrix substrate. As discussed in earlier chapters, VSM cells differentiate into at least two clearly identifiable phenotypes, namely contractile and synthetic. This might provide the explanation. One method of investigating this phenomenon would be to repeat the flow cytometry experiments in combination with antibodies to myosin and α-actin. However, a clear comparison with specific cell membrane receptors would not be possible, as the experiment would require the human VSM cells to be permeabilised to allow the intra-cellular structural proteins to be identified. In its place, the epidermal growth factor (EGF) receptor was targeted as animal studies have identified an increase in VSM cell receptor in stretched cells and the receptor is involved in growth. Unfortunately, this experiment did not confirm the findings in previously published studies, but interestingly almost all human VSM cells with a higher than average expression of EGF receptor had a high expression of AT_1 receptors. Although this does not provide any clear-cut answers, it points to a dedifferentiation of phenotype within

the human VSM cells. Future studies exploring the phenotypic changes by flow cytometry investigation of myosin and α-actin structural proteins are warranted and would complement the earlier studies detailed in chapter 3.

Chapter 8

Discussion

The origin of this thesis was a desire to investigate in greater detail the effect of mechanical strain on the production of extracellular matrix proteins in human VSM cells and potentially to determine the cellular mechanism supporting this observation.

Our cells were human and derived from veins acquired at the time of venous stripping to treat varicose veins. Initial phenotypic characterisation of the cell culture model of human VSM cells by immunofluorescence studies of structural proteins (myosin heavy chain 1 & 2 and α-actin) revealed a true population of human VSM cells with little or no evidence of endothelial cell or fibroblast contamination. This was important as it provided a reassuringly robust population of human VSM cells for further study.

Further studies revealed differences in the phenotypes dependent on the underlying cell culture substrate. Human VSM cells cultured on plastic or glass may be predominantly *synthetic*, whereas cells cultured on collagen type I appear to be *contractile*. However, mechanical strain causes an increase for both contractile microfilaments. Rather than mechanical strain leading to a more proliferative *synthetic* phenotype, these observations highlight the development of a more pronounced *contractile* phenotype. It was this phenotype that was ultimately examined for AT_1 receptor expression.

The consequences of mechanical strain on human VSM cell synthesis of matrix proteins has previously been well characterised and numerous additional factors (e.g. RAS, nitric oxide, sympathetic nervous system, $TGF\beta_1$ etc) have been implicated. In addition, the use of a mechanical strain model for *in vitro* cell culture studies of human VSM cells is fundamental for those cells that are continuously under the influence of mechanical forces *in vivo*. No cell culture model can be perfect; however, the Flexcell® systems applied cyclical strain to cultured cells at the similar rate and pressure to that experienced by cells within blood vessel walls.

Early experiments confirmed published studies, predominantly in animal species, that cyclical mechanical strain and Ang II induced ECM gene expression and protein synthesis by VSM cells via the AT_1 receptor. The only consistent substrate that permitted our population of venous human VSM cells to proliferate and survive the application of mechanical forces was collagen type I. However, an intriguing observation was made when human VSM cells were co-incubated with Losartan (AT_1 receptor blocker) and exposed to mechanical forces: there was a significant attenuation of ECM synthesis and gene expression in cells treated with the AT_1 receptor blocker. No obvious explanation was initially forthcoming and importantly, no exogenous Ang II had been added to the culture media during these experiments; although not confirmed by Gibco, it was likely that some Ang II is present in the growth media. A review of previously published work had highlighted this observation in both VSM cells in rats and cardiomyocytes in rabbits. However, follow-up studies published subsequently to determine a mechanism revealed conflicting conclusions. Sadoshima (Sadoshima et al., 1993) suggested that mechanical forces lead to an increased release of stored Ang II from the cytoplasm of cardiomyocytes, but the methods used to quantify supernatant Ang II were criticised as not enough care was taken to prevent degradation of cell preps from peptidases. In addition, if this observation is translated to human subjects and those with hypertension, there is no evidence that hypertensive individuals have greater circulating or vessel Ang II.

