Hypoxia-mediated human pulmonary arterial fibroblast proliferation is dependent on p38 mitogen-activated protein kinase activity.

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MD Thesis submitted for examination at the University of Glasgow, UK.

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Abstract

Background:

Pulmonary hypertension (PH) is a rare condition that can occur as a primary disease process, Idiopathic Pulmonary Hypertension (IPH) or secondary to other disorders. In Familial IPH mutations have been identified in the bone morphogenetic protein receptor II gene (BMPRII) (chromosome 2q32-31) a member of the Transforming Growth Factor β (TGFβ) (Lane *et al*, 2000). Despite the mutation being present in all cells, vascular wall remodelling is only seen in the pulmonary circulation with marked thickening of the intima and neointimal formation, muscularisation of small-generation resistance vessels and thickening of the adventitial layer together with increased ECM deposition. Similar appearances are noted in the pulmonary circulation's response to hypoxia. Prolonged exposure of the pulmonary circulation to hypoxia results in vasoconstriction and subsequent vascular wall remodelling.

The hypothesis of this work is that the pulmonary circulation's response to hypoxia may be partially explained by the existence of differences in cell signalling pathways in adventitial fibroblasts from pulmonary and systemic arteries. Studies from the

Scottish Pulmonary Vascular (SPVU) Laboratory have shown that pulmonary arterial fibroblasts (PAFB) in bovine and rat models of acute hypoxic exposure preferentially proliferate to hypoxia, whereas systemic arterial fibroblasts (SAFB) do not, that the stress mitogen activated protein kinase p38 MAPK is consistently activated in PAFB exposed to acute hypoxia, and is constitutively upregulated in PAFB cultured from rats exposed to chronic hypoxia (Welsh *et al*, 1998; Welsh *et al*; 2001). This response to hypoxic exposure has been shown to be dependent on p38 MAPK activity, as use of SB203580 can block the hypoxia-mediated proliferative response to acute hypoxia (Scott *et al*, 1998; Welsh *et al*, 2001).

Aims and methods:

We wished to establish whether the pro-proliferative response of PAFB to acute hypoxic exposure previously noted in bovine and rat models could also be demonstrated in a human model. We wished to establish a role for both classic MAPK and stress MAPKs in hypoxia-mediated PAFB proliferation. We also wished to examine the role of hypoxia inducible factor 1 (HIF1) in human arterial fibroblast responses to acute hypoxia. There is a body of literature that documents cross talk between p38 MAPK and the Bone Morphogenetic Protein (BMPR) signalling pathways. We wished to establish whether Smad proteins (involved in the downstream signalling cascade from BMPR) might play a role in human pulmonary and systemic arterial fibroblast proliferation to acute hypoxia.

Following approval from the local Ethics Committee, PAFB were harvested from patients undergoing lobectomy for the treatment of lung cancer. Left internal mammary arteries (SAFB) were harvested from patients undergoing coronary artery

bypass grafting. Cells from systemic and pulmonary arterial fibroblasts were grown in conditions of normoxia or acute hypoxia (PO_2 35 mmHg ~ 5% O_2). Cellular proliferation was assessed using [^{3}H]Thymidine uptake as a surrogate. p38, p44/p42 - ERK1/2 and JNK MAPKs and Smad protein activity was assessed using Western Blotting Techniques with the use of appropriate primary and secondary antibodies and Chemiluminescence to detect the presence of protein. p38 MAPK isoform activity was assessed using Catch and Release® immunophoresis techniques.

Findings and conclusions:

We demonstrated that acute hypoxic exposure results in human PAFB proliferation, associated with increased p44/p42 – ERK 1/2 MAPK activity, but *dependent* on p38 MAPK α activity. We also found that the p38 MAPK γ isoform was expressed in human PAFB following hypoxic exposure but this did not appear to be involved in the hypoxia-mediated proliferative response. p38 MAPK α activity appeared to occur in a bi-phasic pattern with peaks of activity at t = 6 and 16 hours, the second peak was found to be responsible for the hypoxia-mediated proliferation seen in these cells in agreement with previous work from the SPVU laboratory (Scott *et al*, 1998; Welsh et al., 2001). The second peak in p38 MAPK α activity was synchronous with peak HIF1α activity (between t = 8 –16 hours). We demonstrated that HIF1α activity can be abrogated by pre-incubation of human PAFB with SB203580 suggesting a mechanistic link between p38 MAPK α activation and HIF1α in a human model of acute hypoxic exposure.

We have also demonstrated that that BMPR2-associated Smad 1, 5 and 8 activation is increased in hypoxic human SAFB, suggestive of the activation of an *anti-*

proliferative pathway in these cells that is not associated with p38 MAPK activity. To our knowledge this is the first demonstration of an active response in SAFB to acute hypoxic exposure that involves the *active* upregulation of an anti-proliferative pathway in these cells. In addition we have demonstrated that in hypoxic pulmonary arterial fibroblasts phospho Smad 1, 5 and 8 expression is reduced (suggestive of the down-regulation of an anti-proliferative pathway) and can be further abrogated by pre-incubation with SB203580. This suggests that in SAFB Smad 1, 5 and 8 activation occurs independent of p38 MAPK activation while in PAFB, p38 MAPK activity augments Smad 1, 5 and 8 activation.

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Table of contents **Page number**

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I would also like to thank John Gough, my family and friends without whose support this work would not have been possible.

Preface:

I would like to confirm that the following piece of work is my own. It is however based on work previously performed in the Scottish Pulmonary Vascular Laboratory by Drs Pam Scott and David Welsh whose results are discussed in chapters 1 and 6 of this thesis.

Dr David Welsh has shown me how to perform various laboratory techniques including primary cell culture, SDS Page Electrophoresis, immunophoresis and [^{3}H]Thymidine uptake assays.

Dr David Welsh has helped me with data analysis, presentation and statistical analysis.

Publications arising from this work:

Chapters:

Mortimer H, Morse H & Peacock A, 'The genetics of Pulmonary Hypertension', in Pulmonary Hypertension, eds. Peacock A & Rubin L, 2nd Edition, Arnold (2004) pp 62-70.

Papers:

Mortimer H, Patel S & Peacock A, (2004) 'The genetics of High Altitude Pulmonary Oedema', Pharmacology and Therapeutics. 101; 183-192.

Welsh D, Mortimer H, Kirk A & Peacock A, (2005) 'The Role of p38 Mitogen-Activated Protein Kinase in Hypoxia-Induced Vascular Cell Proliferation: An Interspecies Comparison', Chest; 128; 573-574.

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Table of figures and tables:

Chapter 1 – Introduction/Hypothesis

Chapter 2 – Methods and Materials

Chapter 3 – To examine the proliferative behaviour of human pulmonary and systemic arterial fibroblasts under normoxic and acute hypoxic conditions

Chapter 4 – To investigate the relationship between hypoxia-mediated proliferation and stress activated protein kinase (SAPK) signalling in human pulmonary and systemic arterial fibroblast cells.

Chapter 6 – Discussion/Thesis

Chapter 1:

1.1.1. Introduction and Hypothesis

Pulmonary hypertension (PH) is a rare condition that can occur as a primary disease process, Idiopathic Pulmonary Hypertension (IPH) or secondary to other disorders such as congenital heart disease, thyroid dysfunction, liver cirrhosis with portal hypertension, HIV infection or hypoxia. The development of increased pulmonary vascular resistance results in elevated pulmonary artery pressures leading to right ventricular failure. Untreated survival rarely exceeds three years (D'Alsonzo *et al*, 1991).

The diagnosis of PH requires the demonstration of elevated mean pulmonary arterial pressure in excess of 25 mmHg at rest, or 30 mmHg on exercise, during right heart catheterisation (RHC). At The World Symposium on Pulmonary Hypertension held in Venice 2003 PH was re-classified with Group 1 diagnoses including IPH, Familial PH (FPAH) and porto-pulmonary PH but in addition Groups 2 – 4 included many causes of PH not primarily the result of pulmonary vascular pathology such as PH associated with left-sided heart disease (Simonneau *et al*, 2004). The rationale for this was the importance of making a definitive diagnosis in order to establish an appropriate treatment plan. **Table 1.1** details the classification system currently in use for the diagnosis of PH.

Currently there are broadly 3 groups of therapeutic agents for the treatment of PH: prostaglandins including epoprostanol, treprostinil and iloprost, endothelin receptor antagonists such as bosentan, ambrisentan and sitaxentan and phosphodiesterase inhibitors such as sildenafil and tadalafil. Treatment aims to reduce pulmonary

1. Pulmonary arterial hypertension (PAH)
1.1. Idiopathic (IPAH)
1.2. Familial (FPAH)
1.3. Associated with (APAH):
1.3.1. Connective tissue disease
1.3.2. Congenital systemic to pulmonary shunts
1.3.3. Portal hypertension
1.3.4. HIV infection
1.3.5. Drugs and toxins
1.3.6. Other (thyroid disorders, glycogen storage disease, Gaucher's disease, hereditary haemorrhagic telangiectasia, haemoglobinopathies, myeloproliferative disorders, splenectomy)
1.4. Associated with significant venous or capillary involvement
1.4.1. Pulmonary veno-occlusive disease (PVOD)
1.4.2. Pulmonary capillary haemangiomatosis (PCH)
1.5. Persistent pulmonary hypertension of the newborn (PPHN)
2. Pulmonary hypertension associated with left heart diseases
2.1. Left-sided atrial or ventricular heart disease
2.2. Left-sided valvular heart disease
3. Pulmonary hypertension associated with lung respiratory diseases and/or hypoxia
3.1. Chronic obstructive pulmonary disease
3.2. Interstitial lung disease
3.3. Sleep disordered breathing
3.4. Alveolar hypoventilation disorders
3.5. Chronic exposure to high altitude
3.6. Developmental abnormalities
4. Pulmonary hypertension due to chronic thrombotic and/or embolic disease
4.1. Thromboembolic obstruction of proximal pulmonary arteries
4.2. Thromboembolic obstruction of distal pulmonary arteries
4.3. Non-thrombotic pulmonary embolism (tumour, parasites, foreign material)
5. Miscellaneous
Sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels (adenopathy, tumour, fibrosing mediastinitis)

Table 1.1 Venice Classification of Pulmonary Arterial Hypertension. (Simonnaeu *et al*, 2004)

arterial vasoconstriction and prevent vascular remodelling, improving exercise capacity and quality of life. In addition calcium channel blockers (CCB) such as nifedipine are used in the small proportion of patients who prove to be vasodilator responders on RHC (a fall in mean PAP – PAPm of greater than 10mmHg to a level less than 40 mmHg with inhalation of nitric oxide or intravenous administration of other agents such as adenosine) together with oxygen therapy, anticoagulation and diuretics (Lee *et al*, 2005). **Table 1.2** details the current treatment algorithm in use for PH.

1.2. Pulmonary and Systemic Vascular Response to Injury

1.2.1 Histological changes in pulmonary hypertension.

In PH regardless of disease aetiology, the histological changes seen in the pulmonary vasculature appear to be consistent. The vascular wall consists of three layers; the intima, a single layer of endothelial cells (EC) on a basement membrane, the media, vascular smooth muscle cells (VSMC) in a matrix of connective tissue and the adventitia, composed of fibroblasts (FB) and extra cellular matrix (ECM). In PH there is marked thickening of the intima and neointimal formation, muscularisation of small-generation resistance vessels and thickening of the adventitial layer together with increased ECM deposition (**figure 1.2**). The extent of these structural changes varies throughout the pulmonary circulation with medial expansion most marked in the small diameter resistance vessels (< 150μm diameter) and thickening of the adventitia apparent in the larger conduit vessels (Stenmark *et al*, 1999). This results in a narrowed vessel lumen and reduced vessel compliance with secondary elevation

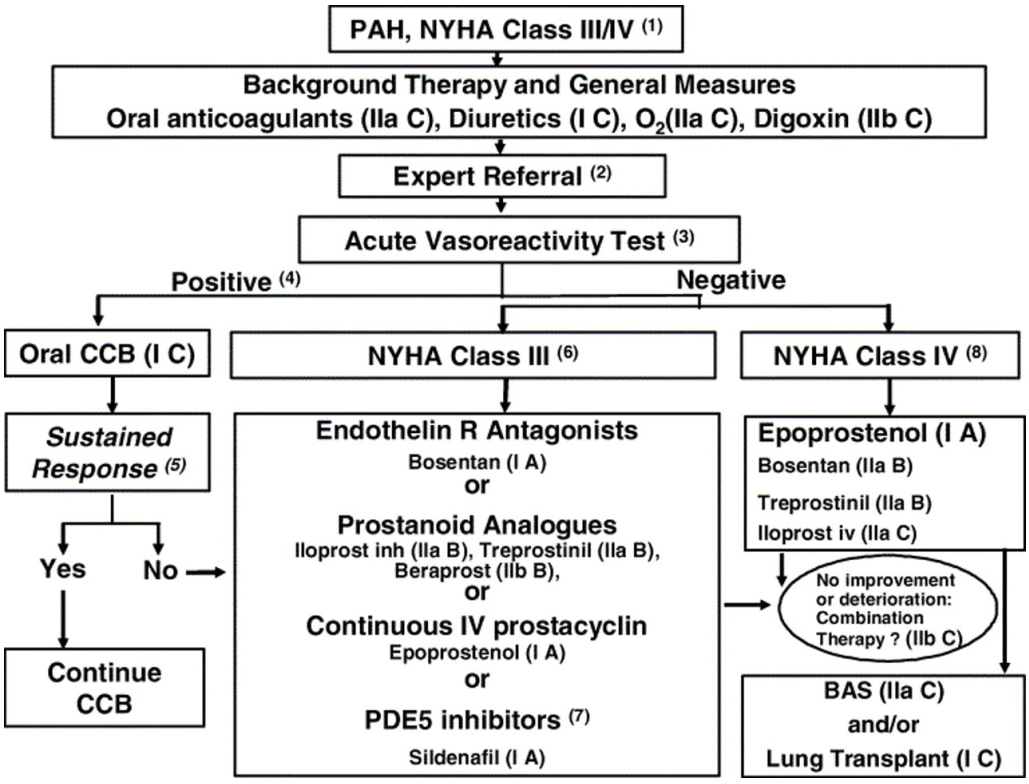


Figure 1.1 Treatment algorithm for pulmonary arterial hypertension (PAH), (Galie *et al*, 2004)

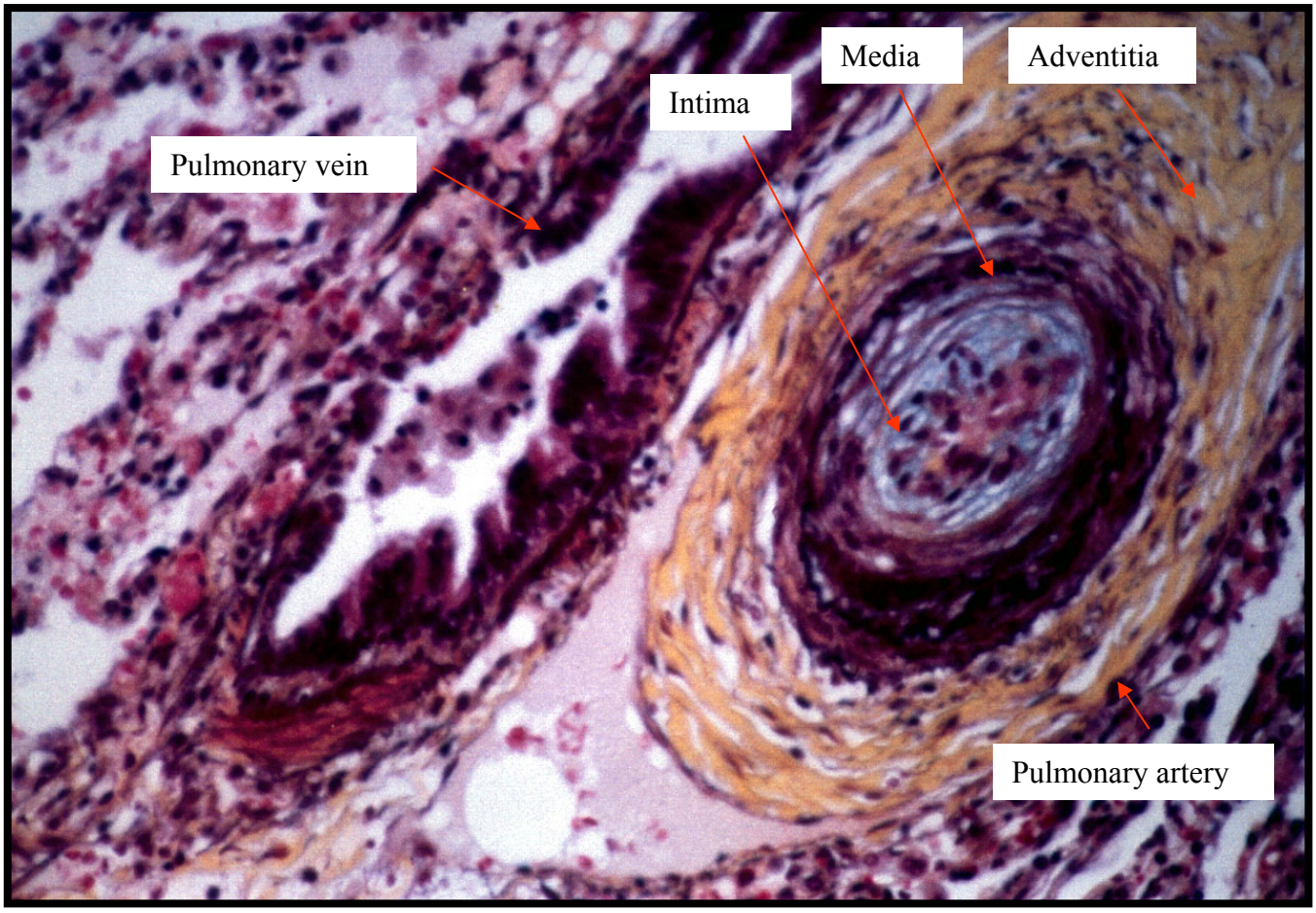


Figure 1.2: Section of pulmonary arterial and vein wall taken from a patient with Familial Pulmonary Arterial Hypertension. This demonstrates marked thickening of the intima and neointimal formation, muscularisation of small-generation resistance vessels and thickening of the adventitial layer together with increased ECM deposition. The adjacent pulmonary vein does not demonstrate these changes.

of pulmonary artery pressure, hypoxaemia (low arterial oxygen tension) and subsequent polycythaemia (over production of red blood cells).

1.2.2. The systemic circulation's response to hypertension.

In a biological context remodelling can be defined as an increase in the number of cells, size or mass of a structure. It may either be appropriate, as in fetal development or growth, or inappropriate, when change continues or persists to the disadvantage of physiological function. Evidence of remodelling in response to altered environment is seen in both the pulmonary and systemic vasculature. Remodelling in pulmonary hypertension appears to involve all layers of the vascular wall; in the systemic circulation the response to injury seems to occur more focally in the intimal and medial layers (Stenmark *et al*, 1997). Intimal hyperplasia with migration of VSMC into the neointimal layer has been demonstrated in vein grafts following coronary artery by-pass surgery, vessels supplying transplanted organs, in arteriosclerotic vessels and in coronary arteries following angioplasty (Newby and Zaltsman, 2000). Remodelling of the vascular wall is also seen in systemic hypertension (Su *et al*, 2001). Changes are variable but occur in the medial and intimal layers in both conduit and resistance arteries.

In systemic hypertension 'remodelling' is considered by some investigators to imply altered shape without an increase in cellular proliferation (Baumbach *et al*, 1993). Mulvany *et al* (1996) detail a highly complex classification of systemic vascular 'remodelling'. They define it as either hypertrophic, (an increase in medial thickness as a result of cellular hypertrophy with subsequent narrowing of the vessel lumen); eutrophic remodelling, (no alteration in medial thickness but involving a reduction in

both external and internal vessel diameter); and hypotrophic remodelling where there is loss of medial wall thickness and reduction in vascular lumen diameter. Mulvany *et al* (1996) hypothesised that the character of vascular wall remodelling may be specific to vessel generation. Work by Dunn *et al* (1998) suggested that patterns of vascular remodelling are not so easily generalised: they demonstrate significant histological differences between 'remodelled' vessels of the same generation - from the mesenteric and cerebral circulations - in genetically hypertensive rats.

In summary, both systemic and pulmonary circulations can remodel as a result of hypertension but vessel wall remodelling can also cause hypertension. However it seems that while pulmonary arteries appear to respond in a histologically consistent manner regardless of aetiology, there is considerable variation in patterns of systemic artery remodelling.

1.2.2. A comparison of pulmonary and systemic arterial response to hypoxia

Von Euler and Liejstrand first demonstrated pulmonary artery vasoconstriction to hypoxia in 1946 using a cat model. The response to hypoxia seemed to be two-fold: acute hypoxia resulted in rapid onset of pulmonary artery vasoconstriction while chronic hypoxia resulted in vascular wall remodelling and the eventual development of pulmonary hypertension. The acute vasoconstrictive response was mediated by an increase in VSMC tone while the chronic changes occurred as a result of increased cellular proliferation and ECM deposition (Meyrick and Reid, 1979). Pulmonary artery vasoconstriction is important in fetal life to maintain blood flow through the *foramen ovale* to the systemic circulation from the placenta. The retention of this response in the adult may assist with ventilation-perfusion matching under hypoxic

conditions (Voelkel, 1996) and as such the development of hypoxic pulmonary hypertension may be viewed as an adaptive response that becomes harmful to the organism (Strauss and Rabinovitch, 2000).

In contrast to the pulmonary circulation, the systemic vascular response to hypoxaemia is marked vasodilatation together with increased cardiac output in order to maintain systemic blood pressure (Wagner and Mitzner, 1988). This is mediated in part by glomus type I cells in the carotid body, which sense partial arterial oxygen pressure (P_aO_2), and by the central respiratory centre which monitors partial arterial carbon dioxide pressure (P_aCO_2) by means of cerebrospinal fluid pH. Peripheral vasodilatation in response to hypoxia serves to increase oxygen supply to hypoxic tissues.

In summary, despite superficial histological similarities in the response to hypertensive injury, there are significant differences between the pulmonary and systemic circulations. They 'remodel' differently in response to a sustained increase in blood pressure. They also behave differently to hypoxic challenge in order to respond appropriately to the physiological demands placed upon them.

1.3.1. Pulmonary vascular response to hypoxia: *Inter*species variation.

The pulmonary vascular response to hypoxia appears to be maintained across all mammals but the extent of this response varies between species. Tucker and Rhodes (2001) distinguished between hyper-responders, species having rapid and dramatic elevations of pulmonary artery pressures (PAPs) on hypoxic exposure: e.g.: cattle and pigs; moderate responders: humans and other primates, rats, mice and cats; and hypo-

responders, those showing little increase in PAPs: camelids (llama, vicunas etc.), sheep, goats and dogs.

A possible explanation for the development of hypoxic pulmonary hypertension lies in the absence of collateral ventilation (CV) leading to pulmonary arteriolar muscularisation. Collateral ventilation is defined in man as the ventilation of alveolar structures through passages or channels that bypass the normal airways (Cetti *et al*, 2006). In man resistance to airflow through these structures is approximately 50 times higher than through normal airways and CV is not thought to have a functional role. CV can occur across inter-alveolar pores, accessory bronchiole-alveolar communications and accessory respiratory bronchioles (Krahl, 1959; Lambert, 1955). Collateral ventilation is variably present in mammals; dogs and sheep exhibit collateral ventilation, horses and man to a lesser extent whereas cattle and pigs do not (Kuriyama *et al*, 1981; Kuriyama *et al*, 1984; Robinson *et al*, 1978). The lack of CV would require dependence on hypoxia-mediated vasoconstriction to protect P_aO_2 in the context of regional hypoxia within the lung resulting in a more muscularised pulmonary arterial media at sea level. Mammals with CV would theoretically be able to preferentially ventilate relatively hypoxic portions of the lung via accessory pathways. This has been demonstrated experimentally in a canine model – a species known to exhibit CV – in comparison to a porcine one (known to lack CV) (Kuriyama, 1984).

Species with highly muscularised media at sea level such as cattle and pigs develop severe pulmonary hypertension on hypoxic exposure whereas those lacking a muscular media, such as camelids, seem more resistant to the effects of relative

hypoxia. Experimental data from the coati, a South American mammal with a highly muscularised arteriolar wall which is thought to lack CV at sea level (therefore theoretically expected to develop PH on hypoxic exposure), provides conflicting data. Hanson *et al* (1993) demonstrated that coati develop acute hypoxic pulmonary hypertension but indices suggestive of PAH: for example histological changes in the pulmonary artery muscularis or evidence of right ventricular hypertrophy, were lacking in animals exposed to chronic alveolar (A) hypoxia (10% P_AO_2 for 6 weeks). The vasoconstrictor response to acute hypoxia however was retained in the chronically hypoxic coati (Hanson *et al*, 2000). It appears that in some species the response to acute hypoxic exposure does not predict the response to chronic hypoxia.

Work from the Scottish Pulmonary Vascular Laboratory has focused on the need to establish a valid cellular experimental model for human hypoxic pulmonary arterial hypertension. Models investigated include bovine, chronically hypoxic rat and human; and have specifically looked at the behaviour of classical (ERK 1/2 ~ p44/p42 mitogen activated protein kinases or MAPKs) and stress activated protein kinases (p38 and JNK MAPK) – enzymes involved in stress and growth responses - in pulmonary artery fibroblasts (**Table 1.2**). There are a variety of activation patterns for both ERK and JNK MAPK but p38 MAPK is consistently activated in all models investigated so far.

1.4.1. Pulmonary vascular response to hypoxia: *Intra*species variation.

Evidence also exists for *intra*species variation in response to both acute and chronic hypoxic exposure. Only a proportion of climbers develop high altitude pulmonary oedema (HAPE) – non-cardiogenic pulmonary oedema - or cerebral oedema,

Table 1.2: Summary of Pulmonary Vascular Unit findings: cross species differences in responses to hypoxia

Species	Fibroblast	Proliferation to Hypoxia	p38 phosphorylation to hypoxia	ERK phosphorylation to hypoxia	JNK phosphorylation to hypoxia
Human	**Pulmonary**	**YES**	**YES**	YES	NO
	Systemic	NO	NO	NO	NO
Bovine	**Pulmonary**	**YES**	**YES**	NO	**YES**
	Systemic	NO	NO	NO	NO
Rat	**Pulmonary**	**YES**	**YES**	YES	NO
	Systemic	NO	NO	NO	NO

Table 1.2: Summary of Pulmonary Vascular Unit findings: cross species differences in responses to hypoxia. All three adult species so far investigated demonstrate pulmonary arterial fibroblast proliferation to hypoxic exposure whereas systemic arterial fibroblasts do not. While there is a variable expression pattern for ERK and JNK MAPKs in adult human, bovine and rat models there is consistent activation of p38 MAPK associated with hypoxic pulmonary arterial fibroblast proliferation in all three adult models.

suggesting a variable response in man to high altitude-induced hypoxia (Sartori *et al*, 2000; Basnyat *et al*, 2003). This is supported by the work of Morrell and colleagues who found that only 6% of Kyrghyz Highlanders develop symptomatic chronic mountain sickness (CMS) – the association of polycythaemia, hypoxaemia and right ventricular failure. If ECG data are taken into consideration 14% of high altitude residents demonstrate evidence of right ventricular strain as a result of chronic hypoxaemia (Morrell *et al*, 1999; Aldashev *et al*, 2000).

1.4.2. Response to hypoxia in man: Phenotypic variation and genetic polymorphisms.

In a small study comparing right heart catheter data from native Tibetans and low altitude Chinese, at rest and on exercise, Groves *et al* (1993) found that the majority of those Tibetans examined had normal pulmonary artery pressures at rest in comparison to the low altitude natives. This perhaps suggests that long-term residence of a population at altitude may predispose towards greater tolerance of hypoxia and hypoxaemia. There is a considerable body of work in the literature that investigates the potential genetic influences on the response to acute and chronic hypoxia.

1.4.2.1 Genetic polymorphism – Candidate genes

i ACE genotype

Following the association of the Angiotensin Converting Enzyme Insertion genotype (ACE I) with enhanced athletic performance (Gayagay *et al*, 1998; Montgomery *et al*, 1998; Myerson *et al*, 1999) and with enhanced performance at high altitude (Woods *et al*, 2002) there has been interest in its potential role in hypoxic adaptation and hypoxic

lung disease in man. ACE, a component of the renin-angiotensin system (RAS), is involved with fluid balance and sodium excretion. It acts by converting angiotensin I (ATI) to the highly active angiotensin II (ATII) causing systemic vasoconstriction and aldosterone secretion. ACE is particularly abundant in the lung and most conversion of AT1 to ATII occurs there. The gene for ACE is located on chromosome 17q23 and takes the form of an insertion (I) or deletion (D) genotype. Homozygotes for the ACE I/I genotype have low levels of circulating ACE while the D/D (deletion) genotype is associated with high ACE levels (Rigat *et al*, 1990). High levels of circulating ACE, causing increased fluid load, could potentially be associated with a worse prognosis in hypoxic pulmonary hypertension.

There have been several studies in differing populations attempting to associate ACE I/D genotype with hypoxic lung diseases such as chronic obstructive pulmonary disease (COPD). The results have been conflicting. Some studies have found a positive association of the ACE D/D genotype with lower PAPs in COPD – suggesting that homozygotes for the deletion genotype are more able to adapt to chronic hypoxia (Abraham *et al*, 1995). Other studies found that the D/D genotype was associated with more severe hypoxic pulmonary hypertension in patients with COPD (Kanazawa, 2002). In the Kyrghyz Highlanders Morrell *et al* (1999) found a positive association between the ACE I/I genotype and the development of CMS. At first this seems surprising given that the I/I genotype is found more commonly in elite mountaineers who have climbed above 7000m without the aid of supplemental oxygen (Montgomery *et al*, 1998) and the finding that on cardiopulmonary exercise testing (CPET) those individuals with I/I genotype had higher minute ventilation and lower end tidal P_ACO_2 when breathing O_2 at a concentration of 12.5% (Patel *et al*,

2003). A possible explanation is Woods' *et al* (2002) finding that ACE genotype only influences performance during rapid ascent. Acute hypoxic exposure causes an imbalance in the ratio between plasma aldosterone and plasma renin while chronic hypoxic exposure results in down-regulation of AT-1 receptor. Morrell *et al* (1999) suggest that the association between CMS (and therefore high altitude PH) and the I/I genotype probably rests on the linkage of this gene with another that predisposed towards the development of PH.

ii Endothelial Nitric Oxide Synthase

Another gene potentially linked with a variable response to hypoxia is that for endothelial Nitric Oxide Synthase (eNOS). So far two polymorphisms have been identified on the NOS gene (chromosome 7q35-6) which may be associated with the development of High Altitude Pulmonary Oedema (HAPE). The association of eNOS polymorphisms with high altitude illness has only been identified in a Japanese population (Droma *et al*, 2002) and not in Europeans (Bartsch *et al*, 2002; Smith *et al*, 2006). Droma *et al* have also identified a higher incidence of wild-type alleles Glu298Asp and eNOS4b/a in Sherpa Nepalis in Kathmandu, which they suggest might be beneficial to high altitude adaptation. They did not find a correlation between eNOS genotype and serum NO suggesting that eNOS polymorphisms are not a reliable indicator of endogenous NO production (Droma *et al,* 2006).

iii Serotonin (5-HT) hypothesis and Serotonin transporter (5-HTT) polymorphisms

Following two separate 'out-breaks' of pulmonary hypertension associated with the use of anorexigens such as dexfenfluramine and fenfluramine (amphetamine

derivatives which interfere with neuronal uptake of serotonin or 5-hydroxytryptamine) in the 60's and 80's; there has been interest in how disordered serotonin handling results in pulmonary hypertension. In addition altered serotonin handling has been implicated in the aetiology of pulmonary hypertension by the association between some glycogen storage disorders – in particular von Gierke's syndrome types 1a and 1b which demonstrate abnormal 5-HT handling - and pulmonary hypertension.

In work on chronically hypoxic mice, Eddahibi *et al* (2000) demonstrated that mice homozygous for a deleted copy of the 5HT transporter (5-HTT ~ or serotonin transporter SERT) had maintained hypoxic pulmonary vasoconstriction to acute hypoxic exposure but demonstrated less right ventricular hypertrophy and a reduced right ventricular systolic pressure following two weeks of hypoxic exposure (10% oxygen) in comparison to wild-type mice exposed to the same conditions. The same investigators demonstrated that the use of specific 5-HTT inhibitors (citalopram and fluoxetine) could produce similar results under identical growth conditions in wild-type mice (Eddahibi *et al*, 2001) and that fluoxetine infusion could completely abrogate monocrotaline-induced pulmonary hypertension in Wistar rats (Guignabert *et al*, 2005). Welsh *et al* (2004) demonstrated that both 5HTT and $5HT_{2A}$ receptors were required for PA fibroblast proliferation in a chronically hypoxic rat model as both fluoxetine and ketanserin (a specific $5HT_{2A}$ receptor inhibitor) pre-incubation were required to completely abrogate the proliferative response in this model. Eddahibi *et al* (2006) demonstrated cross talk between pulmonary arterial EC and SMC in a human model, showing that SMC are stimulated to proliferate by supernatant taken from EC cultured in serum-free conditions. The proliferative effects have been ascribed to the action of 5HTT as EC cultured in serum free

conditions with the addition of fluoxetine but not ketanserin significantly abrogated the proliferative effects noted.

The influence of 5HTT polymorphisms on the development of pulmonary hypertension is less clear. The gene for 5HTT is localised to 17q11.1-q12 and consists of 14 exons spanning a 31kb region (Lesch *et al*, 1996). Within the promoter region long (L) and short (S) functional polymorphisms have been identified. The L/L polymorphism is associated with a 2 to 3 times increase in the expression of 5HTT in comparison to the S allele. Eddahibi *et al* (2003) demonstrated that in a population of patients with moderate/severe COPD those carrying the LL genotype for 5-HTT had higher 5-HTT expression and had higher pulmonary artery pressures: the effect of hypoxia on right ventricular systolic pressure was additive. Heterozygotes (L/S genotype) had an indermediate response to hypoxia. The same group demonstrated that L/L expression was present in 56% of patients with pulmonary hypertension undergoing lung transplantation in comparison to controls (undergoing lobectomy or pneumonectomy for either lung volume reduction surgery or for treatment of carcinoma) (Marcos *et al*, 2004; Marcos *et al*, 2005). These data have not been reproduced by other groups who have looked at patients with PAH rather than COPD associated/hypoxic PAH. Willers *et al* (2006) did not find an association between 5HTT L/S allele expression in patients with IPAH, nor did they find an association between 5HTT polymorphisms and Bone Morphogenetic Protein Receptor II (BMPRII) mutations in patients with FPAH. They did however note that patients with FPAH with the L/L allele presented at an earlier age – however there was no difference in survival data. Machado *et al* (2006) found no association between BMRRII mutations and 5HTT polymorphisms in a large cohort of FPAH

patients. There is clear experimental evidence for the influence of both 5HT and 5HTT in the development of pulmonary hypertension in hypoxic and inflammatory models but the genetic basis for this effect is yet to be elucidated.

iv Transforming growth factor β and Bone Morphogenetic Protein Receptor II polymorphisms

Genetic mutations important in the aetiology of pulmonary hypertension include mutations in the bone morphogenetic protein receptor II gene (BMPRII) (chromosome 2q32-31) (Lane *et al*, 2000) in Familial IPH and mutations in the activin-like kinase (Alk-1) gene (chromosome 12q13) and endoglin (ENG) (chromosome 9) (Trembath *et al*, 2001) in hereditary haemorrhagic telangectasia (HHT). All of these receptors are members of the Transforming Growth Factor β (TGFβ) superfamily and are involved in transmembrane signalling of bone morphogenetic protein (BMP) and TGFβ respectively. It is not known how these mutations result in the development of pulmonary hypertension or whether these mutations also affect an individual's ability to adapt to hypoxia. In Familial IPH there is a high incidence of BMPRII mutations in 'unaffected' relatives. This suggests a 'double hit' hypothesis; where a pre-existing abnormality plus another external insult (such as HIV infection or anorexigens use) results in the development of PH. Alternatively, overt Familial IPH could represent the extreme of a disease spectrum as 'unaffected' carriers of the BMPRII mutation demonstrate elevated pulmonary artery pressures on stress echo implying an abnormal but not symptomatic response to exercise (Grunig *et al*, 2000).

1.5. Hypoxia as an experimental model for pulmonary hypertension.

PAH regardless of aetiology, appears to demonstrate histological consistency although there has been debate about whether pulmonary arterial hypertension secondary to hypoxia or hypoxic lung disease can be said to be truly representative of the PAH vascular phenotype. However there is a body of evidence that suggests a small number of individuals with hypoxic lung disease can develop significant PAPs comparable to those seen in patients with PAH associated with other conditions, and that their pulmonary vasculature appears similarly remodelled to those with PAH accepting the absence of plexiform lesions within the endothelial layer (Naeije and Barbera, 2001; Naeije, 2005; Wilkinson *et al*, 1988). In experimental work the hypoxic model of pulmonary hypertension is well established. It is easily reproducible in the laboratory, both *in vivo* and *in vitro*; moreover the effects of hypoxia on the pulmonary circulation can be studied *in vivo* at altitude both in animal and in human models.

The relevance of hypoxic PH has been questioned because PAH associated with hypoxic lung disease - such as chronic obstructive pulmonary disease (COPD) - typically progresses slowly with mean PAPs rarely reaching the extremes seen in IPH or PAH secondary to other causes (Kessler *et al*, 1999; Kessler *et al*, 2001; Oswald-Mammosser *et al*, 2005). However there is evidence that prolonged exposure to hypoxia at altitude can result in severe hypoxic pulmonary hypertension and secondary right ventricular failure (Stenmark *et al*, 1987; Peacock *et al*, 2007). Morrell *et al* demonstrated symptomatic chronic mountain sickness (CMS) in 6% of Kyrghyz Highlanders with 14% showing evidence of right ventricular strain on ECG (Morrell *et al*, 1999; Aldashev *et al*, 2002). In the Everest II study, Groves *et al*

(1987) demonstrated significant elevations of PAPm that were not reversible with reoxygenation following exposure to hypobaric hypoxia for 40 days in previously healthy adult males. Recent work examining the histological changes seen in peripheral pulmonary arteries taken from a cohort of patients undergoing lung volume reduction surgery demonstrated significant intimal and medial expansion together with migration of adventitial fibroblasts into the medial compartment (Sirico *et al*, 2005). These findings differed from those noted in patients undergoing lobectomy for the treatment of lung cancer. Sirico *et al* (2005) did not note any evidence of plexigenic lesion formation within the intimal regions of these arteries – but they did not include patients within their study who had clinical evidence of pulmonary hypertension.

1.6. Location and nature of the oxygen sensor

1.6.1. The role of K^+ channels in the hypoxic response

The distribution of voltage-sensitive potassium channels (K^+) throughout the vascular tree may provide an explanation for the opposing tonal responses of pulmonary and systemic circulations to hypoxic exposure. Several different isotypes of membrane bound K^+ channels have been identified: K_V, K_{Ca2+}, K_{DR}, K_{IR} and K_{ATP} (Brijj and Peacock, 1998; Gurney, 2002). Their function appears to be closely allied to the control of membrane potential (Voelkel, 1997; Dittrich and Daut, 1999). K^+ channels act by maintaining a constant efflux of K^+ from the cell cytosol. This produces a constant negative internal cell potential in comparison with the positive extra cellular milieu, rendering the cell potentially excitable.

1.6.2. Variable distribution of K^+ channels throughout the vasculature

The relative distribution of K^+ channel isotypes varies throughout the vascular tree. The increased frequency of K_{ATP} channels in VSMC in the systemic circulation may provide an explanation for systemic vasodilatation observed in response to hypoxic exposure (Dittrich and Daut, 1999; Weir and Olschewski, 2006). These channels are not oxygen sensitive but open when intracellular ATP levels fall resulting in a hyperpolarized and less excitable cell. Cell contraction is prevented by the failure of intra-cellular Ca^{2+} release and therefore maintains vasodilatation (Archer and Rich, 2000).

The VSMC in the pulmonary circulation express higher numbers of K_V and K_{DR} channels in comparison to systemic VSMC. Hypoxaemia inhibits the K^+ current through these channels causing the cytosol to become more positive relative to the external environment (Archer *et al*, 1986; Archer and Rich, 2000). This results in depolarisation of the SMC and activation of voltage dependent Ca^{2+} channels. The subsequent influx of Ca^{2+} ions plus the release of Ca^{2+} from intracellular stores as a result of increased $[Ca^{2+}]^i$ stimulates SMC contraction causing vasoconstriction (Weir and Archer, 1995). K^+ channels appear to vary in their sensitivity to oxygen and this adds support to their potential role in pulmonary vascular oxygen sensing (Archer *et al*, 1986; Archer *et al*, 2004; Platoshyn *et al*, 2006; Voelkel, 1997).

1.6.3. Interspecies variation in K^+ channel distribution

There is considerable interspecies variation in K^+ channel type, distribution and responsiveness (Ward and Aaronson, 1999). *In vitro*, Yuan *et al* (1998) have shown that hypoxia down-regulates K_V channels in hypoxic rat pulmonary artery VSMC but

not in mesenteric VSMC cells grown in hypoxic conditions. It has been suggested that conduit vessels in the pulmonary circulation (similar to systemic arteries in structure and responsiveness to hypoxaemia) have more K_{Ca2+} and $Ca^{2+}{}_L$ channels (Archer et al, 1986). The response of conduit vessels to hypoxaemia appears to be one of initial vasoconstriction followed by relaxation. Resistance vessels appear to have higher frequencies of oxygen sensitive K_{DR} channels (Franco-Obregon *et al*, 1996) .

1.6.4. Hypoxia induces rapid changes in vascular tone: adventitial location of primary O_2 sensor?

In the altitude/hypobaric model of hypoxic pulmonary hypertension, changes in vascular tone may occur via the sensing of a low alveolar oxygen tension (P_AO_2) rather than a low arterial oxygen tension (P_aO_2) as increases in vascular tone occur within seconds of exposure to hypoxia (Voelkel, 1996). In support of this, patients who are hypoxaemic as a result of right to left intracardiac shunts do not develop pulmonary hypertension unless the shunt is reversed (Eisenmenger's syndrome). The O_2 sensor may be located on the adventitial rather than the endothelial side of the vascular wall. Meyrick and Reid (1979) noted the earliest and most marked structural changes following hypoxic exposure occurred in the adventitial walls of rat pulmonary arteries and not in the media or intima.

1.6.5. Other proposed models for the hypoxic sensor

A review of the literature suggests another two possible models for the cellular O_2 sensor (Zhu and Bunn, 1999). It is possible that the O_2 sensor is either a membrane-bound flavohaem protein or a mitochondrial haem protein, on the basis that cell

responsiveness to hypoxia can be mimicked in normoxic conditions by both nitric oxide (NO) and carbon monoxide (CO), both of which have high affinity for haemoglobin (Zhu and Bunn, 1999). A membrane-bound receptor that binds O_2 could generate reactive oxygen species (ROS) by the reduction of oxygen to superoxide and hydrogen peroxide. The expression of oxygen-sensitive genes might then be altered. For example, H_2O_2/superoxide results in the degradation of the hypoxia inducible factor 1 α (HIF1α) subunit therefore preventing the upregulation of genes involved in the hypoxic response.

Work on the mitochondrial model has demonstrated that hypoxia results in a reduction of mitochondrial respiration, not as a result of decreased ATP use, but by inhibition of a mitochondrial proton pump (Zhu and Bunn, 1999). Mitochondria are significant producers of ROS. A decrease in mitochondrial respiration would result in reduced production of ROS: ROS production has been shown to be proportional to P_aO_2 (Chandel and Schumacker, 2000). However production ROS has also been shown to be upregulated in hypoxaemic lung parenchyma (Voelkel and Tuder, 2000). Unfortunately differences in the experimental models used by investigators make direct comparisons difficult.

Waypa *et al* (2006) argue that hypoxia results in increased ROS production on the basis that they have demonstrated increased DCF (dichlorfluoresceine-diacetate) fluorescence in patch-clamped pulmonary VSMC to hypoxia. Moudgil *et al* (2005) suggest that isolated cultured cells rapidly lose ion channels *ex vivo* and therefore have reduced oxygen sensitivity. In addition they also comment that DCF also detects NO and is able to produce H_2O_2 independently, which may influence the reliability of

DCF as a model to detect ROS experimentally. Irrespective of current theoretical and experimental disagreements – there do appear to be areas of consensus. It appears that hypoxia-mediated ROS generation is dependent on proximal electron transport chain (ETC) complexes, that the ROS required for hypoxia-mediated pulmonary vasoconstriction are derived from mitochondria, that inhibitors of ETC complexes I and III mimic the effect of hypoxia and that hypoxia-mediated production of ROS does not depend on having a completely functional ETC, as cyanide (a complex IV inhibitor) fails to inhibit hypoxia-mediated vasoconstriction.

Michelakis *et al* (2002), argue for mitochondrial diversity as an explanation for the divergent responses of pulmonary and systemic arteries to hypoxia. They demonstrated that whole lung and pulmonary arterial SMC mitochondria show slower respiratory rates than that seen in whole kidney and renal arterial SMC. They have also demonstrated that lung mitochondria produced more activated oxygen species (AOS) both at baseline and under hypoxic conditions, and that rotenone (an ETC complex I inhibitor) was able to mimic the effects of hypoxic exposure on both systemic (renal) and pulmonary arterial ring models, suggesting that the oxygen sensor is closely allied with early ETC complex function (Michelakis *et al*, 2002).

1.7. Differential responses of vascular wall components to hypoxia

1.7.1. Endothelium

Pulmonary hypertension is characterised by concentric EC proliferation and neointimal formation. Neointimal expansion represents both an increase in total cell

number (hyperplasia) and the size and quantity of surrounding ECM. Intimal hyperplasia is not necessarily a pathological process and occurs within the aorta as part of normal physiological process of aging (Su and Mioa, 2001) (**figure 1.3**).

1.7.1.2. Disruption of endothelial basement membrane as stimulus for proliferative change

A large body of work supports a central role for the endothelial cell in the aetiology of pulmonary hypertension. The EC is the primary barrier between the blood, a potentially hypoxaemic environment and other vascular wall components, together with its capacity to secrete mitogens and growth factors for other cell types following hypoxaemic stimulus. One of the first histological changes noted in the development of pulmonary hypertension is disruption of the endothelial basement membrane (Stenmark *et al*, 1999). It has been suggested that endothelial injury – whether as the result of hypoxaemia, inflammation or alteration of haemodynamic flow – is the stimulus for vascular remodelling. Damage to the endothelial basement membrane would permit plasma-born mitogens and growth factors access to underlying cells and matrix (Cowan *et al*, 2000).

In vivo work performed on neonatal hypoxic calves has demonstrated that hypoxia acts as a stimulus for endothelial proliferation. EC proliferation is magnified and prolonged by hypoxic exposure in neonatal calves (Belknap *et al*, 1997). In addition the plexigenic lesions seen in IPH, but not secondary pulmonary hypertension, represent a monoclonal expansion of the EC layer suggesting disordered growth regulation as is seen in neoplasia (Lee *et al*, 1998). Interestingly, Teichert-Kuliszewska *et al* (2006) have demonstrated that loss of function of Bone

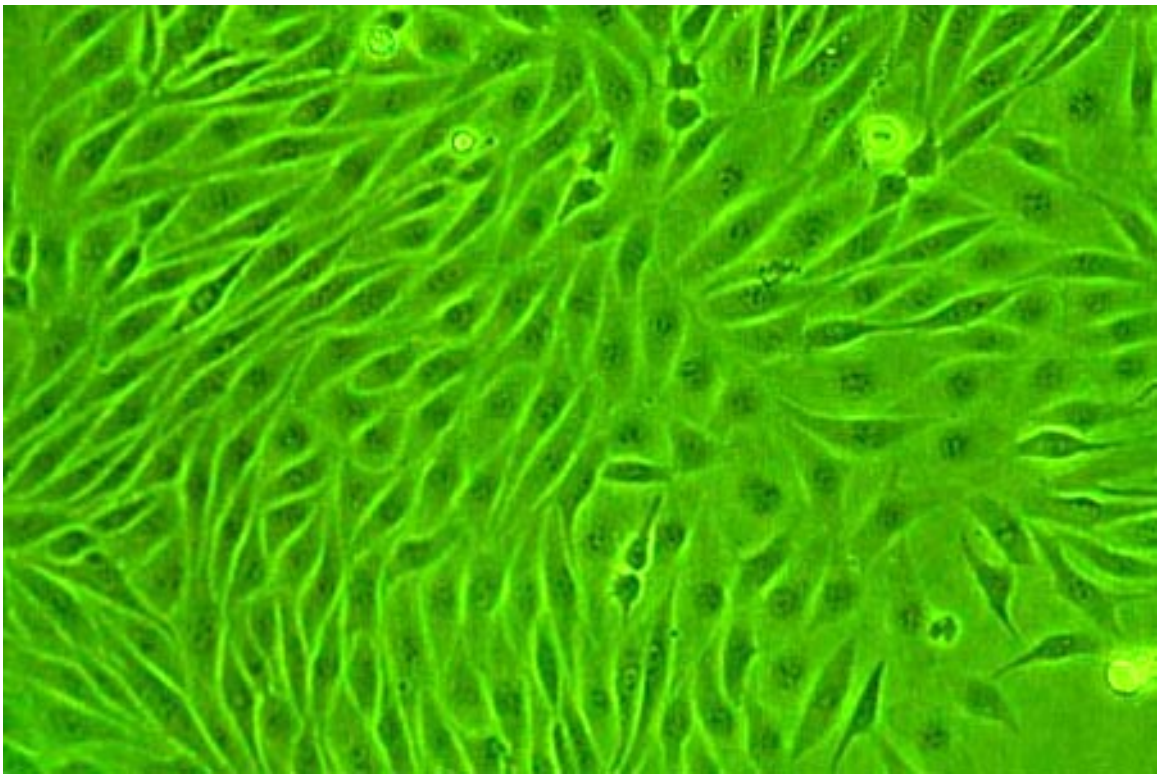


Figure 1.3: Vascular endothelial cells. Vascular EC under magnification showing confluent growth.

Morphogenetic Receptor 2 (BMPR2) is associated with increased apoptosis in EC. The same investigators have also shown that epithelial progenitor cells (EPC) from patients with FPAH are resistant to BMP2-mediated protection to apoptosis. This appears to be contradictory at first sight – but Michelakis (2006) suggests that perhaps pulmonary arterial hypertension may be a disease process of altering cellular function – with EC apoptosis occurring in the early stages allowing mitogens access to the medial and adventitial layers while in later phases of the disease EC proliferation dominates.

1.7.1.3. Endothelial cells produce mitogens for SMC and fibroblasts

Endothelial cells are capable of producing a variety of mitogens for SMC and fibroblasts. They produce platelet derived growth factor (PDGF), Endothelin – 1 (ET-1), vasoactive endothelial growth factor (VEGF) and NO in response to hypoxaemia (Faller, 1999; Veyssier-Belot and Cacoub, 1999). *In vitro* increases in PDGF and steady-state PDGF mRNA in response to hypoxia have been noted in human umbilical vein endothelial cells (HUVEC), rat pulmonary artery and aortic EC and bovine pulmonary artery and aortic EC. Increases in ET-1 have also seen (Kourembanas *et al,* 1997). EC mitogen and growth factor production is not isolated to the pulmonary circulation, however local effects on surrounding tissues may be different to those seen in the systemic circulation.

1.7.1.4. Platelet derived growth factor

PDGF is a growth promoting vasoactive mitogen that is produced by EC and platelets under hypoxaemic conditions. It induces vasoconstriction and proliferation in smooth muscle cells and can also stimulate pulmonary artery fibroblast migration and

proliferation (Veyssier-Belot and Cacoub, 1999). There are three isotypes of PDGF, PDGF AA, AB and BB that have a variety of responses in VSMC (Faller, 1999). PDGF AA is mitogenic for cultured VSMC *in vitro*, the effect being augmented by the addition of PDGF BB (Majack *et al*, 1990). Induction of PDGF-B mRNA is inversely proportional to the degree of hypoxia and has been shown to be reversible on reoxygenation (Faller, 1999). PDGF has actions on other mitogens: upregulating the production of VEGF mRNA in EC via its interaction with specific endothelial PDGF-B receptors (Wang *et al*, 1999). A role for PDGF in the aetiology of pulmonary arterial hypertension has been suggested by recent case studies showing improved functional status in patients with PAH following treatment with imatinib (*Gleevec* – a multikinase/PDGF inhibitor used in the treatment of haematological malignancies) (Ghofrani *et al,* 2005; Patterson *et al*, 2006). Imatinib has been shown to both prevent *and* reverse pulmonary arterial hypertension in a monocrotaline model of pulmonary arterial hypertension (Schermuly *et al*, 2005).

1.7.1.5. Vascular endothelial growth factor

VEGF is a hypoxia-inducible angiogenic factor and is only produced under hypoxaemic conditions. It has been implicated in intimal proliferation. It is produced by EC, SMC, macrophages and epithelial cells (Veyssier-Belot and Cacoub, 1999). Its production in response to hypoxaemia has been shown to be dose and time dependent (Ankoma-Sey *et al*, 2000) and its only known target is the epithelial cell (Veyssier-Belot and Cacoub, 1999). The upregulation of VEGF mRNA is dependent on the presence of Hypoxia Inducible Factor-1 (HIF-1) that is only stable under hypoxic conditions (Voelkel and Tuder, 2000). Other HIF-1 dependent growth

factors/mitogens are erythropoetin (EPO), glucose transporters and glycolytic enzymes.

1.7.1.6. HIF-1 and cellular adaptation to hypoxia

HIF-1 is important in mammalian cellular adaptation to hypoxic conditions (Semenza, 2000). It upregulates genes which enable the cell to adapt both acutely and in the long term, to a hypoxic environment; target genes for HIF-1 include most enzymes involved in the glycolytic pathways to assist with the shift from aerobic to anaerobic respiration together with those for NOS 2, ET – 1, insulin-like growth factor 2 (IGF – 2). The upregulation of EPO and VEGF assist with adaptation to chronic hypoxia. Upregulation of EPO results in an increase in haematocrit assisting oxygen delivery to hypoxic cells while VEGF, which specifically targets EC, results in angiogenesis thereby facilitating oxygen supply to hypoxic tissues. Three HIF isoforms have been identified: HIF1 appears to be ubiquitously expressed, HIF2 appears to be localised to the lung and is approximately 48% homologous to HIF1 while HIF3 appears to act as a HIF1 inhibitor (Semenza, 2007).

1.7.1.7. Structure of HIF-1

HIF-1 is a heterodimer consisting of two basic helix-loop-helix subunits; these subunits combine together to form an active complex HIF-1 that is necessary for activation of transcription. HIF-1 β is a constitutively expressed protein found in normoxic cells (also known as aryl hydrocarbon receptor or ARNT subunit 1). The stability of HIF-1 α, an 826 amino acid peptide, regulates HIF-1 activity. Under normoxic conditions HIF-1 α has a half-life of approximately 5 minutes, rapidly targeted by von Hippel Lindau protein (pVHL) and Factor Inhibiting HIF-1α (FIH-1)

for degradation by E3 ubiquitin ligase in the cytosol. At present HIF-1α is thought to be unique in that molecular oxygen is required for its breakdown under normoxic conditions. The hydroxylation of prolyl and asparaginyl residues at HIF-1α's Oxygen Dependent Domain (ODD) enables pVHL to bind the protein. Under hypoxic conditions HIF-1α remains active, is phosphorylated and forms an active complex with HIF-1β which enables the upregulation of genes possessing a Hypoxia Response Element (HRE) within their promoter region (Semenza, 2000).

1.7.1.8. HIF-1 and pulmonary vascular remodelling

Mice null for HIF-1α and HIF2α die in early life as a result of vascular abnormalities (Yu *et al*, 1999). Mice heterozygous for HIF-1 α exposed to relative hypoxia (10% O_2 for six weeks) develop less right ventricular hypertrophy and have a lower haematocrit than control mice (Semenza, 2000). In addition mice heterozygote for HIF2α appear to be completely protected from hypoxic pulmonary arterial hypertension (Brusselmans *et al*, 2003). This suggests that HIF-1 and HIF-2 have significant roles in the control of hypoxia-mediated pulmonary vascular remodelling. But HIF-1 can also be activated under normoxic conditions, for example via a G protein coupled receptor (GPCR) associated with the Kaposi's Sarcoma-associated Human Herpes Virus 8, via p38 Mitogen Activated Protein Kinase (MAPK) in immortalised fibroblasts (Sodhi *et al*, 2000). Both classical ERK1/2 (p44/p42) MAPK and p38 MAPK have the capacity to phosphorylate HIF-1 α (Berra *et al*, 2000; Minet *et al*, 2001). Activation of HIF-1 under normoxic conditions is rarely of the same magnitude as seen in hypoxia but a pathway known to affect hypoxia-mediated pulmonary vascular remodelling (Welsh *et al*, 2001) also activated under

normoxic conditions could also be involved in pulmonary vascular remodelling without a hypoxic stimulus.

1.7.1.9. Endothelin 1

ET-1 is the most powerful vasoconstrictor in man and is known to be upregulated in pulmonary, but not systemic, hypertension (Giaid, 1998; Grimpen *et al*, 2000). It is produced by EC in both systemic and pulmonary vasculature and is mitogenic for VSMC and fibroblasts. It can also act as a chemotaxin for adventitial fibroblasts (Stenmark and Mecham, 1997). ET-1 is produced from Big ET-1 by Endothelin Converting Enzyme (ECE) and acts via ET – A or B receptors that are mostly localised in VSMC (Veyssier-Belot and Cacoub, 1999). The frequency and distribution of ET A and ET B receptors varies within the vasculature, in a human model ET B receptors predominate in the circulation with the highest density in the alveolar epithelium and VSMC (Henry, 1999). The upregulation of both ET-1 and ET receptors has been demonstrated in both human and rat endothelial cells following hypoxic exposure (Veyssier-Belot and Cacoub, 1999).

1.7.1.10. Nitric Oxide

NO is thought to be central in maintaining low basal blood pressure in normal pulmonary vasculature balancing the effects of ET-1 and thromboxane. It is synthesised by EC by the action of nitric oxide synthase (NOS) on L-arginine. Three isosymes of NOS exist: (endothelial) eNOS, (inducible) iNOS and (neuronal) nNOS. Neuronal NOS is found in vascular endothelial cells but eNOS appears to be the predominant isozyme implicated in pulmonary hypertension – dense immunostaining for eNOS has been found in plexigenic lesions in patients with IPH (Veyssier-Belot

and Cacoub, 1999). NO has an inhibitory effect on platelet aggregation and on SMC and ECM proliferation (Adnot *et al*, 1995). eNOS also downregulates PDGF and ET-1 gene expression at the level of gene transcription (Faller, 1999). In the rat model eNOS expression is upregulated by hypoxic exposure, shear stress and by elevations in $[Ca^{2+}]^i$ in both pulmonary and systemic circulations (Adnot *et al*, 1995). But experimental evidence is conflicting, some investigators have found that both NO and NOS production are down regulated in response to hypoxia (Higenbottam and Cremona, 1993). There is also considerable inter-species variation in NO expression. If NO is produced by the endothelium and pulmonary hypertension is thought to be a disease process at least in part mediated by endothelial dysfunction, then it would be reasonable to infer that reduced NO and eNOS production occurs secondary to damaged endothelium; an increase in eNOS expression does not necessarily imply an increase in NO activity (Veyssier-Belot and Cacoub, 1999).

1.8.1. Vascular Smooth Muscle Cells

Pulmonary hypertension is a disease process characterised by progressive muscularisation of previously non-muscularised small arteries and venules. There is evidence of migration of VSMC into the neointima, their development of a secretory function and of a proliferative role. The alteration of VSMC function is not unique to pulmonary hypertension but also occurs in the systemic circulation; for example in response to increases in systolic blood pressure (Su and Miao, 2001). The adult differentiated VSMC normally resides in a quiescent state; this is maintained by its interactions with neighbouring EC and the matrix proteins that surround it (Newby and Zaltsman, 2000) (**figure 1.4**).

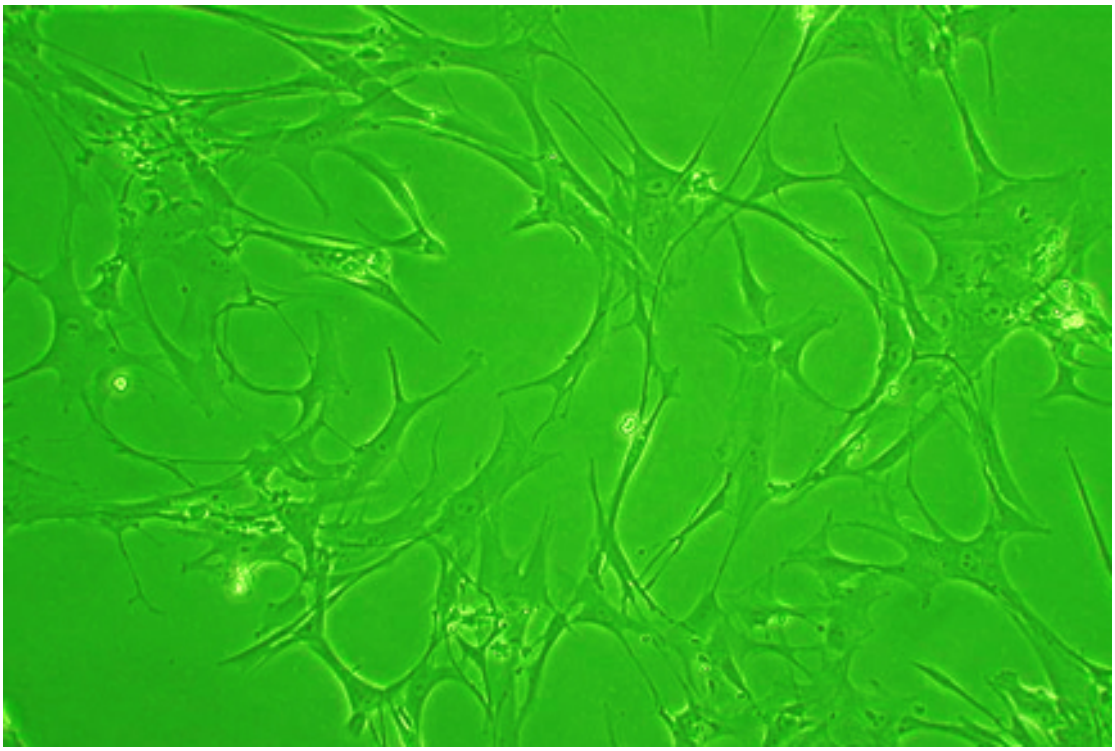


Figure 1.4: Vascular smooth muscle cells. VSMC under magnification.

1.8.2. Hypoxia enhances and prolongs VSMC proliferation

As in EC, hypoxia prolongs and enhances proliferation in VSMC from pulmonary artery tissue (Belknap *et al*, 1997). DNA indices were increased in both the media and adventitia of pulmonary artery tissue from neonatal calves following prolonged hypoxic exposure. VSMC proliferation was most marked in small generation vessels.

1.8.3. Phenotypic switching of resident VSMC in response to hypoxia.

Cowan and Rabinovitch (2000) have suggested that the disruption of EC basement membrane permits growth factors either from the serum or from neighbouring EC to gain access to the underlying VSMC and ECM, thereby enabling a change in function (Cowan *et al*, 2000). Whether this change in function is as a result of the reactivation of an inherent fetal state (Stenmark and Mecham, 1997) or as the result of growth factor stimulation of phenotypically distinct groups of VSMC (Frid *et al*, 1994; Stenmark *et al*, 1999) remains questionable. Hypoxic VSMC have been shown to upregulate elastin and tropoelastin mRNA production; elastin secretion is normally confined to fetal development and is down-regulated before birth. The re-expression of a gene product normally associated with the embryological period suggests a return to a more immature state of development (Stenmark *et al*, 1997). Evidence to support the co-existence of varying SMC phenotypes has been demonstrated *in vitro*. Frid *et al* (1994) were able to identify four different 'types' of VSMC from bovine pulmonary arteries on the basis of immunohistochemical staining following hypoxic culture. This may argue for the emergence of oxygen-sensitive VSMC that respond preferentially to hypoxia in comparison with other VSMC.

1.8.4. VSMC involvement in systemic vascular remodelling

As discussed earlier, VSMC have been implicated in vascular remodelling in systemic hypertension. Substantial structural change in the aorta has been noted in sinuaortic denervated (SAD) rats (Su and Miao, 2001). There is marked increase in total wall thickness and 'wall thickness: internal diameter' ratio. As seen in pulmonary hypertension, there is an increase in total collagen and a relative decrease in elastin content in the aortic media implying an altered synthetic role for VSMC. However on staining, the number of nuclei remains the same in comparison to non-denervated rats. This suggests that in the aorta, the mechanism behind the increase in medial wall thickness, at least in systemic hypertension, is VSMC hypertrophy rather than hypertrophy and hyperplasia, as seen in pulmonary hypertension (Su and Miao, 2001). As discussed previously, the patterns of systemic small artery and arteriolar remodelling in the face of elevated systolic hypertension or increased pulse pressure is variable and not predictable, but in the human model there is evidence for altered VSMC function and behaviour throughout the systemic circulation as a result of endothelial injury (Newby and Zaltsman, 2000).

1.9. Adventitial Fibroblasts

1.9.1. Earliest structural changes in pulmonary vascular wall to hypoxia are seen in the adventitial layer

The earliest and most marked changes in vascular wall remodelling to hypoxic exposure have been seen in the adventitial layer of rat hilar pulmonary artery (Meyrick and Reid, 1979). *In vitro*, hypoxic exposure has a direct effect on fibroblasts, stimulating proliferation without the addition of growth factors (Welsh *et*

al, 1998; Strauss and Rabinovitch, 2000). This makes the adventitial fibroblast unique from other cells in the vascular wall. VSMC can contract independent of other stimuli but require growth factors to enable a functional change (Voelkel, 1997) (**figure 1.5**).

1.9.2. Hypoxia sensitises adventitial fibroblasts to mitogenic stimuli

In addition to enhancing proliferation, Welsh *et al* (1998) have also shown that hypoxia sensitises pulmonary artery adventitial fibroblast to the effects of mitogens and the increase in proliferation is associated with increased inositol 1,4,5-triphosphate (IP_3) production (see section 1.11.2.3). This effect does not extend to hypoxic fibroblasts from systemic arteries (Welsh *et al*, 1998). The addition of growth factors such as Basic Fibroblast Growth Factor (bFGF), PDGF and insulin-like growth factor- 1 (IGF-1) seems to augment the growth response of pulmonary artery fibroblasts to hypoxia (Strauss and Rabinovitch, 2000). This effect can be partially abrogated by the addition of inhibitors to PDGF and ET-1 suggesting that these are important in hypoxia-stimulated fibroblast proliferation (Dawes *et al*, 1994). However, structural changes to the vessel wall are not uniform throughout the pulmonary vascular tree. In smaller generation vessels adventitial thickening is more marked, while large pulmonary arteries appear to develop medial hypertrophy like systemic arteries subjected to elevated systolic blood pressure (Stenmark *et al*, 1999). This heterogeneous response to hypoxia (or an increase in vascular pressure) suggests that 'phenotypic switching' may be an important concept not only for the VSMC but also the adventitial fibroblast.

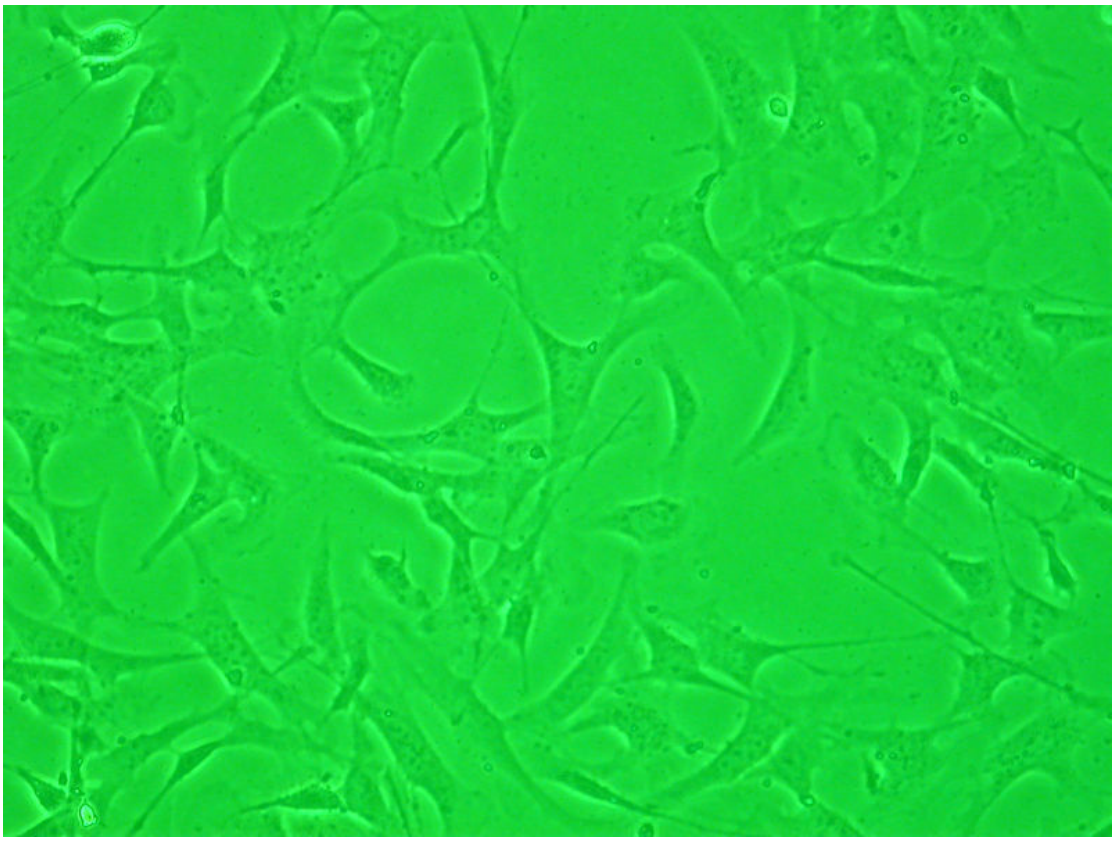


Figure 1.5: Adventitial fibroblast cells. Adventitial fibroblasts under magnification.

1.9.3. Hypoxia enables phenotypic switching of mature fibroblasts

The mature fibroblast is non-mobile and non-contractile. As a result of hypoxic and growth factor exposure, adventitial fibroblasts appear to differentiate into highly active cells. Mecham and Stenmark (1997) note that during embryological development the fibroblast has a very unstable phenotype. They suggest that this enables it to respond in a multiplicity of ways to various stimuli. This potential instability is seen as an advantage in the adult state as it enables a more adaptive response to injury and altered haemodynamics (Mecham *et al*, 1987).

Whether this variable response is as a result of a fibroblast population that contains cells at different stages in the cell cycle, enabling a variety of outcomes from a single stimuli, or whether this represents a variety of phenotypically distinct cells co-existing in the vessel wall capable of responding differently to the same stimulus, is unknown. Evidence for a variety of distinct cell lines is supported by the work of Das *et al* (2001). They compared pulmonary artery fibroblasts with fibroblasts from different parts of the arterial circulation in neonatal calves. Significantly they identified two fibroblast cell lines cultured from hypoxic neonatal calf aortic adventitia that proliferated in response to hypoxic exposure. These cells were stable through a series of passages demonstrating a maintained phenotype (Das *et al*, 2001). This suggests that phenotypic variation is not specific to the pulmonary vasculature but can also occur in the systemic circulation. However, neonatal and fetal cells appear to be more capable of proliferation with any given stimulus than adult cells, and this may provide an explanation for the growth of neonatal aortic fibroblasts under hypoxic conditions (Strauss and Rabinovitch, 2000).

1.10. Extra Cellular Matrix

1.10.1. Hypoxic expansion of ECM is not reversed by normoxia

It has been demonstrated that rat hilar pulmonary artery structure is significantly altered by hypoxic exposure (Meyrick and Reid, 1979). While Meyrick and Reid show that the changes in adventitial and medial wall thickness are reversible on re-oxygenation, the expansion in ECM is not reversed. Hypoxic exposure upregulates the production of elastin and collagen in calf pulmonary arteries matched by steady state upregulation of tropoelastin mRNA (Stenmark and Mecham, 1997). A similar upregulation of ECM proteins has been seen in human umbilical vein endothelial cells (HUVEC) following hypoxic exposure (Stenmark and Mecham, 1997).

1.10.2. ECM makeup varies with the function of the vessel

There is marked variability in the relative proportions of matrix collagen and elastin content throughout the vascular tree. Major arteries that are subjected to large pulsatile pressures: for example the thoracic and abdominal aorta, have a higher proportion of elastin than the small generation resistance vessels. Elastin production also appears to be confined in a time and tissue specific manner to the embryological period (Stenmark and Mecham, 1997). As the distance from the heart increases, so the proportion of elastin in the vascular wall decreases and collagen content increases. Collagen when mature and cross-linked is much stiffer and less distensible that elastin. The structural properties of ECM appear to be dependent not only on the function of the vessel, but also the amount and type of collagen which increases with age. There is much less type 1 collagen in the vascular wall of the neonate than the adult (Stenmark and Mecham, 1997). Therefore it is easy to see how a pathological

process that results in altered blood flow and localised shear stress could have a profound effect on the composition of the underlying ECM.

1.10.3. Hypoxia and matrix metalloproteinases

Vascular wall cells may be maintained in a quiescent phase by their interactions with integrins and proteoglycans in the ECM (Newby and Zaltsman, 2000). Hypoxia appears to result in the upregulation of proteins that disrupt and inhibit this interaction; particularly marked is the increase in matrix metalloproteinases (MMPs). These matrix proteins act by degrading elastin and proteoglycans, adding support to the theory that elastolysis is required prior to cell migration and proliferation (Rabinovitch, 1997; Cowan *et al*, 2000). They also act by releasing growth factors from the ECM such as endogenous vascular elastase (EVE), bFGF and tenascin C. EVE has also been shown to increase bFGF release from ECM in its own right and bFGF has been shown to be mitogenic for SMC (Jones *et al*, 1997). MMPs also induce the production of fibronectin, which can also be stimulated by inflammatory markers such as interleukin 1 (IL-1) and tumour necrosis factor α (TNF α). Upregulation of fibronectin may provide paths along which SMC and fibroblasts are able to migrate (Rabinovitch, 1995) and experimentally-induced pulmonary hypertension can be reversed by MMPs inhibitors (Cowan *et al*, 2000). However there are species differences: MMPs only appear to be upregulated in the rat pulmonary hypertension model in the post-hypoxic period (Strauss and Rabinovitch, 2000).

1.11. Cell signalling mechanisms

1.11.1. Upregulation of genes and gene products in response to hypoxia and other stimuli

Hypoxic exposure results in the upregulation of genes and gene products in the pulmonary circulation that differ substantially from those seen in normoxic vasculature. These genes are not only upregulated in response to hypoxia; but can also occur following the action of shear stress and growth factors. The mechanism of action is thought to be mediated by an extra cellular signal; a growth factor, altered vessel pressure or hypoxia which is then conveyed across the cell membrane via a transmembrane receptor and thence to the nucleus where it exerts its effect on mRNA transcription and protein translation (**figure 1.6**).

1.11.2. Transmembrane Receptors

1.11.2.1. G Protein Coupled Receptors

G protein coupled receptors (GPCR) consist of a seven membrane-spanning unit which associates with an inner membrane bound 'G protein' (Berridge, 1993). The G proteins (heterodimeric guanine nucleotide binding proteins) exist in several isoforms, including β, γ and δ. They vary in their distribution and specificities. G proteins enable phosphorylation of the cytosolic portion of the transmembrane receptor resulting in a conformational change and interaction with other enzymes and proteins located at the plasmalemma surface (Berridge, 1987). An example of a GPCR agonist is antithrombin II (AT II) or ET-1.

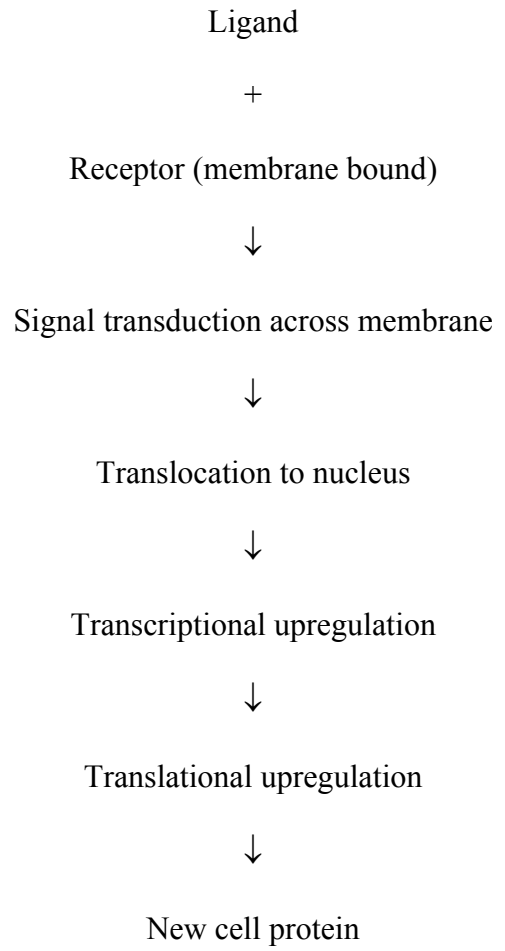


Figure 1.6: Overview of membrane bound receptor signalling. Extracellular ligand binds to the membrane bound receptor enabling conformational change of the intracellular component. This in turn permits the transduction of the signal to the nucleus via a series of intermediary protein signalling molecules where it then affects upregulation of specific gene products and subsequent protein translation.

1.11.2.3. Association of GPCR, G protein and Phospholipase C

The association of the inner membrane complex – phosphorylated GPCR and G protein - enables the translocation of phospholipase C (PLC) from the cytosol to the inner cell membrane where it is then activated by the receptor-G protein complex (Majerus *et al*, 1990). There are several different isoforms of PLC, γ and δ predominate in fibroblasts. The function of these different subtypes is not yet known. PLC's main action is to hydrolyse phosphatidylinositol 1-4-5 bisphosphate (PhtdInst P_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1993).

1.11.3. IP_3 stimulates release of intra cellular Ca^{2+} stores

Soluble IP_3 dissociates from the membrane surface into the cytosol where it binds specific IP_3 receptors on the sarcoplasmic and endoplasmic reticulum (Berridge, 1987). This stimulates the release of intra cellular Ca^{2+} stores (Berridge, 1993). Ca^{2+} acts as a co-factor for many intracellular enzymes, is necessary for muscle contraction and is required for DNA synthesis.

1.11.4. Phosphatidylcholine derivatives also activate DAG

DAG is lipid soluble and remains bound to the inner surface of the cell membrane where it can interact with Protein Kinase C (PKC) (Berridge, 1993). PKC translocates to the membrane from the cytosol as a result of DAG formation. DAG can also be activated by phophatidylcholine derivatives. PKC has a variety of functions: it is involved in smooth muscle contraction, it enhances the action of Na^+/H^+ antiporters at the cell membrane enabling increases in intracellular pH, it also acts as a stimulus for other intracellular signalling pathways including the activation of HIF-1 in hypoxic conditions and in the upregulation of EPO (Berridge, 1987).

Like the G protein family, there are several isosymes for PKC. The differential response of pulmonary artery fibroblasts to hypoxia may be related to an oxygen-sensitive PKC isosyme (Das *et al*, 2000). Hasan *et al* (1996) have demonstrated that PKC_{α} is preferentially upregulated in hypoxic lung fibroblasts. Das *et al* (1997) and Xu *et al (*1997) have demonstrated variation in PKC isoform expression in both adventitial fibroblasts and vascular smooth muscle cells respectively, in neonatal and fetal models of hypoxic pulmonary arterial hypertension. This suggests that there may be intrinsic differences in cell signalling pathways between the hypoxic pulmonary and systemic vasculature (**figure 1.7**).

Figure 1.7 over view of G-Protein coupled receptor signalling. Growth factors bind to the membrane-bound G protein coupled receptor (GPCR) that enables phosphorylation (P) of the inner membrane component and association with a G protein. G protein association subsequently permit phospholipase C (PLC) to move from the cytosol to the cell membrane where it is then activated by the G protein. PLC then hydrolyses phosphatidylinositol 1-4-5 bisphosphate (PhtdInst P_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). Soluble IP_3 stimulates intracellular calcium release from the sarcolemma. PhtdInstP_2 can also activate diacylglycerol (DAG) which in concert with protein kinase C (PKC) influences membrane Na^+/H^+ antiporters and HIF1α.

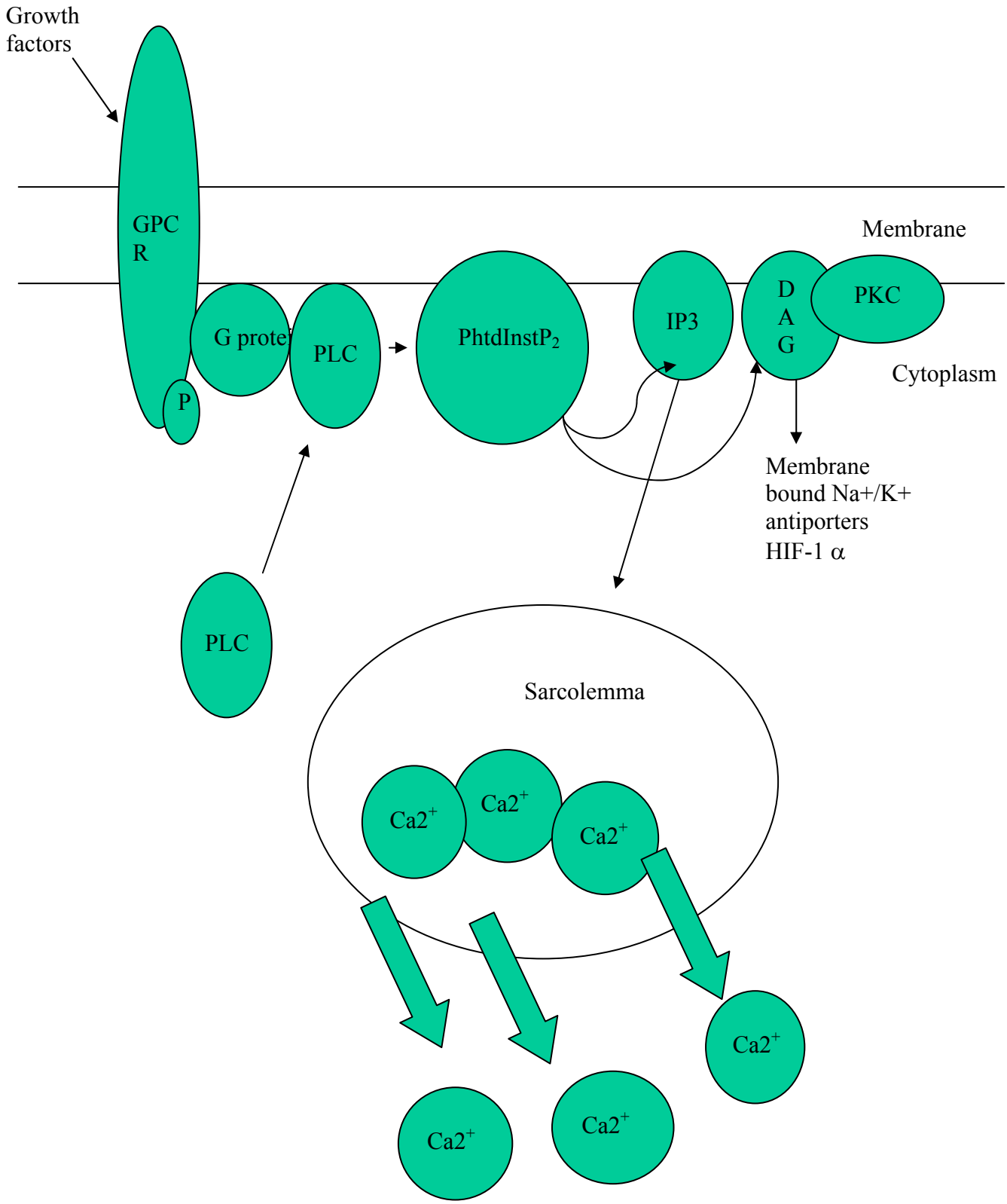


Figure 1.7: Overview of G protein coupled receptor signalling

1.11.5. Tyrosine Kinase Receptors

Tyrosine kinase receptors (TKR) are transmembrane receptors that have intrinsic autophosphorylation ability; they are not dependent on G proteins for activation (Berridge, 1993). The binding of an agonist, for example PDGF, to the extracellular portion of the receptor is sufficient to result in autophosphorylation. The conformational change occurring at the inner membrane surface allows the association of an shc protein that translocates from the cytosol to the inner membrane wall (Obermeier *et al*, 1993). The shc protein does not have any innate catalytic capacity in its own right but mediates the subsequent association of other proteins with the TKR: for example Grb and SOS (Segal and Greenberg, 1996). Grb is a Ras-GTP exchanger enabling the activation of Ras from its GDP bound form to active GTP form. NO is thought to inhibit this reaction by increasing intracellular cGMP (Voelkel, 1997). SOS acts as another nucleotide exchange factor that promotes the activation of Ras in association with Grb. The autophosphorylation of TKR results in the translocation of shc, Grb and SOS migrate to the cell membrane that act in concert to activate Ras. Ras acts as a G protein and enables the translocation and activation of Raf-1 to the cell membrane. Raf-1 is a serine/threonine kinase and is activated by becoming tyrosine phosphorylated. Raf-1 is part of the Raf-1-MAPK pathway that is intimately bound up with intra cellular signalling and has been extensively studied (Marshall, 1994; Marshall, 1996; Marias and Marshall, 1996). The substrate for Raf-1 is MEK 1 kinase or MAP kinase kinase (**figure 1.8**).

Figure 1.8: Overview of tyrosine kinase receptor signalling. The tyrosine kinase receptor (TKR) has autophosphorylative ability and does not require a G protein to enable signal transduction. The resulting conformational change occurring at the inner membrane surface allows the association of an shc protein that translocates from the cytosol to the inner membrane wall which mediates the subsequent association of other proteins with the TKR: for example Grb and SOS. Grb is a Ras-GTP exchanger enabling the activation of Ras from its GDP bound form to active GTP form. SOS acts as another nucleotide exchange factor that promotes the activation of Ras in association with Grb. Shc, Grb and SOS to the cell membrane that act in concert to activate Ras. Ras acts as a G protein and enables the translocation and activation of Raf-1 to the cell membrane. Raf-1 is a serine/threonine kinase and is activated by becoming tyrosine phosphorylated. Raf-1 is part of the Raf-1-MAPK pathway. The substrate for Raf-1 is MEK 1 kinase (MEKK1) or MAP kinase kinase (MKK), which enables signal transduction to the nucleus via MAPK activation and subsequent transcription and translation of relevant gene products with associated transcription factors such as c-myc and ATF-2.

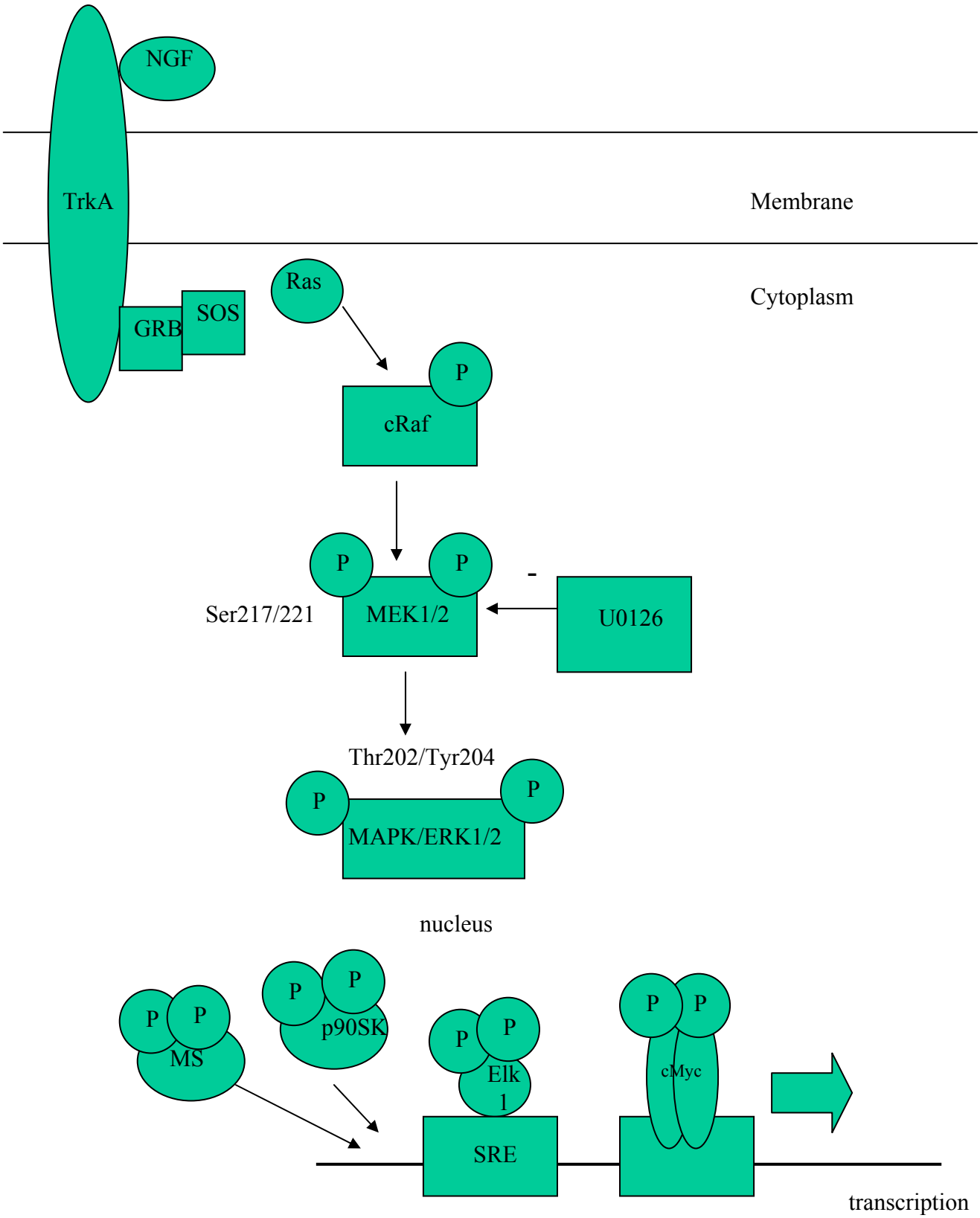


Figure 1.8: Overview of tyrosine kinase receptor signalling (p44/42 mitogen activated protein kinase signalling)

1.11.6. Raf-1-MAP kinase pathway

MAP kinase is the substrate for MEK-1, a dual specificity kinase that has 2 binding sites for Raf-1. MAPKs are dual specificity serine/threonine kinases that appear to be involved in a variety of cellular responses and which can be activated by the Raf-MEK-MAPK cascade, by DAG and PLC and by TAB-1/TAK-1. Two classes of MAPK have been identified so far, the so-called 'classic' MAPK – p44/p42 or ERK 1 and 2 MAPK – and the stress-activated MAPK or SAPK – including p38 and C-Jun N-terminal kinase (JNK) MAPK. The classic MAPKs are involved in cellular proliferation in some cell lines while the SAPK are known to mediate apoptosis in others together with cellular adaptation to altered pH, temperature or oxygen tension (Han *et al*, 1994; Lee *et al*, 1994; Rouse *et al*, 1994; Raingeaud *et al*, 1995; Verheij *et al*, 1996).

There is an increasing body of work investigating the relative roles of MAPK in a variety of human disease, in particular p38 MAPK appears to be involved with inflammatory responses and regulation of the immune system (Hale *et al*, 1999; Ono and Han, 2000). It has also been implicated in ischaemia/reperfusion injury of the brain, in myocardial pre-conditioning and in systemic hypertension (Ono and Han, 2000; Behr *et al*, 2003; Ju *et al*, 2003). However the exact nature of its role in these disease states remains uncertain.

The MAPKs function within highly complex signalling cascades that depend on their dual phosphorylation at threonine and tyrosine residues within their regulatory loop by upstream MEKs that are activated by Raf-1 binding. All MAPKs share threonine and tyrosine residues within this loop but the intervening protein varies between

subtypes and confers their relative specificity to substrate, but not to their upstream kinases. In addition a variety of different MAPK isoforms exist: JNK has three identified isoforms (JNK 1/2/3), while four isoforms of p38 MAPK have so far been isolated: α, β, γ and δ. p38 α and β isoforms appear to be ubiquitously expressed whereas γ and δ isoforms are more tissue specific (Enslen *et al*, 2000; Brancho *et al*, 2003). p38 γ appears to be strongly expressed in skeletal muscle, is involved with the regulation of basal state glucose uptake via its action on GLUT1 and 4 transporters, in addition it may also be involved with muscle cell differentiation (Wang *et al*, 1997; Sweeney *et al*, 1999; Ono and Han, 2000; Fujishiro *et al*, 2001). p38 δ appears to be expressed in glandular tissue – its exact function remains obscure (Ono and Han, 2000). It also appears that isoform expression is not only tissue-type specific but can also vary with developmental stage.

Upstream MEKs exhibit varying specificity for MAPKs – with MEK 1 and 2 phosphorylating p44/p42 MAPK, MEK4 and 7 - JNK isoforms and MEK3 and 6 - p38 isoforms (Brancho *et al*, 2003). There is however a degree of overlap with cells over-expressing MEK4 able to phosphorylate p38 MAPK and MEKs 3 and 6 also able to activate JNK MAPK isoforms. p38 MAPK isoforms also exhibit relative specificity to MEK, with MEK 6 showing high affinity for all isoforms but MEK 3 only showing affinity for α, β and δ (Enslen *et al*, 2000). It appears that MEK 6 is preferentially able to activate p38 MAPK α at significantly lower concentrations than other isoforms demonstrating another level of control within the cellular signalling cascade (Alonso *et al,* 2000). Once activated MAPKs act as serine/threonine kinases on their substrates – MAPKAP kinases – which then are able to phosphorylate and activate a variety of downstream transcription factors - including ATF-1 and 2 and the

AP-1 family of transcription factors (c-jun, c-fos etc.) resulting in transcription and subsequent translation of MAPK-mediated gene products. Control of signalling cascades appears to occur not only as a result of signal transduction at the membrane surface, but also as a result of subcellular localisation of the various signalling cascade components, which is in part dictated by the presence of nuclear export elements within proteins but also as a result of scaffolding proteins such as TAB-1 which can control access that individual signalling components have to each other (Ono and Han, 2000).

Like MEK-1, the MAP kinases have dual specificity phosphorylation sites and following activation, MAP kinase translocates to the nucleus from the cytosol where it acts as a substrate for transcription factors such as Elk-1 and the ribosomal S6 kinase family (Segal and Greenberg, 1996). This results in the upregulation of so-called immediate early genes (IEG), such as c-fos, by association with a serum response element (SRE) that acts as a docking site for Elk-1. This enables increased transcription of mRNA. The MAP kinases can also be activated by DAG/PLC complexes, phorbol esters and by GPCR without the necessity of tyrosine kinase involvement. The Raf-1-MAP kinase pathway appears to be a central cell-signalling pathway enabling the conversion of an extracellular signal into increased transcription of mRNA. p38 and JNK MAPKs are mediated through the action of G proteins; the action of JNK together with ERK can be abrogated by the use of pertussis toxin in neonatal calf pulmonary artery fibroblasts (Das *et al*, 2001). Pertussis toxin is a known inhibitor of $G_{1\alpha/0}$ protein. p38 MAPK is not inhibited by pertussis toxin suggesting that another G protein mediates its action. **Figure 1.9** represents a summary of the p38 MAPK signalling cascade.

Figure 1.9 An overview of p38 MAPK signalling. Tyrosine kinase receptors (TKR) are stimulated by the action of a variety of growth factors resulting in the association at the inner cell membrane of shc, Grb and SOS proteins enabling the activation of Ras. Ras is able to activate p38 MAPK via Mixed Lineage Kinases (MLK) and MAPK kinases (MKKs). p38 MAPK function within highly complex signalling cascades that depend on their dual phosphorylation at threonine and tyrosine residues within their regulatory loop by upstream MEKs that are activated by Raf-1 binding. Once activated p38 MAPK act as serine/threonine kinases on their substrates – MAPKAP kinases and MAPK signal-integrating kinases (MNKs) – which then are able to phosphorylate and activate a variety of downstream transcription factors - including ATF-1 and 2 and the AP-1 family of transcription factors (c-jun, c-fos etc.) resulting in transcription and subsequent translation of MAPK-mediated gene products.