Recently, Zou (Zou et al., 2004) has published similar work investigating mechanically stretched rat neonatal cardiomyocytes. In these studies stretch activation of ERKs was inhibited by Candesartan suggesting activity via the AT_1 receptor. To produce an identical ERK response, 20% mechanical stretch for 8 minutes was roughly equivalent to Ang II at 10^{-8} to 10^{-7} M. In the culture medium, a radioimmunoassay did not detect a

significant increase of Ang II concentrations (10^{-12} M) following the application of stretch, suggesting that Ang II is not released by cardiomyocytes or even if it is, it will be too small to have the effect on ERK activation. Nevertheless the use of Ang II neutralising antibodies incubated at the time of experimentation inhibited the ERK response to Ang II (10^{-8} to 10^{-7} M) but not mechanical stretch. Further studies with HEK293 and COS7 cells showed stretch induced AT_1 receptor activation without the secretion of Ang II. Moreover, Janus Kinase 2, a key component of AT_1 receptor signalling, was activated by stretch. This paper underscores a mechanistic role for stretch induced activation of cardiomyocytes via the AT_1 receptor.

There had not been any published work, which has rigorously examined the role of the AT_1 receptor in stretched human VSM cells. This thesis therefore wished to examine the hypothesis that this observation was a consequence of up regulation of AT_1 receptors on human VSM cell membranes. This would effectively sensitise the VSM cell and augment its biological response to any Ang II present in the cell culture media.

Examining the activation of ERK 1/2 by western blotting provided several key additional results. This secondary messenger acts to translate neuro-humeral stimuli into fibrogenic cellular activity in human VSM cells and both mechanical strain and Ang II were observed to significantly increase its synthesis. Firstly, this provided further evidence that our cell population was a true cohort of VSM cells and our culture model of mechanical strain was robust. Ang II induced ERK 1/2 activation in cells stretched for 24 hours prior to co-incubation with Ang II. This effectively excluded the possibility that stretched human VSM cells synthesise Ang II de novo as the additional Ang II would be unlikely to induce such a significant activation of ERK 1/2. Other explanations for these observations could relate to up-regulation of the ERK 1/2

pathway or the AT_1 receptor function. Subsequent studies examined in greater detail the latter of the two explanations.

At first, the gene expression and protein synthesis of the AT_1 receptor was investigated by northern and western blotting techniques respectively. Unfortunately, the ^{32}-P radiolabelled cDNA probe for the AT_1 receptor proved to be very ineffective even after protocol adaptations. Further experimentation was not justified on the basis of expense and time. Moreover, analysis of total cellular AT_1 receptor protein was robust. This revealed a significant increase in total cellular AT_1 receptor protein in response to mechanical strain. Nevertheless, it was unclear if this increase related specifically to the cell surface receptor as an increase in the cytoplasmic component of the receptor could similarly account for the observations. Although a detailed protocol for cell plasma membrane protein isolation was prepared, concerns over the validity of the technique resulted in a change of direction towards the use of radioligand binding and later immunofluorescence-based techniques.

Radio-ligand binding studies demonstrated a 4-fold increase in stretched human VSM cell AT_1 receptor density as determined by B_{MAX} without a significant change in AT_1 receptor affinity (defined by the dissociation constant). Regrettably, the data exhibited a wide variance around the mean and the saturation binding curves were not complete. Moreover, there is no published data available for comparisons to be made. Therefore these results were not robust enough to draw this work to a firm conclusion.

The predominant factor limiting the value of the radio-ligand binding studies was the very low ^{125}-Iodine isotope count of the cell lysate at the completion of the experiment. Other studies have demonstrated that cultured VSM cells in primary culture lose all their cell membrane AT_2 receptors and the majority of AT_1 receptors. The studies using

cultured human VSM at different passages supported the concept as the data from *Split 1* human VSM cell triplicate samples was the most reproducible.

Immunofluorescence studies revealed a strain-induced increase in human VSM AT_1 cell surface receptors. Unfortunately it was only possible to report qualitative observations. Signal could be identified in the plasma membrane, which represents cell surface receptors and also throughout the cellular cytoplasm, which was likely to represent internalised receptors or receptors synthesised de novo. Unfortunately, attempts to specifically examine the external cell surface receptors failed.

The flow cytometry experiments reveal for the first time the conclusive evidence that mechanical strain up regulated cell surface AT_1 receptors on human VSM cells. This was not unexpected as previous experiments using different techniques had strongly hinted but not proven the hypothesis. Moreover, flow cytometry also excluded the possible bias from internalised or unexpressed receptors and there was clear evidence that stretch did not influence cell size.

Although radio-ligand binding studies report receptor affinity as well as density, these studies have highlighted that fluorescent antibody-based experimental techniques appear to have advantages over radio-ligand binding experiments when the target receptor is expressed in low numbers.

One question remained. It was intriguing to consistently identify two populations of human VSM cells based on their AT_1 receptor status in both static and stretched cells. Further studies with co-incubation of immunofluorescent antibodies to both AT_1 and EGF receptors did not provide an answer, but hinted at the possibility of dedifferentiation of the human VSM cell phenotype in cell culture and therefore

differences of AT_1 receptor expression on *synthetic*, *contractile* and *stretched* VSM cells. This could form the basis of further study.

The role of Angiotensin Converting Enzyme inhibitors (ACEi) and AT_1 receptor blockers (ARB) has been thoroughly investigated in the treatment of cardiovascular disease (including heart failure and hypertension), diabetes and diabetic nephropathy.

The actions of Ang II have been extensively described in chapter 1 and although both ACEi and ARBs reduce the potency of Ang II, there are important differences between these agents. ACEi only inhibit about 70% of Ang II synthesis as non-ACE pathways convert renin or Ang I to Ang II. Nevertheless, ACEi also reduce bradykinin breakdown, which may have beneficial properties such as vasodilatation. In comparison, ARBs block the effect of Ang II at the AT_1 receptor, allowing greater stimulation of the AT_2 receptor and thus potentially promoting nitric oxide, which often antagonises the effects of Ang II.

Not only are these drugs effective and well tolerated blood pressure reducing agents, they have shown long-term reduction in mortality and morbidity in large scale randomised controlled clinical studies, particularly for subjects following myocardial infarction or with heart failure, diabetic nephropathy and hypertensive patients with evidence of left ventricular hypertrophy.

Although for some studies, there has been controversy as to the relative merits of the blood pressure lowering compared to the specific reduction of the non-haemodynamic effects of Ang II, it is broadly agreed that individuals with cardiovascular disease are likely to benefit from therapies that reduce the burden of the RAS on the vasculature. Currently it is not clear whether ACEi have an advantage over ARB drugs. Generally,

head-to-head clinical studies have been equivocal, but concerns about the low dosage of ARB therapy have provided a distraction where these drugs do not fulfil expectations of benefit.

Similarly, in studies specifically investigating the treatment of heart failure with combination ACEi/ARB against single ACEi or ARB have revealed mixed fortunes for different drugs within the class. The most successful study involving lisinopril and candesartan suggested combination therapy significantly reduced cardiovascular events compared to either agent alone (Pfeffer et al., 2003).

Small-scale studies, often supported by industry, have compared the efficacy in terms of blood pressure reduction between classes and meta-analysis of those studies, which have been published, reveal little difference. However, there will never be clinical outcome studies in purely hypertensive subjects comparing ACEi and ARBs. Boehringer Ingleheim supported a 4-year follow-up double-blind RCT investigating 25,000 patients at high cardiovascular risk, but not necessarily hypertensive, to compare an ACEi (ramipril), an ARB (telmisartan) and a combination of the two. This study failed to show a benefit of dual treatment over single drugs (Yusef et al.,2008).

This cyclical strain cell culture model is probably closest to the pathophysiological changes to human blood vessel VSM cells in the early stages of hypertension. Guidelines from the British Hypertension Society have included a reference to the *ACD* rule, which bases first-line anti-hypertensive therapy on the renin status of individuals presenting with high blood pressure. From a small study examining the responses of patients to various single drugs, those with a high renin status (i.e. young Caucasian individuals) are more likely to obtain better blood pressure reduction with drugs that inhibit the RAS. A genetic basis for this phenomenon is well founded, but an additional

factor could clearly be related to the early phenotypic changes that result in AT_1 receptor up regulation within VSM cells proving an obvious target for inhibition.