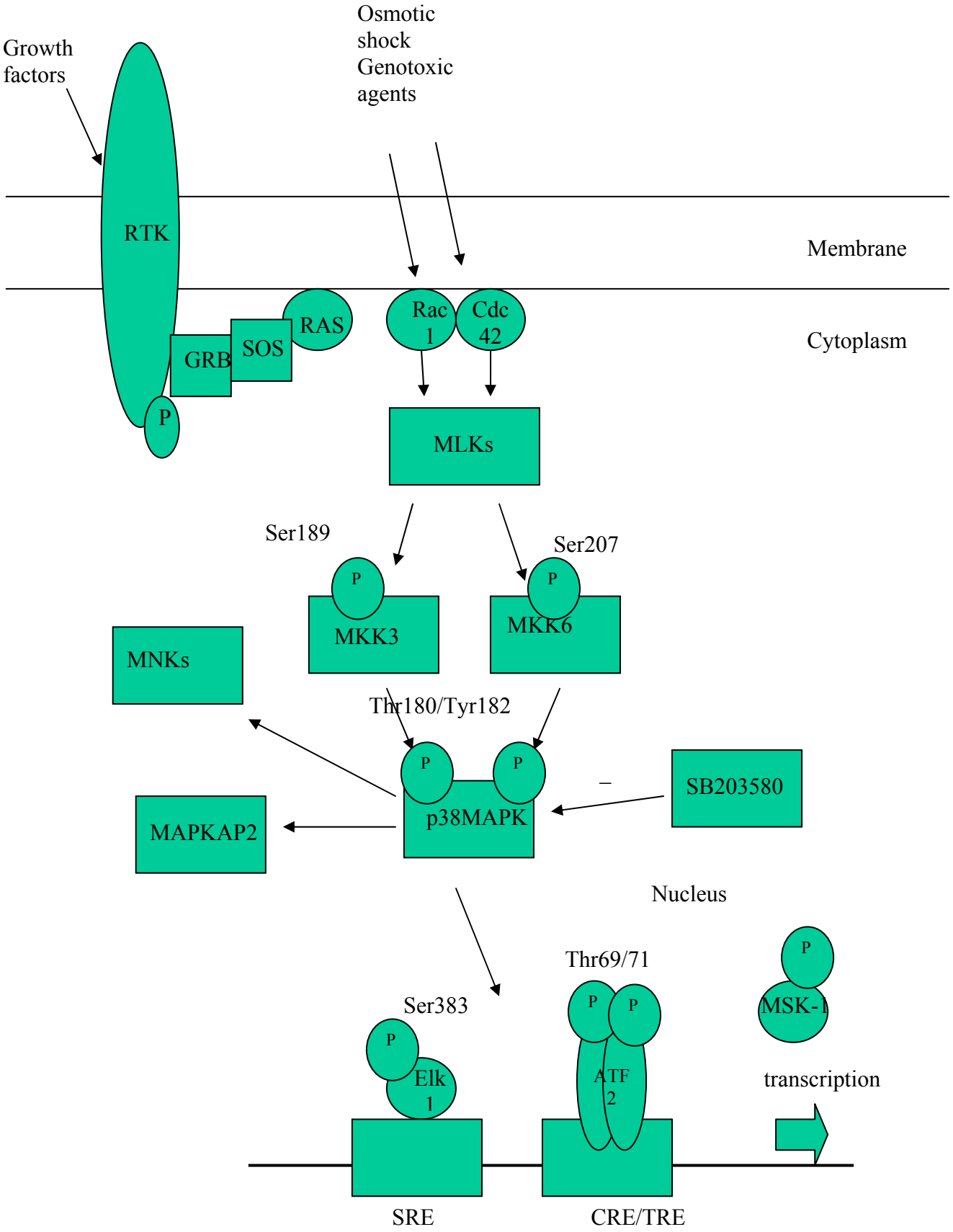


Figure 1.9 An over-view of p38 MAPK signalling

Most recently Welsh *et al* (2001) have demonstrated that p38 and ERK MAPK are constitutively upregulated in pulmonary artery fibroblasts in the chronically hypoxic rat model but not in fibroblasts from systemic arteries. Inhibitors of p38, but not ERK MAPK, abrogate the proliferative response to hypoxia. Unlike Das and colleagues (2001), Welsh *et al* did not demonstrate an upregulation of JNK in hypoxic bovine pulmonary artery fibroblasts. Das *et al* (2001) were using neonatal calf rather than an adult rat model and, as noted above, there appear to be significant differences between mature and neonatal lung physiology. Both species difference and developmental stage may play a role in explaining these conflicting results.

1.12. BMP, BMP receptors and TGF β superfamily

1.12.1. BMPRII and PPH

With the discovery of mutations within BMPRII as the predominant inherited genetic abnormality in IPH (Lane *et al*, 2000) attention has now focused on the function of BMP and its receptor-signalling cascade in the aetiology of PH. BMP, a member of the TGF β superfamily, has an important role in development – particularly osteo- and chondrogenesis. However its role in adult physiology is less certain (Kawabata *et al*, 1998).

1.12.2. BMP mediated signalling

As with other TGF β related ligands, signal transduction occurs via a two-step receptor process at the cell membrane. BMP binds the higher affinity BMPR2 which when activated phosphorylates BMPR1. In turn this results in the downstream

activation of cytosolic SMAD proteins, which act as the intracellular messengers of TGF β superfamily receptors (Kawabata *et al*, 1998). SMAD (Sma – from *Caenorhabditis elegans* - and Mad, Mothers Against Decapentaplegia in *Drosophila*) proteins are the mammalian equivalents of signalling factors downstream of TGF β which were originally identified in *Drosophila*, termed decapentaplegic or Dpp (Hata *et al*, 1997). TGF β related ligands exert a wide variety of effects. Their role tends to be inhibitory for example in EC but can be proliferative in cells of mesenchymal origin.

1.12.3. Smad proteins act as intracellular messengers for TGF β superfamily

Smad proteins are highly conserved between species and can be divided into three categories: Receptor activated Smads (R Smads), Common (Co) Smads and Inhibitory Smads (I Smads). R Smads are phosphorylated by an activated membrane bound type 1 receptor and are then able to bind either Co Smad (Smad 4) enabling nuclear translocation of the R/Co Smad complex and subsequent DNA transcription, or an I Smad (Smad 6/7) which prevents nuclear transport and gene upregulation. Smad 6 appears to be a specific inhibitor of BMP stimulated signal transduction (Hata *et al*, 1997). The specificity of the intracellular signalling cascade appears to be determined by the L45 area of the type 1 receptor's kinase region (Chen *et al*, 1998). TGF β specific Smads appear to be Smads 2, 3 and 7 BMP specific Smads are 1,5, 6 and 8 (Kawabata *et al*, 1998).

1.12.4. Cross talk between TKR and serine/threonine kinase (RSK) receptors in BMP signalling

BMP and TGF β do not appear to work exclusively through this signalling cascade. There is a growing body of evidence documenting cross talk between RSK and TKR (Yue *et al*, 1999). VSMC taken from patients with IPH show abnormally enhanced growth responses to both BMP and TGF β stimulation despite the use of BMP inhibitors (Morrell *et al*, 2001). This suggests a wider disturbance of TGF β related cellular signalling than could be explained by BMPR 2 disruption alone.

1.12.5. ERK1 inhibits R/Co Smad nuclear translocation

TGF β signalling has the capacity to upregulate not only RSK but also the Ras/MEK/ERK1 classic MAP kinase pathway and ERK1 (but not JNK or p38) is able to phosphorylate R Smads resulting in inhibition of nuclear transport (Kretzschmar *et al*, 1997). ERK1 phosphorylation occurs at a different site to that of Smad 4 phosphorylation and does not interfere with R/Co Smad binding (Kretzschmar *et al*, 1997). There is also evidence that TGF β can upregulate p38 MAP kinase signalling via TAK 1 and MKK6 and that this signalling cascade is enhanced by the intervention of Smad2/4 complex. Smad 4 binding to the ATF 2 domain of DNA has been shown to be dependent on p38 phosphorylation (Kretzschmar *et al*, 1997).

1.12.6. BMP receptors exist in two conformational states

BMP receptors 1 and 2 exist in two distinct conformational states: either as isolated membrane bound receptors that are then activated by binding ligand, or as pre-formed membrane bound heterodimers (Nohe *et al*, 2002). The heterodimeric form appears to be unique to BMP receptors amongst the TGF β superfamily. The mechanism by

which these different receptor conformations are activated seems to dictate which intracellular signalling cascade is upregulated. If BMP activates preformed receptor heterodimers the Smad pathway appears to be activated, as a result of ligand mediated receptor conformational change. If BMP binds and then activates, an isolated type 2 receptor- the p38 MAP kinase signalling pathway seems to be preferentially activated. Activation of divergent signalling pathways would be dependent on the relative frequency of preformed heterodimers at the cell membrane, which is in turn dependent on the presence of functional type 2 receptor subunits. If in Familial IPH, the BMPR2 were non-functioning then this would result in a skewing of activation of intracellular signalling cascades away from Smads (an inhibitory pathway in fibroblasts) towards p38 MAPK. This would result in the upregulation of proliferative pathways that had previously been held in check by BMP/Smad dependent signalling (**figure 1.10**).

However there also appears to be degrees of interdependence between the BMP-BMPR2-Smad signalling axis and p38 MAPK activation. There is evidence that p38 MAPK is required for Smad 1 phosphorylation in a human osteoclast model (Noth *et al*, 2003) but p38 MAPK also appears to be required for both early and late TGFβ mediated signalling via TAK-1 and GADD45β respectively. Early activation via TAK-1 seems to be Smad independent (Horowitz *et al*, 2004) while late activation requires Smad activation (Takekawa *et al*, 2002).

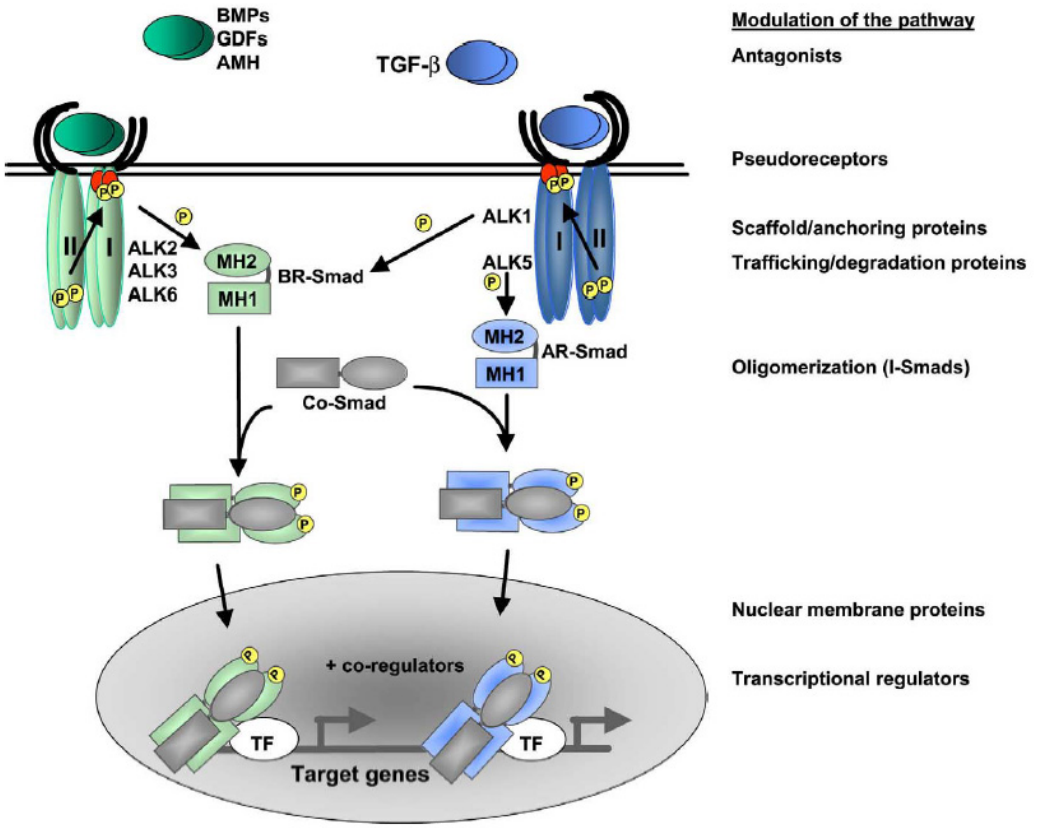


Fig. 1. BMP and TGF-β signaling pathways mediated by BR-Smads. Ligand-induced heteromeric receptor complex formation and phosphorylation of the type I receptor lead to phosphorylation of R-Smads. Activated R-Smads form complexes with co-Smad and accumulate in the nucleus, where they participate, together with transcription factors and co-regulators, in the transcriptional regulation of target genes. It is peculiar that TGF-β binds in endothelium in the presence of TβRII to ALK5 as well as to ALK1. The former activates the classical TGF-β/AR-Smad pathway, whereas ALK1 activates BR-Smads and transmits therefore BMP-like signals. Different levels of modulation of the BMP/BR-Smad signaling cascade are indicated schematically, see for details Table 1 and text.

Figure 1.10: Overview of BMPR/Smad signalling from Zwijsen *et al* (2003)

1.12.7. Inconsistencies in BMP and TGF β cell signalling

It is not known how TGF β exerts a negative influence on Smad signalling via activation of TKR/Ras/MEK/ERK1, releasing the stimulus towards cell proliferation, or why the Smad2/4 complex acts as a cofactor in p38 MAPK-dependent transcription when its previously documented function appears to be to act as an inhibitory pathway to prevent cell growth.

1.13 HYPOTHESIS

Macroscopically, it has been shown that significant differences exist between the pulmonary and systemic arterial responses to both hypertension and to hypoxia. The central hypothesis of this thesis is that these dissimilarities are partially explained by the existence of fundamental differences in cell signalling pathways in adventitial fibroblasts from the pulmonary and systemic circulations. Studies from this laboratory have already shown in a bovine model that IP_3 is upregulated in fibroblasts from pulmonary arteries in comparison with those from the mesenteric vasculature when exposed to acute hypoxia (Welsh *et al*, 1998) and that the stress activated kinases p38 and JNK (rather than ERK 1 and 2) are upregulated in pulmonary artery fibroblasts that have been exposed to acute hypoxia. In a chronic hypoxic adult rat model (5% 0_2 for 2 weeks), p38 MAPK is constitutively upregulated in pulmonary but not systemic artery fibroblasts. In addition pulmonary artery fibroblasts appear to have been phenotypically altered by a chronic hypoxic exposure, demonstrating increased proliferative activity even when subsequently cultured under normoxic conditions. This proliferative response is not seen in fibroblasts taken from the systemic circulation (Welsh *et al*, 2001).

In this work 'acute' hypoxia is defined as 24 hours of continuous hypoxic exposure while 'chronic' hypoxia is understood as at least 2 weeks of continuous hypoxia. These definitions differ from those employed in experiments investigating the mechanisms of HPV, a process that begins within seconds of hypoxic exposure and becomes sustained over a period of minutes (Gurney, 2002). Because this work aims to examine the proliferative behaviour of human pulmonary and systemic arterial fibroblasts to hypoxic exposure, a necessary consideration in designing the experimental model is the duration of the cell cycle in arterial fibroblasts: work from Scott *et al* (1998) confirms this to be between 20 and 24 hours in an adult bovine model. As noted above work by Meyrick and Reid (1979: 1980) and Welsh *et al* (2001) have demonstrated that long-term 'chronic' hypoxic exposure results in sustained structural change in the pulmonary vasculature in comparison to the systemic circulation. This work aims to examine the pulmonary circulation's 'early' proliferative response to hypoxia in a human model.

The literature shows that there is remarkably little work that has been performed on human cells despite the numbers of studies performed looking at hypoxic vascular remodelling. What has been done in animal models shows significant interspecies variation. Scott *et al* (1998) demonstrated upregulation of JNK and p38 MAPK, but not ERK 1 and 2 MAPK, in a hypoxic adult bovine model. In an adult rat model of chronic hypoxia both ERK and p38 but not JNK MAPK, were upregulated (Welsh *et al*, 2001) in comparison to similar work performed by Das *et al* (2000) in neonatal calves. Regardless of inter-species variations, there appear to be fundamental differences in cell signalling response to hypoxia between pulmonary and systemic circulations. Given this, it is important to investigate cell-signalling systems in a

hypoxic human model to establish whether the same pathways are used and to attempt to find a suitable animal model comparison.

Aims:

1) To investigate the role of hypoxia in both human pulmonary and systemic artery fibroblast proliferation.
2) To investigate a potential role for the stress activated and classic MAPK in hypoxia mediated fibroblast proliferation and a possible role for HIF-1α in this process.
3) To investigate the relative roles of various SMAD proteins involved in BMPR signal transduction in human pulmonary and systemic arterial fibroblasts exposed to acute hypoxia and attempt to identify any possible interaction with both stress activated and classic MAPK.

Chapter 2:

Materials and Methods

2.1. Materials

All general chemicals were of Analar grade and were supplied by Merck (Thornliebank, Glasgow) or Sigma (Poole, Dorset) unless otherwise stated. All tissue culture plastics were from Greiner Labortechnick Ltd (Gloucestershire.UK). All components of tissue culture medium were purchased from Gibco Life Technologies (Paisley, Scotland). [^{3}H]thymidine was from Amersham (Little Chalfont, Bucks). Antibodies used for Western Blot analysis were from New England Biolabs (Hertfordshire, UK). Gel electrophoresis equipment was purchased from Bio-Rad Laboratories (Herts, UK). Incubators were supplied by LEEC (Nottingham, UK).

2.2. Cell and tissue culture

Cells derived from human pulmonary and left internal mammary arteries were used. Following ethical approval (R+D No. 03RM004VRM) and full written consent (see appendix), tissue was obtained fresh on the day of experimentation from patients undergoing surgical treatment for lung cancer. All patients were smokers but had normal spirometry at the time of surgery; tissue from patients with abnormal spirometry or with any known respiratory condition was not used. Tissue was transported from theatres at the Western Infirmary, Glasgow to the Pathology Department where suitable samples were removed by pathology staff. The samples were then taken directly to the laboratory in a container filled with chilled Krebs-Henseleit Solution (NaCl 118mM, $NaHCO_3$ 25mM, KCl 4.7mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $CaCl_2$ 2.5mM, and Glucose 11mM).

2.3. Primary Cell Culture

Fibroblasts used throughout these studies were obtained from primary culture by explant.

Since the conditions (warm, humid and nutrient rich) necessary for culturing the cells are an ideal environment for promoting fungal and bacterial growth, extreme caution must be taken to avoid contamination. All steps such as making up solutions, changing media, etc were conducted under sterile conditions, that is, within a clean, Microflow laminar flow hood (model number M25121/1) (**figure 2.1**). The flow hood was dismantled and cleaned regularly and before use each day was sprayed liberally with 70% (v/v) ethanol. Anything taken inside the flow hood (i.e. pipettes and reagent bottles) was also sprayed with ethanol and sterile gloves were worn throughout. Pipette tips and distilled water were sterilised using a prestige Medical "Omega" autoclave (model number 220140).

2.3.1. Primary fibroblast culture

Lobar pulmonary artery was dissected free from the lung tissue and then cut longitudinally into a flat sheet (**figure 2.2** and **2.3**). Pulmonary and systemic artery fibroblasts were prepared using the technique of Freshney (1983), with some modifications. Muscular tissue and endothelial cell layers were removed by gentle abrasion of the vessel using a sterile razor blade (**figure 2.4**). The remaining tissue (adventitia) was then dissected into 5mm^2 portions. Approximately 25 portions of tissue were evenly distributed over the base of a 25cm^2 culture flask containing 2ml of DMEM with 20% FCS, supplemented with penicillin/streptomycin (400iu/ml and 400μg/ml) and amphotericin B (5μg/ml) (**figure 2.5**). The explants were incubated in a humidified atmosphere of 5% CO2 in air at 37^{o}C. Within a few days cells were

Figure 2.1: Microflow laminar hood (model number M25121/1)

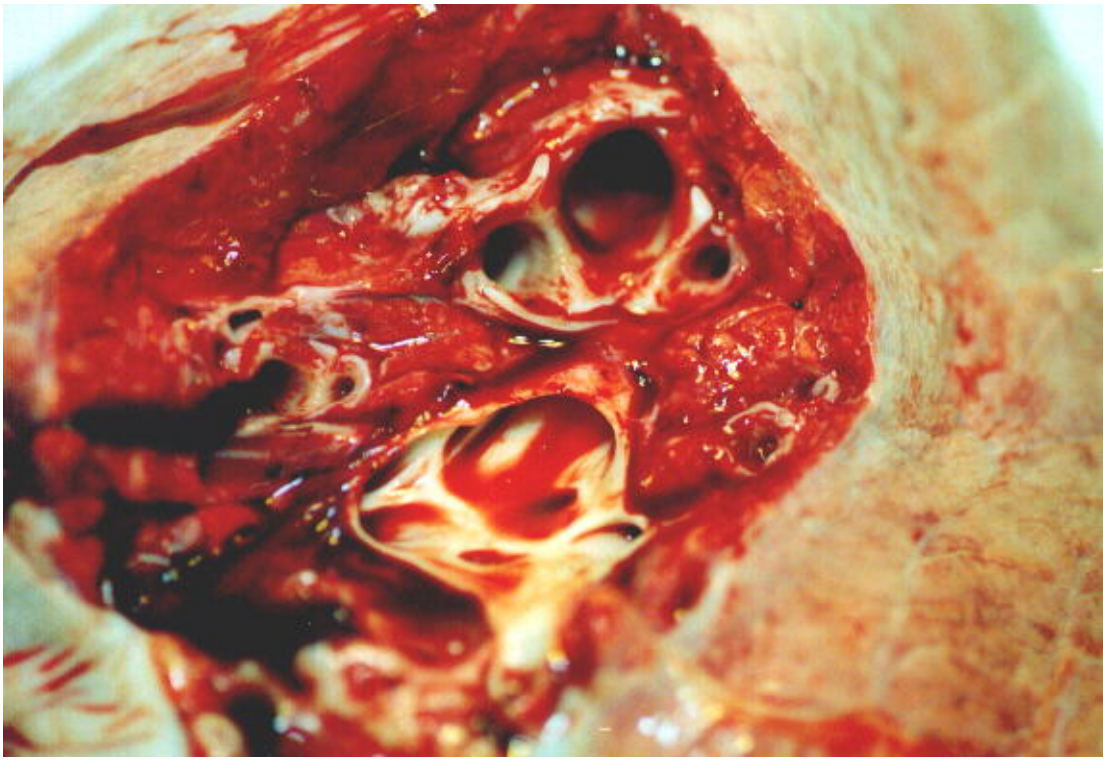


Figure 2.2: Lobar pulmonary artery *in situ.*

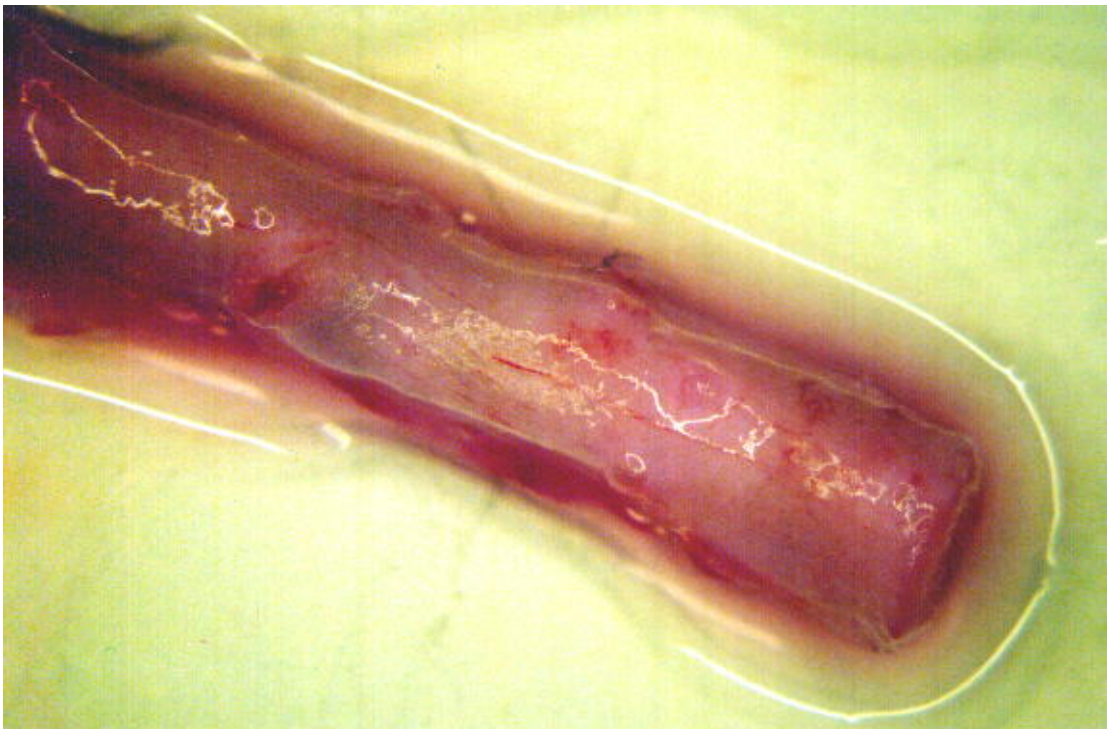


Figure 2.3: Pulmonary artery dissected from gross specimen

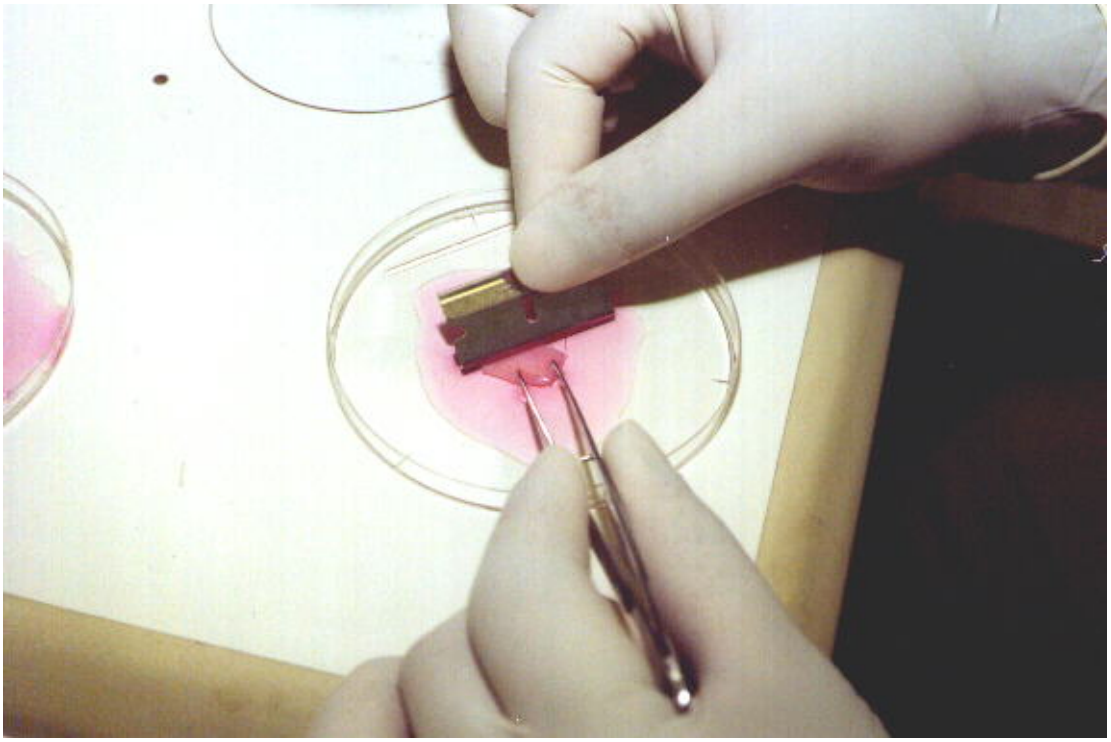


Figure 2.4: Dissection of longitudinally sectioned pulmonary artery using sterile razor blade

Figure 2.5: Sections of pulmonary artery seeded into 25cm^3 culture flask

observed growing out from the tissue fragments (**figure 2.6**). Once a monolayer of cells had partially covered the flask, cells were lifted from the flask by trypsinisation as described in Chapter 2.3.1. Tissue fragments were removed by aspiration.

The main branch of the pulmonary artery was used to acquire pulmonary artery fibroblasts. To study fibroblasts from the systemic circulation, the internal mammary artery was dissected – this artery is typically 3mm in diameter and is consistent with the dimensions of systemic control vessels previously used by this laboratory (rat aorta and bovine mesenteric arteries) (Scott *et al*, 1998; Welsh *et al*, 1998; 2001; 2004; 2006).

Cells were not selected for experimentation. The only criteria used were that the cells did not stain positively for either smooth muscle actin or for Von Willebrand Factor – markers for smooth muscle cells and endothelial cells respectively and that they proliferated. Cells that did not proliferate from explant and stained positively for other cell markers were discarded. **Figure 2.7 (a)** and **(b)** demonstrate cell staining for smooth muscle actin and Von Willebrand Factor (VWF). Cells were used between passage 2 and 10 – there appeared to be no significant alteration in proliferative response in the later passages.

2.3.2. Routine Cell Maintenance

Cells were grown routinely in 75cm^2 culture flasks in Dulbeccos modification of Eagles medium (DMEM), supplemented with penicillin (200 units/ml) and streptomycin (200 ug/ml), L-glutamine (27 mg/ml) and 10% foetal calf serum (FCS).

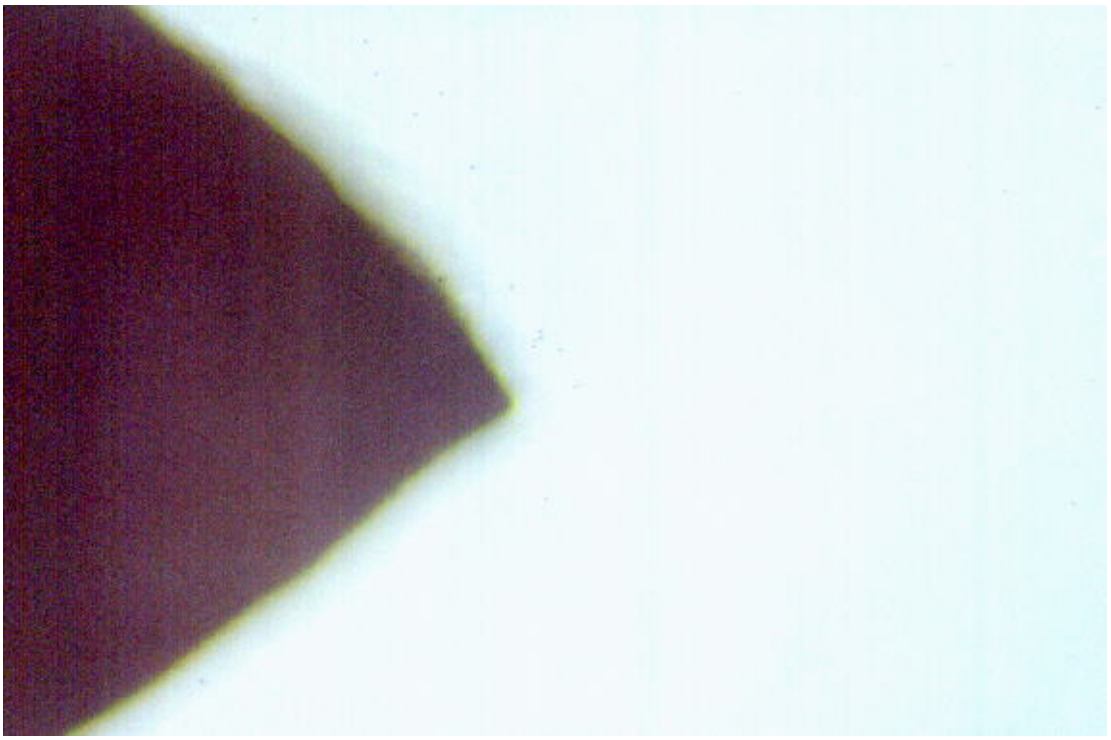
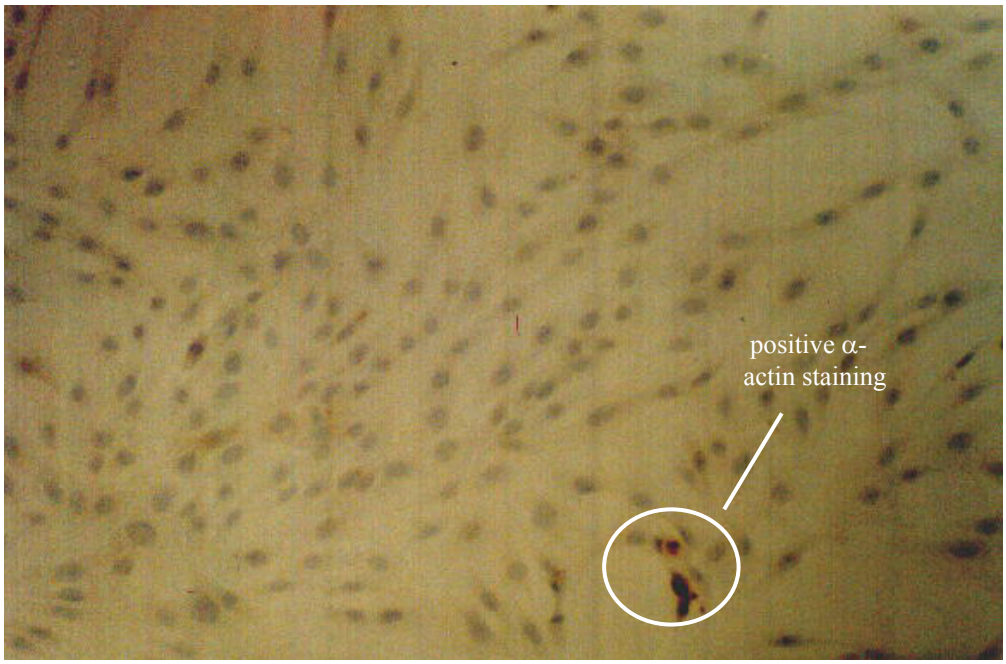


Figure 2.6: Fibroblast cells growing from explant tissue under magnification

(a)



(b)

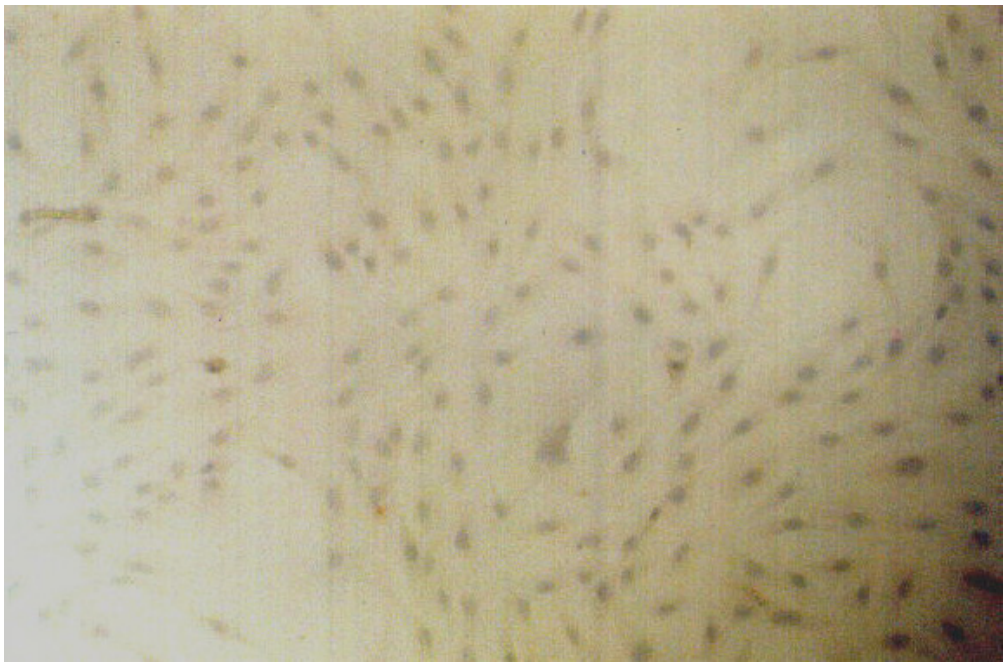


Figure 2.7 (a) α-Actin and (b) VWF Staining of Human Pulmonary Artery Fibroblasts.

The cultures were kept in a humidified atmosphere of 5% CO_2 in air at 37^{o}C. Culture medium was changed every 2 days and cells were passaged just prior to confluency.

Cell passage was performed by removing the culture medium and washing the cells with 2mls pre-warmed trypsin solution (0.05% trypsin / EDTA 0.02%) which was then aspirated. A further 2ml of trypsin solution was then added and left on the surface of the cells for approximately 10s before aspiration. The cells were then incubated at 37^{o}C for approximately 15 minutes or until they had began to detach from the surface of the flask. This was observed under a light microscope (Olympus CK2) (**figure 2.8**). Gentle tapping of the dish was used to dislodge the cells and 10ml of DMEM containing 10% FCS was added to the flask to re-suspend the cells. A portion of this cell suspension (0.3ml) was then aliquoted into new flasks containing another 10mls of fresh medium. At this stage cells could be plated out onto dishes as required for experimentation. Cells were used for experimentation between passage 3-10.

2.3.3. Cell Freezing/Thawing

A cell suspension was collected from a 75cm^2 flask by trypsinisation (Chapter 2.3.1) in 10ml of culture medium. Cells were centrifuged at 1000g for 10 minutes. The cell pellet was then resuspended in 1ml Cryopreservation Medium growth medium (DMEM containing 10% FBS and 10% DMSO). The resuspended cells in the freezing medium were placed in a 2ml cryotube and left in a fridge for 20 minutes followed by a –80^{o}C freezer overnight. The tube was then transferred to the vapour phase of the liquid nitrogen for 1h, then placed directly into the liquid nitrogen.

Figure 2.8: Light microscope (Olympus CK2)

Frozen cells were removed from liquid nitrogen and thawed rapidly in a water bath at 37^{o}C. The cryotubes were then swabbed with tissue paper soaked in 70% ethanol and the caps loosened. Holding the vial in one hand, the cap was removed and the contents taken up into a pipette, then placed in a 10ml centrifuge tube. The cell suspension was diluted slowly with 5mls of fresh growth medium and the tubes centrifuged at 1000g for 10mins. The cells were resuspended in 10mls fresh growth medium and seeded onto a 25cm^2 flask. After approximately 4h the cells attached to the flask. The medium was replaced with fresh medium.

2.4 Hypoxia: Methods for studying acute hypoxic fibroblast cells *in vitro*

2.4.1. Generation of Hypoxic Environment

A humidified temperature controlled incubator (Model GA156; LEEC, Colwick, Nottingham, UK) (**figure 2.9**) was used to produce a hypoxic environment. This incubator allows control of internal oxygen levels between 0 and 21% using medical grade nitrogen, while the CO_2 level is simultaneously controlled at 5%. Due to the large volume of nitrogen required to sustain a suitable degree of hypoxia, nitrogen cylinders were linked using a Pneuchange automatic gas cylinder change over unit (NTC, Woulton, Liverpool, UK), which activated a fresh supply of nitrogen as required.

2.4.2. Measurement of Hypoxia

The levels of hypoxia achieved within the environment of the incubator could be monitored with the oxygen probe that was an integral part of the unit. Measurements from within the bathing medium of the cells were also analysed with a portable

Figure 2.9: Humidified temperature controlled incubator (Model GA156; LEEC, Colwick, Nottingham, UK)

oxygen probe (Jenway: 9015. Dunmon, Essex, UK) to determine the rate of gaseous diffusion, and the pH of the medium analysed throughout the course of hypoxic exposure with the use of a portable pH probe (Mettler Delta 340, Hanstead, UK). Cells were routinely maintained for acute hypoxia in an atmosphere of 5% O_2 (35mmHg) and 5% CO_2 at 37^{o}C.

2.5. Assessment of Cell Proliferation

2.5.1 Cell counting

Cell counts were confirmed manually using a haemocytometer following fibroblast cell staining with propodium iodide.

2.5.1 [^{3}H] Thymidine uptake assays

Cell proliferation was measured by determining the uptake of [^{3}H]thymidine into DNA. Cells were seeded at a density of 5x10^3 cells / well into 24-well plates in 500µl of culture medium. Cells were grown to 70% confluency in 24-well plates and then growth-arrested for 24h by replacing the medium with 500µl serum-free DMEM. Cells were then stimulated with appropriate agonists and incubated for 24h, either in a normal CO_2 incubator (5% CO_2) or in the hypoxic incubator to obtain an acute hypoxic exposure (Chapter 2.4). In the latter case, the O_2 content of the atmosphere was reduced from 21% to 5% by flooding with N_2.

For the final 4h of agonist stimulation, cells were labelled with [^{3}H]thymidine (0.1µCi/well). The reaction was stopped by washing the cells twice in ice-cold PBS

(500µl/well). Proteins were precipitated by washing three times with 500µl/well 5% trichloroacetic acid (TCA) and lipid fractions were solubilised by washing twice with 90% ethanol (500µl/well). The remaining cell contents were incubated in 0.3M NaOH (500µl/well) for 30mins. The contents of each well were transferred to scintillation vials, to each of which was added 3mls of Ecoscint A (Ecoscint, Atlanta, Georgia, USA) scintillation fluid. Vials were vortexed briefly before radioactivity was measured by scintillation counting using a Wallac scintillation counter. Results are expressed as disintegrations per minute (DPM) and were confirmed manually using a haemocytometer following fibroblast cell staining with propodium iodide demonstrating that [^{3}H]Thymidine uptake was a good proxy for cellular proliferation.

2.6 Detection and Analysis of Proteins

2.6.1 Preparation of samples for SDS-PAGE and immunoblotting

Cells were seeded at a density of $5x10^3$ cells/well onto 6-well dishes. After the cells had reached 60% confluency, they were growth-arrested in serum-free DMEM for a period of 24h. After the cells had been agonist-stimulated, the medium was removed, the cells placed on ice and the cell monolayer washed 2x with 500µl ice-cold PBS. The cells are then lysed with 50µl RIPA buffer. The samples were left on ice for 15 mins. (Tris-HCl 50mM pH 7.4, NP-40 1%, $C_{24}H_{39}O_4Na$ 0.25%, NaCl 150mM, EGTA 1mM, PMSF 1mM, Na_3VO_4 1mM, NaF 1mM, CLAP 1/1000, pH 7.4).

The cells were scraped on ice into the RIPA buffer and the contents placed in microcentrifuge tubes. The lysates were centrifuged at 14,000g at 4^{o}C for 15mins. The supernatant was then transferred to fresh tubes, aliquoted and stored at –70^{o}C.

2.6.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Pre prepared gel plates (Amersham) were used for protein electrophoresis. Each gel plate was removed from its packaging and rinsed thoroughly with dH_2O. The combs were removed and the wells rinsed with Electrophoresis Buffer (Tris-base 25mM, glycine 192mM and SDS 0.1%). Unloaded gels were then placed in the BioRadTM electrophoresis unit and the central well filled with Electrophoresis buffer containing 500 µl electrophoresis antioxidant.

2.6.3. Electrophoresis conditions

All protein samples to be examined by SDS-PAGE were first diluted to 22µl by the addition of 10µl of sample buffer and 2µl of rainbow marker to the original 10µl of sample. Samples were heated to 70^{o}C for 10 min to denature proteins and disrupt disulphide bonds. The required volume was loaded into the individual wells of the electrophoresis gel using loading tips. Pre-stained SDS protein molecular weight markers (Biorad) of known size were also placed in lanes either side of the loaded gel. The outer chambers of the electrophoresis unit was filled with Electrophoresis Buffer and the polyacrylamine gel subjected to electrophoresis for approximately 50 min at a constant current of 200 mA.

The molecular weight of proteins was estimated by comparing their mobility to that of pre-stained SDS-PAGE standards. The standards used were myosin (205kDa), β-galactosidase (120kDa), bovine serum albumin (87kDa) and ovalbumin (48kDa).

2.6.4. Western blot analysis

2.6.4.i Transblotting to nitrocellulose.

Following completion of electrophoresis, polyacrylamide gels were removed from the apparatus and washed once. Proteins were transferred from the polyacrylamide gel to the nitrocellulose by assembling a transfer cassette with the nitrocellulose juxtapositioned between the polyacrylamide gel and the cathode. By this method negatively charged proteins were transferred to nitrocellulose for 1h at a constant 30-amp current.

2.6.4.ii. Immunoblotting

Samples (15μg of protein) were subjected to SDS-PAGE (Chapter 2.8.3) and the proteins transferred to nitrocellulose by Western blotting (Chapter 2.8.4.1). The nitrocellulose blots were washed in PBS/T (PBS containing 0.02% Tween-20 (v/v)) and blocked for non-specific binding for 1h on a rocking platform at room temperature in PBS/T supplemented with 10% non-fat milk (Marvel) (w/v). Blots were incubated for 1h in PBS/T supplemented with 5% Marvel (w/v) containing the appropriate dilution of primary antibody. The primary antibody dilutions used for detection of specific antigens is detailed in **table 2.1**. The nitrocellulose blots were rinsed in PBS/T, and washed with 1 litre of PBS/T over an hour. The blots were then incubated for a further 60mins in PBS/T containing 5% Marvel (w/v) containing the appropriate secondary antibody dilution. The blots were then washed as before. Following completion of washing procedure proteins were detected using a method of Enhanced Chemilumenescence (ECL). Blots were incubated in ECL solution (Amersham) for 30s and sandwiched between acetate film. Care was taken to ensure

all air bubbles were removed. Blots were then placed in an X-ray cassette and light emission from the HRP enzymatic action on its substrate contained within the ECL solution was detected following exposure (10 mins – 24 h) of nitrocellulose blots to a piece of X-ray film using a KODAK M35-M X-OMAT processor**.**

2.6.4.iii. Immunoprecipitation

Using Catch and Release® Immunoprecipitation Kit (Upstate) cell lysates were prepared for Western Blotting as follows: 2.5mls of x1 Catch and Release® wash buffer was prepared. The snap off bottom plug was removed from the spin column and inserted into the capture tube. The screw cap was then removed from the spin column and was then centrifuged at 2000 x g for 15-30 seconds to remove the resin slurry buffer. The resin was then washed x 2 with 400 µl of x 1 wash buffer. The capture tube was emptied and the bottom end of the column was re-plugged with the inverted snap-off plug. 500µg cell lysates were added to the tube together with 4 µg of specific p38 MAPK isoform antibody together with 10µl of antiserum, 10µl antibody capture affinity ligand and diluted to a total volume of 500 µl with x 1 wash buffer. Samples were added to the spin tube in the following order: x 1 wash buffer, cell lysates, specific primary antibody followed by antibody capture affinity ligand. The spin tube was re-capped and then mixed at room temperature for 30 minutes ensuring that the slurry remained suspended throughout. The snap-off bottom was discarded from the spin tube and was placed on a fresh capture tube, the screw on cap removed and was then centrifuged at 1,500 x g for 15 –30 seconds; the flow through was discarded. The column was washed x 3 with 400 µl of x 1 wash buffer and spun at 1,500 x g for a further 15-30 seconds for each wash. The spin column was then placed on a fresh capture tube, 70 µl of x 1 non-denaturing elution buffer was then

added to the spin column which was then centrifuged at 1,500 x g with the elutant being reserved for Western blotting. This last step was repeated x 3 – 4 in order to obtain maximum retrieval. Samples were then processed as for Western blotting and immunophoresis as described above.

Table 2.1

Analysed Protein	Antibody Type	Dilution
phospho p38	rabbit monoclonal anti-phospho p38 IgG antibody	1:500
whole p38	rabbit monoclonal anti- whole p38 IgG antibody	1:500
phospho p42/44	rabbit monoclonal anti- phospho p42/44 IgG antibody	1:500
Whole p42/44	rabbit monoclonal anti- whole p42/44 IgG antibody	1:500
phospho Jnk	rabbit monoclonal anti- phospho Jnk IgG antibody	1:500
whole Jnk	rabbit monoclonal anti- whole Jnk IgG antibody	1:500
HIF 1	rabbit monoclonal anti- phospho HIF 1 IgG antibody	1:500
phospho Smad 1/5/8	rabbit monoclonal anti-whole Smad 1/5/8 IgG antibody	1:5000
phospho Smad 2	rabbit monoclonal anti-whole Smad 2 IgG antibody	1:500
Whole Smad 6	goat monoclonal anti-whole Smad 6	1:1000
Whole Smad 7	goat monoclonal anti-whole Smad 7	1:750

Table 2.1 Primary antibody dilutions utilised for Western blotting

2.6.4.iv. Re-probing nitrocellulose membranes

In instances where the same membrane was used to probe for different proteins, primary and secondary antibodies were stripped from the nitrocellulose by incubating in Stripping Buffer (100mM B-mercaptoethanol, 2% SDS and 62.5mM Tris-HCl, pH

2.7) for 1 hour at room temperature with agitation. Blots were then rinsed with PBS/T overnight before the immunodetection protocol was repeated (Chapter 2.6.3.2).

2.7 Densitometric analysis of blots

Densitometric analysis of blots was carried out using a computer programme that allowed for comparison of blot density in graphical form (Quantiscan).

2.8 Data analysis

Data are expressed as mean ± S.D. for replicate plates from the same experiment. Experiments were repeated in cells from 4 different individuals. The statistical significance of differences between mean values from control and treated groups were determined by Student's t-tests, unpaired and paired where applicable, using OXTAT software. Two-tailed probability values of less than 0.05 ($p<0.05$) were considered to be significant.

Chapter 3:

To examine the proliferative behaviour of human pulmonary and systemic arterial fibroblasts under normoxic and acute hypoxic conditions

3.1. Introduction

In the Everest II study, Groves *et al* (1987; 1993) demonstrated that graded and chronic hypobaric hypoxia over a period of 40 days, resulted in significant elevations in mean pulmonary artery pressure (> 60mmHg) in healthy male adults that could not be fully reversed with adequate reoxygenation. This implied that the rise in mean PAP was not only the result of hypoxic vasoconstriction, but also the result of structural remodelling within the pulmonary vascular tree. In a rat model, Meyrick and Reid (1979) demonstrated that chronic hypoxic exposure resulted in structural change in all compartments of the vascular wall: intimal, medial and adventitial, but with the earliest, most marked and irreversible changes occurring within the adventitia. The persistence of adventitial thickening following reoxygenation was in part the result of extracellular matrix deposition, but also the result of increased numbers of adventitial fibroblasts.

In a recent review article, Stenmark *et al* (2006) commented that there are features that are nearly universally present in the pulmonary vasculature of animals that develop PAH secondary to hypoxic exposure: namely intimal hyperplasia, medial thickening and extension of the muscularis into previously unmuscularised arterioles, together with adventitial expansion. These authors also noted that there are significant differences between animal models in terms of proximal and distal vascular structural changes. Specifically they remarked on the different structural responses to hypoxia seen in the conduit vessels of large mammals where medial

hyperplasia predominates with minimal associated adventitial expansion, compared to the distal vessels, where smooth muscle cells appear to be more resistant to proliferation and there is more marked adventitial thickening. This review is written on a background of increased interest in the phenotypic diversity of cells that make up the vascular wall not only in the medial compartment, but also in the adventitia that provide a pulmonary circulation that is much more plastic in its response to physiological stress.