In light of the work reported in this thesis, if hypertensive vessels become more sensitive to the effects of Ang II by up-regulation of the AT_1 receptor, then a clinical marker of this is better blood pressure reduction. But this would not take into account the relative roles of bradykinin in those treated with ACEi. The relative benefits of ACEi and ARB therapy on hypertensive vessels may not be exclusively determined by the role of the RAS and obtaining clinical justification for a more aggressive inhibition of Ang II in patients with hypertension may not be forthcoming. Nevertheless, this thesis highlights a clear mechanistic advantage for ARBs in reducing the deleterious effects of Ang II on the vasculature and thus will secure their future as potent therapies in the treatment of cardiovascular disease.

8.2 Reflection

Over the duration of the thesis, there have been some improvement in the technology that is cheaply and more freely available. With hindsight, I would have developed rtRCR technology to gauge gene expression not only of ECM proteins, but also angiotensin receptor expression. More use of the flow cytometry would be employed to underscore the changes to cell-surface angiotensin receptor expression on human VSM from different sources as ultimately this proved the most successful technique.

More recently a number of studies have investigated the roles of mechanical strain and the renin-angiotensin system on VSM cell biology predominantly in animals.

The role of the cell adhesion kinase (CAK) β was investigated by Iwasaki et al. They identified that mechanical strain, applied to cultured rat VSM cells, led to activation of CAKβ which in turn led to ERK 1/2 activation and cell growth (Iwasaki et al., 2003).

The role of ERK 1/2 activation in stretch-induced VSM cells is further supported by Morita. By applying a transmural pressure of 160 mmHg to human VSM cells for 3 hours led to activation of ERK and c-JUN N-terminal kinase, which was suppressed by ACE inhibition (Morita et al., 2004).

Tsai et al investigated the mechanism, which in part promotes the phenotypic shift of VSM cells from a contractile to synthetic state (Tsai et al., 2009). They applied shear stress to endothelial cells and investigated its paracrine effects on VSM cells. They identified endothelial cell-induced release of prostacyclin led to VSM cell activation of peroxisome proliferator-activated receptor α/δ, which in turn promoted phenotypic modulation.

In similar work to this thesis, Iizuka et al applied mechanical strain to cultured human aortic cells revealing strain-induced up-regulation of ACE mRNA and enzyme activity. These effects were also abolished by the co-administration of an AT_1 receptor blocker (Iizuka et al., 2008). Their studies also reveal strain-induced cell proliferation and ERK 1/2 activation.

VSM cell production of reactive oxygen species is well recognised; Hitomi et al measured VSM cell expression of p22-phox and Nox-1 mRNA in response to Ang II and mechanical strain. It required synergy between Ang II and strain to affect a significant increase in p22-phox and Nox-1 mRNA and this was associated with an increase in NAPDH oxidase activity. They also identified strain-induced VSM cell protein synthesis of the AT1 receptor (Hitomi et al., 2006).

Zeidan et al investigated the role of leptin in cultured rat portal vein cells stretched constantly for three days by the application of a 0.6g load. Leptin production was increased in stretched cells, but in addition, the co-administration of exogenous leptin led to cellular hypertrophy and ERK 1/2 activation (Zeidan et al., 2005).

Shyu et al investigated the effect on mechanical strain on rat VSM cell expression of the discoid domain receptor 2 (DDR2) mindful of the latter's potential role in the regulation of ECM. They used the FX-2000 to apply cyclical mechanical strain and identified up-regulation of mRNA expression and protein synthesis of DDR2. Through further experimentation, they demonstrated DDR2 protein synthesis expression was mediated by TGFβ2 and p38 MAP kinase (Shyu et al., 2005).

Chapter 9

Conclusion

The integrity of the vasculature is vital for the prevention of atherosclerosis or arteriosclerosis in hypertension and thus a reduction in the risk of stroke, myocardial infarction or renal failure.

The role of the RAS in human blood pressure physiology is well characterised and thus became a target for novel drug treatment in the 1970s. However, it is only more recently that the detrimental effects of Ang II on blood vessels have been fully described and it has been argued that Ang II is effectively a poison in those individuals at higher cardiovascular risk from any cause. Indeed, although it has been well recognised that effective blood pressure lowering significantly reduces cardiovascular disease and overall mortality, there has been much debate as to the relative merit of the different classes of drugs to lower blood pressure, particularly whether therapies that antagonise the RAS (ACEi and ARBs) have benefits over and above their blood pressure lowering effect. Recent British Hypertension Society guidelines have promoted the use of these drugs as first-line therapy in young Caucasian individuals with a presumed high renin status.

This thesis developed the hypothesis that higher pressures applied to vascular smooth muscle cells within blood vessels, promoted up-regulation of AT_1 receptors on the cell surface and thus increased sensitivity of these cells to the effects of Ang II. Historically, this has been a controversial area of research due to the experimental and technical difficulties in proving definitively the relationship between cyclical mechanical strain and the RAS.

By using a model for the application of cyclical mechanical strain in a cell culture system, a series of studies using a variety of experimental techniques finally concluded that strain induced an increase in AT_1 cell surface receptors that provides a mechanism for earlier observations. In addition, immunocytochemistry studies revealed modification of the VSM cell cytoskeletal proteins α-actin and myosin after the application of strain, which may reflect a 'hypertensive' VSM phenotype. Currently the link between these two findings is circumstantial.

These laboratory based mechanistic studies have taken place at the same time as clinical studies that have examined the relative benefits of anti-hypertensive drugs. Although it is not be possible to answer every question in terms of conducting large-scale RCTs, this work imparts useful and robust scientific evidence to guide clinical practice. If this laboratory model of *hypertensive* VSM cells, showing up-regulation of key AT_1 cell surface receptors, best represents the early changes to VSM cells in a hypertensive individual, it provides further support to the use of therapies that inhibit the synthesis of Ang II and/or antagonise the AT_1 receptor.

Appendix A - Reagents

General

Sterile glassware baked at 160^{o}C for at least 6 hours

Sterile plastic baked at 120^{o}C for at least 6 hours

Autoclaving – 121^{o}C for 15 minutes

RNAase treat with 2% Hydrogen peroxide

DEPC-H_2O: 1ml DEPC per 1000 ml H_2O, leave 2 hours, autoclaved.

Cell Culture

15% Foetal Calf Serum

RPMI 1640		330 ml	
FCS		60 ml	
CEE		2 ml	
Penicillin / Streptomycin	(10,000 iu/ml)	4 ml	}GIBCO
Glutamine (100x)		4 ml	}

Complete MEM x1

Sterile H_2O		422 ml	
MEM x10		50 ml	
Hepes (1M)		12 ml	
NaOH (1M)		1 ml	
Penicillin / Streptomycin	(10,000 iu/ml)	10 ml	}GIBCO
Fungizone (250 ug /ml)		5 ml	}

Plasmid Preparation

2YT (50ml)

1.6% Bactro-tryptone	1.6 g
1% Yeast Extract	1.0 g
1% NaCl	1.0 g
2M Glucose	0.5 ml

3M Sodium Acetate pH 6 (25ml)

NaAOC	10.2 g
H_2O	25 ml

Correct to pH 6 and treat with DEPC

TE Buffer (100ml)

10 mM TRIS pH 7.4
0.1 mM EDTA pH 8

QIAGEN:

Re-suspension buffer

50mM TRIS-HCl pH 8
10mM EDTA
100 µg / ml RNase A

Lysis buffer

200mM NaOH
1% SDS

Neutralizing buffer

3M potassium acetate pH 5.5

Elution buffer

1.25M NaCl;
50mM TRIS-HCl pH 8.5
15% isopropanol

RNA Extraction

Phosphate Buffered Saline
For 2 litres:

NaCl	160 g
Na_2HPO_4	23 g
KCl	4 g
KH_2PO_4	4 g

Solution A:

Mix in ratio of 1:1:0.1 the following:

- water saturated phenol/tris saturated phenol pH 7.0
- Solution B: Guanidine thiocyanate 4M (50 g/ 100 ml)
  Tri-sodium citrate 25mM (0.735 g/ 100 ml)
  pH 7.0
- NaOAc pH 4.0

Plus 750 μl Beta-mercaptoethanol/100ml of solution (Sterile glassware)