Das *et al* (2002) cultured a variety of fibroblast subpopulations taken from conduit pulmonary arterial adventitia in a chronically hypoxic neonatal calf model. These cells demonstrated a variety of morphologies and proliferative responses when compared with adventitial fibroblasts from normoxic calves. Neither morphology nor smooth-muscle actin expression predicted the proliferative response to further hypoxic exposure, but Das *et al* identified twice as many pro-proliferative fibroblasts in chronically hypoxic calves than controls. Previously they had identified two fibroblast cell lines cultured from hypoxic neonatal calf aortic adventitia that not only proliferated in response to hypoxic exposure, but also were stable through a series of passages demonstrating a maintained phenotype (Das *et al*, 2001). This suggests that phenotypic variation is not specific to the pulmonary vasculature but can also occur in the systemic circulation albeit in an immature experimental animal model (Das *et al*, 1995; Das *et al*, 2000; Das *et al*, 2001; Das *et al*, 2002).

Pulmonary arterial fibroblast proliferation to hypoxic exposure has been demonstrated in a number of animal models (fetal, neonatal and adult bovine, adult rat and adult human) by a number of different investigators. In an adult bovine model, Welsh *et al* (1998) have demonstrated that pulmonary arterial fibroblasts proliferate vigorously to

hypoxic exposure compared to the same cells cultured in normoxia, or systemic arterial fibroblasts cultured in hypoxia. The increased proliferation seen in pulmonary arterial fibroblasts associated with upregulation of the IP_3/DAG pathway. In a chronically hypoxic rat model the same group have demonstrated that pulmonary arterial fibroblasts cultured in normoxia develop a permissively proliferative phenotype when compared to fibroblasts from the systemic circulation (Welsh *et al*, 2001). In adult bovine and human models, the Scottish Pulmonary Vascular Unit Laboratory has shown that pulmonary arterial fibroblasts can proliferate to hypoxia without additional serum stimulation. In an adult rat model however, adventitial fibroblasts require serum to enable the hypoxic proliferative response (Welsh *et al*, 1998). Other laboratories have been able to demonstrate increased DNA synthesis in pulmonary arterial fibroblasts to hypoxia alone, but have not been able to show increased proliferation (Short *et al*, 2005). This may be as a result of differing experimental models as fibroblast and smooth muscle cell behaviour to hypoxia appears to change with increasing age (Das *et al,* 1997; Xu *et al*, 1997).

There is a body of work documenting the alteration in pulmonary arterial adventitial fibroblast behaviour to chronic hypoxic exposure in a variety of models (Xu *et al*, 1997; Das *et al*, 2000; Welsh *et al*, 2001). Until recently investigators have focused on the 'phenotypic switching' of resident fibroblasts – enabling a *gain of function* towards a pro-proliferative phenotype - but latterly there has been interest in the role of circulating progenitor stem cells in pulmonary adventitial expansion.

In a chronically hypoxic neonatal bovine model Davie *et al* (2004) have demonstrated a significant increase in the density of *vasa vasorum* at all levels of the pulmonary circulation; the differences being most marked in the distal resistance vessels. They

noted that these new blood vessels were immature and leaky (as indicated by the presence of erythrocytes both within the adventitia, and in the adventitial/medial interface) allowing access to the media by circulating mitogens and stem cells. They noted an increase in the number of cells staining positive for the tyrosine kinase marker *c-kit* (a marker for circulating mononuclear (MNC) cells) within the adventitia of pulmonary arteries from chronically hypoxic calves compared to calves raised in normoxia. These MNC when cultured in enriched media were able to differentiate into either EC or SMC – with the transformed EC demonstrating a similar expression profile to EC resident within the *vasa vasorum* or within the vessels. Davie *et al* theorised that the pluripotent MNC either represented external seeding of the adventitia by circulating stem cells, or expansion of a *c-kit* positive subclass of fibroblasts already resident within the adventitia.

Circulating MNC appear to make a significant contribution to adventitial thickening as a result of chronic hypoxic exposure. Using weanling Wistar-Kyoto rat and neonatal bovine models, Frid *et al* (2005; 2006) demonstrated that circulating MNC contributed to approximately one third of proliferating cells within the adventitia. In addition some MNC stained positively for smooth muscle actin and collagen type I (suggesting a myofibroblast phenotype). By blocking MNC production in the bone marrow these investigators have demonstrated a marked reduction in the degree of adventitial expansion to hypoxic exposure in a neonatal rat model (Davie *et al*, 2006).

In spite of the current debate about the origins of cells proliferating within the pulmonary adventitia, there is little to explain why the pulmonary and systemic circulations should behave so differently to both acute and chronic hypoxic exposure. Previous work has shown that pulmonary and systemic arteries behave in a different

manner following hypoxic exposure: pulmonary arteries vasodilatate and systemic arteries vasoconstrict suggesting that there are significant differences between the two circulations (Von Euler and Liljestrand, 1946; Wagner and Mitzner, 1988). This is born out by the results of experimental work examining the relative behaviours of fibroblasts cultured from pulmonary and systemic arteries following both acute and chronic hypoxic exposure in a variety of animal models. Previous work from the Scottish Pulmonary Vascular Unit Laboratory has demonstrated that pulmonary artery fibroblasts harvested from both adult bovine and rat models show a permissively proliferative response to hypoxic exposure, whereas fibroblasts taken from the systemic circulation do not when grown under identical conditions (Scott *et al*, 1998; Welsh *et al*, 1998; Welsh *et al*, 2001). So far this work has only been performed in animal models; we wished to see whether pulmonary and systemic arterial fibroblasts in a human model behaved similarly in order that we might better be able to confirm or refute the extrapolation of results from animal experiments to hypoxic pulmonary vascular disease in man.

3.2. Methods

Cells were obtained from pulmonary and systemic arteries of consented patients undergoing lobectomy for the treatment of lung cancer or coronary artery bypass surgery respectively. Fibroblasts were grown by primary cell culture using an explant technique as described above (chapter 2.3). Human pulmonary (HPAF) and mammary (HMAF) artery fibroblast cells were grown to 60% confluency in 24 well plates. The cells were quiesced for 24 hours using serum-free media; they were then grown in normoxic conditions for 24 hours with the addition of incremental serum concentrations (0, 0.1, 1, 3, 5 & 10%). [^{3}H]Thymidine uptake was used as a surrogate marker for DNA synthesis, cell counts having confirmed this method as being representative of fibroblast proliferation (chapter 2.5).

We wished to examine the relative behaviour of HPAF cells to increasing serum concentrations under both hypoxic growth conditions and those of normoxia for the same time period. HPAF cells were grown to 60% confluency in 24 well plates. The cells were quiesced using serum-free media for a period of 24 hours. They were then cultures in conditions of acute hypoxia (35mmHg PO_2) for 24 hours or normoxia with increasing serum concentrations (0, 0.1, 1, 3, 5 & 10 %). Proliferation was assessed by [^{3}H]Thymidine uptake.

Following this we wished to examine the behaviour of human mammary arterial fibroblast (HMAF) cells under conditions of acute hypoxia. As above HMAF cells were grown to 60% confluency in 24 well plates. They were quiesced using serum-free media for 24 hours. They were then grown in conditions of normoxia or hypoxia (35mmHg PO_2) for a period of 24 hours. The cells were incubated with incremental

serum concentrations (0, 0.1, 1, 3, 5 & 10 %). Cellular proliferation was assessed using [^{3}H]Thymidine uptake as above.

A visual assessment of pulmonary arterial fibroblast proliferation was made. In addition cell counts were performed on both systemic and pulmonary arterial fibroblast cells cultured in both normoxic and hypoxic conditions with or without 5% serum stimulation for a period of 24 hours. This confirmed that [^{3}H]Thymidine assays were a good proxy for cellular proliferation – as a result of this concurrence further cell counts were not performed for subsequent experiments.

All experiments were repeated 4 times in the same individual – the results shown are representative of the mean of those experiments. The experiments were repeated in a total of 4 individuals.

3.3. Statistics

Results are expressed as the mean + S.D. with statistical analysis being carried out as described in Chapter 2.6 with * indicating a statistically significant result ($p<0.05$).

3.4. Results

Human pulmonary arterial fibroblasts demonstrate dose-response to increasing serum concentrations under normoxic growth conditions for 24 hours

HPAF cells were grown in normoxic conditions in 24 well plates to 60% confluency. They were then serum starved for a period of 24 hours and were then grown in normoxic conditions with increasing serum concentrations (0, 0.1, 1, 3, 5 & 10%) for a further 24 hours. HPAF proliferation was assessed by [^{3}H]Thymidine up take as described above (chapter 2.5.1). [^{3}H]Thymidine incorporation by human pulmonary artery fibroblasts showed a dose-response to increasing serum concentrations under normoxic growth conditions for 24 hours (**figure 3.1.a**). Cellular proliferation was also assessed visually over a 24h period **figure 3.1.b**. **Figure 3.1.b A** and **B** shows serum-starved normoxic fibroblast cells at t = 0. **Figure 3.1.b C** and **D** shows fibroblast cells at 12 and 24h later after 5% serum stimulation. Cell counts were confirmed manually using a haemocytometer following fibroblast cell staining with propodium iodide (chapter 2.5.1). Visual assessment and cell counting techniques confirmed that [^{3}H]Thymidine uptake was a reliable indicator of proliferation as a result of this further cell counts were not performed in subsequent experiments. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD with * indicating a statistically significant result ($p<0.05$).

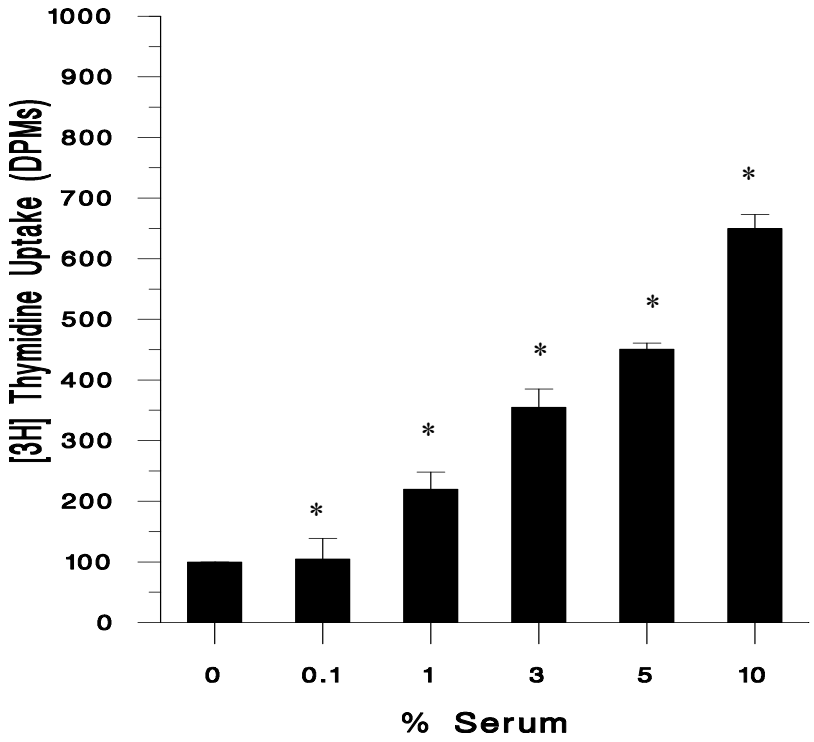


Figure 3.1a: Human pulmonary arterial fibroblasts demonstrate dose-response to increasing serum concentrations under normoxic growth conditions for 24 hours. Human pulmonary artery fibroblasts proliferated in a dose-response manner to increasing serum concentrations (0, 0.1, 1, 3, 5 & 10%) under normoxic growth conditions for 24 hours with proliferation being assessed by [^{3}H]Thymidine counts (DPM ~ disintegrations per minute). The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD with * indicating a statistically significant result ($p<0.05$).

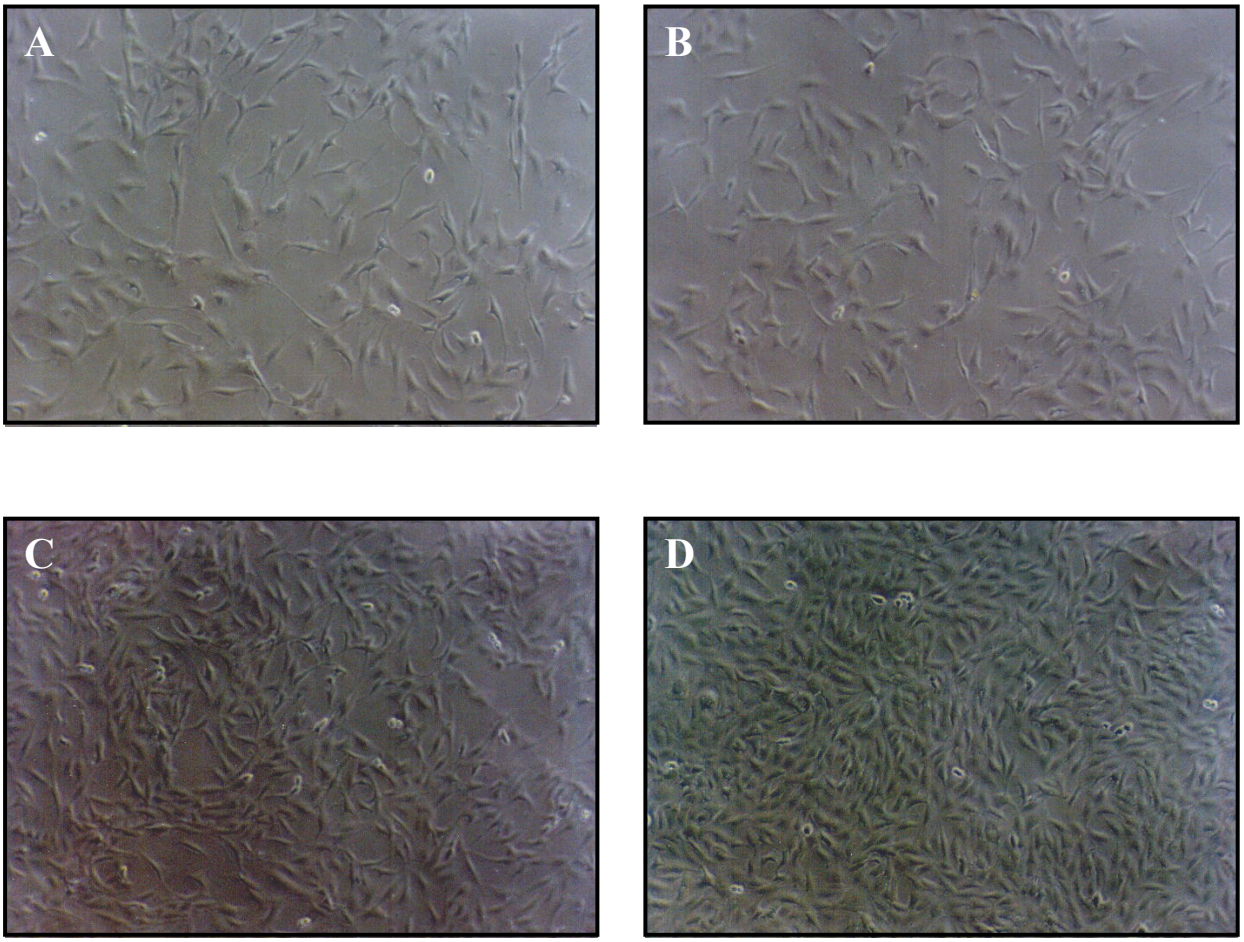


Figure 3.1.b. Visual assessment of human pulmonary arterial fibroblast proliferation. Visual confirmation of the hypoxic proliferation of these cells over a 24h period when compared with those maintained in normoxia can be seen in **figure 3.1.b**. **Figure 3.1.b A** and **B** shows serum-starved normoxic fibroblast cells at t = 0. **Figure 3.1.b C** and **D** shows fibroblast cells 12 and 24h later after 5% serum stimulation.

Human mammary arterial fibroblasts demonstrate dose-response to increasing serum concentrations under normoxic growth conditions for 24 hours

HMAF cells were grown in 24 well plates under normoxic conditions to 60% confluency. They were then quiesced with serum-free medium for 24 hours. They were then cultured with increasing serum concentrations (0, 0.1, 1, 3, 5 & 10%) under normoxic growth conditions for 24 hours. **Figure 3.2** demonstrated that [^{3}H]Thymidine incorporation by human systemic artery fibroblasts showed a dose-response to increasing serum concentrations. The growth response appeared similar to that seen in human pulmonary arterial fibroblasts cultured under similar conditions. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD.

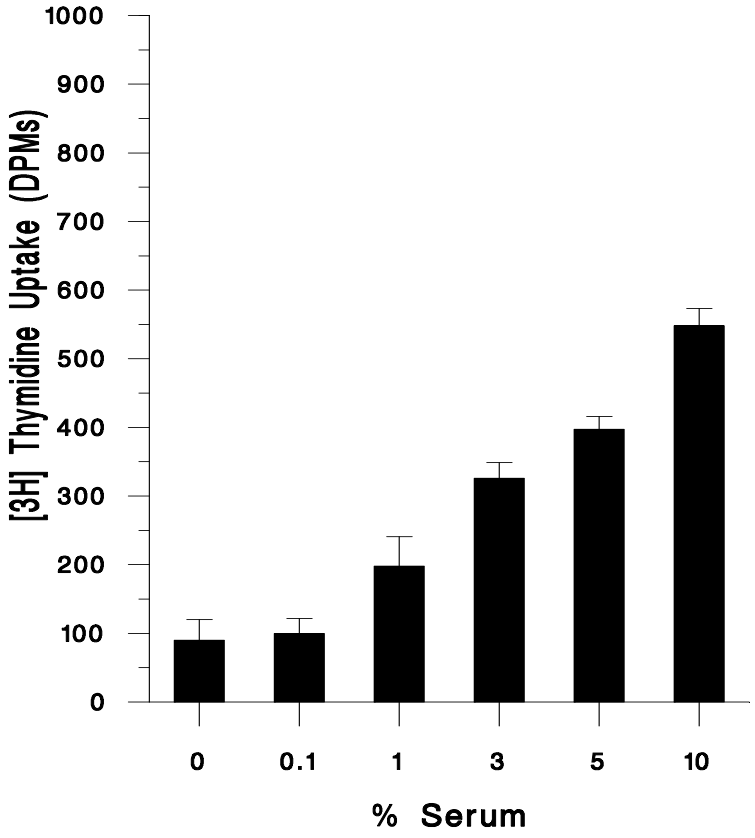


Figure 3.2: Human mammary arterial fibroblasts demonstrate dose-response to increasing serum concentrations under normoxic growth conditions for 24 hours

Human systemic artery fibroblasts proliferate in a dose-response manner to increasing serum concentrations (0, 0.1, 1, 3, 5 & 10%) under normoxic growth conditions for 24 hours. Cellular proliferation was assessed by [^{3}H]Thymidine uptake measured in DPM ~ disintegrations per minute. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD.

Human pulmonary arterial fibroblasts demonstrate increased proliferation to acute hypoxia compared with normoxic growth conditions for 24 hours.

HPAF cells were grown in 24 well plates to 60% confluency under normoxic conditions. They were then quiesced in serum free medium for a period of 24 hours. They were then cultured with incremental serum concentrations under either normoxic or hypoxic growth conditions for a further 24 hours. **Figure 3.3** demonstrates that under 24 hours normoxic conditions human pulmonary artery fibroblasts demonstrated increased proliferation (as indicated by an increase in [^{3}H]Thymidine incorporation) in a dose-dependent manner to increasing serum concentrations (**figure 3.1**). HPAF grown in conditions of acute hypoxia demonstrated increased proliferation to incremental serum concentrations (0, 0.1, 1, 3, 5 & 10%) when compared to identical cells grown in normoxic conditions as evidenced by increased [^{3}H]Thymidine incorporation. Hypoxia alone without additional serum stimulation was enough to cause increased proliferation in HPAF cells. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.05$).

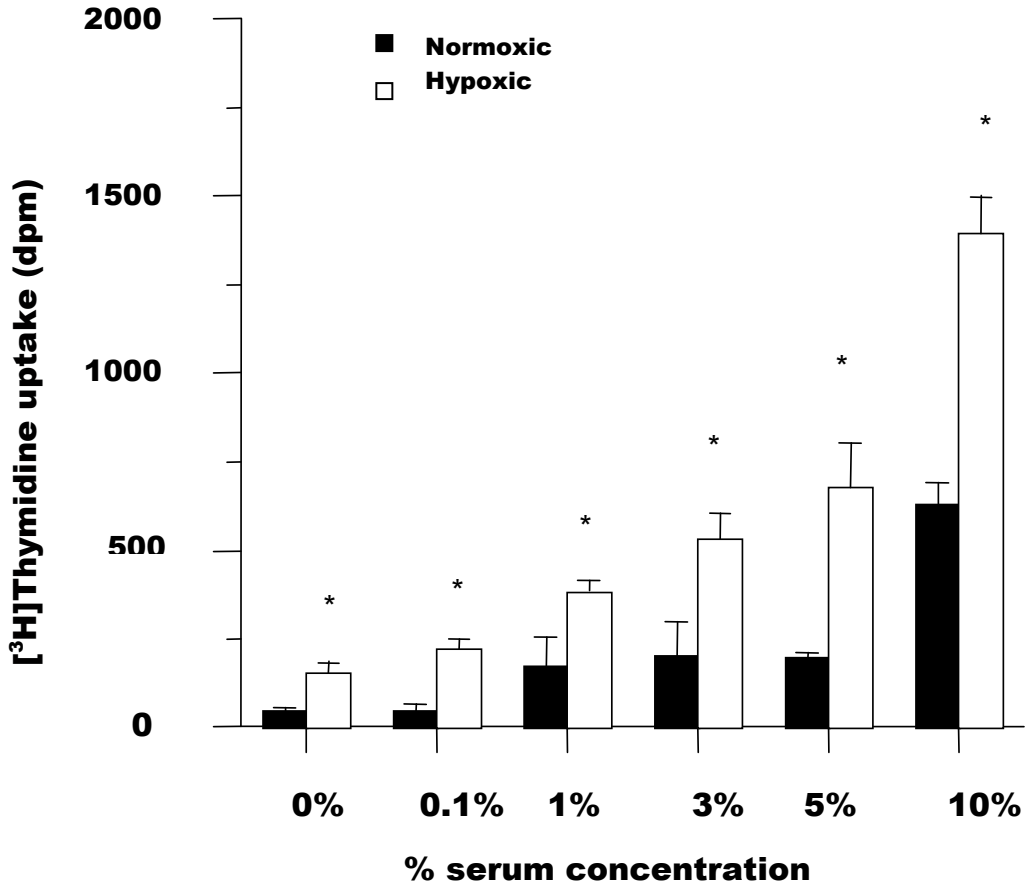


Figure 3.3: Human pulmonary arterial fibroblasts demonstrate increased proliferation to acute hypoxia compared with normoxic growth conditions for 24 hours. Hypoxic HPAF cell (white bar) show increased proliferative response to increasing serum concentrations (0, 0.1, 1, 3, 5 & 10%) when compared to identical cells cultured under normoxic conditions (black bar). Cellular proliferation was assessed by [^{3}H]Thymidine uptake – (DPM) disintegrations per minute. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.05$).

Human mammary arterial fibroblasts do not demonstrate increased proliferation to 24 hours hypoxia (PO_2 35 mmHg).

HMAF cells were grown in 24 well plates until 60% confluent under conditions of normoxia. They were then quiesced with serum free media for a period of 24 hours. They were then cultured in conditions of either normoxia or hypoxia for a further 24 hours with increasing serum concentrations (0, 0.1, 1, 3, 5 & 10%). **Figure 3.4** shows that although HMAF cells demonstrated incremental proliferation to increasing serum concentrations under normoxic (black bar) growth conditions (24 hours) no increased proliferation to acute hypoxia 5% PO_2 ~ 35 mmHg (white bar) was demonstrated. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between normoxic (black bar) and hypoxic (white bar) groups.

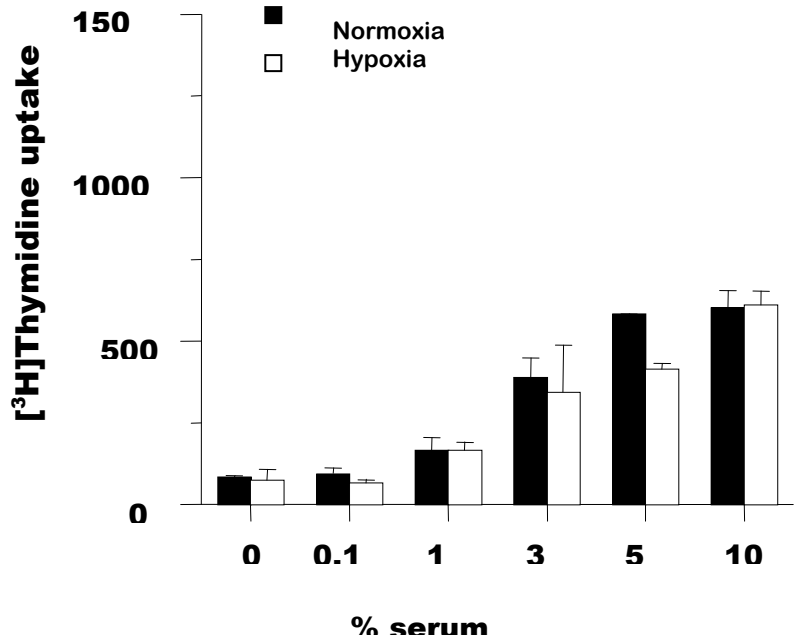


Figure 3.4: Human mammary arterial fibroblasts do not demonstrate increased proliferation to acute hypoxia – 24 hours 5% PO2 ~ 35mmHg. HMAF cells show a dose response to increasing serum concentrations (0, 0.1, 1, 3, 5 & 10%) under both hypoxic (white bar) and normoxic (black bar) growth conditions. Cellular proliferation was assessed by [^{3}H]Thymidine uptake – disintegrations per minute (DPM). The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between the 2 groups.

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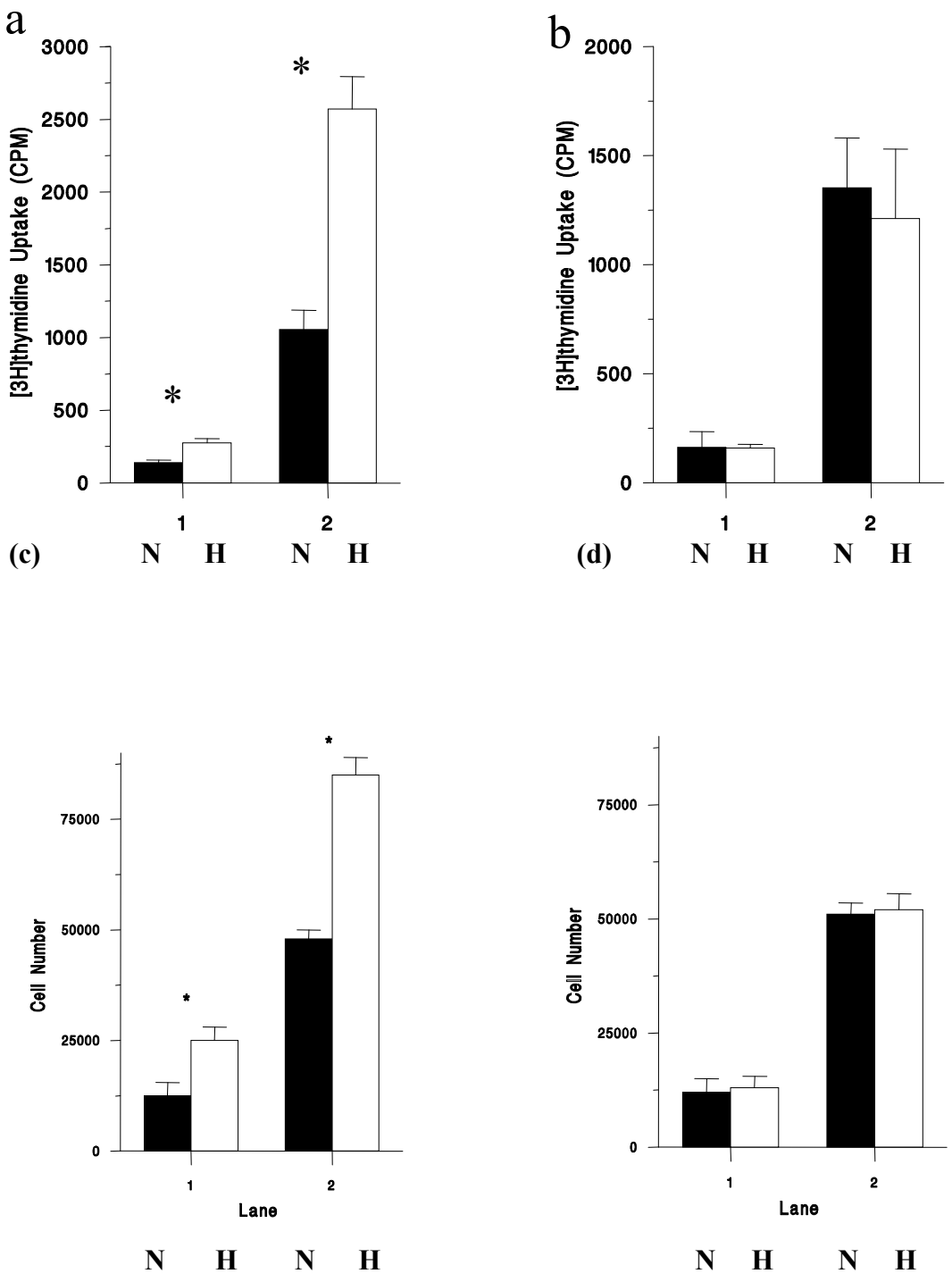
Effect of Hypoxia on proliferation of human pulmonary and systemic artery fibroblasts. a, pulmonary artery fibroblasts were maintained in normoxic **(black bar)** or hypoxic **(white bar)** conditions in the absence **(1)** or presence **(2)** of 5% serum. **Figure 3.5** shows that hypoxia alone could induce a significant proliferative response that was greatly augmented in the presence of 5% serum. **b,** systemic (mammary) artery fibroblasts were treated in the same way as the pulmonary fibroblasts. Hypoxia, whether in the presence or absence of serum, had no effect on proliferation of the systemic artery fibroblasts. **Figures 3.4 c & d** demonstrate the rate of fibroblast proliferation using cell counting in response to hypoxia and that [^{3}H]thymidine uptake is a good proxy for cellular proliferation. The data shown are representative data of 4 experiments performed in the same individual. The experiment was performed in 4 individuals. The results are expressed as mean +/- 2 SD, * signifies a statistically significant result ($p < 0.05$).

Figure 3.5 a shows that hypoxia (H) alone could induce a significant proliferative response that was greatly augmented in the presence of 5% serum in pulmonary arterial fibroblasts when compared to normoxic (N) growth conditions. This response was not seen in systemic (mammary) artery fibroblasts **(b)** which were treated in the same way as the pulmonary fibroblasts. Hypoxia (H), whether in the presence or absence of serum, had no effect on proliferation of the systemic artery fibroblasts. **Figures 3.4 c & d** demonstrate the rate of fibroblast proliferation using cell counting in response to hypoxia and that [^{3}H]Thymidine uptake is a good proxy for cellular proliferation. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.05$).

Figure 3.5


a
b
[3H]thymidine Uptake (CPM)
3000
2500
2000
1500
1000
500
0
2000
1500
1000
500
0
*
*
1
2
N H N H
(c)
(d)
Cell Number
75000
50000
25000
0
Lane
1
2
N H N H

3.5. Discussion

In this chapter we have demonstrated that both human pulmonary and systemic arterial fibroblasts proliferate to increasing concentrations of serum in a dose-dependent manner. We have also demonstrated that human pulmonary arterial fibroblasts demonstrate an additional proliferative response when exposed to acute hypoxia; a capacity to proliferate that can occur in the absence of serum stimulation. Hypoxia-related proliferation is not seen in human systemic arterial fibroblasts cultured under identical conditions.

These findings agree with other experimental data from the Scottish Pulmonary Vascular Unit Laboratory which demonstrate that pulmonary arterial fibroblasts show a permissively proliferative phenotype when exposed to hypoxia (both acute and chronic) in both adult bovine and rat models when compared to identical cells cultured from the systemic circulation (Scott et al, 1998; Welsh *et al*, 1998; Welsh *et al*, 2001; Welsh *et al*, 2006). This concurs with work from other laboratories, albeit using different models. Krick *et al* (2005) have demonstrated human pulmonary arterial fibroblast proliferation to acute hypoxic exposure (as assessed by cell cycle progression and reduced apoptosis), when compared to renal arterial fibroblast control cells. These investigators, using pulmonary arterial fibroblasts taken from small vessels (< 1mm diameter), found that these cells proliferated maximally when cultured in 1.5% oxygen and noted an increase in the apoptotic rate of renal arterial fibroblasts cultured under identical conditions. In a neonatal bovine model, Das *et al* (2001) demonstrated a significantly higher proportion of replicating pulmonary arterial fibroblast cells when grown under conditions of acute hypoxia than identical cells grown in normoxic conditions; they also noted that fibroblasts taken from neonatal bovine aorta generally did not proliferate to hypoxic exposure. Of interest

however is that these investigators were able to identify some aortic adventitial fibroblasts that were able to proliferate to hypoxia, suggesting a degree of plasticity in the immature circulation that is not seen in the adult. Consistent with differences in developmental stage and species, work from the Scottish Pulmonary Vascular Unit Laboratory has repeatedly demonstrated that pulmonary arterial fibroblasts from adult bovine and human models are able to proliferate to acute hypoxia alone without additional serum stimulation (Scott *et al*, 1998; Welsh *et al*, 1998). Other investigators have only been able to show increased DNA synthesis – as assessed by [^{3}H]Thymidine uptake – but not increased proliferation to hypoxia alone in a juvenile model (Short *et al*, 2005).

There has been a robust debate about the origin of pulmonary arterial fibroblasts that proliferate to hypoxia. It is not known whether the vigorous proliferative response seen in pulmonary arterial fibroblasts to hypoxia represents a global increase in proliferative potential of all resident cells, or whether it results in the preferential growth of subpopulations of fibroblasts more able to proliferate to hypoxia, or whether circulating precursor cells also contribute to hypoxia-mediated adventitial expansion. Given the functional and structural heterogeneity of fibroblasts (Jordana *et al*, 1987; Raghu *et al*, 1988; Goldring *et al*, 1990; Rodemann and Muller, 1990; Derdak *et al*, 1992; Hakkinen and Larjava, 1992) it seems likely that adventitial expansion is the result of selective expansion of resident fibroblast subpopulations that are hypoxia-responsive.

In the attempt to characterise pulmonary arterial adventitial fibroblasts, Das *et al* (2002) using limited dilutional cloning techniques, were unable to demonstrate a reliable means of predicting adventitial fibroblast growth characteristics in a

chronically hypoxic neonatal calf model, based on cell morphology. They did show that fibroblasts taken from chronically hypoxic animals demonstrated a higher proportion of proliferating cells than their control littermates when these cells were grown under normoxic conditions. Previously, the same laboratory had demonstrated that chronic hypoxic exposure resulted in increased smooth-muscle actin expression in cultured neonatal bovine pulmonary arterial fibroblasts (Stenmark *et al*, 2002). Smooth muscle actin is considered a marker for myofibroblast cells – so-called 'activated fibroblasts' or 'hybrid non-muscle' muscle cells – and increased smooth-muscle actin expression is synchronous with increased myofibroblast number in a systemic balloon angioplasty injury model – suggesting the development of a more active fibroblast phenotype (Sartore *et al*, 2001). However in a chronically hypoxic neonatal bovine model, Das *et al* (2002) found that fibroblast smooth-muscle actin expression was a poor predictor of adventitial fibroblast behaviour to hypoxia. In a human model, we were unable to demonstrate increased smooth-muscle actin expression following acute hypoxic exposure - a finding that has been confirmed by other investigators (Krick *et al*, 2005). The differences in smooth-muscle actin expression may be as a result of inter-species differences in the regulatory regions of the smooth-muscle actin gene (Min *et al*, 1990) but also highlight the difficulties of attempting to sub-type adventitial fibroblasts and the significant diversity apparent when using non-human experimental models.

Significant diversity occurs not only between species, but also within the same species model at different developmental stages. Illustrative of this point, work from Das *et al* (1997) demonstrated that pulmonary arterial fibroblasts from adult cattle proliferated less vigorously to acute hypoxia than identical cells from fetal or neonatal animals. These investigators found that the variation in proliferative response was

associated with differing expression of Protein Kinase C (PKC) isozymes, with more immature cells expressing largely Ca^{2+} dependent isozymes α and βII, with only PKC ζ (a Ca^{2+} independent isozyme) being reproducibly expressed across all generations. Work from the same laboratory also demonstrated a similar age-related variation in proliferative potential in a pulmonary arterial smooth muscle cell model (Xu *et al*, 1997). Of note is the fact that smooth muscle cells from animals exposed to chronic hypoxia appeared to have been phenotypically modified, showing increased proliferative potential to normoxia when compared to identical cells cultured from normoxic controls. Xu *et al* theorised that this represented a specific *gain of function* rather than a reversion to a more immature phenotype, as indicated by differing PKC isozyme expression patterns.

In spite of the differences that exist between animal models, chronic hypoxic exposure appears to exert a reliably modulatory effect on pulmonary vascular cell phenotype. Work from the Scottish Pulmonary Vascular Unit Laboratory in a rat model has demonstrated that chronic hypoxic exposure results in increased proliferative potential in pulmonary arterial fibroblasts when cultured in normoxia compared to normoxic controls (Welsh *et al*, 2001). The increase in proliferative potential in this model was associated with constitutive activation of p38 MAPK, a SAPK that has been shown to have a significant role in pulmonary arterial fibroblast proliferation to acute hypoxia in adult bovine and rat models (Welsh *et al*, 1998; Welsh *et al*, 2006). In a chronically hypoxic adult rat model Welsh *et al* (2001) did not demonstrate any further proliferative response when pulmonary arterial fibroblasts were exposed to further hypoxia. Das *et al* (2000) demonstrated increased proliferation of pulmonary arterial fibroblasts from chronically hypoxic neonatal calves when grown in normoxia but also found that further acute hypoxic exposure

resulted in an augmented proliferative response. The increased proliferative response of pulmonary arterial fibroblasts to further hypoxic exposure in a chronically hypoxic neonatal calf model may be a result of the plasticity of an immature experimental model.

Recent work has investigated the contribution of circulating precursor cells to adventitial compartment expansion to chronic hypoxic exposure. Davie *et al* (2004) have identified accumulation of cells positive for *c-kit* – a transmembrane spanning receptor expressed by cells of bone marrow origin – in the adventitia and extravascular adventitial portions of pulmonary arteries taken from neonatal cattle exposed to chronic hypoxia. In addition, mononuclear cells (MNC) taken from chronically hypoxic animals were able differentiate into smooth-muscle actin expressing cells using conditioned media. Work from the same laboratory has shown that in primary cell culture, pulmonary adventitial fibroblast cells did not express *c-kit*, nor did they express markers for MNC such as CD45 (Frid *et al*, 2005; Frid *et al*, 2006). This suggested that a significant proportion of proliferating adventitial fibroblasts may have originated outside the vascular wall. Work from Hayashida *et al* (2005) confirmed this in a lethally irradiated mouse model repopulated with radiolabelled bone marrow cells. Radiolabelled cells were found to accumulate in the pulmonary arteries of irradiated mice, but not in the systemic circulation, nor in any other organ when compared to control animals. A significant expansion in the *vasa vasorum* of pulmonary, but not systemic arteries taken from chronically hypoxic neonatal calves, particularly marked in resistance vessels, would permit access by MNC to the adventitia (Davie *et al*, 2004; Davie *et al*, 2006). The expanded *vasa vasorum* appears to be functionally immature – allowing extravasation of circulating erythrocytes into the adventitial compartment and adventitial perivascular

compartment – enabling access by circulating mitogens from both autocrine and paracrine sources.

There is evidence to demonstrate that pulmonary arterial fibroblasts proliferate to hypoxic growth conditions whereas identical cells taken from the systemic circulation do not. Our findings from an adult human model under conditions of acute hypoxia concur with these data. It is not certain whether this increased proliferation is as a result of an increase in proliferative potential of all pulmonary arterial fibroblasts or whether this represents the expansion of subpopulations within the adventitia that are more responsive to hypoxic stimuli. Unlike other laboratories we have demonstrated that hypoxia alone is adequate to stimulate pulmonary arterial fibroblast proliferation without the addition of other mitogens in both human and bovine adult models. These findings are likely to result from differences in experimental model. Further speculation regarding the origin of fibroblasts within the adventitia following chronic hypoxic exposure is less certain.

Chapter 4:

To investigate the relationship between hypoxia-mediated proliferation and stress activated protein kinase (SAPK) signalling in human pulmonary and systemic arterial fibroblast cells.

4.1. Introduction

In chapter 3 we demonstrated that human pulmonary arterial fibroblast cells proliferate to acute hypoxic exposure while human mammary arterial fibroblast cells grown in identical conditions do not. This is in keeping with previous work not only from the SPVU Laboratory but also from other investigators. We have shown increased pulmonary arterial fibroblast proliferation to hypoxic exposure in adult bovine and rat models (Scott *et al*, 1998; Welsh *et al*, 1998; Welsh *et al*, 2001). Other investigators have produced similar results in both fetal and neonatal bovine models under both acute and chronically hypoxic conditions (Das *et al*, 1995; Das *et al*, 2000; Das *et al*, 2001).

The mechanism behind the differential response of systemic and pulmonary arterial fibroblasts to hypoxia has not been fully elucidated. Earlier work from our laboratory demonstrated a link between hypoxia-mediated proliferation in adult bovine pulmonary arterial fibroblasts with increased inositol tri-phosphate (IP_3) generation – a protein component of GPCR signalling which is responsible for mediating Ca^{2+} release from sarcoplasmic and endoplasmic reticulum which is a necessary cofactor for many intra-cellular enzymes (Welsh *et al*, 1998) (Introduction 1.11.2.3). In the same experimental model pulmonary arterial fibroblast proliferation to hypoxia was

also found to be associated with increased levels of mitogen activated protein kinases (MAPKs) (Scott *et al*, 1998).

As discussed previously (Introduction 1.11.6), MAPKs are dual specificity serine/threonine kinases that appear to be involved in a variety of cellular responses and which can be activated by a variety of up-stream signalling cascades. The classic MAPKs (p44/p42 MAPK ~ ERK 1/2) are involved in cellular proliferation in some cell lines while the SAPK (p38 MAPK and JNKs) are known to mediate stress responses (Han *et al*, 1994; Lee *et al*, 1994; Rouse *et al*, 1994; Raingeaud *et al*, 1995; Verheij *et al*, 1996). There is evidence that p38 MAPK is involved with inflammatory responses and regulation of the immune system (Hale *et al*, 1999; Ono and Han, 2000) in addition it has also been implicated in a variety of vascular disease processes (Ono and Han, 2000; Behr *et al*, 2003; Ju *et al*, 2003).

Four isoforms of p38 MAPK have so far been identified: α, β, γ and δ with p38 α and β isoforms ubiquitously expressed whereas γ and δ isoforms demonstrating tissue specificity (Enslen *et al*, 2000; Brancho *et al*, 2003). The γ isoform is strongly expressed in skeletal muscle and may have a role in basal state glucose uptake; it may also be involved with muscle cell differentiation (Wang *et al*, 1997; Sweeney *et al*, 1999; Ono and Han, 2000; Fujishiro *et al*, 2001). The δ isoform is expressed in glandular tissue – its exact function remains unknown (Ono and Han, 2000). Isoform expression may also vary with developmental stage in addition to varying within tissue type.

Previous work from the SPVU laboratory has demonstrated an important role for MAPKs in the pulmonary arterial fibroblast hypoxia-mediated response. Scott *et al* (1998) identified that increased pulmonary arterial fibroblast proliferation to hypoxia was associated with increased p38 and JNK but not p44/p42, activation in an adult bovine model. In a chronically hypoxic adult rat model, Welsh *et al* (2001) demonstrated constitutive activation of p38 MAPK that was associated with a permissively proliferative phenotype in pulmonary arterial fibroblasts. The same investigators also noted constitutive activation of p44/p42 MAPK but noted that this also occurred in the systemic circulation, making the significance of this less certain. It appeared that pulmonary arterial fibroblasts cultured from chronically hypoxic animals had in someway been modified and that their enhanced proliferative potential was linked to alterations of p38 MAPK activation as levels of whole p38 MAPK remained stable in both experimental and control animals.

While pulmonary arterial fibroblasts appear to exhibit enhanced growth potential to hypoxia – whether acute or chronic – the exact mechanism by which hypoxia-mediated signalling occurs is still uncertain. There is a body of work that investigates the relative contribution of hypoxia inducible factors-1, 2 and 3 (HIF1/2/3) in oxygen sensing. HIF-1 is ubiquitously expressed and is known to upregulate gene products that enable cellular adaptation to hypoxic environments, either in the short term – for example enzymes involved in anaerobic metabolism – or long-term hypoxia – EPO and VEGF (Semenza, 2000a). The expression of HIF-2 and 3 appears to be more restricted with HIF-2 expression being most marked within the lungs (Krick *et al*, 2005; Eul *et al*, 2006). HIF-1 is a two subunit basic helix-loop-helix protein complex, the β subunit of which is constitutively expressed in all cells. Control of HIF-1

activity is achieved via the rapid breakdown of the HIF-1α subunit under normoxic conditions by proteosomal ubiquitinylation. In normoxia HIF-1α is targeted for enzymatic degradation by the interaction of molecular oxygen, iron and the von Hippel Lindau Protein (pVHL) and under these conditions HIF-1α has a half-life of less than 5 minutes (Yu *et al*, 1998). However under hypoxic conditions pVHL is unable to bind HIF-1α – a direct result of low cellular oxygen concentrations – enabling the α and β subunits to combine to form an active protein which is then able to upregulate transcription of genes containing hypoxia response elements (HRE) (Lee *et al*, 1997; Semenza, 2000a; Semenza, 2000b; Semenza, 2001).

Experimental work has demonstrated that mice heterozygote for HIF1 $\alpha^{+/-}$ develop less right ventricular hypertrophy and pulmonary vascular resistance to hypoxic exposure compared to controls (Semenza, 2000b) while mice heterozygote for HIF2$\alpha^{+/-}$ appear to be completely protected from hypoxic pulmonary hypertension (Brusselmans *et al*, 2003). Given that HIF-1α inactivation requires molecular oxygen and that hypoxia-mediated proliferation in pulmonary arterial fibroblasts has been linked to p38 MAPK activation in a number of animal models, a functional relationship may exist between the two. Previous work from the SPVU laboratory has demonstrated that pulmonary arterial fibroblasts appear to demonstrate enhanced proliferation to hypoxic exposure - whether acute or chronic – whereas identical cells from the systemic circulation do not. This has been demonstrated in adult bovine and rat models. In animal models increased proliferative potential has been associated with upregulation of MAPK activity – specifically p38 MAPK – while the relative roles of other MAPKs are less consistent. We wished to establish whether MAPK were also involved in the human pulmonary artery fibroblast proliferation

demonstrated in chapter 3. We also wished to determine the relative contribution of p38 MAPK isoforms in hypoxia-mediated proliferation and whether any link could be demonstrated between p38 MAPK activation and HIF1α expression.

4.2. Methods

4.2.1. Stress activated protein kinase (SAPK) (p38 and JNK MAPK) and classic (ERK 1/2) MAPK expression in human pulmonary (HPAF) and systemic (HMAF) artery fibroblasts grown in normoxia and acute hypoxia.

Cells were obtained from pulmonary arteries of consented patients undergoing lobectomy for the treatment of lung cancer and systemic arteries from consented patients undergoing coronary artery by-pass grafting. Cells were prepared by the method described above (Chapter 2.3). Cells were grown to 60% confluence in 6 well plates and were then quiesced using serum-free media for a period of 24 hours. They were then grown in conditions of normoxia or hypoxia (35mmHg PO_2 ~ 5%) for a total period of 48 hours with the addition of 5% serum stimulation. Cell lysates were prepared at a variety of time points (t = 0, 1, 2, 4, 8, 16, 24, 32 and 48 hours) using the method previously described (chapter 2.6). MAPK phosphorylation was assessed using Western blotting with retardation on SDS-PAGE gels as a marker of phosphorylation using the appropriate primary and secondary antibodies on nitrocellulose membranes and a Chemiluminescence detection system (chapter 2.6).

We wished to assess the relative activation of both classic (ERK 1/2) and stress (JNK and p38) MAPKs in HMAF and HPAF cells under conditions of normoxia and acute hypoxia. Membranes were loaded with cell lysates from HMAF and HPAF cells grown under conditions of normoxia and acute hypoxia at a variety of time points (t = 0, 1, 2, 4, 8, 16 and 24 hours) and probed using a 1:500 dilution of a rabbit anti-phospho ERK monoclonal IgG antibody with a 1:1000 dilution of a flagged anti-rabbit monoclonal IgG secondary antibody. The membrane was then stripped (using the method described above) and re-probed using a 1:500 dilution of a rabbit anti-

whole ERK monoclonal IgG antibody and a 1:1000 dilution of a flagged anti-rabbit monoclonal IgG secondary antibody. This experiment was repeated looking for expression of phospho-JNK MAPK using a 1:500 dilution of a rabbit anti-JNK monoclonal IgG antibody and a 1:1000 dilution of a flagged anti-rabbit monoclonal IgG secondary antibody. The membrane was then stripped and re-probed for whole JNK activity using a 1:500 dilution of a rabbit anti-whole JNK IgG monoclonal antibody and a 1:1000 dilution of a flagged anti-rabbit monoclonal IgG secondary antibody. This procedure was repeated for expression of phospho-p38 MAPK using a 1:500 dilution of a rabbit anti-phospho-p38MAPK monoclonal IgG antibody and a 1:1000 dilution of a flagged anti-rabbit monoclonal IgG secondary antibody. The membrane was then stripped and re-probed using a 1:500 dilution of a rabbit anti-whole p38MAPK monoclonal IgG antibody using a 1:1000 dilution of a flagged anti-rabbit monoclonal IgG secondary antibody.