50x TAE (1000mls)

Tris	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

(pH 7.6, with conc HCl or NaOH)

Northern Blotting

STOP Solution

0.5% bromophenol blue
1 mM EDTA pH 8.0
50% glyerol

20X MOPS (2000ml)

0.2 M MPA	83.6 g
50 mM Sodium Acetate	13.6 g
10 mM EDTA	7.44 g
	Adjust to pH 7

Gel (125 mls):

Agarose (1.2%)	1.5 g
DEPC-H_2O	96 ml
MOPS (20x)	6 ml
Formaldehyde	21.5 ml
Ethidium bromide	1.5µl

Sample buffer (20.2µl):

20x MOPS	0.5 µl
STOP	5.7 µl
Formamide	10 µl
Formaldehyde	3.5 µl
DEPC H_2O	0.5 µl

20X SSC (1000ml)

3 M Sodium Chloride	175.3 g
300 mM Sodium Citrate	88.2 g
Adjust to pH 7	

20X SSPE (500ml)

3.6 M Sodium Chloride	105.2 g
0.02 M EDTA	3.72 g
0.2 M Na Disphpos pH 7.7	15.6 g

Denhart's solution

2% BSA
2% Polyvinylpyrrolidone
2% Ficoll
(In Distilled H_2O)

Re-hybridisation Buffer and Hybridisation Solution

PEG 6000	1.5 g
20X SSPE (or SSC)	6.25 ml
DEPC-H_2O	2.25 ml
10 % SDS	1.25 ml
Denhart's solution	1.25 ml
Formamide - deionized	12.5 ml

Washing of the blot (2X SSPE / 1.5% SDS) – 1000ml:

20x SSPE	100 ml
10% SDS	30 ml
Distilled water	870 ml

RNA Marker Exposure

0.5M NaOAC pH 5.4
0.04% w/v Methyl Blue

Stripping Solution (1000ml)

10%SDS	20 ml
1M Tris HCl pH 7.4	2 ml
(In distilled water)	

ELISA - Fibronectin

Nunc immuno plates: Maxisorp, flat bottom

Rabbit anti-human fibronectin antibody (Sigma F3648)

Standard fibronectin (Sigma F0895)

Mouse monoclonal anti fibronectin (Sigma F7387)

HRP conjugated rabbit anti mouse IgG (DAKO P260)

Citric Phosphate Buffer (See Protocol)**:**

Disodium orthophosphate - $Na_2HPO_4.12H_20$
Hydrogen peroxide - H_2O_2
Citric acid
OPD tablets – fridge

1M Sulphuric acid

Washing buffer (WB)

1x PBS
Tween (0.1%)

Coating buffer pH 9.6

1 x 0.05 M Na_2CO_3
9 x 0.05 M $NaHCO_3$

10 x PBS (2 litres):

NaCl	160 g
Na_2HPO_4	23 g
KCl	4 g
KH_2PO_4	4 g

CHART 1 - Fibronectin Standards

(Use 1 ml of *1/50,000 ng/ml* ministock by adding 50 μl of fibronectin stock)

- Make up 1 ml of *1/2000 ng/ml soln* from by adding 40 μl of ministock (for 1 plate)
- Make up 1 ml of *1/200 ng/ml soln* from by adding 100 μl of 1/2000 ng/ml soln (for 4 plates)
- Make up 150 μl for each sample (giving duplicates of **50 μl/well**)

Sample	**Concentration ng/ml**	**1:200 Soln μl**	**PBS μl**
1	0	0	150
2	25	18.75	131.25
3	50	37.5	112.5
4	100	75	75

Sample	**Concentration ng/ml**	**1:2000 Soln μl**	**PBS μl**
5	250	18.75	131.25
6	500	37.5	112.5
7	750	56.25	93.75
8	1000	75	75
9	1200	90	60
10	1500	112.5	37.5
11	2000	150	0

CHART 2 – Substrate phosphate Buffer

Plates	**2**	**3**	**4**	**5**	**6**
H_2O (ml)	12	18	24	30	36
Citric acid (g)	0.09	0.14	0.18	0.23	0.27
$Na_2HPO_4.12H_20$ (g)	0.29	0.44	0.58	0.73	0.87
OPD tablets	4	6	8	10	12
H_2O_2 (μl)	5	7.5	10	12.5	15