4.2.2. Stress activated p38 MAPK expression at different time points over 24 hours of hypoxic exposure.

HPAF cells were grown in 6 well plates to 60% confluency under normoxic growth conditions. They were quiesced for a period of 24 hours using serum-free media. They were then exposed to hypoxia for a range of time points (time = 0, 2, 3, 4, 6, 8, 10, 12, 16, 18 and 24 hours) with the addition of 5% serum. Cell lysates were prepared as previously described; solubilised protein was transferred to nitrocellulose membrane using SDS-PAGE. Phospho and whole p38 MAPK expression were assessed using the appropriate primary and secondary antibodies (at concentrations listed above) using Western Blotting techniques.

4.2.3. Influence of phospho-ERK and phospho p38 MAPK inhibitors on HMAF and HPAF hypoxia-mediated growth.

HMAF and HPAF cells were grown in 24 well plates to 60% confluency and were then quiesced using serum-free medium for a period of 24 hours. The cells were then subjected to either normoxic or hypoxic (35mmHg PO_2) growth conditions for 24 hours in the absence of serum, the presence of 5% serum or the presence of 5% serum plus a specific phospho-ERK inhibitor U0126 added 6 hours prior to the end of the experiment. Four hours before the end of the experiment the cells were labelled with [^{3}H]Thymidine (0.1μCi/well). The samples were processed as described above (Methods 2.5.1) with radiation being measured by scintillation counting and results being expressed as disintegrations per minute (DPM). This experiment was repeated adding SB203580 - a specific p38 MAPK inhibitor - 6 hours prior to the end of the experiment.

4.2.4. Reoxygenation study to establish temporal relationship between phospho-p38 MAPK expression and hypoxia-mediated proliferation in HPAF cells.

HPAF cells were cultured in 24 well plates until 60% confluent. They were then quiesced for a period of 24 hours using serum free medium. 5% serum was added to each well and the cells were then grown in conditions of either normoxia or hypoxia over a variety of time points (t = 6, 16, 20 and 24 hours). Those cells grown under hypoxic conditions were returned to normoxia for the duration of the 24-hour experimental period. Four hours prior to the end of the experiment [^{3}H]Thymidine was added to each well and the samples were processed as described above.

4.2.5. Phospho-p38 MAPK isoforms and hypoxia-mediated proliferation in HPAF cells.

HPAF cells were grown in 6 well plates to 60% confluency. They were then quiesced for 24 hours using serum-free medium. 5% serum was added to each well and the cells were grown in hypoxic conditions (35mmHg PO_2) for 16 hours. Cell lysates were then prepared (as described above). As there were no specific primary antibodies to the α, β, γ and δ isoforms of p38 MAPK at the time of the experiment, cell lysates were subjected to Catch and Release® immunoprecipitation (Methods 2.6.3.3) using primary antibody capture ligands specific for the α, β, γ and δ isoforms. The relevant isoforms were released using elution buffer and the resulting samples were then processed as Western Blots probed for phospho-p38 MAP with sorbitol being used as a positive control. The membrane was then stripped as described above and re-probed for whole p38 MAPK expression.

4.2.6. Relationship between phospho-p38 MAPK expression and HIF-1 α in HMAF and HPAF cells exposed to hypoxic growth conditions.

HPAF and HMAF cells were grown in 6 well plates to 60% confluency – they were then transferred to either normoxic or hypoxic (35mmHg PO_2) growth conditions for 16 hours with the addition of 5% serum. SB203580 – a specific p38 MAPK inhibitor at t = 0, 2, 4 and 6 hours prior to the start of the experiment. Cell lysates were prepared and processed as previously described by immunophoresis and Western Blotting techniques. The membrane was stripped and then probed for whole-p38 MAPK (as described above).

4.3 Densitometry.

Densitometric analysis of blots was carried out using a computer programme that allowed for comparison of blot density in graphical form (Quantiscan) with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control.

4.4. Statistics

All experiments were repeated 4 times in the same individual and were performed in a total of 4 individuals. The results presented are representative of the mean of experiments performed in a single individual. Results are expressed as the mean +/- 2 S.D. and statistical analysis was undertaken as described in chapter 2.8.

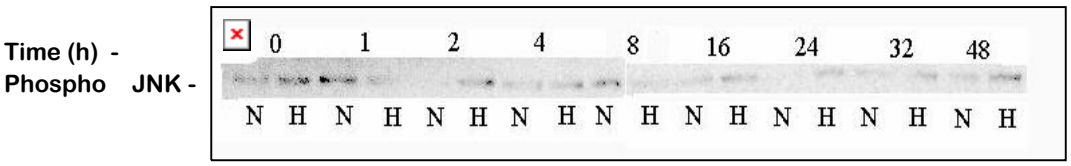
4.4. Results

Human pulmonary arterial fibroblasts do not express increased levels of phosphorylated JNK under either normoxic or hypoxic growth conditions t = 0 – 48 hours.

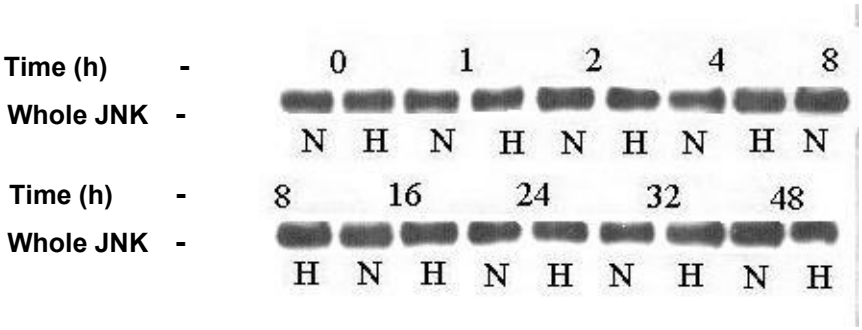
Pulmonary arterial fibroblasts were grown in normoxia and 5% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 48 hours and lysates were prepared for Western Blot analysis as outlined in Chapter 2.6. As seen in **figure 4.1** there was no expression of phosphorylated JNK in HPAF cells grown in either normoxic (N) or hypoxic (PO_2 ~ 35mmHg) (H) growth conditions at any time point examined **(a)**. The membrane probed for whole JNK expression demonstrates the consistent presence of inactive JNK at all time points and demonstrates equal protein loading **(b).** These results were confirmed by densitometry **(c)** with values expressed as a percentage of proliferation using conditions of normoxia without 5% serum stimulation as a control. The results illustrated are representative of 4 experiments performed in the same individual, the experiment was performed in n = 4 individuals, with results expressed as mean +/- 2 SD. There was no statistical difference between the 2 groups.

Figure 4.1. Human pulmonary arterial fibroblasts do not express increased levels of phosphorylated JNK under either normoxic or hypoxic growth conditions. (a) No phosphorylated JNK was detected in HPAF cells under either normoxic (N) or hypoxic (H) growth conditions over a variety of time points (t = 0, 1, 2, 4, 8, 16, 24, 32, 48 hours. **(b)** The membrane was re-probed for whole JNK demonstrating the presence of consistent whole JNK expression at all time points under both normoxic (N) and hypoxic (H) growth conditions demonstrating equal protein loading. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) using conditions of normoxia without 5% serum stimulation as a control **(c)**. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the individual with results expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) and hypoxic (H) groups.

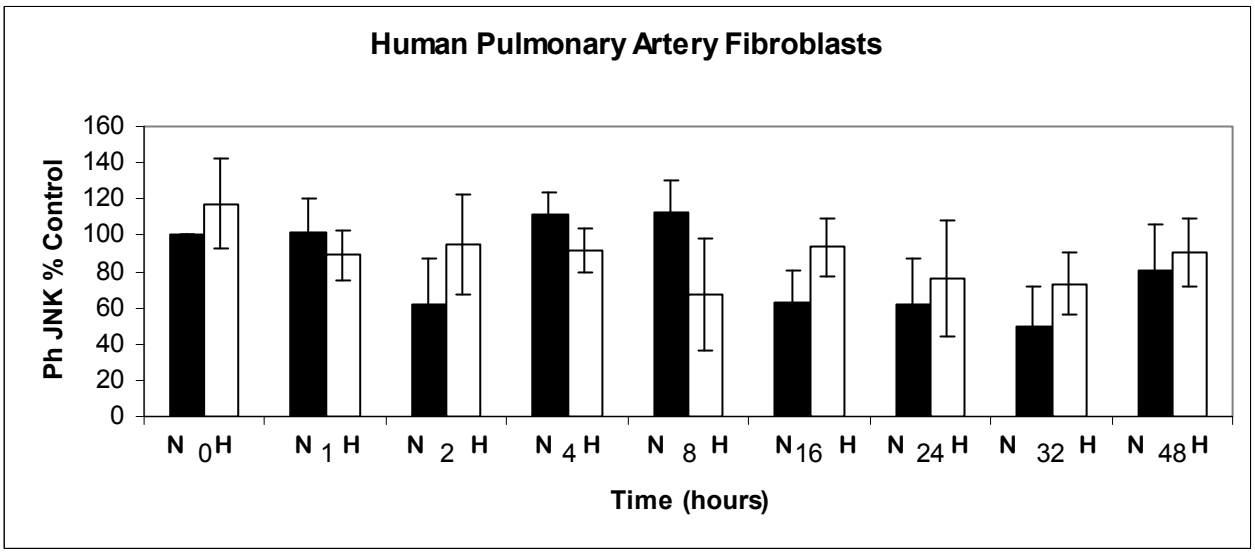
(a)


Time (h) -
Phospho JNK -
0 1 2 4 8 16 24 32 48
N H N H N H N H N H N H N H N H N H

(b)


Time (h) -
Whole JNK -
0 1 2 4 8
N H N H N H N H N
Time (h) -
Whole JNK -
8 16 24 32 48
H N H N H N H N H

(c)

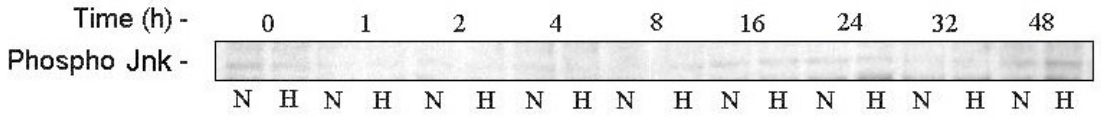

Human Pulmonary Artery Fibroblasts
Ph JNK % Control
160
140
120
100
80
60
40
20
0
N 0 H
N 1 H
N 2 H
N 4 H
N 8 H
N 16 H
N 24 H
N 32 H
N 48 H
Time (hours)

Human systemic arterial fibroblasts do not express increased levels of phosphorylated JNK under either normoxic or hypoxic growth conditions

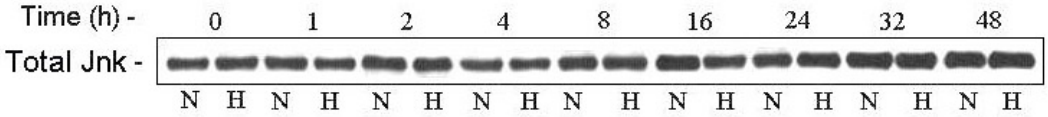
Systemic arterial fibroblasts were grown in normoxia and 5% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 48 hours and lysates were prepared for Western Blot analysis as outlined in Chapter 2.6. **Figure 4.2** demonstrates that here was no expression of phosphorylated JNK in human mammary artery fibroblasts in either normoxic or hypoxic (35mmHg PO_2) growth conditions **(a)**. The membrane probed for whole JNK demonstrated equal expression of whole JNK at all time points and equal protein loading **(b)**. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments from the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between the 2 groups.

Figure 4.2: Human systemic arterial fibroblasts do not express increased levels of phosphorylated JNK under either normoxic or hypoxic growth conditions. (a) No phosphorylated JNK was detected in HMAF cells under either normoxic (N) or hypoxic (H) growth conditions over a variety of time points (t = 0, 1, 2, 4, 8, 16, 24, 32, 48 hours. **(b)** The membrane was re-probed for whole/total JNK demonstrating the presence of consistent whole JNK expression at all time points under both normoxic (N) and hypoxic (H) growth conditions demonstrating equal protein loading. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) and hypoxic (H) groups.

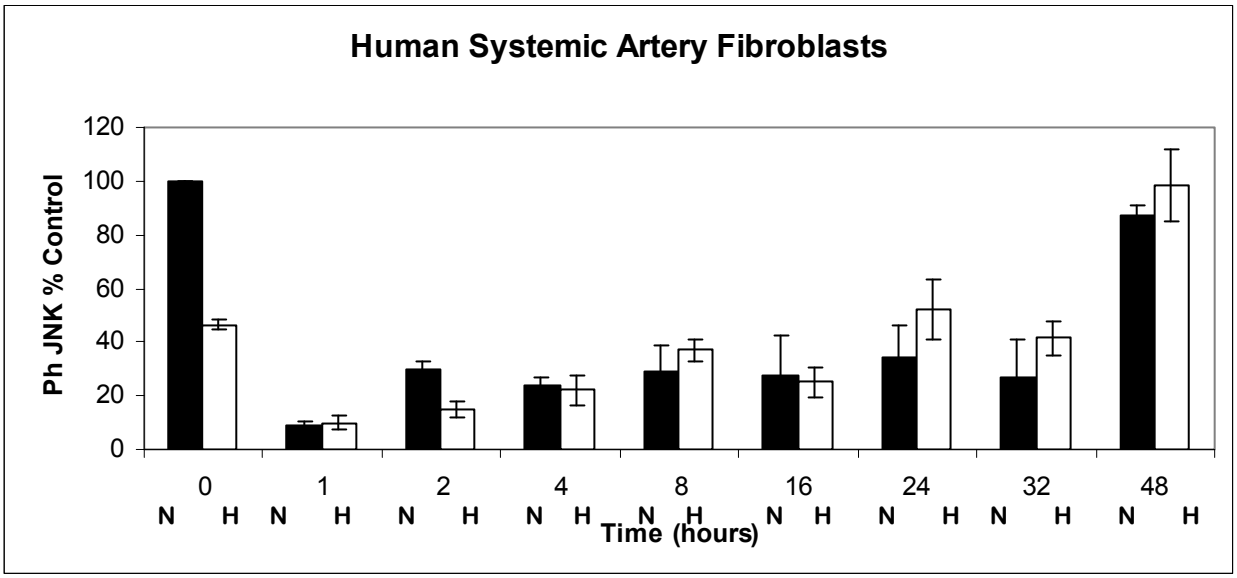
(a)


Time (h) -
0 1 2 4 8 16 24 32 48
Phospho Jnk -
N H N H N H N H N H N H N H N H N H N H

(b)


Time (h) -
0 1 2 4 8 16 24 32 48
Total Jnk -
N H N H N H N H N H N H N H N H N H N H

(c)

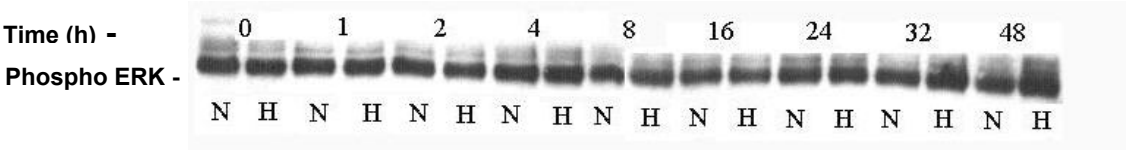

Human Systemic Artery Fibroblasts
Ph JNK % Control
120
100
80
60
40
20
0
0 1 2 4 8 16 24 32 48
N H N H N H N H N H N H N H N H N H
Time (hours)

Human pulmonary arterial fibroblasts show equal activation of phosphorylated ERK1/2 mitogen activated protein kinase (MAPK) under both normoxic and hypoxic growth conditions

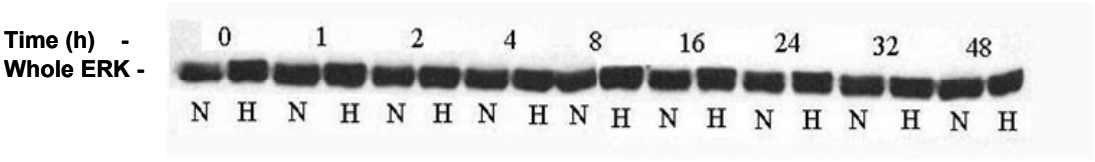
Pulmonary arterial fibroblasts were grown in normoxia and 5% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 48 hours and lysates were prepared for Western Blot analysis as outlined in Chapter 2.6. **Figure 4.3** demonstrates equal phospho-ERK expression in HPAF cells at all time points under both normoxic and hypoxic growth conditions **(a)**. The membrane probed for whole ERK demonstrates consistent presence of whole/total ERK at all time points and confirms equal protein loading **(b)**. These results were confirmed by densitometry – cells cultured in normoxia (N) (black box) and hypoxia (H) (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between the 2 groups.

Figure 4.3: Human pulmonary arterial fibroblasts show equal activation of phosphorylated ERK 1/2 mitogen activated protein kinase (MAPK) under both normoxic and hypoxic growth conditions. **(a)** Phosphorylated ERK was equally expressed in HPAF cells under both normoxic (N) or hypoxic (H) growth conditions over a variety of time points (t = 0, 1, 2, 4, 8, 16, 24, 32, 48 hours. **(b)** The membrane was re-probed for whole ERK demonstrating the presence of consistent whole JNK expression at all time points under both normoxic (N) and hypoxic (H) growth conditions demonstrating equal protein loading. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no significant statistical difference between normoxic (N) and hypoxic (H) groups.

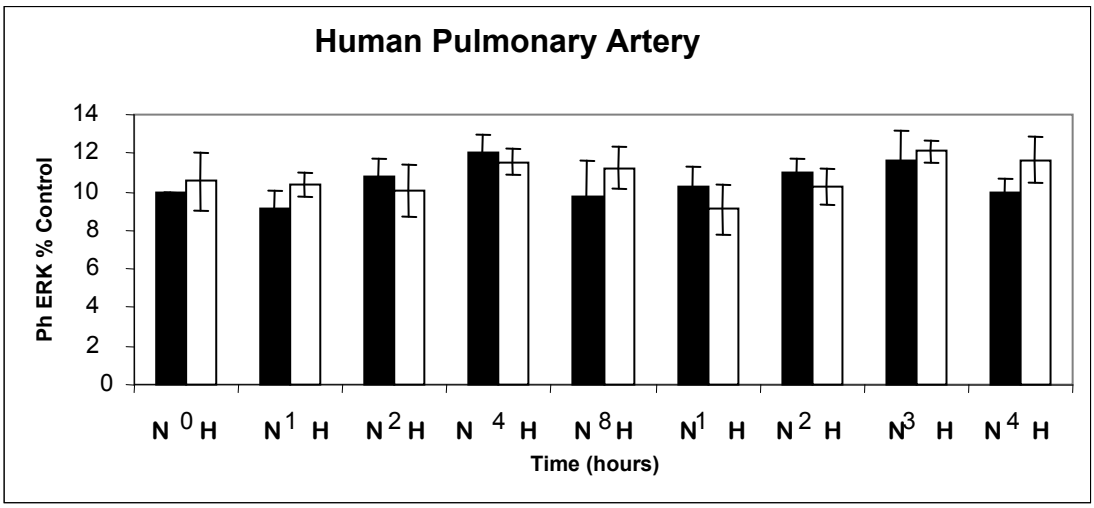
(a)


Time (h) -
Phospho ERK -
0 1 2 4 8 16 24 32 48
N H N H N H N H N H N H N H N H N H N H

(b)


Time (h) -
Whole ERK -
0 1 2 4 8 16 24 32 48
N H N H N H N H N H N H N H N H N H N H

(c)

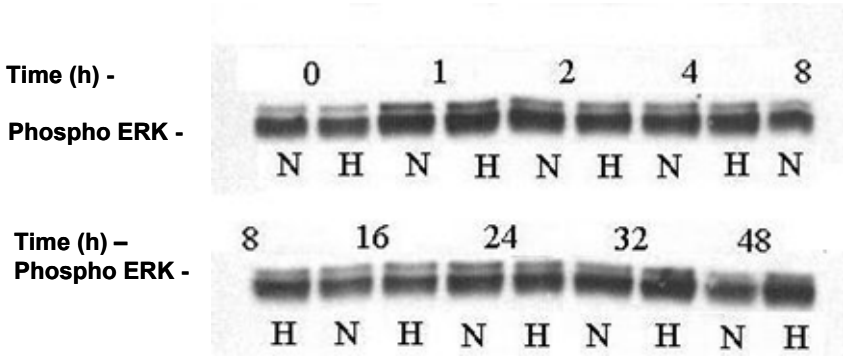

Human Pulmonary Artery
Ph ERK % Control
0
2
4
6
8
10
12
14
N 0 H
N 1 H
N 2 H
N 4 H
N 8 H
N 1 H
N 2 H
N 3 H
N 4 H
Time (hours)

Human systemic arterial fibroblasts demonstrate phosphorylated ERK1/2 MAPK under both normoxic and hypoxic growth conditions

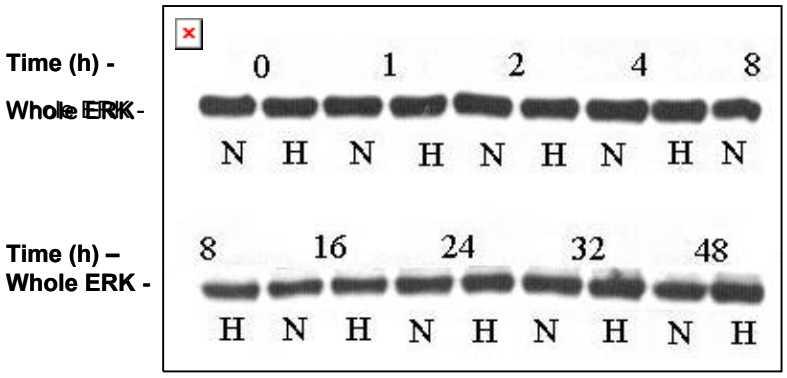
Systemic arterial fibroblasts were grown in normoxia and 5% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 48 hours and lysates were prepared for Western Blot analysis as outlined in Chapter 2.6. **Figure 4.4** demonstrates equal phospho-ERK expression in HMAF cells at all time points under both normoxic and hypoxic growth conditions **(a)**. The membrane probed for whole ERK demonstrates consistent presence of whole/total ERK at all time points and confirms equal protein loading **(b)**. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between the 2 groups.

Figure 4.4: Human systemic arterial fibroblasts demonstrate equal phosphorylated ERK 1/2 MAPK under both normoxic and hypoxic growth conditions. **(a)** Phosphorylated ERK was equally expressed in HMAF cells under both normoxic (N) or hypoxic (H) growth conditions over a variety of time points (t = 0, 1, 2, 4, 8, 16, 24, 32, 48 hours). **(b)** The membrane was re-probed for whole ERK demonstrating the presence of consistent whole JNK expression at all time points under both normoxic (N) and hypoxic (H) growth conditions demonstrating equal protein loading. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) and hypoxic (H) groups.

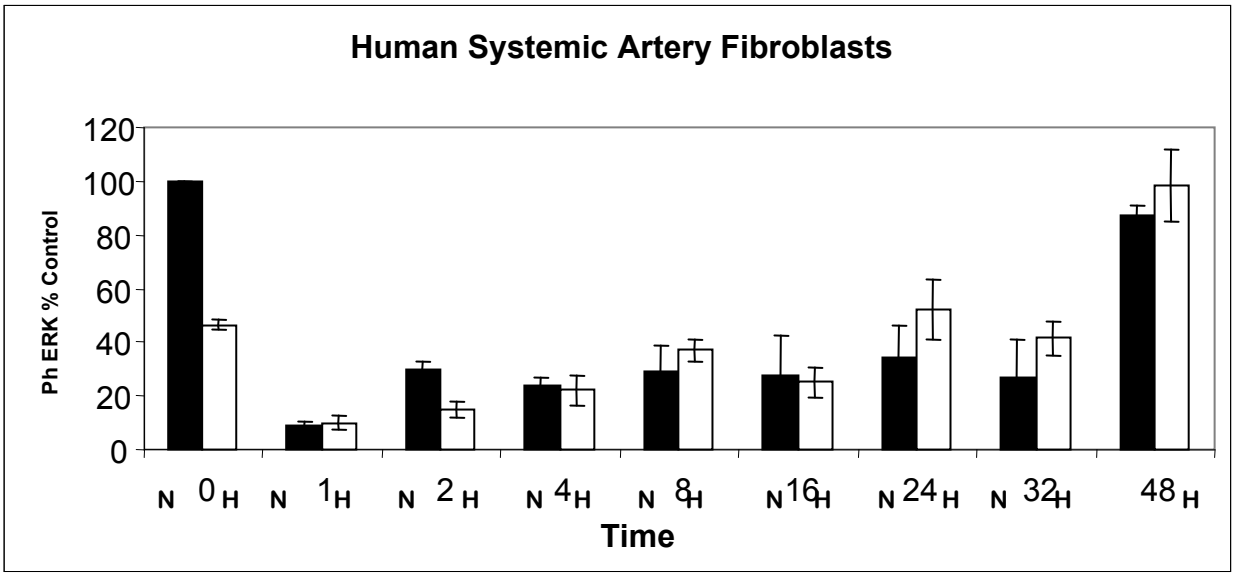
(a)


Time (h) -
0 1 2 4 8
Phospho ERK -
N H N H N H N H N
Time (h) –
Phospho ERK -
8 16 24 32 48
H N H N H N H N H

(b)


Time (h) -
0 1 2 4 8
Whole ERK -
N H N H N H N H N
Time (h) –
Whole ERK -
8 16 24 32 48
H N H N H N H N H

(c)


Human Systemic Artery Fibroblasts
Ph ERK % Control
120
100
80
60
40
20
0
N 0 H N 1 H N 2 H N 4 H N 8 H N 16 H N 24 H N 32 H 48 H
Time

Human pulmonary arterial fibroblasts show increased p38 MAPK phosphorylation at t = 6 and t = 16 hours under hypoxic growth conditions

Pulmonary arterial fibroblasts were grown in normoxia and 5% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 48 hours and lysates were prepared for Western Blot analysis as outlined in Chapter 2.6. **Figure 4.5** demonstrates that there was little expression of phosphorylated p38 at any time point (t = 0, 3, 6, 8, 16 and 24 hours) under normoxic (N) conditions but under hypoxic (H) growth conditions there were peaks of expression at 6 and 16 hours of hypoxic exposure **(a)**. Expression of whole (unphosphorylated) p38 was uniform at all time points and both growth conditions **(b)**. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD with * denoting a statistically significant result ($p<0.01$).

Figure 4.5: Human pulmonary arterial fibroblasts show increased p38 MAPK phosphorylation at t = 6 and t = 16 hours under hypoxic growth conditions. (a) There was no identified expression of phosphorylated p38 MAPK under normoxic (N) growth conditions (t = 0, 3, 6, 8, 16 and 24 hours)**.** Under hypoxic (H) growth conditions there were peaks of p38 MAPK activity at t = 6 and 16 hours**. (b)** Expression of whole (unphosphorylated) p38 was uniform at all time points and under both hypoxic (H) and normoxic (N) growth conditions and demonstrates equal protein loading throughout. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 other individuals; the results illustrated are representative of 4 separate experiments performed in the same individual with results expressed as mean +/- 2 SD, and where * indicates a statistically significant result ($p<0.01$).

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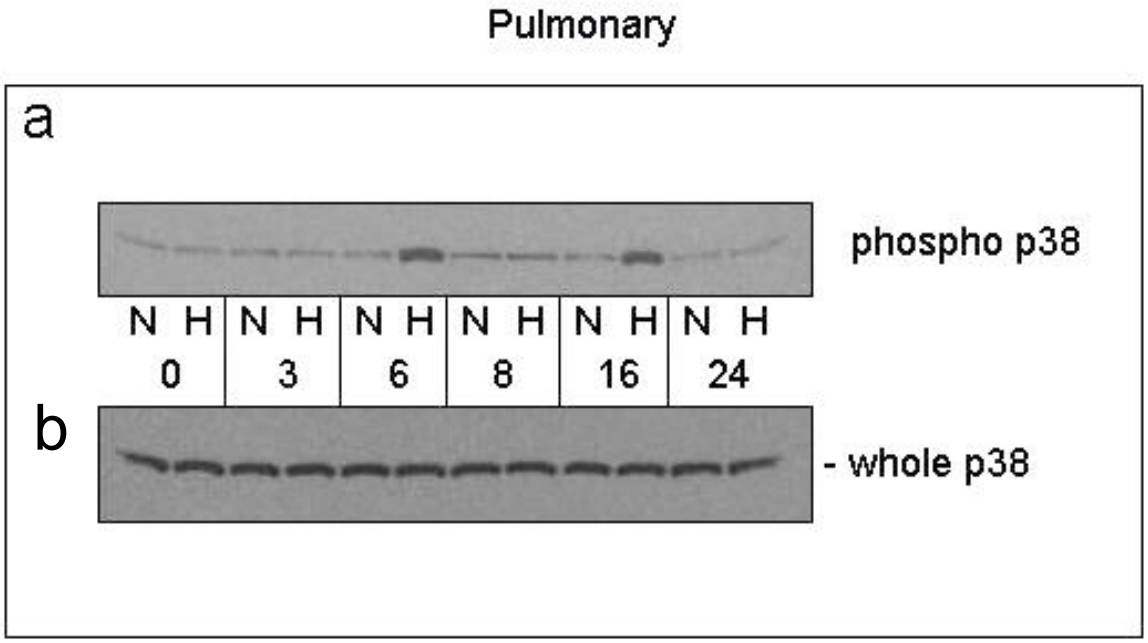
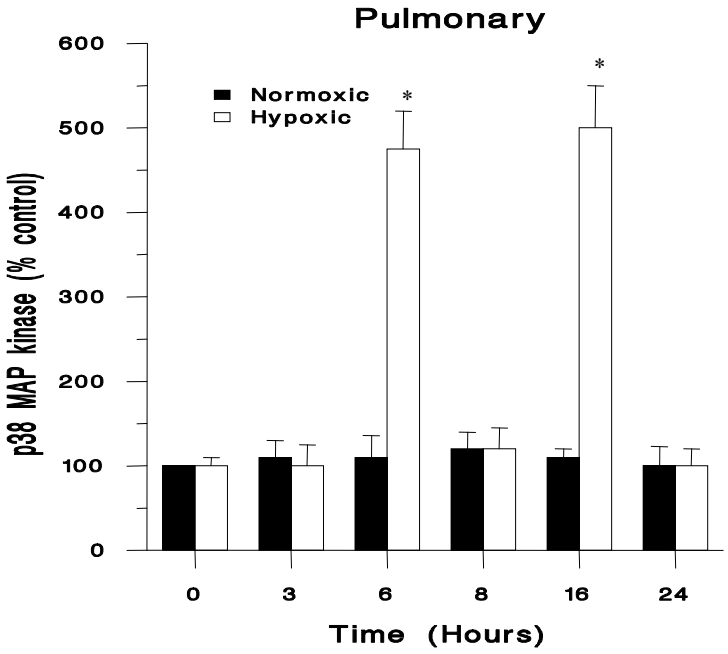

Pulmonary
a
phospho p38
N H N H N H N H N H N H
0 3 6 8 16 24
b
- whole p38

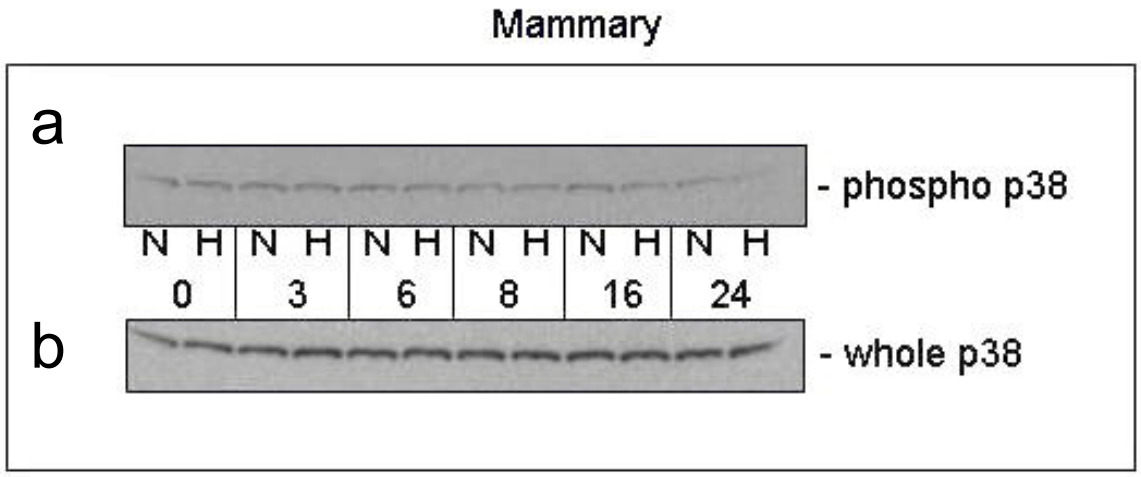
(c)


Pulmonary
Normoxic
Hypoxic
p38 MAP kinase (% control)
600
500
400
300
200
100
0
*
*
0 3 6 8 16 24
Time (Hours)

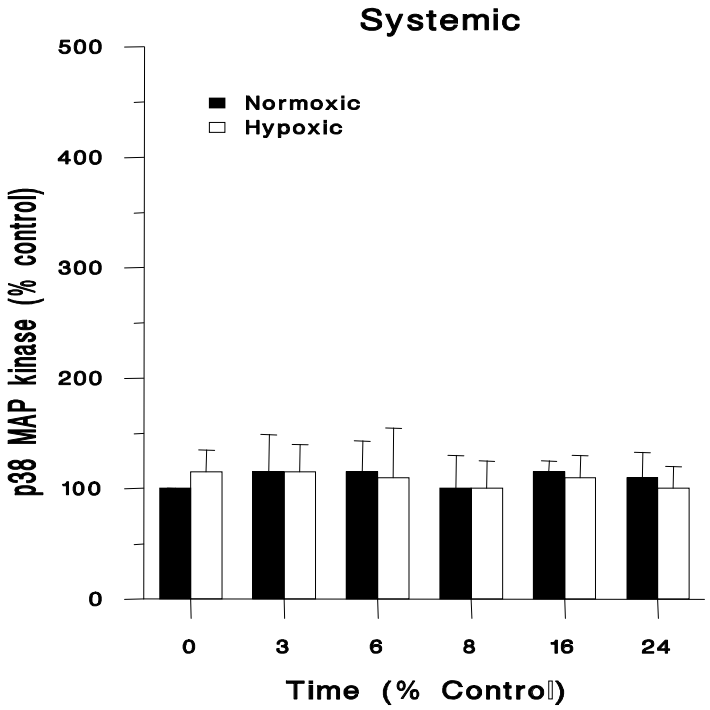
Human systemic arterial fibroblasts demonstrate no phosphorylation of p38 MAPK under either normoxic or hypoxic growth conditions

Systemic arterial fibroblasts were grown in normoxia and 10% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 48 hours and lysates were prepared for Western Blot analysis as outlined in Chapter 2.6. **Figure 4.6** demonstrates that there is little phospho-p38 MAPK expression in HMAF cells in either growth condition at any time point (t = 0, 3, 6, 8, 16 and 24 hours) **(a)**. The membrane probed for whole p38 MAPK expression demonstrates the consistent presence of inactive p38 MAPK and demonstrates equal protein loading throughout **(b)**. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments performed in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between the 2 groups.

Figure 4.6: Human systemic arterial fibroblasts demonstrate no phosphorylation of p38 MAPK under either normoxic or hypoxic growth conditions. **(a)** There is little phosphorylated p38 MAPK expression in HMAF cells under either normoxic (N) or hypoxic (H) growth conditions at any time point (t = 0, 3, 6, 8, 16 and 24 hours). **(b)** There is equal expression of whole/total p38 MAPK in HMAF cells under both normoxic (N) and hypoxic (H) growth conditions at all time points investigated demonstrating equal protein loading throughout. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(c)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) and hypoxic (H) 2 groups.


Mammary
a
- phospho p38
N H N H N H N H N H N H
0 3 6 8 16 24
b
- whole p38

(c)


Systemic
Normoxic
Hypoxic
p38 MAP kinase (% control)
500
400
300
200
100
0
0 3 6 8 16 24
Time (% Control)

Human pulmonary arterial fibroblasts demonstrate peaks of p38 MAPK activation between t = 4 –6 and t = 16 –18 hours.

Pulmonary arterial fibroblasts were grown in normoxia and 10% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 24 hours and lysates were prepared for Western Blot analysis as outlined in Chapter 2 6. **Figure 4.7 (a)** demonstrates that there was little expression of phosphorylated p38 MAPK at any time point (t = 0, 2, 3, 4, 5, 6, 10, 12, 16, 18, 20, 24 hours) under normoxic (N) conditions but under hypoxic (H) growth conditions there were peaks of expression at t = 4-6 and t = 16-18 hours of hypoxic exposure. The membrane probed for whole p38 MAPK expression demonstrates the consistent presence of inactive p38 MAPK and demonstrates equal protein loading throughout. These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(b)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments performed in the same individual with results expressed as mean +/- 2 SD with * denoting a statistically significant result ($p<0.01$).

Figure 4.7: Human pulmonary arterial fibroblasts demonstrate peaks of p38 MAPK activation between t = 4 –6 and t = 16 –18 hours. Under normoxic (N) growth conditions there was no demonstrated increase in phosphorylated p38 MAPK activity at any time point (t = 0, 2, 3, 4, 5, 6, 10, 12, 16, 18, 20, 24 hours). However under hypoxic (H) growth conditions there were peaks of activity at t = 4 –6 hours and t = 16 – 18 hours (**figure 4.7.a**). These results were confirmed by densitometry – cells cultured in normoxia (black box) and hypoxia (white box) **(b)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.01$).

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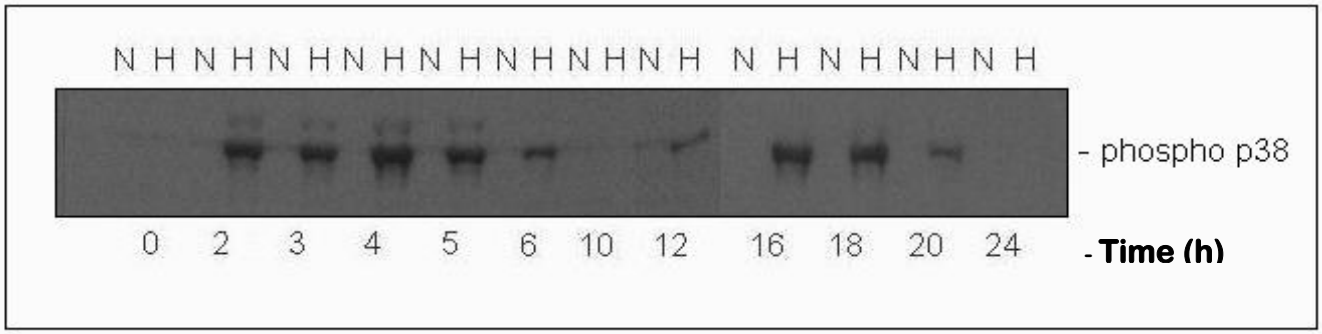
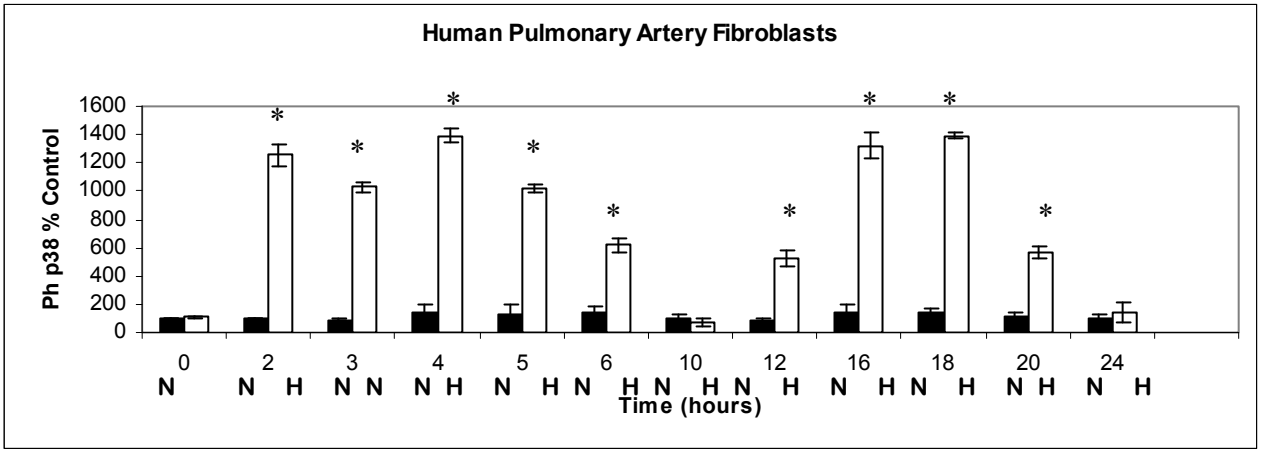
(a)


N H N H N H N H N H N H N H N H N H N H N H N H N H N H
- phospho p38
0 2 3 4 5 6 10 12 16 18 20 24
- Time (h)

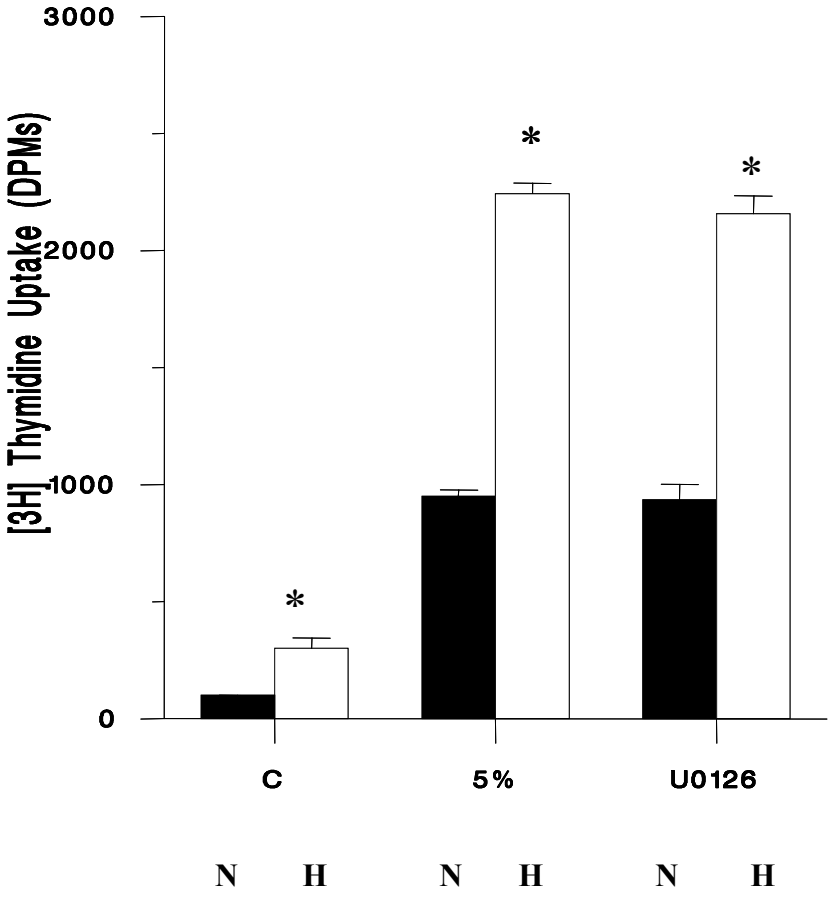
(b)


Human Pulmonary Artery Fibroblasts
Ph p38 % Control
1600
1400
1200
1000
800
600
400
200
0
0 2 3 4 5 6 10 12 16 18 20 24
N N H N N N H N H N H N H N H N H N H N H N H
Time (hours)

Preincubation with U0126 – a specific p 44/p42 - ERK1/2 MAPK inhibitor - has no effect on human pulmonary arterial fibroblast cell proliferation to acute hypoxia.

Pulmonary arterial fibroblasts were grown in normoxia and 5% serum in 24 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 24 hours and were cultured with either 0% serum (C), 5% serum (5%) or 5% serum plus 50 μl U0126 (U0126) which was added six hours prior to the end of the experiment. The cells were then returned to either normoxic or hypoxic growth conditions. Samples were prepared for [^{3}H]Thymidine uptake assays as outlined in Chapter 2.5. **Figure 4.8** demonstrates that there is a significant increase in proliferation witnessed under hypoxic growth conditions (white bar) without the addition of serum (C), a further marked increase in proliferation is noted relative to HPAF grown in normoxic conditions (black bar) with the addition of 5% serum to the cell sample grown under hypoxic conditions (5%). The addition of U0126 – a specific phospho-ERK inhibitor has no influence on the growth behaviour of HPAF grown under either hypoxic or normoxic conditions (U0126). The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.01$).

Figure 4.8: Preincubation with U0126 – a specific ERK 1/2 MAPK inhibitor - has no effect on human pulmonary arterial fibroblast cell proliferation to acute hypoxia. There is increased proliferation under hypoxic growth conditions (H) (white bar) compared to normoxic growth (N) (black bar) with or without the addition of 5% serum. The addition of U0126 had no effect of HPAF growth behaviour under hypoxic conditions. The results illustrated are representative of 4 separate experiments in the same individual with the experiment being repeated in 4 individuals. The figures are representative of the mean of 4 experiments in the same individual with results expressed as mean +/- 2 SD with ∗ denoting a statistically significant result ($p < 0.01$).


3000
2000
1000
0
[3H] Thymidine Uptake (DPMs)
*
*
*
C
5%
U0126
N
H
N
H
N
H

Preincubation with U0126 has no effect on human systemic arterial fibroblast cell proliferation under either normoxic or hypoxic growth conditions

Systemic arterial fibroblasts were grown in normoxia and 5% serum in 24 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 24 hours. They were cultured with either 0% serum (C), 5% serum (5%) or 5% serum plus 50 μl U0126 (U0126) which was added six hours prior to the end of the experiment. The cells were then returned to either normoxic or hypoxic growth conditions. Samples were prepared for [^{3}H]Thymidine uptake assays as outlined in Chapter 2.5. **Figure 4.9** demonstrates that there is an increase in proliferation witnessed with the addition of 5% serum under normoxic (N) (black bar) conditions in keeping with the initial growth response curve to increasing serum concentrations (see **figure 1.1**). There is no significant difference in growth under hypoxic (H) (white bar) conditions. The addition of U0126 – a specific phospho ERK inhibitor – has no influence on HMAF proliferation. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistically significant difference between the 2 groups.

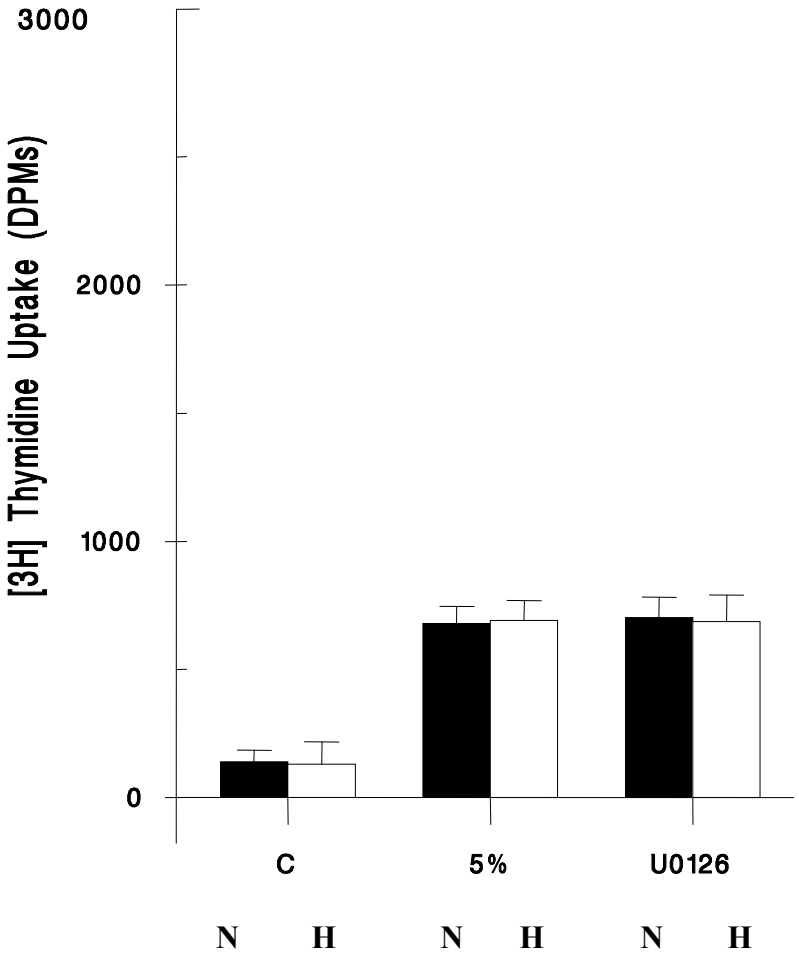


Figure 4.9: Preincubation with U0126 has no effect on human systemic arterial fibroblast cell proliferation under either normoxic or hypoxic growth conditions. There was no difference in HMAF proliferation under either normoxic (N) (black bar) or hypoxic (H) (white bar) growth conditions. The addition of 5% serum resulted in a dose response increase in HMAF proliferation in both normoxic and hypoxic growth conditions. The addition of U0126 made no difference to the proliferative response under either growth condition. Proliferation was assessed by [^{3}H]Thymidine uptake ~ disintegrations per minute (DPM). The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistically significant difference between the 2 groups.