Protein Extraction

50x TE (100ml):

1M Tris	12.1 g	
0.1M EDTA	3.725 g	pH to 7.4

Lysis buffer

Compound	[stock]	Volume
H_2O		9.7 ml
50x TE		200 µl
10 µg/ml Leupeptin (Sigma L2023)	1 mg/ml (H_2O)	100 µl
20 µM E64 (Sigma E3132)	2 mM (H_2O)	100 µl
2 µg/ml Aprotinin	2 mg/ml (H_2O)	10 µl
1 µM Pepstatin A (Sigma P5318)	1 mM (DMF)	10 µl
50 mM Sodium fluoride (Na F)		21 mg
2.5 mM Sodium orthovanadate ($Na_3 VO_4$)		4.6 mg
62.5 mM β-glycerophosphate		135 mg
0.1 % Triton X-100		10 µl
Immediately prior to use:		
1 mM PMSF	100 mM (propan-2-ol)	100 µl

2x Reducing Buffer (16 ml)

H_2O	800 µl
1 M Tris pH 6.8	2 ml + 2 ml H_2O (Stacking buffer)
Glycerol	3.2 ml
10% SDS	6.4 ml
0.2% Bromophenol blue	Tiny amount

2x reducing sample buffer (8 ml) - used for Westerns

Water	4.0 ml
0.5 M Tris-HCl pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
0.2% (w/v) bromophenol blue	0.2 ml
2-β mercaptoethanol	0.4 ml
Store at room temperature	

2% Na_2CO_3

BSA (10 mg/ ml to 1 mg/ ml)

1% $CuSO_4.5H_2O$

2% K/Na Tartrate

Western Blotting

Ponceau S Make up as 2 ml per 100 ml (Distilled H_2O)

10 ml of 10 % Polyacrylamide running gel:

Water	4.0 ml	
30 % acrylamide	3.3 ml	
Buffer A (1.5 M Tris, pH 8.8)	2.5 ml	
10 % SDS	100 µl	
10 % APS	100 µl	} Add immediately
TEMED	4 µl	} before pouring

10 ml of 5% Polyacrylamide stacking gel

Water	6.8 ml	
30% acrylamide	1.7 ml	
Buffer B (1.0M Tris, pH 6.8)	1.25 ml	
10% SDS	100 µl	
10% APS	100 µl	} Add immediately
TEMED	10 µl	} before pouring

10x Upper Electrode Buffer (1000ml)

Tris	30.28 g
Glycine	144 g
SDS	1.9 g
Water	Do not adjust pH (≈8.6)

10x Lower Electrode Buffer (1000ml)

Tris	60.56 g
Water	pH to 8.3 with HCl

Blotting Buffer (1000ml):

39 mM glycine	2.9 g
48 mM Tris	5.8 g
0.037% SDS	0.39 g
20% methanol	200 ml
Water	

Nitrocellulose membrane (0.45μm Protein Nitrocellulose, Cat. No. 401196, Schleicher & Schnell [distributed by Anderman % Co.])

Strip Solution (500ml)

62.5 mM Tris	3.78 g
2 % (w/v) SDS	1 g
Distilled water	

β-mercaptoethanol (final concentration - 100 mM) added prior to use

PBS-T (500ml):

Na_2HPO_4	5.75 g	
NaH_2PO_4	1.48 g	
NaCl	2.92 g	
0.1% Tween-20	0.5 ml	pH 7.5

TBS-T (500ml):

20 mM Tris	2.42 g	
137 mM NaCl	8 g	
1 M HCl	3.8 ml	
0.05% Tween-20	0.5 ml	pH 7.6

Blocking buffer 10% Marvel in PBS-T

Antibody Buffer 5% Marvel in PBS-T

ECL Reagents Reagents A & B mixed equally – Use 500 μl to 750 μl

Radioligand binding Studies

50x TE (100ml):

1M Tris	12.1 g	
0.1M EDTA	3.725 g	pH to 7.4

Lysis Buffer:

Compound	[stock]	Volume
H_2O		9.7 ml
50x TE		200 µl
10 µg/ml Leupeptin (Sigma L2023)	1 mg/ml (H_2O)	100 µl
20 µM E64 (Sigma E3132)	2 mM (H_2O)	100 µl
2 µg/ml Aprotinin	2 mg/ml (H_2O)	10 µl
1 µM Pepstatin A (Sigma P5318)	1 mM (DMF)	10 µl
50 mM Sodium fluoride (Na F)		21 mg
2.5 mM Sodium orthovanadate (Na_3 VO_4)		4.6 mg
62.5 mM β-glycerophosphate		135 mg
0.1 % Triton X-100		10 µl
Immediately prior to use:		
1 mM PMSF	100 mM (propan-2-ol)	100 µl

Binding buffer (1000 ml):

100 mM NaCl	5.84 g
50 mM Tris – HCl	6.1 g
10 mM KCl	0.75 g
5 mM $MgCl_2$	1.02 g
0.5 mg /ml Bacitracin (Amersham US)	
0.25% BSA	2.5 g

pH 7.4

Harvest Solution (1000 ml):

0.1% SDS	1 g
0.1 M NaOH	4 g

Immunocytochemistry

PBS pH 8.5

TBS/ 0.5% Triton X-100

Tyrode Buffer (Glucose 5mmol/l) - 500 mls:

135 mM Sodium Chloride (NaCl)	3.94 g
5 mM Potassium Chloride (KCl)	0.186 g
0.33 mM Sodium Dihydrogen Phosphate (NaH_2PO_4)	0.026 g
5 mM Glucose	0.45 g
1 mM Magnesium Chloride ($MgCl_2$)	0.102 g
10 mM HEPES	1.30 g
pH to 7.3-7.4	

Flow cytometry

P/B/A (500ml)

Phosphate Buffered Saline	500 ml
0.1% Bovine Serum Albumin	0.5 g
0.05% Sodium Azide	0.25 g

Appendix B - Presentations and Publications

PUBLICATION

Stanley AG, Patel H, Knight AL, Williams B. Mechanical strain-induced vascular matrix synthesis: The role of Angiotensin II. Journal of the Renin-Angiotensin-Aldosterone System 2000; 1:32-5.

ABSTRACTS – Personal Oral Presentations

Stanley AG, Patel H, Williams B. Mechanical Strain increases matrix synthesis in cultured Human Vascular Smooth Muscle Cells via an Angiotensin II-Dependent Pathway. J Hypertens. 1999; 17(S3):S18. (Personal oral presentation - EHS)

Stanley A, Patel H, Williams B. Evidence for the role of Angiotensin II and Mechanical Strain in vascular matrix production by human vascular smooth muscle cells. J Human Hypertens. 1999; 13:4. (Personal oral presentation - BHS).

Stanley A, Patel H, Williams B. Synergy between Angiotensin II and Mechanical Strain in matrix synthesis by human vascular smooth muscle cells. Journal of the Renin-Angiotensin-Aldosterone System. 2000; 1:57. (Personal oral presentation - AIIA).

Stanley AG, Knight AL, Williams B. Mechanical Strain sensitizes Human Vascular Smooth Muscle Cells to Angiotensin II. Am J Hypertens. 2000; 13(No 4(2)):12A. (Personal oral presentation - ASH).

Stanley A, Varo N, Patel H, Williams B. Mechanical Strain promotes Angiotensin II-induced Matrix Metalloproteinase-2 Activity in Human Vascular Smooth Muscle Cells. Am J Hypertens. 2000; 13(No 4(2)):26A. (Personal oral presentation - ASH).

Stanley AG, Patel H, Knight AL, Williams B. Mechanical Strain Upregulates the AT1 Receptor and sensitizes Human Vascular Smooth Muscle Cells to Angiotensin II. J Human Hypertens. 2000; 14:2. (Personal oral presentation - BHS).

Stanley AG, Patel H, Knight AL, Williams B. Mechanical Strain induces Vascular Matrix Synthesis by Upregulation of the AT1 Receptor in Human Vascular Smooth Muscle Cells. Circ. 2000; 102:1105 (Personal oral presentation - AHA).

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