Preincubation with SB203580 – a specific p38 MAPK α and β isoform inhibitor – abrogates hypoxia-mediated proliferation in human pulmonary arterial fibroblast cells

Pulmonary arterial fibroblasts were grown in normoxia and 5% serum in 24 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 24 hours. They were cultured with either 0% serum (C), 5% serum (5%) or 5% serum plus 50 μl SB203580 (SB203580) which was added six hours prior to the end of the experiment. The cells were then returned to either normoxic or hypoxic growth conditions. Samples were prepared for [^{3}H]Thymidine uptake assays as outlined in Chapter 2.5. **Figure 4.10** demonstrates that HPAF cells were grown in the absence of serum under hypoxic conditions (H) (white bar) show increased proliferation compared to HPAF cells grown without serum stimulation under normoxic conditions (N) (black bar), the presence of 5% serum further enhances this response. The addition of 5% serum and SB203580– a specific p38 MAPK inhibitor - results in the abrogation of the hypoxia-mediated proliferative response. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.01$).

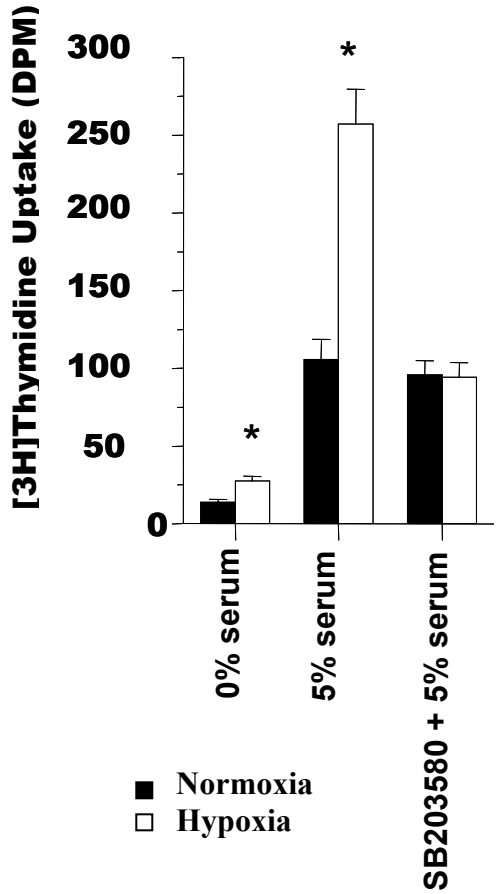


Figure 4.10: Preincubation with SB203580 – a specific p38 MAPK α and β isoform inhibitor – abrogates hypoxia-mediated proliferation in human pulmonary arterial fibroblast cells. HPAF cells preferentially proliferate to hypoxia (white bar) compared to normoxia (black bar) with or without the addition of 5% serum. The addition of SB203580 and 5% serum completely abrogated the hypoxia-mediated proliferative response. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.01$).

Preincubation with SB203580 has no effect on human systemic arterial fibroblast proliferation under either normoxic or hypoxic growth conditions

Systemic arterial fibroblasts were grown in normoxia and 10% serum in 24 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 0 – 24 hours. They were cultured with either 0% serum (C), 5% serum (5%) or 5% serum plus 50 μl SB203580 (SB203580) which was added six hours prior to the end of the experiment. The cells were then returned to either normoxic or hypoxic growth conditions. Samples were prepared for [^{3}H]Thymidine uptake assays as outlined in Chapter 2.5. **Figure 4.11** demonstrates that there is a no significant increase in proliferation in HMAF cells grown in hypoxic conditions (white bar) without serum stimulation (C) compared to cells grown in normoxic conditions (black bar) or with the addition of 5% serum (5%). The addition of 5% serum and SB203580 to HMAF cells has no influence on proliferative behaviour whether under normoxic or hypoxic conditions (SB203580). The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between the 2 groups.

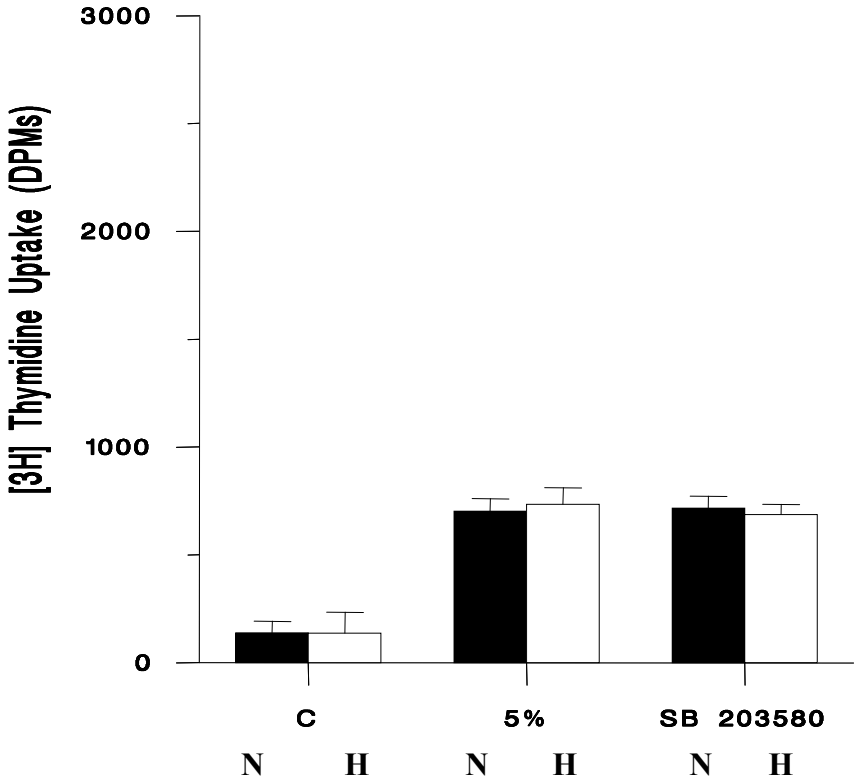


Figure 4.11: Preincubation with SB203580 has no effect on human systemic arterial fibroblast proliferation under either normoxic or hypoxic growth conditions. There was no significant difference in proliferative response in HMAF cells grown under either normoxic (N) (black bar) or hypoxic (H) (white bar) conditions with or without 5% serum. The addition of SB203580 + 5% serum did not affect the growth response of HMAF under either growth condition. Cellular proliferation was assessed by [^{3}H]Thymidine uptake ~ disintegrations per minute (DPM). The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) and hypoxic (H) groups.

Reoxygenation of human pulmonary fibroblast cells after 6 hours abrogates hypoxia-mediated proliferation

Pulmonary arterial fibroblasts were grown in normoxia and 5% serum in 24 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of acute hypoxia for t = 0, 6, 16, 20 and 24 hours with the cells being returned to normoxia following hypoxic exposure to complete a total culture time of t = 24 hours. Samples were prepared for [^{3}H]Thymidine uptake assays as outlined in Chapter 2.5. **Figure 4.12** demonstrates that there was no difference in [^{3}H]Thymidine uptake in HPAF cells grown in normoxic conditions for the whole 24-hour period (C) and those HPAF cells grown in hypoxic conditions for 6 hours. [^{3}H]Thymidine uptake was significantly increased in HPAF cells grown in hypoxic conditions for between t= 16, 18 and 20 hours. A slight decrease in [^{3}H]Thymidine uptake was noted in HPAF cells grown in hypoxic conditions for 24 hours in comparison to those grown for t = 16 to 20 hours in hypoxia. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.05$).

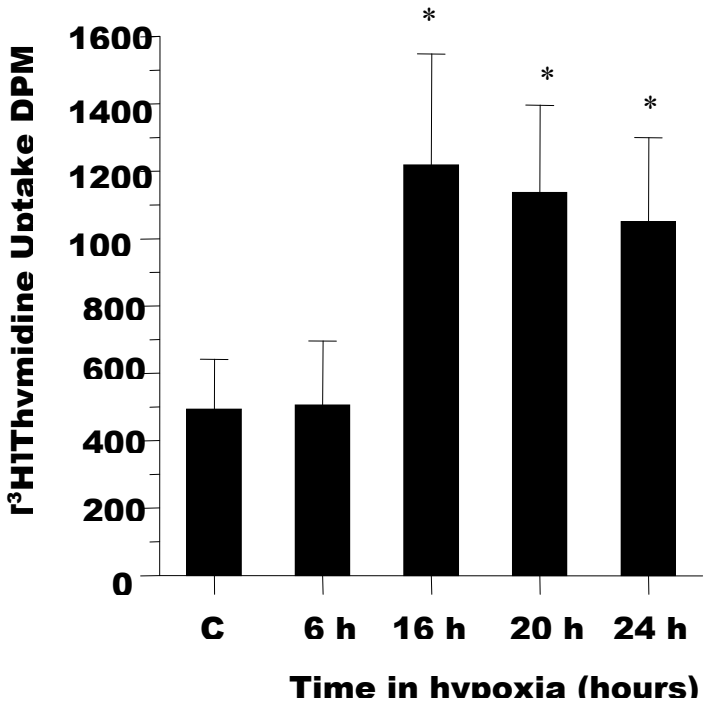


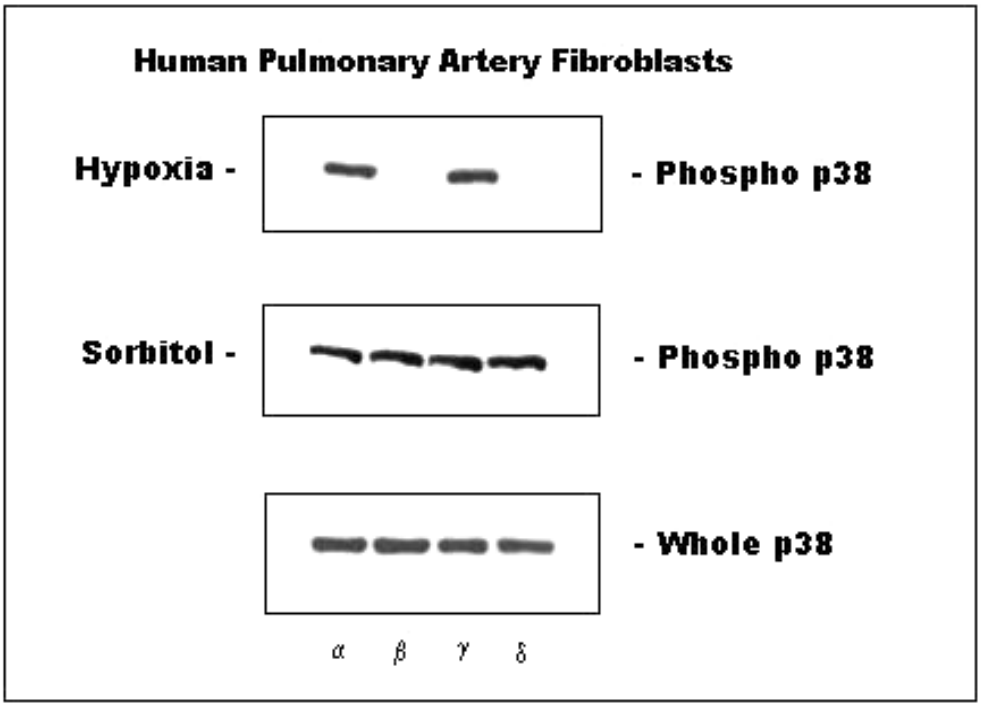
Figure 4.12: Reoxygenation of human pulmonary fibroblast cells after 6 hours abrogates hypoxia-mediated proliferation. HPAF cells grown in normoxic conditions for t = 24 hours (C) and reoxygenated following t = 6 hours of hypoxic exposure exhibited similar reduced proliferative responses when compared with HPAF cells grown in hypoxic conditions for t = 16 and 20 hours with subsequent return to normoxia for the remainder of the 24 hour period. Cellular proliferation as assessed by [^{3}H]Thymidine uptake ~ disintegrations per minute (DPM). [^{3}H]Thymidine uptake was significantly increased in HPAF cells grown in hypoxic conditions for between t = 16, 18 and 20 hours. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD and where * indicates a statistically significant value ($p<0.05$).

Human pulmonary arterial fibroblasts express phosphorylated p38 MAPK α and γ isoforms in response to hypoxic growth conditions

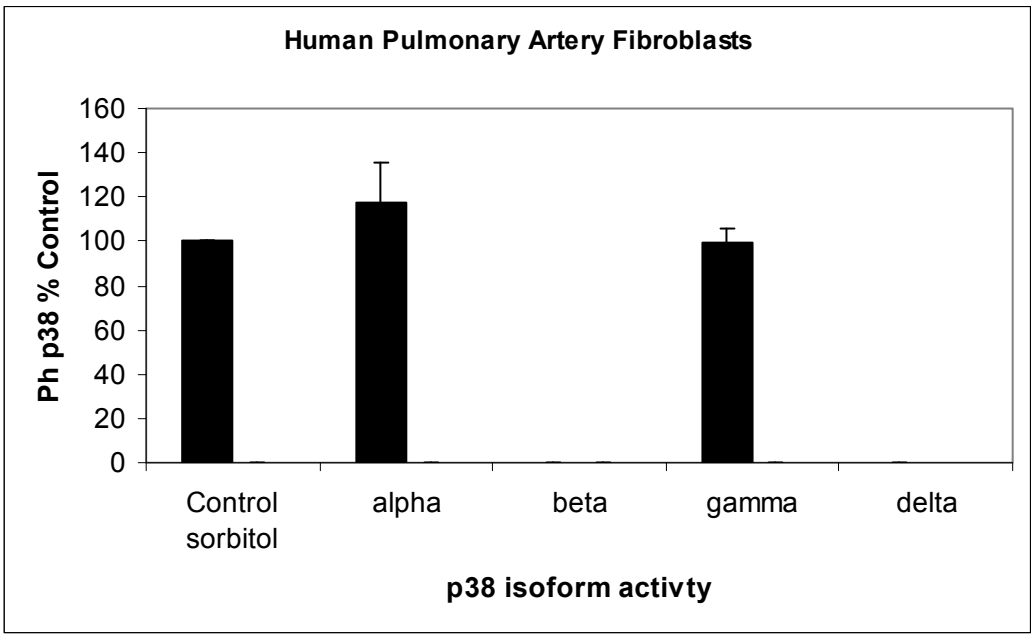
Pulmonary arterial fibroblasts were grown in normoxia and 5% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of d acute hypoxia (5% PO_2 ~ 35mmHg) for t = 16 hours and lysates were prepared for Western Blot analysis as outlined in Chapter 2.6. **Figure 4.13 (a)** demonstrates that α and γ isoforms of phospho-p38 MAPK are expressed in HPAF cells grown in hypoxia for t = 16 hours. Sorbitol acting as a positive control demonstrates that inactive forms of all 4 isoforms are present - probing for whole-p38 MAPK demonstrates equal protein loading. These results were confirmed by densitometry **(b)** with values expressed as a percentage of sorbitol induced p38 MAPK phosphorylation as a positive control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD.

Figure 4.13: Human pulmonary arterial fibroblasts express phosphorylated p38 MAPK α and γ isoforms in response to hypoxic growth conditions. (a) HPAF cells were grown in hypoxia for t = 16 hours. α and γ isoforms of p38 MAPK were expressed in hypoxic HPAF cells but not β or δ isoforms. Sorbitol was used as a positive control for all phosphorylated p38 MAPK activity. Probing for whole/total p38 MAPK demonstrated equal protein loading throughout. These results were confirmed by densitometry **(b)** with values expressed as a percentage of sorbitol induced p38 MAPK phosphorylation as a positive control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD.

(a)


Human Pulmonary Artery Fibroblasts
Hypoxia -
- Phospho p38
Sorbitol -
- Phospho p38
- Whole p38
α β γ δ

(b)

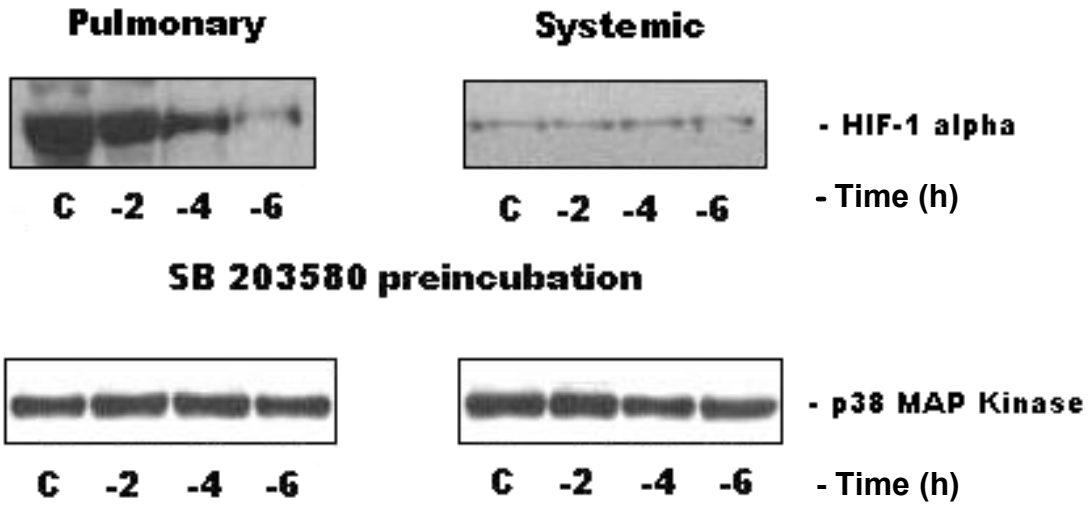

Human Pulmonary Artery Fibroblasts
Ph p38 % Control
160
140
120
100
80
60
40
20
0
Control sorbitol
alpha
beta
gamma
delta
p38 isoform activty

Human pulmonary arterial fibroblasts express HIF1α to hypoxic exposure that is inhibited by preincubation with SB203580. Human systemic arterial fibroblasts demonstrate no activation of HIF1α to hypoxic exposure

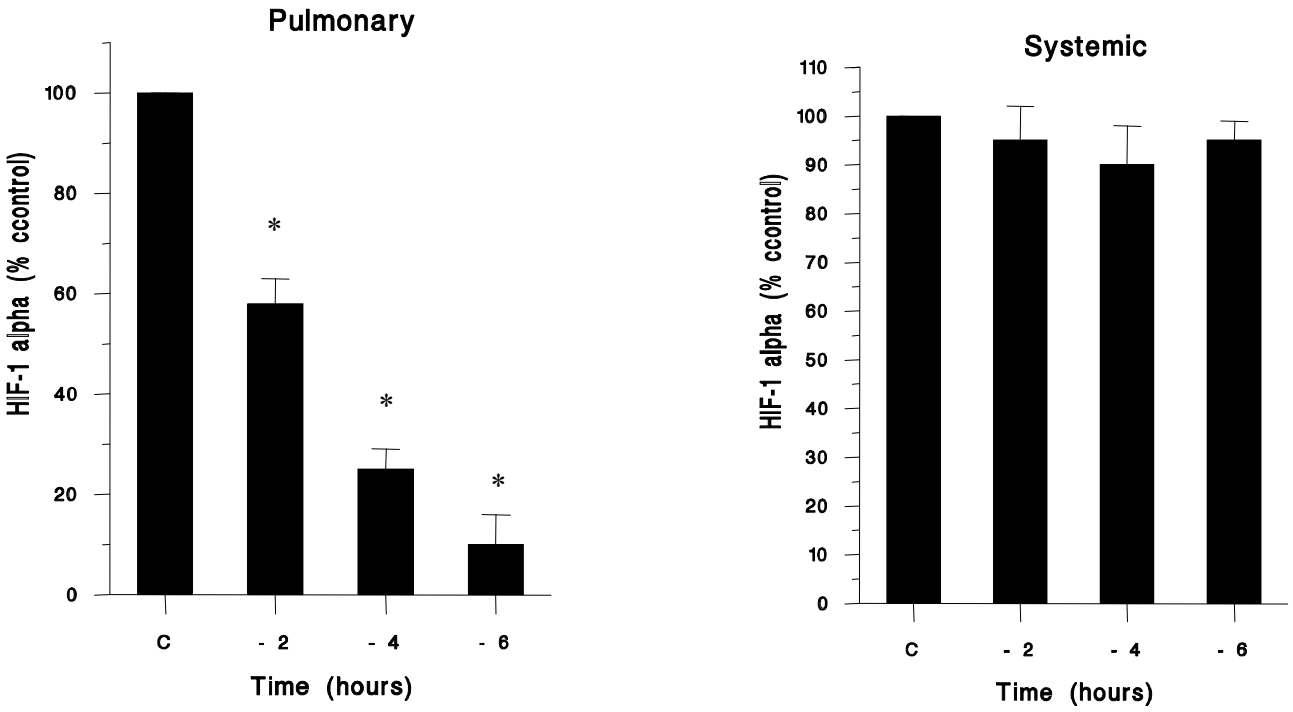
Pulmonary and systemic arterial fibroblasts were grown in normoxia and 5% serum in 6 well plates until 60% confluent they were then quiesced in serum-free media for 24 hours. The cells were then kept in conditions of normoxia and acute hypoxia (5% PO_2 ~ 35mmHg) for t = 16 hours. Both HMAF and HPAF cells were pre-incubated either without (C) or with SB203580 for a variety of time points (t = 2, 4 and 6 hours before the end of the experiment). Lysates were prepared for Western Blot analysis as outlined in Chapter 2.6. **Figure 4.14 (a)** demonstrates that there is no HIF-1α expression in HMAF cells at any time point examined. In HPAF cells HIF-1α expression was inhibited by the presence of SB203580, but maximally at 6 hours pre-incubation. The lower panel demonstrates even expression of whole p38 MAPK and equal protein loading. These results were confirmed by densitometry **(b)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD.

Figure 4.14: Human pulmonary arterial fibroblasts express HIF1α to hypoxic exposure that is inhibited by preincubation with SB203580. Human systemic arterial fibroblasts demonstrate no activation of HIF1α to hypoxic exposure. (a) HMAF (systemic) cells did not demonstrate any evidence of HIF1α activity under either normoxic or hypoxic growth conditions with preincubation with SB203580 for a variety of time points at t = 0, 2, 4 and 6 hours prior to the start of the experiment. HPAF (pulmonary) cells demonstrated that HIF1α expression could be progressively abrogated – with SB203580 preincubation which was maximal when added at t = 6 hours prior to the start of the experiment. The membrane was stripped and then probed for whole-p38 MAPK which demonstrated equal protein loading throughout. These results were confirmed by densitometry **(b)** with values expressed as a percentage of proliferation under conditions of normoxia without 5% serum stimulation as a control. The experiment was repeated 4 times in the same individual and performed in 4 individuals; the results illustrated are representative of 4 separate experiments in the same individual with results expressed as mean +/- 2 SD.

(a)


Pulmonary
Systemic
- HIF-1 alpha
C -2 -4 -6
C -2 -4 -6
- Time (h)
SB 203580 preincubation
- p38 MAP Kinase
C -2 -4 -6
C -2 -4 -6
- Time (h)

(b)


Pulmonary
HIF-1 alpha (% ccontrol)
100
80
60
40
20
0
*
*
*
C
- 2
- 4
- 6
Time (hours)
Systemic
HIF-1 alpha (% ccontrol)
110
100
90
80
70
60
50
40
30
20
10
0
C
- 2
- 4
- 6
Time (hours)

4.5. Discussion:

In chapter 3 we have demonstrated that human pulmonary arterial fibroblasts proliferate to acute hypoxic exposure whereas systemic arterial fibroblasts do not (with or without serum stimulation). In this section we have demonstrated that both p38 MAPK and p44/p42 – ERK 1/2 MAPK are activated in hypoxic human pulmonary arterial fibroblasts (HPAF) and in systemic arterial fibroblast (HMAF) cells but that hypoxia-mediated proliferation in HPAF cells is associated with p38 MAPK activity alone. We have also demonstrated that JNK MAPK is not activated in either hypoxic or normoxic HPAF and HMAF cells. In addition we have demonstrated that inhibition of p38 MAPK in HPAF cells with SB203580 (a specific p38 MAPK α and β inhibitor) results in complete abrogation of the hypoxia-mediated proliferative response observed in HPAF, in contrast to HMAF cells which show no change in behaviour under identical growth conditions.

Phospho-p44/42 – ERK 1/2 expression was activated under both normoxic and hypoxic growth conditions in human pulmonary arterial fibroblasts at all time points with no significant differences in activity witnessed at any of the time points examined. Pre-incubation of HPAF cells with U0126 (a p44/42 – ERK 1/2 MAPK/MEK inhibitor) did not affect the hypoxia-mediated response witnessed in HPAF cells. This suggests that p38 MAPK and p44/p42 – ERK 1/2 MAPK may have different roles in hypoxic HPAF cells: p38 MAPK appears to be crucial for the hypoxia-mediated proliferative response seen in these cells and this agrees with observations from other animal models (Scott *et al*, 1998; Das *et al*, 2001; Welsh *et al*, 2001). The role of p44/42 – ERK 1/2 MAPK is less certain – classic MAPKs may be involved in cell hypertrophy in response to the hypoxic stimulus in this model.

Other investigators have found that both p44/p42 – ERK 1/2 and JNK MAPK to be important in hypoxia-mediated proliferation of pulmonary artery fibroblasts in a neonatal bovine model (Das *et al*, 2001). Scott *et al* (1998) found evidence of both p38 and JNK MAPK activation in an adult bovine model, however in a human model we did not find any evidence of JNK MAPK activation in human pulmonary artery fibroblasts although whole JNK MAPK was equally expressed in both normoxic and hypoxic HPAF and HMAF cells. This would suggest that while JNK MAPK is present in this model, it does not take an active role in human pulmonary or systemic artery fibroblast response to hypoxia

In human pulmonary arterial fibroblasts subjected to acute hypoxia p38 MAPK activity appears to be biphasic in nature with peaks of activity at t = 6 hours and at 16 hours. This finding concurs with previous work from the SPVU laboratory: Scott *et al* (1998) demonstrated that increases in p38 MAPK activity occurred in a biphasic manner at t = 6 hours and 24 hours in an adult bovine model. It also concurs with work from other investigators using a neonatal bovine model (Das *et al*, 2001). In addition work from the SPVU laboratory has also demonstrated constitutive activation of p38 MAPK in pulmonary arterial fibroblasts to chronic hypoxic exposure that display a permissively proliferative phenotype (Welsh *et al*, 2001). However our results differ from those seen in animal models in that we were unable to demonstrate any phospho-JNK activity in human arterial fibroblasts exposed to acute hypoxia. In an adult bovine model Scott *et al* (1998) demonstrated that pulmonary arterial fibroblast proliferation was also associated with JNK activation. In a neonatal bovine model, Das *et al* (2001) demonstrated that phospho-JNK, p44/42 – ERK1/2 and p38 MAPK were all required for hypoxia-mediated pulmonary arterial fibroblast

proliferation to acute hypoxia. They also demonstrated that SB203580 (a p38 MAPK α and β isoform inhibitor) was not able to fully abrogate hypoxia-mediated proliferation witnessed in this model whereas pertussis toxin (a $G_{\alpha i/o}$ protein inhibitor) completely abrogated neonatal bovine pulmonary arterial fibroblast proliferation implying a crucial role for JNK1 and p44/42 – ERK1/2 in hypoxia-mediated proliferation in this model.

It seems likely that the relative differences in MAPK activation patterns witnessed in bovine, rat and human models rest on intrinsic inter-species differences. They may also be the result of development differences – Das and Xu *et al* (1995; 1997) demonstrated significant differences in PKC isoform expression in pulmonary arterial fibroblasts and vascular smooth muscle cells to acute hypoxia between fetal, neonatal and adults in a bovine model. In addition Hale *et al* (1999) demonstrated that p38 MAPK isoform expression in human inflammatory cell lineages was not ubiquitous but was controlled at different developmental stages. Despite the significant differences that exist *inter*species, and *intra*species at different developmental stages, p38 MAPK activation appears to be a constant and important feature of all models that we have so far investigated. Moreover it appears to behave consistently in a biphasic manner to acute hypoxic exposure with activity peaks at t = 6 hours and around 16-24 hours – a finding which has been demonstrated in neonatal and adult bovine models, and now also in man (Scott *et al*, 1998; Das *et al*, 2001).

The functional significance of biphasic p38 MAPK activity in hypoxic pulmonary arterial fibroblasts is not certain. Following reoxygenation experiments we have demonstrated that in a human model the second peak of p38 MAPK activity is

responsible for hypoxia-mediated proliferation in pulmonary arterial fibroblasts as fibroblasts reoxygenated after only 6 hours of hypoxic exposure fail to show the same proliferative response as identical cells cultured for 16 hours in hypoxic conditions. This concurs with previous work from the SPVU laboratory that demonstrated that the second peak of p38 MAPK activity could be abrogated following reoxygenation after 6 hours (Scott *et al*, 1998). It appears both from human and bovine models that the second peak of p38 MAPK activity is required for the hypoxia-mediated proliferative response witnessed in pulmonary arterial fibroblasts. Scott *et al* (1998) noted that the second peak of p38 MAPK activity coincided with DNA synthesis in a fibroblast model. This finding is at odds with work from Lavoie *et al* (1996) who demonstrated that p38 MAPK inhibited cell cycle progression from G_0 to G_1 in a fibroblast cell line. Contrary to our findings, Lavoie *et al* found that p44/p42 – ERK 1/2 activation appeared to be crucial for cell cycle progression via activation of cyclin D1, 2 and 3 in conjunction with cyclin dependent kinases (cdk) 4 and 6. p38 MAPK over-expression inhibited cell cycle progression, causing cell cycle arrest that could be overcome with pre-incubation with SB203580. Previous work from this laboratory has not been able to demonstrate a role for p38 MAPK inhibition of cell cycle progression via cyclin D1 in an acute hypoxic adult rat model (Welsh *et al*, 2006) and this is in keeping with other work from our laboratory demonstrating a consistent association of pulmonary arterial fibroblast proliferation with p38 MAPK activity. The variance in results may be a result of Lavoie *et al's* use of immortalised cell lines rather than primary cell culture.

Given the finding by other investigators that p38 MAPK α and γ isoform activity were upregulated by hypoxic exposure in PC12 cells (phaeochromocytoma cell

lineage) (Conrad *et al*, 1999), and work from the SPVU laboratory that implicated p38 MAPK α isoform activity in adult rat pulmonary arterial fibroblasts following acute hypoxic exposure (Welsh *et al*, 2006), we wished to establish which of the p38 MAPK isoforms were involved with pulmonary arterial fibroblast proliferation to hypoxia in a human model. p38 MAPK isoform expression appears to vary with tissue type and with developmental stage (Ono and Han, 2000). p38 MAPK α and β appear to be ubiquitously expressed whereas γ and δ isoform expression appears to be more restricted. SB203580 is a p38 MAPK inhibitor of the pyridinyl-imidazole class with a high level of activity against α and β isoforms by blocking their ATP binding site – but it has little activity against γ and δ isoforms. The effective abrogation of hypoxia-mediated proliferation in human pulmonary arterial fibroblast by pre-incubation with SB203580 strongly implicates α and β isoform involvement. Using immunophoresis techniques we identified α and γ as the major isoforms expressed in acutely hypoxic human pulmonary arterial fibroblasts which concurs with the findings of Conrad *et al* (1999) in PC12 cells and those of the SPVU laboratory. In this work we have demonstrated that α isoform is responsible for pulmonary arterial fibroblast proliferation in a human model.

The role of p38 MPAK α appears to be crucially linked with vascular development in the fetus – mice homozygous for null/deleted p38 MAPK α die *in utero* at approximately 10 days of development as a result of vascular and placental abnormalities. A similar phenotype is found in mice homozyote for null/deleted MEK3/6 (upstream activators of p38α) (Emerling *et al*, 2005). HIF1α $^{-/-}$mice also die *in utero* as a result of vascular anomalies (Yu *et al*, 1999) however mice heterozygote for HIF1α $^{+/-}$ are relatively protected against hypoxic pulmonary arterial

hypertension – developing less right ventricular hypertrophy and less pulmonary vascular resistance. Given the association of p38 MAPK activation and pulmonary arterial fibroblast proliferation to hypoxic exposure we wished to establish whether a functional link might exist between p38 MAPK and HIF1α activity in a human model.

Interestingly Emerling *et al* (2005) demonstrated that mouse embryonic fibroblast cells null for p38 α were unable to stabilise HIF1 α to hypoxic exposure (1.5% O_2) – these cells could however stabilise HIF1α to iron chelators (desferrioximine DFO) and anoxia – implying that HIF1α stabilisation under these circumstances involves a p38 MAPK independent mechanism. Embryonic fibroblasts null for p38 MAPK α could be rescued by adenoviral transfection with p38 MAPK α. Fibroblasts that expressed p38 MAPK α, exposed to electron transport chain (ETC) complex III inhibitors such as anisomycin, were also unable to stabilise HIF1α to hypoxia neither were they able to activate p38 MAPK α. This suggests a strong mechanistic link between p38 MAPK activation and HIF1α in a murine model.

In this series of experiments we have demonstrated that whole HIF-1 activity is increased in hypoxic pulmonary artery fibroblasts alone. There is no expression of whole HIF-1 activity in hypoxic HMAF cells. We have also demonstrated a temporal link between p38 MAPK and whole HIF-1 activity coincident with the second peak of p38 MAPK activity at t = 16 hours. The exact nature of this relationship is not clear but may be mechanistic and would be in keeping with work by Sodhi *et al* (2000) who were able to demonstrate that SB203580 was able to inhibit HIF-1α phosphorylation in a Hep3B cell line. Work from Richard *et al* (1999) however proved a central role

for p44/p42 – ERK 1/2 MAPK in HIF-1 activation in a variety of different cell lines and were unable to prove a role for p38. This work was performed in immortalised cells. However Stroka *et al* (2001) demonstrated that HIF-1 expression demonstrated a considerable degree of variation between organs and it may well be that HIF-1 activation shows not only species, but also organ specificity in the pattern of its activation.

Previous experimental models have demonstrated that HIF-1 is rapidly upregulated within minutes of hypoxic exposure (Yu *et al*, 1998; Stroka *et al*, 2001) so it is unusual that we only found evidence of whole HIF-1 activity in HPAF and that there was no evidence of HIF-1 activity at all in HMAF cells grown in hypoxic conditions. Other investigators have demonstrated that HIF-1 activity can vary in a time and tissue dependent manner in hypoxic mice (Stroka *et al*, 2001), it may well be that pulmonary artery fibroblasts that are normally subjected to a lower P_aO_2 than present in systemic arterial blood may express HIF-1 at more extreme levels of hypoxaemia in man.

Yu *et al* (1999) demonstrated that mice heterozygous for HIF1$\alpha^{(+/-)}$ did not demonstrate comparable responses to chronic hypoxic exposure as homozygotes. Heterozygous mice did not demonstrate an increase in haematocrit, an increase in right ventricular mass or pressure – nor did they develop as dramatic neomuscularisation of their pulmonary arteries to prolonged hypoxic exposure in comparison to wild-type littermates. Interestingly mice heterozygote for HIF2α $^{(+/-)}$ appear to be completely protected from hypoxic pulmonary arterial hypertension, suggesting that certainly in a murine model HIF2α may be the predominant isoform

present in the lung. This does in fact concur with work from Eul *et al* (2006) who demonstrated that both HIF1α and HIF2α were required for pulmonary arterial proliferation to hypoxia in a human model and that HIF1α in isolation appeared to mediate pulmonary arterial fibroblast migration suggesting that HIF α isoforms play critical roles in hypoxia-mediated the signal transduction.

The nature of the apparent interaction between p38 MAPK and HIF-1 is not certain. HIF-1 could represent a down-stream effector of p38 MAPK as suggested by Sodhi *et al* (2000), alternatively p38 MAPK could stabilise HIF-1α, the rate-limiting factor that dictates HIF-1 activity, in hypoxic HPAF cells. It seems unlikely that p38 MAPK acts by increasing protein synthesis as control of HIF-1 activity is achieved by reduced degradation on HIF-1α (Kallio *et al*, 1999).

In a human model we have demonstrated that hypoxia results in HIF1α activation in pulmonary arterial fibroblasts exposed to acute hypoxia. This finding concurs with work from other laboratories (Krick *et al*, 2005). We have demonstrated that pre-incubation of human pulmonary arterial fibroblast cells with SB203580 inhibits HIF1α stabilisation which is maximal at t = 6 hours preincubation and is congruent with the second peak of p38 MAPK activity in these cells. It is possible that the first peak of p38 MAPK activity is responsible for the upregulation of gene products with HRE responsive to HIF1α that enables the proliferative response resulting from the second peak of p38 MAPK activity.

In summary we have shown:

1) Human pulmonary artery fibroblasts proliferate to acute hypoxic exposure whereas systemic arterial fibroblasts do not.
2) Hypoxic pulmonary arterial fibroblast proliferation in a human model is associated with increased p44/p42 and p38 MAPK activity.
3) p38 MAPK activity is responsible for hypoxia-mediated pulmonary artery fibroblast proliferation in a human model.
4) p38 MAPK α and γ isoforms are expressed in hypoxic pulmonary arterial fibroblast cells.
5) p38 MAPK α is responsible for hypoxia-mediated pulmonary arterial fibroblast proliferation in a human model.
6) That p38 MAPK activity is associated with HIF1α activity and that SB203580 can inhibit HIF1α activity in human pulmonary arterial fibroblasts exposed to hypoxia.

Chapter 5:

To examine the contribution of Smad signalling in human pulmonary and systemic arterial fibroblast cell proliferative responses to acute hypoxic exposure

5.1. Introduction

Two separate groups mapped the chromosomal locus of the PPH1 gene to chromosome 2q31,32 (Morse *et al*, 1997; Nichols *et al*, 1997) which was later found to code for the Bone Morphogenetic Protein Receptor II gene (BMPRII) (Deng *et al*, 2000; Lane *et al*, 2000). In hereditary haemorrhagic telangectasia (HHT), a condition also associated with pulmonary hypertension, mutations were found in the activin-like kinase (Alk-1) gene (chromosome 12q13) and endoglin (ENG) (chromosome 9) (Trembath *et al*, 2001). BMPR2, Alk-1 and ENG receptors are members of the Transforming Growth Factor β (TGFβ) superfamily and are involved in transmembrane signalling of bone morphogenetic protein (BMP) and TGFβ respectively.

TGFβ superfamily signalling is highly complex, exerting variable effects on differing cell types – effects that can vary with the cell's developmental and metabolic states. A relatively simple two-step membrane-bound receptor combination represents only one level of signalling control which also includes regulation of extra-cellular ligand binding, variable specificity of intracellular signalling proteins and differing combinations of transcriptional co-activators and co-repressors. It is through this multi-faceted system of checks and balances that the TGFβ superfamily is able to produce a wide variety of effects from ligand-receptor interaction (Massague *et al* 2000; Massague and Wooton, 2000; Miyazono, 2000).

The TGFβ receptor superfamily can be sub-divided into either TGFβ receptors 1, 2 and 3, or BMPR 1 and 2 (Blobe *et al*, 2000). Activation of the receptor complex occurs via ligand binding and subsequent phosphorylation of a type 2 receptor, which is then able to bind to, and activate, a type 1 receptor. Downstream intracellular signalling effectors for the TGFβ superfamily receptors are the Smad signalling proteins. Smad proteins are divided into subclasses: Receptor or R-Smads (Smads 1-3, 5 and 8) responsible for binding to the activated type 2 TGFβ or BMP receptor at the membrane surface, the Common or Co Smad (Smad 4) which enables nuclear transport of the phosphorylated R Smad and Inhibitor or I-Smads (Smad 6 and 7) which block Co-Smad binding to the activated R-Smad subunit. Smads 2 and 3 are specific R-Smads for TGFβ receptor signalling, Smads 1, 5 and 8 for BMPR signalling (Chen *et al*, 1998; Massague, 1990; Massague *et al*, 2000; Massague and Wooton, 2000).

Recent work from Nohe *et al* (2002; 2003; 2004) has suggested that BMP receptor conformation at the membrane surface dictates the subsequent intracellular signalling cascade upregulated by BMP stimulation. BMP receptors either exist as preformed hetero-oligomeric receptor complexes (PFC) where ligand binding results in activation of the Smad signalling pathway, or as homo-oligomers where ligand binding to a type 2 receptor subunit results in recruitment and phosphorylation of a type 1 receptor and subsequent activation of the p38 MAPK pathway.

Initially only 55% of all families with Familial PAH were found to carry a single mutated copy of the BMPRII gene and a functional analysis of these mutations appeared to be associated with reduced BMPR2 expression at the cell surface

membrane, reduced kinase ability or truncation of the long C-terminal chain which is unique to BMPR2 (Atkinson *et al*, 2002; Rudarakanchana *et al*, 2002). The mutation is thought to act either by haploinsufficiency or by a dominant negative effect. More recent work looking at both intronic and exonic regions has identified that the frequency of mutation with the BMPRII gene may be as high as 70% within affected families (Cogan *et al*, 2006). Exonic or duplication mutations were identified in 48% of all BMPRII mutations within a large number of affected families who had not been identified as carrying a mutation in previous studies, this is in comparison to a frequency of 14% in individuals with IPH. This work further underlines the significance of BMP-BMPR2-Smad/p38MAPK signalling in the aetiology of pulmonary hypertension.

The primary ligands for BMPR2 are Bone Morphogenetic Proteins 2, 4 and 7. The effects of BMP signalling largely appear to be that of anti-proliferation, anti-differentiation and anti-apoptosis, but as in TGF β mediated signalling the response is cell specific. BMP 2 has been shown to inhibit proliferation of human aortic VSMC via p21 preventing G_1/S phase transition through its action on cyclins (Wong *et al*, 2003). BMP 2 has also been shown to induce apoptosis in mouse hybridoma MH60 cell lines via p38 MAPK (Kimura *et al*, 2000).

A functional analysis of mutated BMPR2 has identified reduced receptor expression either as a result of failure of a truncated receptor to localise to the cell membrane or as a result of a kinase domain mutation causing signal transduction failure (Rudarakanchana *et al*, 2002). The effect of BMPR2 mutation appears to have effects that extend beyond the immediate influence of BMPR2-BMPR1 interaction, with TGF β signalling (not normally a ligand for BMPR2) enhanced as a result of reduced

BMPR2 function (Morrell *et al*, 2001). Nohe *et al* (2002) have demonstrated that the frequency of receptor units at the cell surface membrane predicts the downstream signalling cascade activated with differential activation of either Smad or p38 MAPK signalling. Support for this argument is demonstrated by recent work from Yu *et al* (2005) which shows that disruption of BMPR2 signalling in a mouse VSMC model which is haploinsufficient for BMPRII, enables signal transduction via an alternative pathway via activin type 2 receptors (ActR2 - a TGF beta superfamily receptor) that is normally suppressed in wild-type animals. Reduction in BMP4-mediated signalling resulted in an increase in BMP6 and predominantly BMP7-mediated signalling leading to increased Smad 1, 5 and 8 phosphorylation and p38 MAPK activation.

Morrell *et al* (2001) have demonstrated altered cell responsiveness to BMP4 and TGF β in VSMC from patients with Familial PAH. They demonstrated that TGF β stimulation resulted in an increase in VSMC proliferation in cells cultured from the proximal pulmonary arteries of patients with FPAH, but not in patients with secondary PAH or controls. In addition BMP4 did not, as expected, suppress proximal VSMC proliferation in patients with FPAH in comparison to VSMC cultured from patients with secondary PAH. The same group also demonstrated that VSMC cultured from human proximal pulmonary arteries were growth-inhibited by BMP4 stimulation, but that VSMC from distal pulmonary arteries were protected from apoptosis and proliferated to BMP4 stimulation (Yang *et al*, 2005). The differential growth response did not appear to be as a result of activation of an alternative signalling pathway as both Smad and p38 MAPK pathways appeared to be equally upregulated in VSMC from both proximal and distal pulmonary arteries. Yang *et al* (2005) also demonstrated that VSMC from the proximal pulmonary arteries could be converted to a pro-proliferative phenotype by transfection with a

dominant-negative Smad 1 protein, suggesting that abnormalities within BMPR2 mediated signalling could result in deficient Smad signalling. Interestingly the pro-proliferative effects of BMP4 on peripheral VSMC appeared to be p38 MAPK and ERK1/2 dependent. VSMC known to express BMPR2 with mutations within the kinase domain demonstrated reduced Smad signalling *and* reduced Smad signalling has been demonstrated in both patients with FPAH and IPAH. It is possible that defective Smad signalling could result in unchecked p38MAPK/ERK1/2 signalling, resulting in abnormal cellular proliferation. This is in part born out but the finding that in pulmonary artery VSMC, BMPR2 stimulation by BMP2 results in divergent gene activation in patients with IPAH in comparison to controls (Fantozzi *et al*, 2005).

Recent work from Teichert-Kuliszewska *et al* (2006) demonstrates that the effects of disordered BMPR2 signalling are not only confined to the media. BMP-mediated signalling appears to be largely proapoptotic for VSMC but inhibits apoptosis in EC. When BMPR2 expression is reduced by approximately 50%, a 3 to 4 fold increase in EC apoptosis was demonstrated. In Epithelial Progenitor Cells (EPC) from both patients with IPAH and controls there was no difference in BMPR2 expression levels but there was a significant difference in response to BMP stimulation: BMP2 stimulation was only able to reduce apoptosis in EPC from controls but not from patients with IPAH where the loss of function of BMPR2 resulted in a marked increase in EPC apoptosis. Unfortunately this study did not include any information regarding the genotype of the study group. Michelakis (2006) suggests that perhaps primary pulmonary hypertension should be considered not only as a disorder involving different vascular compartments but also that the behaviour of these compartments may vary with time. He suggested that an early increase in EC

apoptosis may allow mitogens access to the medial layer resulting in VSMC proliferation and that later EC may develop an antiapoptotic phenotype resulting in the plexigenic lesions.

Using hypoxia as a model for pulmonary arterial hypertension, Frank *et al* (2005) demonstrated that BMP4 was upregulated in the lungs of mice exposed to chronic hypoxia. They went on to investigate the behaviour of pulmonary arterial VSMC cultured from mice haploinsufficient for BMP4. $BMP4^{+/-}$ mice were protected from developing hypoxia-related pulmonary hypertension when exposed to conditions of chronic hypoxia. In addition they demonstrated that these animals showed less VSMC proliferation and significantly less pulmonary arterial remodelling, together with a reduction in Smad 1, 5 and 8 phosphorylation. This finding is counter-intuitive, as a reduction in total BMP4 would be expected to release VSMC from anti-proliferative control thereby causing VSMC proliferation and vascular remodelling. The investigators also noted an increase in BMP2 mRNA in the $BMP4^{+/-}$ mouse in response to hypoxia – they theorised that the reduction in total BMP4 allowed BMP2 to become the dominant signal.

There is scant work published on the role of BMP-BMPR2-Smad signalling in fibroblasts. Jeffrey *et al* (2005) demonstrated that pre-incubation of fetal whole lung fibroblasts with BMP4 in combination with serum resulted in reduced proliferation that was associated with an increase in Smad 1 phosphorylation and nuclear translocation. They also noted an increase in smooth muscle actin (SMA) and myosin light chain (MLC) expression in these cells, suggesting a switch to a VSMC phenotype – a response that was specific to BMP4 pre-incubation and not found when cells were pre-incubated with BMP2 or 7. BMP4 stimulation was also associated

with an increase in p21 expression that controls G_1/S phase transition and increases in p38, JNK and ERK1/2 phosphorylation. Jeffrey *et al* also demonstrated that fetal whole lung fibroblasts transfected with a dominant negative Smad 1 were released from their anti-proliferative phenotype, an effect that could be partially abrogated by pre-incubating the cells with a JNK inhibitor, but not SB203580 or U0126 specific p38 and ERK1/2 inhibitors respectively.

Given that BMPRII mutations are known to be associated with Familial IPH and that we have previously demonstrated that p38 MAPK activation is required for hypoxia-mediated pulmonary artery proliferation (see chapter 4) we wished to assess the role of Smad signalling in human pulmonary and systemic artery responses to acute hypoxia; in particular concentrating on Smads 1, 5 and 8 – R Smads directly down-stream from BMPR 1 and 2. We theorised that as the BMP-BMPR-Smad signalling cascade has an inhibitory influence in mesenchymal cells (Massague and Wooton, 2000), Smad 1, 5 and 8 signalling would be down-regulated in proliferating hypoxic pulmonary artery fibroblasts when compared with identical cells grown under normoxic conditions. We also wished to examine any potential interaction between the stress activated MAPK, p38 MAPK, classic MAP kinases ERK 1/2 MAPK and Smad signalling. We also wished to examine the contribution of TGFβ-specific R Smad 2 and Inhibitory Smads 6 and 7 in hypoxic-mediated pulmonary artery fibroblast proliferation; Smad 6 being the specific inhibitory signalling protein for the BMP-BMPR-Smad cascade via its phosphorylation of Smad 4 (Hata *et al*, 1997; Imamura *et al*, 1997; Miyazono, 2000) and on the p38 MAPK cascade via its influence on TAK-1.

5.2. Methods

5.2.1. Activation of Smad proteins in human pulmonary and systemic arterial fibroblasts under conditions of normoxia and acute hypoxia

Cells were obtained from pulmonary and systemic arteries of consented patients undergoing lung reduction surgery for the treatment of lung cancer or chronic obstructive pulmonary disease. Cells were prepared by the method described above (Chapter 2.3). Cells were grown to 60% confluence in 6 well plates and were then quiesced using serum-free media for a period of 24 hours. They were then grown in conditions of normoxia or hypoxia (35mmHg PO_2 ~ 5%) for a total period of 24 hours with the addition of 5% serum stimulation. Cell lysates were prepared following 24 hours exposure to either normoxia or hypoxia using the method previously described (chapter 2.6). Smad 1, 5 and 8, Smad 2, Smad 4 together with Smad 6 and 7 phosphorylation was assessed using Western blotting using retardation on SDS-PAGE gels as a marker of phosphorylation using a 1:500 dilution of a rabbit anti-Smad IgG primary antibody and a 1:1000 dilution of a flagged anti-goat secondary antibody on nitrocellulose membranes and a Chemiluminescence detection system (chapter 2.6). Each experiment was repeated 4 times in the same individual and in a total of 4 individuals and the results shown were representative of the mean from a single individual. The results were confirmed with densitometry (chapter 2.7).

5.2.2. Influence of stress (p38) MAP kinase inhibitor (SB203580) on Smad protein expression in human pulmonary and systemic arterial fibroblasts under conditions of normoxia and acute hypoxia.

Human pulmonary and systemic arterial fibroblast cells were prepared for culture as described above. They were grown to 60% confluency in 6 well plates and were then quiesced for a period of 24 hours using serum-free media. Cells were then stimulated with 5% serum and were then grown under conditions of normoxia or hypoxia (35mmHg PO_2 ~ 5%) for a period of 24 hours. 50 μl of a specific p38 MAPK inhibitor SB203580 was added to each well 6 hours before the end of the experiment. Cell lysates were then prepared and protein expression assessed by means of SDS-gel electrophoresis and Western Blotting techniques using a 1:500 dilution of a rabbit anti-Smad IgG primary antibody together with a 1:1000 dilution of an anti-goat secondary antibody (as described above). This experiment was then repeated using anti Smad 2, 6 and 7 primary antibodies using the same dilutions. Each experiment was repeated 4 times in the same individual and in a total of 4 individuals and the blots demonstrated were representative of the mean from a single individual

5.2.3. Influence of classic (ERK) MAP kinase inhibitors (U0126) on Smad protein expression in human pulmonary and systemic arterial fibroblasts under conditions of normoxia and acute hypoxia.

Cells were prepared as described above, grown to 60% confluence in 6 well plates and were then quiesced using serum-free media for 24 hours. Cells were then stimulated with 5% serum and were then grown in conditions of either normoxia or acute hypoxia (35mmHg PO_2 ~ 5%) for a period of 24 hours. Six hours prior to the end of the experiment 50 μl of a specific ERK 1/2 MAP kinase inhibitor – U0126 –

was added to each well. Cell lysates were prepared as previously described (Chapter 2.6) and protein expression was assessed by Western Blotting techniques and by using a 1:500 dilution of a rabbit anti-Smad IgG primary antibody and a 1:1000 dilution of an anti-goat secondary antibody. This experiment was repeated using anti Smad 2, 6 and 7 antibodies using the same dilutions. Each experiment was repeated 4 times in the same individual and in a total of 4 individuals and the blots demonstrated were representative of the mean from a single individual

5.2.4 Densitometric analysis of blots

Densitometric analysis of blots was carried out using a computer programme that allowed for comparison of blot density in graphical form (Quantiscan). The results are expressed as a percentage of normoxic control without the addition of 5% serum stimulation.

5.3. Statistics

Results are expressed as the mean + S.D. and statistical analysis was undertaken as described in chapter 2.8.

5.4. Results

Smad 5 and 8 activation is increased in human systemic arterial fibroblasts (HMAF) and decreased in human pulmonary arterial fibroblasts (HPAF) to acute hypoxic exposure

HPAF and HMAF cells were grown in 6 well plates until 60% confluent. They were then quiesced in serum-free media for a period of 24 hours. Cells were then grown under conditions of normoxia or acute hypoxia (35mmHg ~ 5% PO_2) for a period of 16 hours without the addition of serum, with 5% serum and with 5% serum plus SB203580 a specific p38 MAPK inhibitor (which was added 6 hours prior to the end of the experiment). Cell lysates were prepared for Western Blotting as per chapter 2.6. **Figure 5.1 (a)** demonstrates that phospho Smad 5 and 8 activation was increased in normoxic pulmonary artery fibroblasts with the addition of 5% serum but was down-regulated under hypoxic conditions. In human mammary artery fibroblasts phospho Smad 5 and 8 activation was increased under hypoxic conditions following the addition of 5% serum. Pre-incubation of HPAF cells with SB203580 reduces the expression of phospho Smad 5 and 8 to the level seen in hypoxic conditions The membranes were probed for whole p38 MAPK that is expressed uniformly in both cell types - irrespective of growth condition - in order to act as a control. These results were confirmed by densitometry – HMAF cells grown in normoxia (N) (black bar) and hypoxia (H) (white bar) **(b)** and **(c)** with results expressed as a percentage of normoxic control. The experiment was repeated 4 times in the same individual with the results illustrated being representative of 4 separate experiments. The experiment was repeated in 4 individuals. The results are expressed as mean +/- 2 SD with * signifying a statistically significant result ($p < 0.05$).

Figure 5.1(a): Smad 5 and 8 activation is increased in human systemic arterial fibroblasts (HMAF) and decreased in human pulmonary arterial fibroblasts (HPAF) to acute hypoxic exposure. HMAF and HPAF cells were grown in 6 well plates until 60% confluent. They were then quiesced with serum free media for a period of 24 hours. They were then grown in conditions of normoxia (N) or hypoxia (H) for 16 hours. Cells were grown in serum-free media (C), with 5% serum (S) or with 5% serum plus SB203580 (S + SB). Phospho Smad 5 and 8 expression was increased in HMAF cells grown in hypoxia with 5% serum stimulation, SB203580 did not alter phospho Smad 5 and 8 expression: **figure 5.1 (a)**. In HPAF cells there was a relative reduction in phospho Smad 5 and 8 expression under conditions of hypoxia with 5% serum stimulation: **figure 5.1 (a).** Preincubation with SB203580 appeared to reduce phospho Smad 5 and 8 expression in these cells. These results were confirmed by densitometry – cells grown in normoxia (black bar) and hypoxia (white bar) **(b), (c), (d) and (e)** with results expressed as a percentage of normoxic control**.** The experiment was repeated 4 times in the same individual with the results illustrated being representative of 4 separate experiments. The experiment was repeated in 4 individuals. The results are expressed as mean +/- 2 SD with * signifying a statistically significant result ($p < 0.05$).

(a)

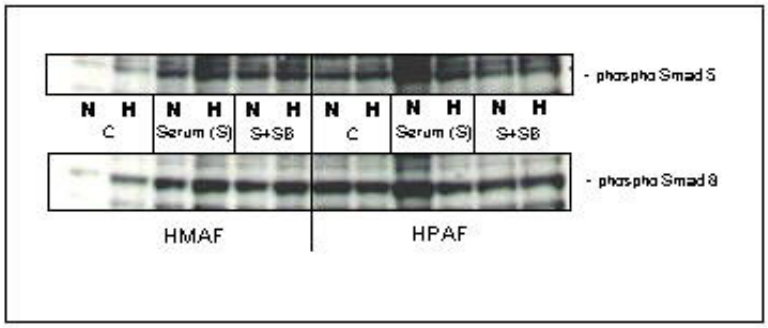
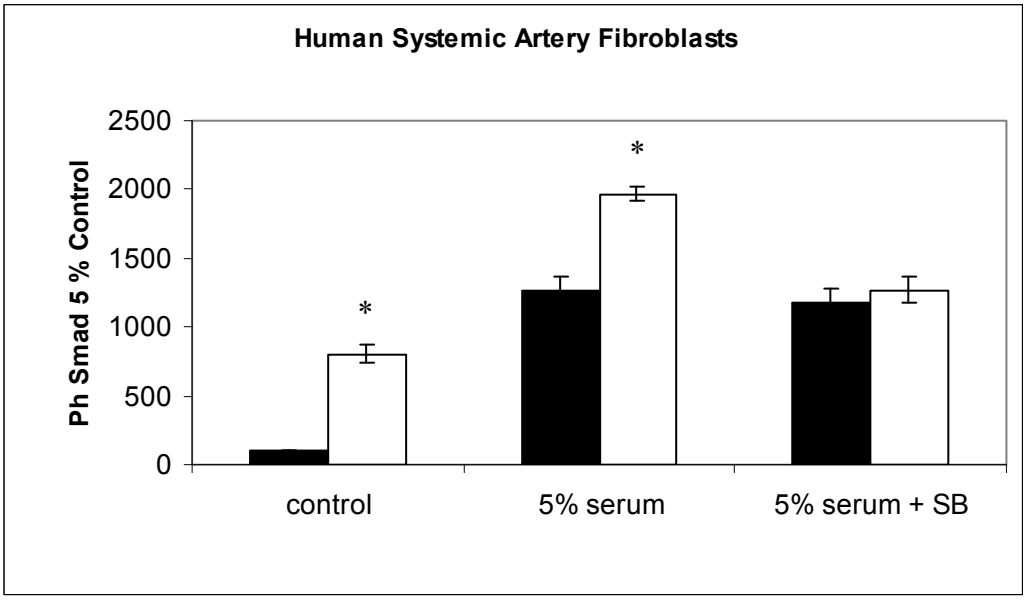

- phospho Smad 5
N H N H N H N H N H N H
C
Serum (S)
S+SB
C
Serum (S)
S+SB
- phospho Smad 8
HMAF
HPAF

Figure 5.1(a) (b) and (c): Smad 5 and 8 activation is increased in human systemic arterial fibroblasts (HMAF) and decreased in human pulmonary arterial fibroblasts (HPAF) to acute hypoxic exposure. HMAF cells were grown in 6 well plates until 60% confluent. They were then quiesced with serum free media for a period of 24 hours. They were then grown in conditions of normoxia (N) or hypoxia (H) for 16 hours. Cells were grown in serum-free media (C), with 5% serum (S) or with 5% serum plus SB203580 (S + SB). Phospho Smad 5 and 8 expression was increased in HMAF cells grown in hypoxia with 5% serum stimulation, SB203580 did not alter phospho Smad 5 and 8 expression: **Figure 5.1 (a).** These results were confirmed by densitometry – cells grown in normoxia (N) (black bar) and hypoxia (H) (white bar) **(b), (c),** with results expressed as a percentage of normoxic control. The experiment was repeated 4 times in the same individual with the results illustrated being representative of 4 separate experiments. The experiment was repeated in 4 individuals. The results are expressed as mean +/- 2 SD with * signifying a statistically significant result ($p < 0.05$).

(b)


Human Systemic Artery Fibroblasts
Ph Smad 5 % Control
2500
2000
1500
1000
500
0
*
*
control
5% serum
5% serum + SB

(c)

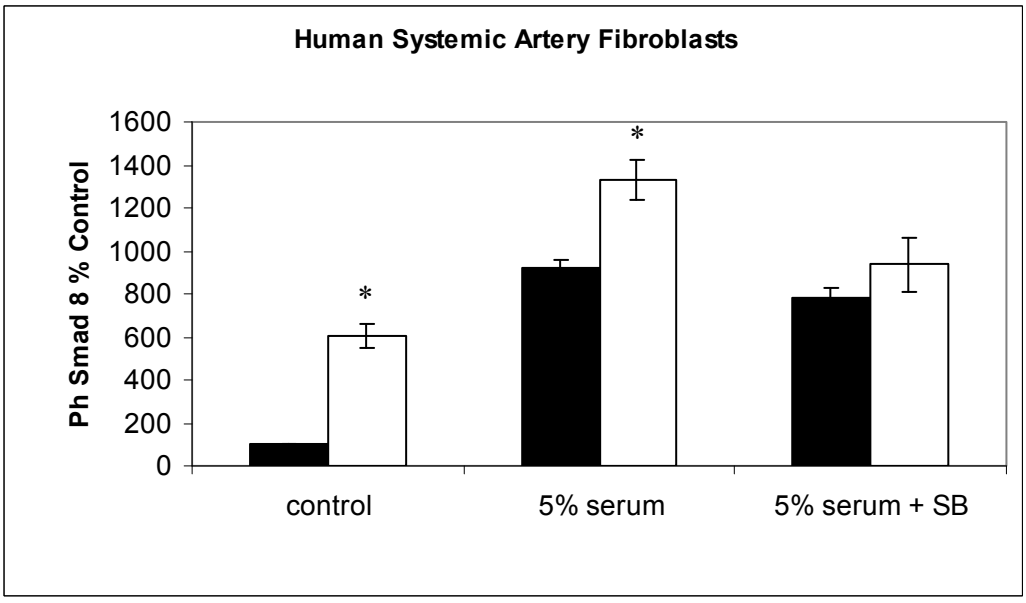
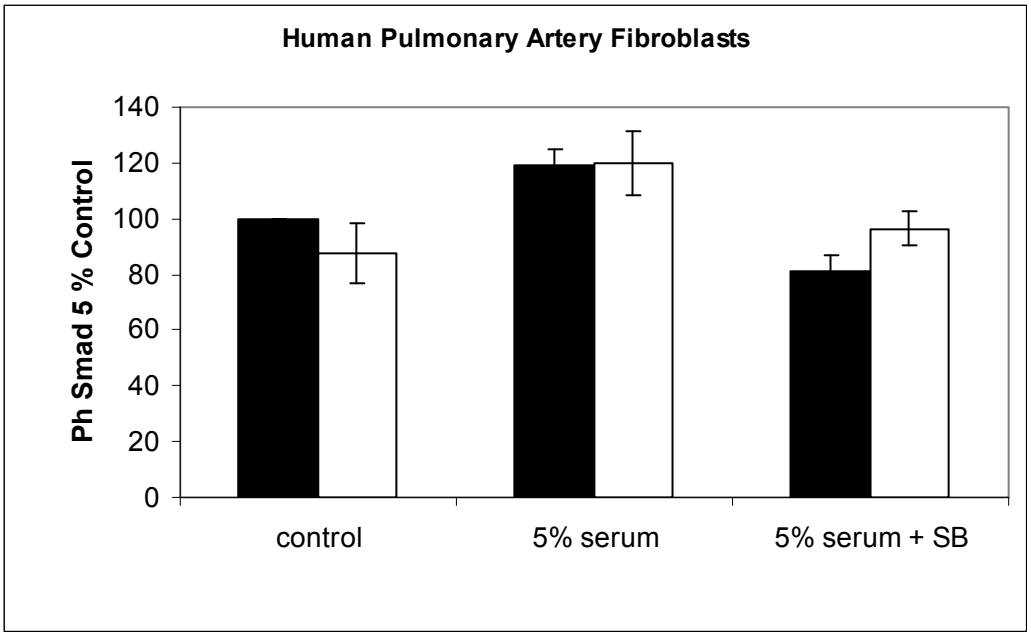
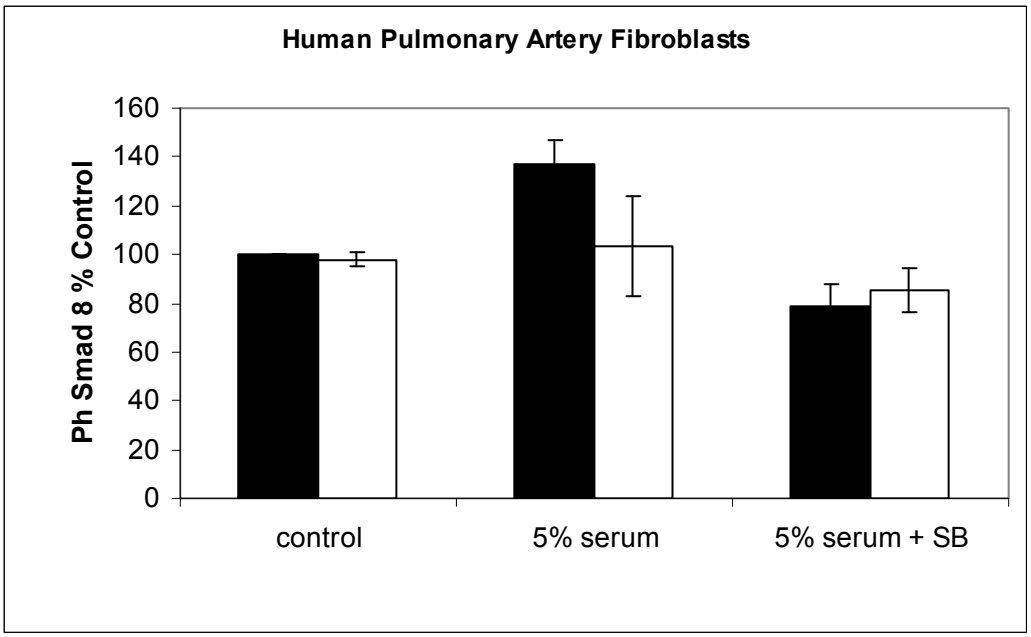

Human Systemic Artery Fibroblasts
Ph Smad 8 % Control
1600
1400
1200
1000
800
600
400
200
0
*
*
control
5% serum
5% serum + SB

Figure 5.1(a), (d) and (e): Smad 5 and 8 activation decreased in human pulmonary arterial fibroblasts (HPAF) to acute hypoxic exposure. HPAF cells were grown in 6 well plates until 60% confluent. They were then quiesced with serum free media for a period of 24 hours. They were then grown in conditions of normoxia (N) or hypoxia (H) for 16 hours. Cells were grown in serum-free media (C), with 5% serum (S) or with 5% serum plus SB203580 (S + SB). In HPAF cells there was a relative reduction in phospho Smad 5 and 8 expression under conditions of hypoxia with 5% serum stimulation which did not reach statistical significance. These results were confirmed with densitometry: **figures 5.1 (a) (d) (e).** Preincubation with SB203580 appeared to reduce phospho Smad 5 and 8 expression in these cells. These results were confirmed by densitometry – cells grown in normoxia (black bar) and hypoxia (white bar) **(d) and (e)** with results expressed as a percentage of normoxic control**.** The experiment was repeated 4 times in the same individual with the results illustrated being representative of 4 separate experiments. The experiment was repeated in 4 individuals. The results are expressed as mean +/- 2 SD.

(d)


Human Pulmonary Artery Fibroblasts
Ph Smad 5 % Control
140
120
100
80
60
40
20
0
control
5% serum
5% serum + SB

(e)


Human Pulmonary Artery Fibroblasts
Ph Smad 8 % Control
160
140
120
100
80
60
40
20
0
control
5% serum
5% serum + SB

Human pulmonary and systemic arterial fibroblasts demonstrate no activation of Smads 2, 6 or 7 under either normoxic or hypoxic growth conditions

HPAF and HMAF cells were grown in 6 well plates until 60% confluent. They were then quiesced in serum-free media for a period of 24 hours. Cells were then grown under conditions of normoxia or acute hypoxia (35mmHg ~ 5% PO_2) for a period of 16 hours without the addition of serum, with 5% serum and with 5% serum plus U0126 a specific p44/42 MAPK inhibitor (which was added 6 hours prior to the end of the experiment). Cell lysates were prepared for Western Blotting as per chapter 2.6. **Figure 5.2 (a)** demonstrates that there was no alteration in activated Smad 2 expression irrespective of growth condition. There was no alteration in whole Smad 6 or 7 activation in either cell type irrespective of growth conditions. Pre-incubation with U0126 did not alter the expression of whole Smad 6 or 7. These results were confirmed by densitometry – cells grown in normoxia (N) (black bar) and hypoxia (H) (white bar) **(b), (c), (d) and (e)** with results expressed as a percentage of normoxic control**.** The experiment was repeated 4 times in the same individual with the results illustrated being representative of 4 separate experiments. The experiment was repeated in 4 individuals. The results are expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) or hypoxic (H) growth conditions in either human pulmonary or systemic arterial fibroblast cells.

Figure 5.2(a): Human pulmonary and systemic arterial fibroblasts demonstrate no activation of Smads 2, 6 or 7 under either normoxic or hypoxic growth conditions. HPAF and HMAF cells were grown in 6 well plates until 60% confluent. They were then quiesced in serum-free media for a period of 24 hours. Cells were then grown under conditions of normoxia or acute hypoxia (35mmHg ~ 5% PO_2) for a period of 16 hours without the addition of serum (C), with 5% serum (S) and with 5% serum plus U0126 a specific p44/42 MAPK inhibitor (S + U0126) which was added 6 hours prior to the end of the experiment. Cell lysates were prepared for Western Blotting as per chapter 2.6. HPAF and HMAF demonstrated no increased activity of phospho Smad 2, whole Smad 6 and whole Smad 7 **(figure 5.2a)**. These results were confirmed by densitometry – cells grown in normoxia (N) (black bar) and hypoxia (H) (white bar) **figures 5.2(b) – (g)** with results expressed as a percentage of normoxic control**.** The experiment was repeated 4 times in the same individual for both systemic and pulmonary arterial fibroblasts with the results illustrated being representative of 4 separate experiments. Each experiment was repeated in a total of 4 individuals. The results are expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) or hypoxic (H) growth conditions in either human pulmonary or systemic arterial fibroblast cells.

(a)

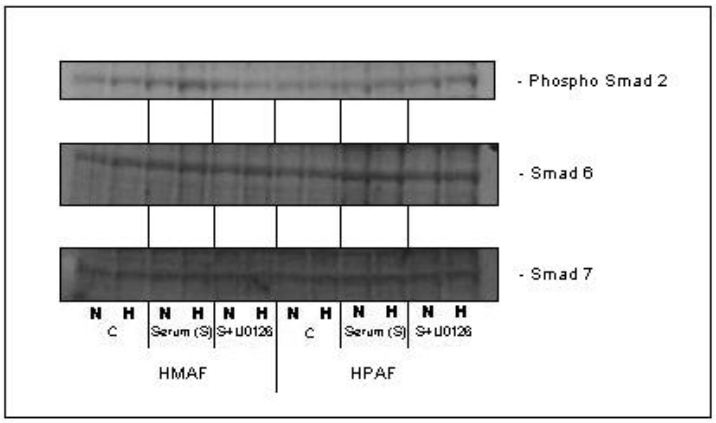
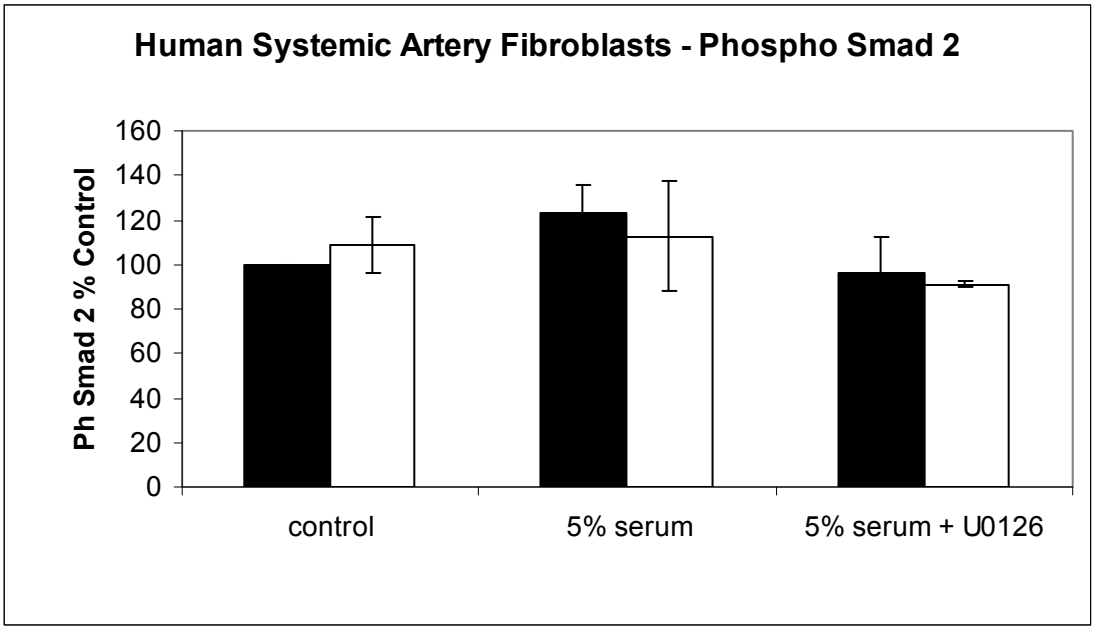

- Phospho Smad 2
- Smad 6
- Smad 7
N H N H N H N H N H N H
C
Serum (S)
S+U0126
C
Serum (S)
S+U0126
HMAF
HPAF

Figure 5.2(a), (b) and (c): Human pulmonary and systemic arterial fibroblasts demonstrate no activation of Smad 2 under either normoxic or hypoxic growth conditions. HPAF and HMAF cells were grown in 6 well plates until 60% confluent. They were then quiesced in serum-free media for a period of 24 hours. Cells were then grown under conditions of normoxia or acute hypoxia (35mmHg ~ 5% PO_2) for a period of 16 hours without the addition of serum (C), with 5% serum (S) and with 5% serum plus U0126 a specific p44/42 MAPK inhibitor (S + U0126) which was added 6 hours prior to the end of the experiment. Cell lysates were prepared for Western Blotting as per chapter 2.6. HPAF and HMAF demonstrated no increased activity of phospho Smad 2 **(figures 5.2 a, b and c).** These results were confirmed by densitometry – cells grown in normoxia (N) (black bar) and hypoxia (H) (white bar) **(b) – (c)** with results expressed as a percentage of normoxic control**.** The experiment was repeated 4 times in the same individual with the results illustrated being representative of 4 separate experiments. The experiment was repeated in 4 individuals. The results are expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) or hypoxic (H) growth conditions in pulmonary or systemic arterial fibroblast cells.

(b)


Human Systemic Artery Fibroblasts - Phospho Smad 2
Ph Smad 2 % Control
160
140
120
100
80
60
40
20
0
control
5% serum
5% serum + U0126

(c)

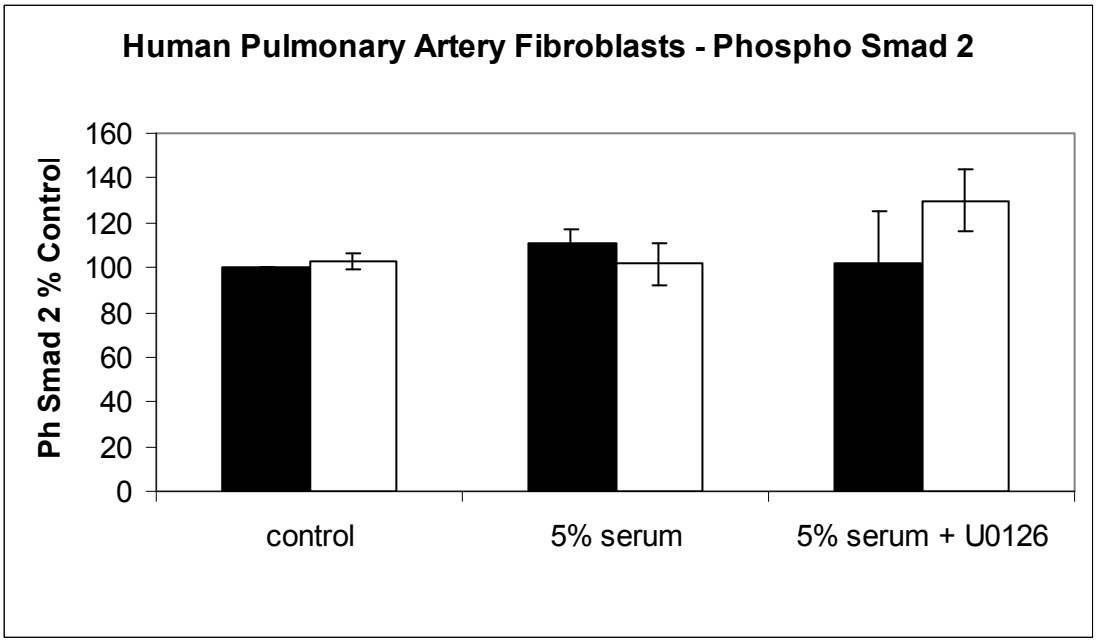
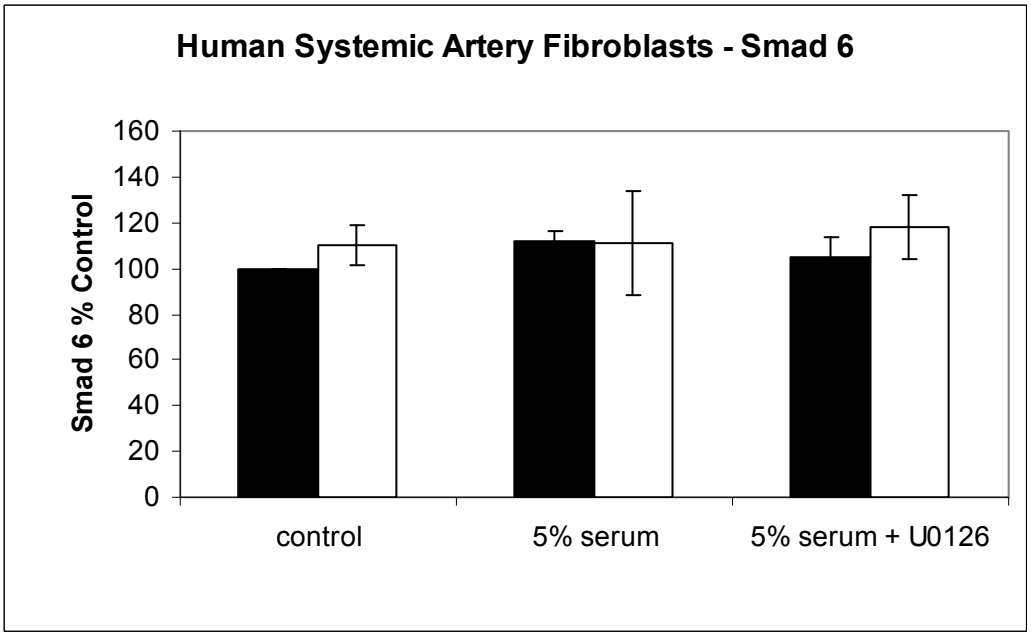

Human Pulmonary Artery Fibroblasts - Phospho Smad 2
Ph Smad 2 % Control
160
140
120
100
80
60
40
20
0
control
5% serum
5% serum + U0126

Figure 5.2(a), (d) and (e): Human pulmonary and systemic arterial fibroblasts demonstrate no activation of Smad 6 under either normoxic or hypoxic growth conditions. HPAF and HMAF cells were grown in 6 well plates until 60% confluent. They were then quiesced in serum-free media for a period of 24 hours. Cells were then grown under conditions of normoxia or acute hypoxia (35mmHg ~ 5% PO_2) for a period of 16 hours without the addition of serum (C), with 5% serum (S) and with 5% serum plus U0126 a specific p44/42 MAPK inhibitor (S + U0126) which was added 6 hours prior to the end of the experiment. Cell lysates were prepared for Western Blotting as per chapter 2.6. HPAF and HMAF demonstrated no increased activity of whole Smad 6 **(figures 5.2, a, d, e)**. These results were confirmed by densitometry – cells grown in normoxia (N) (black bar) and hypoxia (H) (white bar) **(d) - (e)** with results expressed as a percentage of normoxic control**.** The experiment was repeated 4 times in the same individual with the results illustrated being representative of 4 separate experiments. The experiment was repeated in 4 individuals. The results are expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) or hypoxic (H) growth conditions in human pulmonary or systemic arterial fibroblast cells.

(d)


Human Systemic Artery Fibroblasts - Smad 6
Smad 6 % Control
160
140
120
100
80
60
40
20
0
control
5% serum
5% serum + U0126

(e)

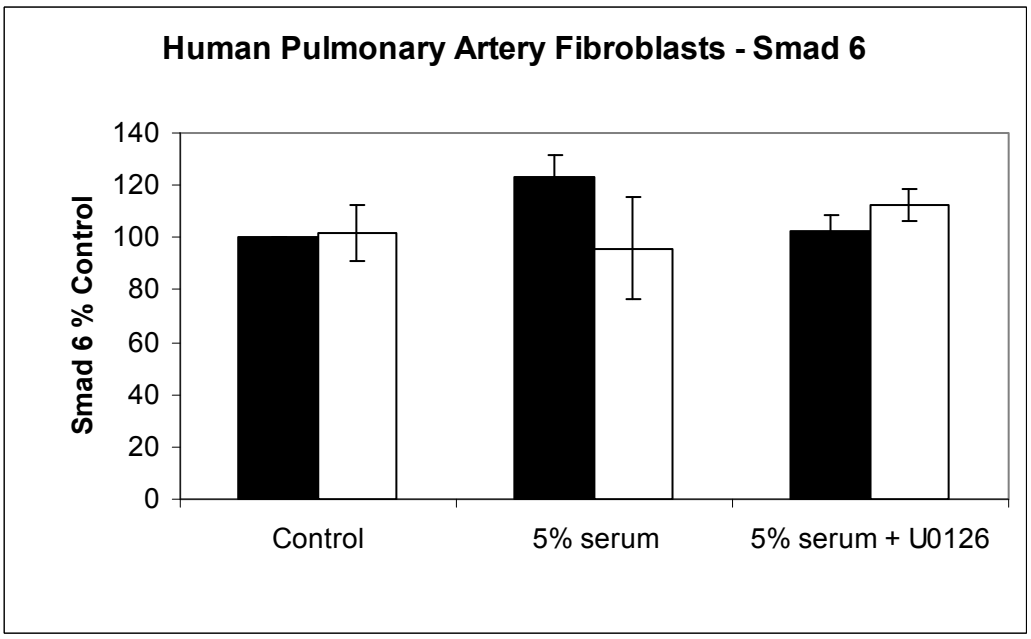
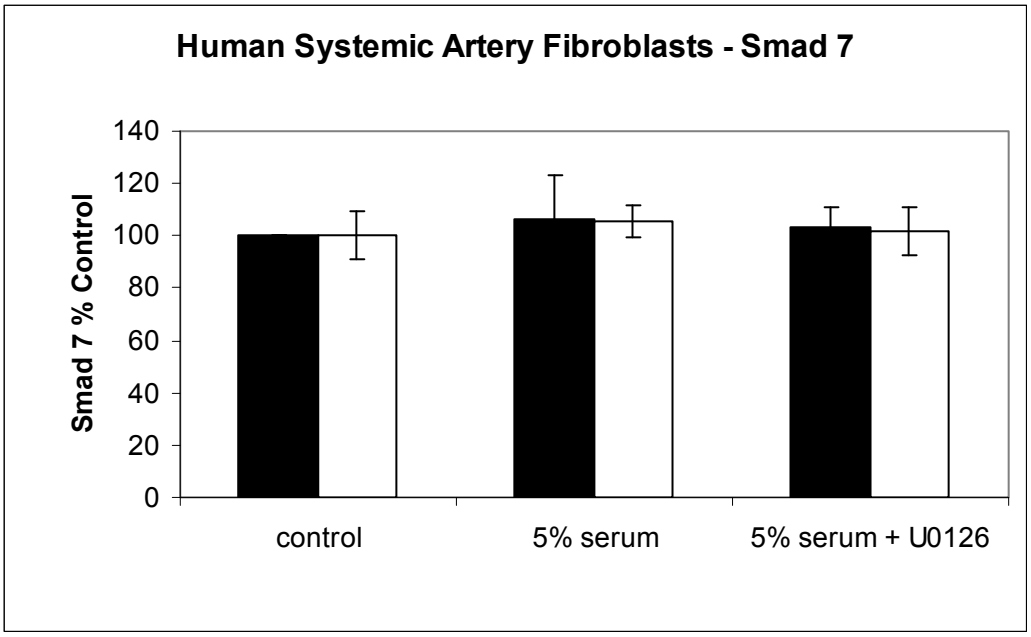
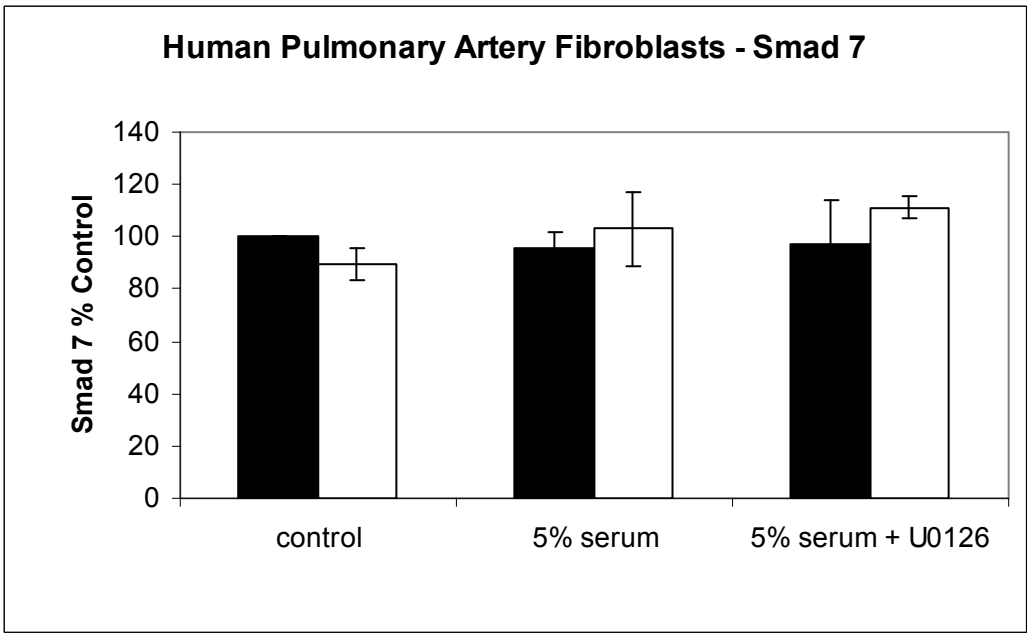

Human Pulmonary Artery Fibroblasts - Smad 6
Smad 6 % Control
140
120
100
80
60
40
20
0
Control
5% serum
5% serum + U0126

Figure 5.2(a, f and g): Human pulmonary and systemic arterial fibroblasts demonstrate no activation of Smad 7 under either normoxic or hypoxic growth conditions. HPAF and HMAF cells were grown in 6 well plates until 60% confluent. They were then quiesced in serum-free media for a period of 24 hours. Cells were then grown under conditions of normoxia or acute hypoxia (35mmHg ~ 5% PO_2) for a period of 16 hours without the addition of serum (C), with 5% serum (S) and with 5% serum plus U0126 a specific p44/42 MAPK inhibitor (S + U0126) which was added 6 hours prior to the end of the experiment. Cell lysates were prepared for Western Blotting as per chapter 2.6. HPAF and HMAF demonstrated no increased activity of whole Smad 7 **(figures 5.2, a, f, g)**. These results were confirmed by densitometry – cells grown in normoxia (black bar) and hypoxia (white bar) **(f) - (g)** with results expressed as a percentage of normoxic control**.** The experiment was repeated 4 times in the same individual with the results illustrated being representative of 4 separate experiments. The experiment was repeated in 4 individuals. The results are expressed as mean +/- 2 SD. There was no statistical difference between normoxic (N) or hypoxic (H) growth condition in either human pulmonary or systemic arterial fibroblast cells.

(f)


Human Systemic Artery Fibroblasts - Smad 7
Smad 7 % Control
140
120
100
80
60
40
20
0
control
5% serum
5% serum + U0126

(g)


Human Pulmonary Artery Fibroblasts - Smad 7
Smad 7 % Control
140
120
100
80
60
40
20
0
control
5% serum
5% serum + U0126

5.5. Discussion

We have demonstrated that human pulmonary artery fibroblast proliferate to acute hypoxia is associated with increased activity of both phosphorylated p44/p42 – ERK 1/2 and p38 MAPKs but that the proliferative response is dependent on phosphorylation of p38 MAPK. The BMP-BMPR-Smad pathway acts to inhibit proliferation in mesenchymal cells and therefore the finding that phosphorylated Smad 1, 5 and 8 is reduced in hypoxic HPAF in comparison to identical cells grown in normoxia is in keeping with the theory that these cells are maintained in a pro-proliferative state when grown in conditions of acute hypoxia. This would suggest active down-regulation of growth-inhibitory pathways with concomitant upregulation of pro-proliferative signalling in these cells.

To date we have not been able to show that there is any response in human systemic artery fibroblast cells to acute hypoxic exposure. We have found upregulation of p44/p42 – ERK1/2 MAPK and have found no expression of p38 MAPK in hypoxic systemic arterial fibroblast cells. This is in keeping with the lack of proliferative response to hypoxic stimuli seen in this cell type. However our investigation of Smad signalling in hypoxic pulmonary artery fibroblasts has led to the surprise discovery that Smad signalling is upregulated in human systemic arterial fibroblast cells grown under conditions of both normoxia and acute hypoxia when compared to human pulmonary arterial fibroblasts grown under identical conditions. The increase in Smad 1, 5 and 8 phosphorylation in human systemic arterial fibroblast cells grown under hypoxic conditions was even more marked compared to identical cells grown in normoxic conditions. This increase was further augmented by the addition of 5% serum.

We were not able to demonstrate any differential expression of whole Smad 6 and 7 in either cell type under any growth condition investigated. We were unable to examine the potential effects of phosphorylated Smad 6 and 7 in these cells because at the time of experimentation primary antibodies did not exist for these proteins and previous attempts to transfect these cells with active proteins via an adenoviral vector had proved unsuccessful resulting in premature cell death. There appeared to be no alteration in expression of phosphorylated Smad 2 (a TGFβ associated R-Smad) in either cell type under either growth condition. This suggests that phospho-Smad 2 does not have an active role to play in human pulmonary or systemic arterial fibroblast behaviour in response to acute hypoxia.

Our findings concur with work performed by Jeffrey *et al* (2005), who found that in whole fetal lung fibroblast cells BMP4 stimulation resulted in a reduction in proliferation which was associated with increased phospho Smad 1 expression. In this model phospho Smad 1-mediated fibroblast growth inhibition was associated with increases in p38 MAPK, JNK and ERK 1/2 expression whereas in our adult arterial fibroblast hypoxic model increases in these MAPKs – both stress and classical - were associated with cellular proliferation in pulmonary arterial fibroblasts only. There is a significant body of work examining the effects of hypoxia on BMP/BMPR/Smad signalling but it is largely based on murine models where it is easier to modify the underlying genotype in order to investigate specific components of the BMP/BMPR/Smad signalling cascade.

The mouse models so far investigated are either haploinsufficient for the BMPR2 receptor (Beppu *et al*, 2004; Jeffery *et al*, 2005; Long *et al*, 2006) or express a dominant negative BMPR2 (West *et al*, 2004; Young *et al*, 2006) or are heterozygous

for BMP4 (Frank *et al*, 2005). Although these investigators examine the effects of disordered BMP/BMPR/Smad signalling on vascular smooth muscle cells or endothelial cells under hypoxic conditions, some parallels can be drawn between these models and the hypoxic human arterial fibroblast model. Frank *et al* (2005) found that Smad 1, 5 and 8 expression was reduced in VSMC cultured from mice heterozygous for BMP4 when exposed to chronic hypoxia (4 weeks at 10% oxygen) in comparison to wild-type controls. The reduction in Smad 1, 5 and 8 was associated with less right ventricular hypertrophy and a reduced number of muscularised arteries in heterozygotes. These investigators demonstrated that BMP4 secretion from endothelial cells from $BMP4^{+/-}$ animals was reduced following hypoxic exposure but that there was increased BMP2 secretion from these cells in comparison to wild-type controls. Beppu *et al* (2004) also demonstrated reduced Smad 1, 5 and 8 expression in a haploinsufficient mouse model following 3 weeks exposure to 10% oxygen compared to wild-type controls. They showed that while Smad 1, 5 and 8 phosphorylation did not differ between wild type and $BMPR2^{+/-}$ animals at base-line there was a significant downregulation of BMPR2 associated Smads 1, 5 and 8 at 3 weeks. This was associated with attenuated vessel remodelling at 3 weeks compared to controls. Neither of these models demonstrated the spontaneous development of pulmonary arterial hypertension under normoxia.

The results from both Frank *et al* (2005) and Beppu *et al* (2004) appear to be counter-intuitive. Following the discovery that BMPRII mutations appear to be the predominant genetic abnormality in patients with FPAH (Cogan *et al*, 2006), that BMPR2 expression is reduced in patients with PPH (Atkinson *et al*, 2002) and that BMPRII mutations are associated with disordered BMP/BMPR2/Smad signalling – in particular with an absolute reduction in Smad 1 expression (Rudarakanchana *et al*,

2002; Yang *et al*, 2005), it seems strange that animals which demonstrate disordered BMP/BMPR2/Smad signalling should show less right ventricular remodelling and less muscularisation of the pulmonary vasculature to chronic hypoxic exposure. In comparison to Frank *et al* (2005) and Beppu *et al* (2004), West *et al* (2004) demonstrated the development of spontaneous pulmonary hypertension without the additional hypoxic stimulus, in a mouse model expressing a dominant negative BMPR2. They demonstrated the presence of mutated BMPR2 in both pulmonary and systemic vasculature using immunohistochemistry but only morphological changes associated with hypoxic PAH in the pulmonary and not systemic circulations. This underlines the functional differences between the two circulations despite their identical genotype. West *et al* (2004) demonstrated that there was a significant increase in right ventricular weight and in right ventricular systolic pressure in comparison to wild-type controls under normoxic conditions and that these differences were further enhanced by mild hypoxia (PO_2 ~ 75 – 80 mmHg).

In contrast, work from other laboratories has not shown reduced Smad 1, 5 and 8 expression on exposure to hypoxia. Long *et al* (2006) demonstrated that Smad 1 and 5 expression was increased in VSMC from both wild type mice and mice heterozygote for BMPRII following hypoxic exposure. They also demonstrated that Smad 1 and 5 expression was reduced as a result of hypoxia plus infused 5HT that was associated with an increase in VSMC proliferation in $BMPR2^{+/-}$ mice but not wild-type controls. The increased proliferation could be inhibited by the addition of ketanserin (a $5HT_{2A}$ receptor antagonist) but not by $5HT_{2B}$ or 5HTT inhibitors. This is in keeping with previous work demonstrating a link between PAH and 5HT (Welsh *et al*, 2004). Takahashi *et al* (2006) demonstrated no change in Smad 1, 5 and 8 expression in VSMC in a chronically hypoxic rat model without disordered

BMP/BMPR2/Smad signalling following 21 days of hypoxic exposure when compared to normoxic controls. These investigators found a transient increase in p38 MAPK expression at day 0.5 – 3 that then fell to below control levels at day 7 – 21. This is in contrast to work from the SPVU laboratory that has demonstrated constitutive activation in p38 MAPK activation in pulmonary arterial fibroblasts following chronic hypoxic exposure in a rat model (Welsh *et al*, 2001).

Meaningful comparison between disparate models is difficult. There are significant variations in the results obtained from mice genetically engineered to express dysfunctional BMP/BMPR2/Smad signalling. The dominant negative BMPR2 model used by West *et al* (2004) appeared to develop pulmonary hypertension spontaneously without any additional stimulus. Mice heterozygote for BMPRII, appeared either to display reduced or enhanced Smad 1, 5 and 8 phosphorylation to hypoxic exposure (Beppu *et al*, 2004; Long *et al*, 2006) and mice heterozygote for BMP4 appeared to show reduced Smad 1, 5 and 8 signalling on exposure to hypoxia (Frank *et al*, 2005).

Yang *et al* (2005) demonstrated that pulmonary artery SMC taken from patients with PPH are deficient in BMP/BMPR2 Smad signalling – specifically Smad 1 – in addition to this they found that VSMC cultured from proximal and distal pulmonary arteries demonstrated divergent behaviour to BMP4 stimulation. This work was performed on the background of altered TGFβ and BMP signalling in SMC cultured from proximal pulmonary arteries taken from patients with IPH. Morrell *et al* (2001) found that proximal PASMC taken from IPH patients proliferated to TGFβ stimulation – not a known ligand for BMPR2 – in comparison with similar cells cultured from patients with secondary PAH. These investigators also found that

BMP4 was not able to inhibit proximal VSMC proliferation in IPH patients compared to identical cells cultured from patients with secondary PAH suggesting a wider dysfunction in TGFβ superfamily signalling. This has been confirmed by Yu *et al* (2005) who found that disrupted BMPR signalling in VSMC cultured from mice haploinsufficient for BMPRII, resulted in a net gain of function in TGFβ superfamily receptors Akt2/3 that do not normally act as receptors for BMP-mediated signalling. These investigators found that consistent with a haploinsufficient genotype, cells expressed approximately half the number of BMPR2 receptors seen in wild-type cells. In addition they were further able to modify the genotype of these cells so that BMPR2 was not expressed at all. Despite the absence of BMPR2 they were able to demonstrate Smad 1, 5 and 8 phosphorylation but at 30 –50% of wild-type level, implying that there exist alternative signalling mechanisms for BMP signal transduction. In this model Smad activation occurred preferentially via BMP 6 and 7 via ActR2a and was associated with increased p38 MAPK phosphorylation. Signalling was not found to be a result of residual BMPR or mutant BMPR, neither was it associated with alterations in inhibitory Smad 6 and 7 expression. Wild-type cells did not transduce BMP signal in this manner.

This concurs with work by Nohe *et al* (2002; 2004) that examines the interaction of BMPR 1 and 2 at the cell membrane surface. As previously discussed receptor oligomerisation at the cell membrane appears to dictate the intracellular signalling cascade activated by BMP ligand. Ligand stimulation of preformed heterodimers of BMPR1 and 2 transduce signal via Smad signalling cascade whereas ligand that binds an individual receptor prior to forming a hetero-oligomer activates the p38 MAPK cascade (Nohe *et al*, 2002; Nohe *et al*, 2004). The majority of BMPR exist as

heteroligomers allowing preferential Smad activation whereas TGFβ receptors exist as homo-oligomers (Gilboa *et al*, 2000). Hetero-oligomerization of BMPR appears to be dependent on a functional C chain – therefore BMPR2 mutants without a functional kinase domain are unable to form hetero-oligmers with BMPR1a or 1b, again allowing preferential p38 MAPK signalling. In addition the presence of BMPR2 affects the dispersal of BMPR1 at the membrane surface with BMPR1 being widely dispersed without ligand stimulation of BMPR2 (Nohe *et al*, 2003). This is significant because many of the BMPRII mutations identified in FPAH involve a dysfunctional or truncated kinase domain (Nishihara *et al*, 2002; Rudarakanchana *et al*, 2002) and because much of the work examining the effects of dysfunctional BMPR2 signalling has identified upregulation of p38 MAPK in a variety of experimental models and cell types (Jeffery *et al*, 2005; Takahashi *et al*, 2005; Yang *et al*, 2005; Yu *et al*, 2005). In addition co-activation of both Smad and p38 MAPK signalling pathways by BMP ligand has been observed in limb bud development (Zuzarte-Luis *et al*, 2004).

In the hypoxic human fibroblast model we have also identified a link between Smad 1, 5 and 8 signalling and p38 MAPK in pulmonary arterial fibroblasts. We have demonstrated that p38 MAPK appears to influence the expression of Smad 1, 5 and 8 in these cells under normoxic conditions in that preincubation with SB203580, a specific p38 MAPK inhibitor, was able to partially abrogate Smad 1, 5 and 8 phosphorylation. So-called classic MAPKs ERK 1/2 are known to inhibit Smad 4/Smads 2/3 nuclear translocation by binding to the Smad 4 linker region between MH1 and MH2 domains (Kretzschmar *et al*, 1999). Brown *et al* (1999) have also demonstrated that ERK1/2 can also prevent Smad4/Smad 1 nuclear translocation in an endothelial cell model. There is also a body of work that demonstrates significant

cross-talk between p38 MAPK and Smad signalling. Noth *et al* (2003) show that p38 MAPK activation is required for Smad 1 phosphorylation and subsequent nuclear translocation in a human osteoclast model. Inhibition of p38 MAPK blocked BMP2 mediated signal transduction. TGFβ signalling is known to cause simultaneous activation of Smad signalling and early p38 MAPK phosphorylation via a Smad-independent mechanism through TAK-1 (Horowitz *et al*, 2004). In addition Takakawa *et al* (2002) have also demonstrated late-phase activation of p38 MAPK by TGFβ via GADD45β protein in a Smad-dependent fashion in a human pancreatic cell model. There is also evidence that p38 MAPK is involved in the regulation of the recently discovered Smad phosphatases (Knockaert *et al*, 2006; Sapkota *et al*, 2006), increasing Smad 4 half-life and increasing Smad 4/R Smad mediated gene product transcription (Ohshima and Shimotohno, 2003). It therefore appears that far from being a comparatively simple choice between preferential Smad and p38 MAPK signal transduction based on receptor conformation at the cell surface membrane (Gilboa *et al*, 2000; Nohe *et al*, 2002; Nohe *et al*, 2003), p38 MAPK regulation of Smad-mediated intracellular signalling appears to occur at many different levels. In addition the influence of Smad signalling cascades on p38 MAPK and vice versa cannot be easily predicted and are strongly dependent on cell type and the sequence in which the two cascades coincide.

In our model it appears that Smad expression is increased in HMAF in hypoxic conditions – acting as a break on cellular proliferation - while in HPAF Smad signalling appears to be reduced allowing a more permissively proliferative phenotype. The reduced expression of BMP related Smads 1, 5 and 8 in HPAF cells

is synchronous with upregulation of p38 MAPK. This would agree with accepted roles for BMP mediated signalling in mesenchymal tissue (**figure 5.3**).

Figure 5.3: Summary of BMP/BMPR/Smad signalling in human pulmonary and systemic arterial fibroblasts in hypoxic growth conditions Ligand binds BMPR2 at the cell surface membrane enabling the formation of a BMPR1/2 complex. This results in the activation of downstream Smad 1, 5 and 8 phosphorylation with subsequent signal transduction to the nucleus and upregulation of relevant gene products by transcription with the aid of co-activators and co-repressors and later translation to new cellular protein. In a human systemic arterial model, Smad 1, 5 and 8 phosphorylation is associated with an anti-proliferative phenotype, whereas in the human pulmonary arterial fibroblast it is associated with a pro-proliferative phenotype.

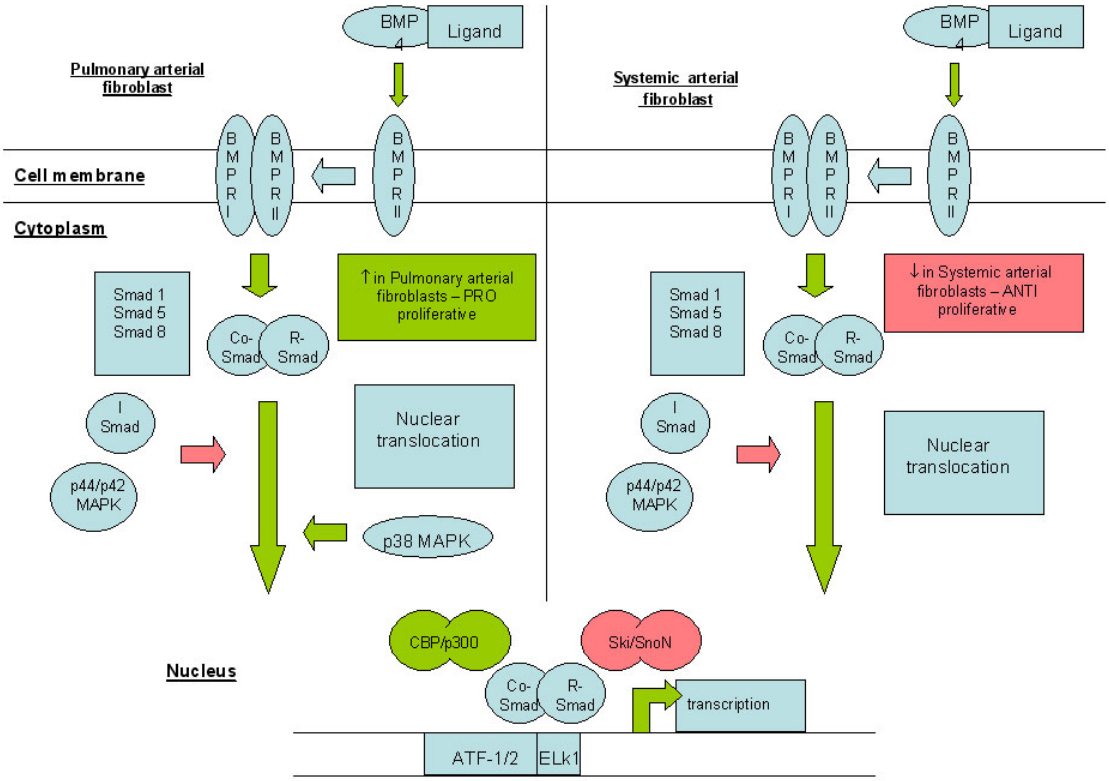


Figure 5.3: Summary of BMP/BMPR/Smad signalling in human pulmonary and systemic arterial fibroblasts in hypoxic growth conditions

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Chapter 6:

Discussion – Thesis:

6.1. Human pulmonary arterial fibroblasts proliferate to acute hypoxic exposure whereas systemic arterial fibroblasts do not.

The hypothesis underlying this project is that significant differences exist between the pulmonary and systemic arterial circulations in terms of their response to hypoxia. Macroscopically Von Euler and Liljestrand (1946) demonstrated that the pulmonary circulation vasoconstricts, while Wagner and Mitzner (1988) showed that the systemic circulation vasodilatates, to acute hypoxic exposure, in cat and dog models respectively. Histologically Meyrick and Reid (1979; 1980) demonstrated that chronic hypoxic exposure resulted in significant structural changes in the pulmonary arterial vessel wall in a rat model. These changes were not confined to the intima or media but involved all three layers of the vessel wall – with the first and most significant changes being witnessed in the adventitial layer. The changes in this compartment were not completely reversible on re-exposure to a normoxic environment unlike those in the media and intima.

Previous studies from the SPVU laboratory have established fundamental differences in arterial adventitial fibroblast proliferative behaviour to hypoxic exposure in a number of animal models under conditions of both acute and chronic hypoxia. Welsh *et al* (1998) established that pulmonary arterial fibroblasts from an adult bovine model proliferated to acute hypoxic exposure whereas mesenteric arterial fibroblasts did not. Similar findings were noted in an adult rat model to acute hypoxic exposure (Welsh *et*

al, 2006). We wished to establish whether these findings could be reproduced in an adult human model.

Our work has demonstrated that in an adult human model, pulmonary arterial fibroblasts preferentially proliferate to acute hypoxic exposure whereas systemic arterial fibroblasts do not – as assessed by both cell counts and [^{3}H]Thymidine uptake; this work has been confirmed by other investigators in an adult human model. Krick *et al* (2005) found that pulmonary arterial fibroblasts cultured from small vessels (< 1mm diameter) proliferated vigorously to 1% oxygen when compared to cells taken from renal arteries of similar generation. They found that cells taken from systemic arteries showed an increased rate of apoptosis when cultured under identical conditions. Das *et al* (2001) found similar proliferative responses in pulmonary arterial fibroblasts cultured from a neonatal calf model of acute hypoxic exposure.

We have also established that hypoxia-mediated proliferation in human pulmonary arterial fibroblasts occurred without the need for additional serum stimulation. This finding has also been demonstrated by our group in an adult bovine model (Welsh *et al*, 1998). This finding differs from those of other investigators who have been able to demonstrate increased BrdU uptake (a marker for DNA synthesis and therefore cell replication) in pulmonary arterial fibroblasts to acute hypoxic exposure, but have not been able to demonstrate increased cell counts (Short *et al*, 2005). This work was performed in a juvenile calf model. The differences are not easily explainable. Work from this same laboratory has shown that pulmonary arterial fibroblasts from fetal and neonatal calves proliferate more vigorously to hypoxic exposure than similar cells from adult cows (Das *et al*, 1997). They have also demonstrated that pulmonary

arterial fibroblasts taken from neonatal calves exposed to chronic hypoxia from birth show an enhanced proliferative response when cultured in normoxic conditions but that this proliferative response can be augmented by further hypoxic exposure (Das *et al*, 2000). Using a chronic hypoxic adult rat model, Welsh *et al* (2001) were only able to demonstrate increased proliferation under normoxic conditions; they were not able to show any increased proliferative potential to further hypoxic exposure. Investigators from Denver demonstrated that patterns of PKC isosyme expression altered with increasing age and with chronic hypoxic exposure in pulmonary arterial smooth muscle cells cultured from fetal and neonatal calves (Xu *et al*, 1997). The same investigators also noted varying PKC expression patterns in adventitial fibroblasts in neonatal and fetal calves (Das *et al*, 1997). They speculated that developmental changes in hypoxia-responsive PKC isosymes might explain the varying behaviour of the maturing pulmonary circulation.

Overall, it appears that there is a degree of plasticity in the fetal and neonatal mammalian pulmonary circulation that is not seen in the adult, which makes our finding that adult pulmonary arterial fibroblasts proliferate to hypoxic exposure alone more puzzling. A possible explanation is that cells cultured in our laboratory were taken from adult cattle and humans living at sea level (Glasgow, UK). It is known that cattle develop severe pulmonary arterial hypertension on hypoxic exposure (Tucker and Rhodes, 2001). The Denver group consistently use Holstein calves sourced from Fort Collins, Colorado ~ 1,540 metres above sea level (Das *et al*, 2002; Frid *et al*, 2006). It is possible that this may have affected the responsiveness of pulmonary arterial fibroblast cultured from these animals to hypoxia alone.

In summary we have demonstrated that adult human pulmonary arterial fibroblasts proliferate to acute hypoxic exposure whereas systemic arterial fibroblasts do not. We have now demonstrated this finding in adult bovine, rat and now human models of acute hypoxia. These findings have been confirmed by other investigators: in not only an adult human but also, in neonatal and juvenile bovine models. In addition we have also demonstrated that hypoxia-mediated proliferation in both adult bovine and human models can occur without serum stimulation (see **figure 6.1**).

6.2. Hypoxia-mediated proliferation in human pulmonary arterial fibroblasts is dependent on p38 MAPK activity and associated with HIF1α activity.

Work from the SPVU laboratory has demonstrated that pulmonary arterial fibroblast proliferation to hypoxia is associated with increased mitogen activated protein kinase (MAPK) activity. Scott *et al* (1998) demonstrated that in an adult bovine model pulmonary arterial fibroblast proliferation was linked with increased JNK and p38 MAPK activity but not p44/p42 – ERK1/2 MAPK activity. In a chronically hypoxic adult rat model, Welsh *et al* (2001) demonstrated that chronic hypoxic exposure resulted in constitutive activation of p38 MAPK in pulmonary arterial fibroblasts under normoxic growth conditions – suggestive that these cells had in some way been phenotypically altered by prolonged hypoxic exposure. Interestingly Welsh *et al* (2001) showed that chronic hypoxic exposure resulted in constitutive activation of p44/p42 – ERK1/2 MAPK in both pulmonary and systemic arterial fibroblasts in a rat model. They speculated that p44/p42 –ERK1/2 might be linked with cell hypertrophy

Figure 6.1 Summary of MAPK and BMPR signalling in human pulmonary and systemic arterial fibroblast cells under hypoxic growth conditions. The G protein Coupled Receptor (GPCR), the Tyrosine Kinase Receptor (TKR) and BMPR/Smad signalling cascades are shown alongside each other. Relevant ligand binds the membrane bound receptor resulting in conformational change of the inner membranous portion of the receptor. Second messengers are either activated via G protein in the case of GPCR or via autophosphorylation in the case of TKR or phosphorylation of BMPR1 by BMPR2. Signal transduction is effected by downstream signalling cascades via Ras, MEK and MAPKs or via Smads resulting in the upregulation, transcription and translation of relevant gene products and may involve the assistance of co-activators or co-repressors in the case of Smad mediated signalling.

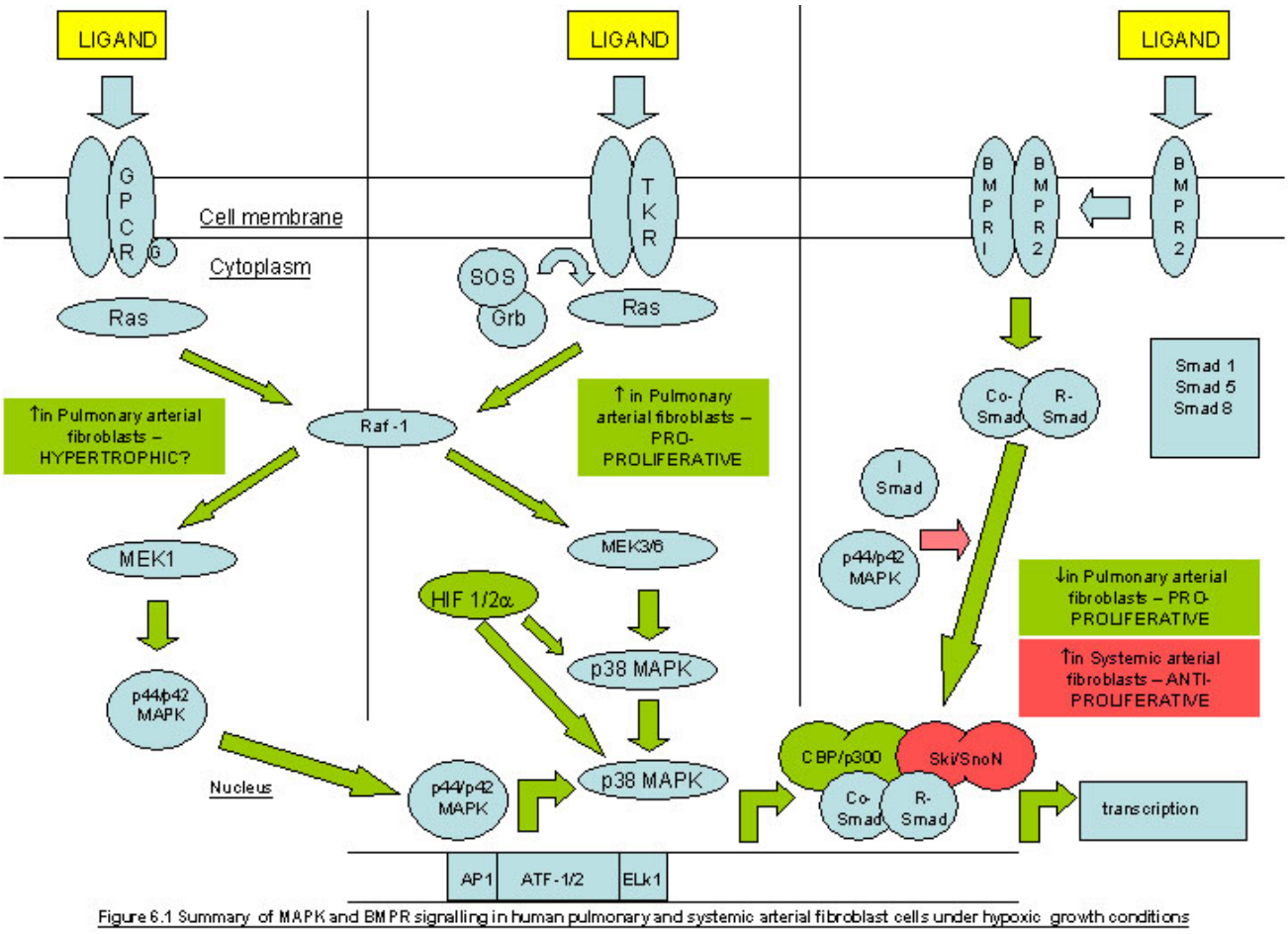

Figure 6.1 Summary of MAPK and BMPR signalling in human pulmonary and systemic arterial fibroblast cells under hypoxic growth conditions

Figure 6.1: Summary of MAPK and BMPR signalling in human pulmonary and systemic arterial fibroblast cells under hypoxic growth conditions

rather than proliferative responses as the p44/p42 – ERK 1/2 MAPK inhibitor U0126 had no effect on pulmonary arterial fibroblast proliferation in this model.

Recent debate has focused on the origin of cells within the pulmonary vascular wall exposed to chronic hypoxia – there is a body of evidence to suggest that circulating mononuclear cells (MNC) originating within the bone marrow may make a significant contribution to pulmonary vascular remodelling. Davie *et al* (2004) found increased *vasa vasorum* formation in the resistance vessels of neonatal calves exposed to chronic hypoxia. They suggested that this allowed access by mitogens and MNC to the adventitial layer permitting an environment enabling further remodelling. Evidence to consolidate this view was provided by Hayashida *et al* (2005) in a lethally irradiated mouse model. These investigators demonstrated that radiolabelled cells accumulated within the pulmonary circulation of mice whose bone marrow had been replaced with labelled cells. This response was only noted in the pulmonary circulation and not in any other organ in the body. Meanwhile Frid *et al* (2005, 2006) demonstrated that circulating MNC made up approximately one third of proliferating cells within the adventitia of chronically hypoxic weanling rats and neonatal calves. Whether proliferating pulmonary arterial fibroblasts to chronic hypoxia originate within the bone marrow or in the local expansion of a pro-proliferative cell type remains uncertain. Work from this laboratory and from Denver has demonstrated that p38 MAPK is consistently involved with proliferative responses in both adult and neonatal mammalian models (Scott *et al*, 1998; Welsh *et al*, 2001; Das *et al*, 2001; Welsh *et al*, 2006).

In an adult human model we have demonstrated that acute hypoxic exposure results in p38 and p44/42 – ERK 1/2 MAPK activation in pulmonary arterial fibroblasts. P44/42 MAPK is also activated in systemic arterial fibroblasts grown under hypoxic conditions but this does not appear to have any modifying effect on systemic arterial fibroblast proliferation. Hypoxia-mediated proliferation in human pulmonary arterial fibroblasts is dependent on p38 MAPK activation as pre-incubation of these cells with SB203580 – a specific p38 MAPK α and β isoform inhibitor – was able to abrogate the proliferative response. Pre-incubation with U0126 – a p44/p42 – ERK 1/2 MAPK activity inhibitor – had no effect on human pulmonary arterial fibroblast behaviour to acute hypoxic exposure. We did not find any evidence of JNK MAPK activity in either pulmonary or systemic arterial fibroblasts under either hypoxic or normoxic growth conditions.

In a human model, p38 MAPK expression in hypoxic pulmonary arterial fibroblasts appeared to be bi-phasic in nature with peaks of activity at t = 6 hours and 16 hours. This agrees with previous work from the SPVU laboratory using an adult bovine model (Scott *et al*, 1998) and also from other investigators using a neonatal bovine model (Das *et al*, 2001). By using re-oxygenation techniques, we have established that in an adult human model the second peak of p38 MAPK activity is responsible for pulmonary arterial fibroblast proliferation to acute hypoxic exposure, as re-oxygenation after 6 hours failed to elicit the same proliferative response. This again concurs with previous work from the SPVU laboratory using an adult bovine model (Scott *et al*, 1998). The significance of the first peak of p38 MAPK activity is not certain but may be associated with HIF1α activity – the second peak appears to coincide with DNA replication in a fibroblast model, although we have not been able

to establish a definite link between p38 MAPK activity and cell cycle progression in a adult rat model of acute hypoxia (Welsh *et al*, 2006). Our findings in the adult human model show some variance with those from other adult animal models used in the SPVU laboratory. These results are summarised in **table 1.2**.

Scott *et al* (1998) demonstrated that in an adult bovine model of acute hypoxic exposure both JNK and p38 MAPK were involved in hypoxia-mediated pulmonary arterial fibroblast proliferation. In all other models so far investigated by the SPVU laboratory only p38 MAPK activity appears to be consistently associated with pulmonary arterial fibroblast proliferation. This is not in agreement with work from other investigators who found that p44/p42 – ERK 1/2, p38 and JNK MAPK activity were all required for hypoxia-mediated pulmonary arterial fibroblast proliferation in a neonatal bovine model (Das *et al*, 2001). Pre-incubation with SB203580 was only able to partially abrogate the hypoxia-mediated proliferation seen in this model whereas pre-incubation with pertussis toxin – a $G_{\alpha i/0}$ protein inhibitor – prevented pulmonary arterial fibroblast proliferation, implying central roles for JNK and p44/p42 – ERK1/2 MAPK in a neonatal bovine model which signal via this G protein. As previously discussed, these investigators noted varying patterns of PKC isosyme activity in pulmonary arterial smooth muscle cells and adventitial fibroblasts at different developmental stages (Das *et al*, 1997; Xu *et al*, 1997). It seems likely that the differences in MAPK expression patterns to hypoxia are as a result not only of inter-species differences, but also of developmental stage. The SPVU laboratory has used adult mammalian models exclusively and our work has demonstrated a consistent role for p38 MAPK activity in hypoxic pulmonary arterial fibroblast proliferation irrespective of species and duration of hypoxic exposure. This suggests

Table 1.2 Summary of Scottish Pulmonary Vascular Unit findings: cross species differences in responses to hypoxia

Species	Fibroblast	Proliferation to Hypoxia	P38 phosphorylation to hypoxia	ERK phosphorylation to hypoxia	JNK phosphorylation to hypoxia
Human	Pulmonary	YES	YES	YES	NO
	Systemic	NO	NO	NO	NO
Bovine	Pulmonary	YES	YES	NO	YES
	Systemic	NO	NO	NO	NO
Rat	Pulmonary	YES	YES	YES	NO
	Systemic	NO	NO	NO	NO

Table 1.2: Summary of Pulmonary Vascular Unit findings: cross species differences in responses to hypoxia. All three adult species so far investigated demonstrate pulmonary arterial fibroblast proliferation to hypoxic exposure whereas systemic arterial fibroblasts do not. While there is a variable expression pattern for ERK and JNK MAPKs in adult human, bovine and rat models there is consistent activation of p38 MAPK associated with hypoxic pulmonary arterial fibroblast proliferation in all three adult models.

that certainly in the adult mammalian model, p38 MAPK activation is fundamental to pulmonary arterial fibroblast proliferation to hypoxia.

There are four known isoforms of p38 MAPK: α, β, γ and δ. Conrad *et al* (1999) demonstrated that hypoxic exposure resulted in activation of p38 MAPK α and γ isoforms in a human phaeochromocytoma (PC12) cell model. Work from the SPVU laboratory has previously established a role for the ubiquitously expressed p38 MAPK α in pulmonary arterial fibroblast proliferation to acute hypoxic exposure in an adult rat model (Welsh *et al*, 2006). Here we have confirmed a role for p38 MAPK α activation in an adult human model of acute hypoxic exposure using immunophoresis techniques. p38 MAPK α appears to be crucial for vascular development: mice null for p38 MAPK α die *in utero* as a result of significant vascular anomalies (Emerling *et al*, 2005); a similar phenotype is seen in mice null for HIF1α (Yu *et al*, 1999). p38 MAPK α also appears to be required for HIF1α stabilisation under hypoxic conditions – as fibroblasts cultured from mice null for p38 MAPK α are unable to stabilise HIF1α to hypoxic exposure but can be rescued by adenoviral transfection with p38 MAPK α. In addition fibroblasts cultured with anisomycin (a known mitochondrial electron transport chain – ETC – complex III inhibitor) also prevented HIF1α stabilisation (Emerling *et al*, 2005).

Here we have established a link between p38 MAPK α activity and HIF1 α in an adult human model of acute hypoxic exposure and have demonstrated that HIF1α activity was associated with p38 MAPK α activity. We have also demonstrated that HIF1α activity in these cells could be completely abrogated by 6-hour pre-incubation

with SB203580 – a specific p38 MAPK α and β isoform inhibitor. This concurs with work by other investigators who have established that SB203580 was also able to inhibit HIF1α activity in a Hep3B cell line (Sodhi *et al*, 2000). The exact nature of the interaction between p38 MAPK α and HIF1α is not clear but may be linked to mitochondrial function in that Emerling *et al* (2005) were able to reproduce the effects of fibroblasts null for p38 MAPK α by the use of anisomycin – an electron transport chain (ETC) complex III inhibitor - in a murine model. The association may also be an functional one: hypoxia-mediated proliferation in human pulmonary arterial fibroblasts is dependent on the second peak in p38 MAPK activity and it has been demonstrated that this second peak of p38 MAPK activity is synchronous with DNA synthesis in a fibroblast model (Scott *et al*, 1998). The maximal inhibition of HIF1α by SB203580 at 6 hours suggests that both p38 MAPK and HIFα are required for hypoxia-mediated proliferative responses in human pulmonary arterial fibroblasts.

Interestingly we demonstrated HIF1α activity in human pulmonary arterial fibroblasts alone and we were not able to demonstrate any HIF1α activity at all in human systemic arterial fibroblasts grown under hypoxic conditions. The reason for this is not entirely clear given that HIF1α is rapidly stabilised under hypoxic conditions (Yu *et al*, 1998; Stroka *et al*, 2001). Stroka *et al* (2001) demonstrated that HIF1α expression patterns showed significant variation in different organs and so it is reasonable to postulate that pulmonary arterial fibroblasts, normally exposed to low partial oxygen pressures of mixed venous blood, may only activate HIF1α at significantly lower P_aO_2 than might occur in other organs. A possible explanation may lie in cellular diversity. Mickelakis *et al* (2002) have demonstrated significant variability in mitochondrial respiration rate in cells taken from pulmonary and renal

arteries – with mitochondria from the pulmonary arteries exhibiting a lower respiratory rate both at base-line and on hypoxic exposure. They also noted that both hypoxia and proximal inhibitors of the mitochondrial ETC resulted in reduced production of activated oxygen species (AOS) and reduced K^+ channel activity in pulmonary, but not in renal arteries; thus providing further evidence that significant signalling and metabolic differences exist between the two circulations. In our work the preparation of cell lysates was not performed in a hypoxic environment, perhaps allowing for the oxygen-mediated degradation of active HIF1α by von Hippel Lindau protein (pVHL) and subsequent ubiquitinylation (see chapter 4). There is still no adequate explanation for the absence of HIF1α in human systemic arterial fibroblasts grown in conditions of acute hypoxia but perhaps differences in baseline mitochondrial respiration may provide a partial explanation.

In summary we have found that acute hypoxic exposure results in human pulmonary arterial fibroblast proliferation, which is associated with p44/p42 – ERK 1/2 MAPK activity, but which is *dependent* on p38 MAPK α activity. p38 MAPK α activity appears to occur in a bi-phasic pattern with peaks of activity at t = 6 and 16 hours, the second of which is responsible for the hypoxia-mediated proliferation seen in these cells. The second peak in p38 MAPK α activity is synchronous with HIF1α activity. We have also demonstrated that HIF1α activity can be abrogated by pre-incubation of human pulmonary arterial fibroblasts with SB203580 suggesting a strong mechanistic link between p38 MAPK α activation and HIF1α in a human model (see **figure 6.1**).

6.3. Lack of proliferation of human systemic arterial fibroblasts to acute hypoxia is associated with increased Smad 1/5 and 8 activity.

Familial pulmonary arterial hypertension is associated with mutations within the Bone Morphogenetic Protein Receptor II gene (BMPRII) in approximately 70% of cases (Cogan *et al*, 2006). BMPR2 is a member of the Transforming Growth Factor β (TGFβ) receptor superfamily that appears to be ubiquitously expressed and whose effects are protean. Atkinson *et al* (2002) have demonstrated that BMPR2 expression occurs predominantly in the intima and media of the human pulmonary artery – although some expression also occurs within the adventitia.

An extensive body of work has established that BMPRII mutations found in FPAH are largely associated with a loss of function that results not only in reduced efficiency of intracellular signalling cascades associated with BMPR2 signal transduction (Rudarakanchana *et al*, 2002), but also extends to affect other components of TGFβ superfamily signalling (Morrell *et al*, 2001). The effect of fully functional BMPR2 signalling results in a growth-inhibitory phenotype, whereas TGFβ-mediated signalling appears to be pro-proliferative in mesenchymal cells (Massague, 1990). While the effects of BMPR2 signalling appear to vary with cell type, they also appear to vary within cell type. Morrell *et al* (2001) demonstrated altered growth responses to BMP4 in pulmonary vascular smooth muscle cells in patients with FPAH compared to secondary causes of pulmonary hypertension with VSMC from resistance vessels in FPAH being resistant to the growth-inhibitory effects of BMP4. This would therefore result in a permissively proliferative VSMC

phenotype perhaps explaining the medial expansion seen in the pulmonary resistance vessels of those with mutation positive FPAH.

Given the association of BMPRII mutations with FPAH we wished to establish the contribution of BMPR2 mediated signalling in human pulmonary and systemic arterial fibroblast proliferation to acute hypoxia. The SPVU laboratory has consistently demonstrated pulmonary arterial fibroblast proliferation to hypoxic exposure but has not managed to elicit any positive response from systemic arterial fibroblasts under similar growth conditions. Here we have demonstrated that acute hypoxic exposure results in upregulation of phospho Smad1, 5 and 8 activity in human systemic arterial fibroblasts, which may suggest the active upregulation of a growth-inhibitory pathway in these cells. We found that phospho Smad 1, 5 and 8 activity was down regulated in hypoxic pulmonary arterial fibroblasts when compared to hypoxic systemic arterial fibroblasts grown under identical conditions; this would again suggest that BMPR2 – Smad signalling in arterial fibroblasts exerts a growth inhibitory influence. To our knowledge this is the first demonstration of an active anti-proliferative response in human systemic arterial fibroblasts to hypoxic exposure. These findings are in keeping with work from Jeffrey *et al* (2005) who demonstrated that BMP4 stimulation of whole fetal lung fibroblasts resulted in growth inhibition in these cells associated with upregulation of phospho Smad 1. However in this model whole lung fibroblast growth inhibition was associated with increased p38 MAPK activity together with JNK and p44/p42 – ERK 1/2 MAPK expression. In contrast we have found that p44/p42 – ERK 1/2 MAPK expression is associated with proliferating human pulmonary arterial fibroblasts to hypoxia, but that the proliferative response is dependent on p38 MAPK activation. The differences may be explained by the fact

that Jeffrey *et al* (2005) were using whole lung fibroblasts taken from a human fetal model whereas we used adult human pulmonary arterial fibroblasts. We were not able to identify a role for phospho Smad 2 (predominantly a TGFβ-responsive Smad), nor for Smads 6 and 7 (inhibitory Smads for BMPR2 and TGFβ receptor signalling respectively) in either cell type under either growth condition.

In a human model we have been able to demonstrate that pre-incubation with SB203580 – a specific p38 MAPKα and β - influences the relative expression of activated Smad 1, 5 and 8 in hypoxic human pulmonary arterial fibroblasts. There is a significant body of evidence which documents cross talk between p38 MAPK signalling and BMPR2-Smad signalling. Nohe *et al* (2002; 2004) have documented that BMP receptor conformation at the cell membrane dictates the down-stream signalling cascade activated by BMP ligand. In addition there appear to be several levels of control within the signal transduction cascade where MAPK and Smads influence each other. Noth *et al* (2003) have demonstrated that p38 MAPK is required for Smad 1 phosphorylation and nuclear translocation in a human osteoclast model, a discovery which would be consistent with our finding that p38 MAPK inhibition appears to down-regulate phospho Smad 1, 5 and 8 in human pulmonary arterial fibroblasts in a hypoxic model. In addition there is also evidence of early activation of p38 MAPK by TGFβ via a Smad-independent cascade via TAK-1 (Horowitz *et al*, 2004). TGFβ is also able to activate p38 MAPK in a Smad-dependent manner via its action on GADD45β (Takekawa *et al*, 2002). In addition recent work from Upton *et al* (2008) also argues for an as-yet-to-be-identified membrane-bound protein – possibly similar to Noggin or BAMBI (see Chapter 1) -

which exerts a further level of control on the intracellular signalling cascade activated by BMP4.

In summary we have shown that BMPR2 associated Smad 1, 5 and 8 activation is increased in hypoxic human systemic arterial fibroblasts, suggestive of the activation of an anti-proliferative pathway in these cells that is not associated with p38 MAPK activity. In addition we have shown that in hypoxic pulmonary arterial fibroblasts phospho Smad 1, 5 and 8 expression is reduced – suggestive of the active down-regulation of an anti-proliferative pathway - and can be further abrogated by pre-incubation with SB203580. This suggests that in systemic arterial fibroblasts Smad 1, 5 and 8 activation occurs independent of p38 MAPK activation while in pulmonary arterial fibroblasts p38 MAPK activity augments Smad 1, 5 and 8 activation (see **figure 6.1**).

6.4. Limitations of study.

In this work we have examined the behaviour of adult human pulmonary arterial fibroblasts to hypoxic exposure, using hypoxia-related pulmonary arterial hypertension as a proxy for pulmonary arterial hypertension proper. Despite significant remodelling of all components of the pulmonary vascular wall to prolonged hypoxic exposure (Meyrick and Reid, 1979; 1980) pulmonary arterial hypertension developed as a result of hypoxia *does not* appear to demonstrate the typical plexigenic lesions seen in both FPAH and PH secondary to other causes. In this respect the monocrotaline experimental model of pulmonary arterial hypertension is more accurately able to reproduce the histological changes expected with

pulmonary arterial hypertension. The intravenous administration of monocrotaline in animal models has been shown to rapidly induce pulmonary arterial hypertension that is rapidly fatal and which is histologically consistent with PH (Yip *et al*, 2008). However because monocrotaline is highly toxic it can only be used *in vivo* in animal models. Hypoxia is a useful model precisely because it can be applied to both *in vitro* and *in vivo* work, both in animals and in man, but it must be accepted that it does not accurately represent the pathology of FPAH.

In this work we have used atmospheric partial pressure of oxygen (21 kPa ~ 140mmHg) as a control in experiments to assess cellular proliferation and to quantify MAPK and Smad activation patterns. It should be borne in mind that atmospheric PO_2 is not truly representative of arterial P_aO_2 which in the pulmonary veins of a healthy adult would be in the region of 13.3 kPa ~ 100mmHg (allowing for the effects of humidification in the upper airways (saturated water vapour pressure of 6.2 kPa), the continuous exchange of CO_2 for O_2 at the alveolar membrane and the presence of a small physiological shunt). The use of atmospheric P_AO_2 does not take into consideration that PO_2 varies throughout the vasculature with normal venous PO_2 being in the range of 6 kPa ~ 40 mmHg and the average P_aO_2 within the renal cortex being within the region of 26 mmHg ~ 4 kPa (O'Connor *et al*, 2007). Work from Moudgil *et al* (2005) demonstrates variable cellular mitochondrial respiration with pulmonary arteries showing significantly lower mitochondrial respiratory rates than mitochondria from renal arteries. This suggests that cells within the human body are attuned to oxygen gradients that bear little resemblance to clinically accepted values for P_aO_2. Stroka *et al* (2001) have shown that HIF1 α activation patterns vary significantly between different organs and within different mammalian species again

suggesting that accepted arterial PO_2 values do not represent a true picture of organ oxygenation. However, our use of 5% PO_2 ~ 35 mmHg as a hypoxic model would represent significant arterial hypoxaemia in a pulmonary arterial model. Grocott *et al* (2009) demonstrated that the P_aO_2 of climbers at 7100m ranged between 19.1 – 29.5 mmHg ~ 2.55 – 3.93 kPa – demonstrating that the use of 5% PO_2 ~ 35 mmHg for hypoxic cellular proliferation and enzyme activation assays is physiologically relevant. In addition it is a model that has been utilised by other laboratories (Krick *et al*, 2005; Eul *et al*, 2006).

There is brisk debate about the significance of pulmonary arterial hypertension associated with hypoxic lung disease. PH seen in conjunction with hypoxic lung diseases such as chronic obstructive airways disease (COPD) is demonstrated to be typically mild (mean pulmonary arterial pressures > 20mmHg), and slowly progressive in those with mild/moderate airways obstruction in association with mild resting hypoxaemia (Chaouat *et al*, 2001; Kessler *et al*, 2001). It is also poorly responsive to re-oxygenation (Naeije, 2005). However hypoxia-associated pulmonary hypertension secondary to altitude exposure can result in significant elevations in pulmonary arterial pressures that approach those seen in FPAH but which are more readily reversible with correction of altitude and oxygen therapy (Groves *et al*, 1987; Morrell *et al*, 1999; Aldashev *et al*, 2002; Dinh-Xuan *et al*, 2002; Naeije, 2005). Moreover it is difficult to assess contribution and extent of pulmonary hypertension in patients with severe COPD, as these individuals are often too unstable for invasive investigation. Vizza *et al* (1998) found that right ventricular failure (defined as an ejection fraction < 45% on transthoracic echo) in isolation, was relatively common in those individuals referred for lung transplantation as a result of COPD, cystic fibrosis,

interstitial lung disease or pulmonary arterial hypertension. Significant elevations of pulmonary arterial pressure were noted in 59% of those patients with COPD referred for lung transplantation. Left ventricular systolic dysfunction was noted in only 6.5% of all patients investigated. Scharf *et al* (2002) found a high prevalence of pulmonary arterial hypertension (found on right heart catheter) in patients with severe COPD (average FEV_1 ~ 27% predicted). Meanwhile Raeside *et al* (2002) demonstrated that patients with resting hypoxaemia secondary to COPD showed significant elevations of pulmonary arterial pressures overnight without the benefit of supplemental oxygen using ambulatory right heart data.

The relative contribution that hypoxic pulmonary arterial hypertension may make to the underlying lung function of patients with COPD is not quantifiable, but significantly patients referred for lung volume reduction surgery (LVRS) - typically displaying severe air flow limitation, initially demonstrate improved total lung capacity (TLC) values post-operatively consistent with significant reduction in dynamic hyperinflation. The failure of these patients to improve in terms of their functional status despite significant improvements in lung function suggests that while LVRS may improve pulmonary function, the surgery itself does little to affect the underlying vascular changes commensurate with the degree of underlying lung disease and resting hypoxaemia (Haniuda *et al*, 2003; Higenbottam, 2005). It can therefore be argued that despite its limitations the hypoxic model of pulmonary arterial hypertension is the most common and physiologically relevant model. It is applicable to other disease states besides PH, can be investigated *in vitro* and *in vivo*, and most importantly, can be investigated *in vivo* in a human model.

Pulmonary vascular tissue used for explant and primary cell culture techniques in this work was taken from patients undergoing lobectomy for the treatment of lung carcinoma. The systemic vessels were harvested from the left internal mammary arteries of patients under-going coronary artery by-pass grafting as previously noted the vessel calibre was consistent with previously utilised systemic controls used by the SPVU laboratory. In the case of the pulmonary vessels, the vascular tissue was sampled at a distance from the site of the primary tumour in those patients with lung carcinoma and for the most part consisted of inter-lobar arteries. The source of our tissue may have influenced the results of our work in several ways: in terms of the generation of pulmonary vessel investigated and the potential influence of cigarette smoking on fibroblast cell behaviour to hypoxic cell culture.

Pulmonary arterial hypertension is a disease condition that predominantly affects smaller resistance arteries, arteries that become increasingly muscularised with disease progression with additional adventitial and intima thickening. Our experimental work has been based on adventitial fibroblasts cultured primarily from conduit vessels. There is a significant body of work that documents the difference in behaviour of conduit and resistance vessels to hypoxic exposure: resistance vessels typically exhibit sustained vasoconstriction to hypoxia whereas conduit vessels show an initial constrictive response followed by vasodilatation (Archer *et al*, 1986; Franco-Obregon and Lopez-Barneo, 1996). In addition Meyrick and Reid (1979; 1980) demonstrated that conduit and resistance vessels displayed different histological responses to prolonged hypoxic exposure – with adventitial expansion predominating in conduit vessels and medial and intimal expansion in resistance vessels. It has been shown that the distribution of voltage-sensitive K^+ channels varies throughout the

vascular tree with a high concentration of oxygen-sensitive Kv $_{1.2, 1.5}$ and $_{2.1}$ channels in resistance compared to conduit vessels that may partially explain the differences in their relative behaviours to hypoxic exposure (Platoshyn *et al*, 2004; Weir and Olschewski, 2006). Yang *et al* (2005) have demonstrated that vascular smooth muscle cells taken from resistance vessels in IPAH patients are less responsive to the growth-inhibitory effects of BMP4 stimulation when compared to patients with secondary PAH or to controls. This suggests that vessel generation may exert a significant effect on the behaviour to varying stimuli. The use of pulmonary arterial fibroblasts cells cultured from human conduit vessels may therefore not provide an accurate picture of hypoxic pulmonary arterial fibroblasts cultured from resistance vessels. In defence of our work – other investigators have found that pulmonary arterial fibroblasts cultured from small vessels (diameter < 1mm) also proliferate to hypoxic exposure (Eul *et al*, 2006).

As a result of the nature of the surgeries, most of the vessels sampled were retrieved from smokers. There is documentary evidence to suggest that smoking in isolation may have a significant effect on the pulmonary vasculature. Santos *et al* (2002) found that patients with COPD and smokers without evidence of airflow limitation demonstrated significant pulmonary arterial intimal thickening in comparison to non-smokers. Cigarette smoke is known to be toxic to pulmonary vascular endothelial cells and airway epithelial cells, with cigarette smokers having lower levels of eNOS, higher levels of circulating endothelin-1, reduced vasoreactivity to hypoxic exposure and lower levels of circulating epithelial progenitor cells (EPC) (Yamakami *et al*, 1997; Santos *et al*, 2002; Kondo *et al*, 2004). It is not known whether the reduction in circulating EPC represents an absolute depletion of these cells in cigarette smokers or

increased margination of these cells in order to repair vascular damage. It has not been possible in this work to control for these potential influences on primary adventitial fibroblast culture.

6.5. Future work

This work has established that the pro-proliferative changes to acute hypoxia previously demonstrated by the SPVU laboratory in adult bovine and rat pulmonary arterial fibroblasts are also seen in an adult human model. In addition we have identified a consistent role for p38 MAPK across all three adult mammalian species so far investigated (see **table 1.2**).

With this in mind therefore, a natural extension of this work would be to establish whether p38 MAPK was involved in pulmonary arterial adventitial fibroblast proliferation in a patient population – those with FPAH both with and without, identified BMPRII mutations, and those with pulmonary arterial hypertension secondary to other disease processes. In addition, given our finding of a distinct temporal relationship between HIF1α and p38 MAPK activation in hypoxic human pulmonary arterial fibroblasts it would be of interest to examine whether the same relationship existed in a patient population and to see whether, as in the work from the Geissen laboratory, HIF2α is the predominantly important isoform for PH-related pulmonary arterial fibroblast proliferation.

The finding that mice heterozygous for HIF1α develop only minimal pulmonary arterial remodelling to hypoxic exposure and that mice heterozygote for HIF2α

appear to be completely protected from hypoxia-induced pulmonary arterial hypertension and right ventricular impairment (Yu *et al*, 1999; Brusselmans *et al*, 2003), suggests a central role for HIF isoforms in the adaptation of the pulmonary circulation to prolonged hypoxia. It appears that the HIF2α isoform is predominantly expressed in the lung, while HIF1α is more ubiquitously expressed (Eul *et al*, 2006). Closer examination of HIF isoform function reveals that HIF1α and HIF2α are approximately 48% homologous and are able to regulate the transcription of some of the same genes with HRE. However their expression patterns vary throughout the developmental process with HIF2α being crucial for embryological vascular epithelial cell development. Mice null for HIF2α die in utero as a result of gross vascular abnormalities (Hu *et al*, 2003). HIF1α appears to be responsible for the upregulation of proteins involved with glycolysis that would explain its importance in the response to hypoxia. HIF2α appears to be responsible for the upregulation of proteins involved in the response to hypoglycaemia (Brusselmans *et al*, 2001; Carroll and Ashcroft, 2006). In addition to finding HIF1α activation to hypoxic exposure, Eul *et al* (2006) have also noted a role for HIF2α in human pulmonary arterial fibroblasts cultured in hypoxic conditions. These investigators found that hypoxia-mediated proliferation in human pulmonary arterial fibroblasts was dependent on HIF2α with HIF1α appearing to exert an influence over fibroblast migration under hypoxic conditions (Eul *et al*, 2006). An examination of HIF1α and HIF2α activation patterns and function in a patient model of PH – particularly in view of the finding that HIF2α heterozygote mice are completely protected from hypoxia-mediated pulmonary vascular remodelling – might provide possible therapeutic strategies for the treatment of pulmonary hypertension.

There is an extensive body of work documenting cross talk between BMP/BMPR2/Smad and p38 MAPK signalling pathways, and between p38 MAPK and HIF1α activation, as previously discussed. Of interest is the finding by Yu *et al* (2005) that vascular smooth muscle cells cultured from mice haploinsufficient for BMPRII and further modified to be null for BMPRII in culture, could activate both p38 MAPK and Smad 1,5 and 8 via an alternative BMP signal transduction pathway involving BMPs 6 and 7 and TGFβ receptor type 2 subtype ActR2a. This suggests a potential role for disordered p38 MAPK signalling in a model known to exhibit aberrant BMP/BMPR2/Smad signalling. We did not demonstrate increased Smad 1, 5 and 8 activation in human pulmonary arterial fibroblasts but did show upregulated Smad 1, 5 and 8 signalling in systemic arterial fibroblasts to hypoxic exposure. This appears to be a novel development as we have not until this point been able to establish any positive response to hypoxic exposure in systemic arteries. Further investigation of Smad signalling pathways in systemic arteries may provide additional evidence as to the fundamental differences between the two circulations.

Given our finding that p38 MAPK is consistently activated in pulmonary arterial fibroblasts to hypoxic exposure, the pharmacological inhibition of p38 MAPK therefore might represent an attractive treatment strategy in the prevention of pulmonary vascular remodelling, certainly in the context of hypoxic lung disease but also possibly for PH. Currently there are two potential areas of pharmacological interest: the development of kinase inhibitors - specific p38 MAPK inhibitors and multi-kinase inhibitors such as imatinib (*Gleevec*) and sorafenib, and the use of small interfering RNA, or siRNA, to effect targeted post-translational gene silencing.

6.5.1. p38 MAPK inhibitors

There are several p38 MAPK inhibitors undergoing trials currently (Lee and Dominguez, 2005). There is interest in their role as anti-inflammatory agents – they have been shown to reduce joint destruction in murine arthritis model (Badger *et al*, 1996). There is some evidence that p38 MAPK inhibitors may help in pain control, delaying time to rescue medication in a human model. Some agents are currently undergoing trials to assess their benefit in the treatment of myelodysplastic disease (Lee and Dominguez, 2005) and COPD (Barnes, 2008). There have been concerns regarding the variable tolerability of these agents, with significant problems occurring related to liver toxicity and in some cases, central nervous system toxicity. Despite these problems there are still some agents undergoing phase II trials (Lee and Dominguez, 2005).

6.5.2. Multi-targeted kinase inhibitors

Current theories that pulmonary arterial hypertension may represent a disorder of non-metastasising dysfunctional cellular proliferation have been raised following the unexpected association of pulmonary hypertension with myelodysplastic disease in some individuals (Gupta *et al*, 2006). Work by Schermuly *et al* (2005) has examined the role of imatinib (*Gleevec*) – a tyrosine kinase receptor inhibitor/PDGF B receptor inhibitor used in the treatment of haematological malignancies – in reversing monocrotaline induced pulmonary arterial hypertension in a rat model. Ghofrani *et al* (2005) and Patterson *et al* (2006) have reported case studies where imatinib has been used as rescue therapy for patients with NYHA stage IV heart failure secondary to pulmonary hypertension with good effect. In addition further work has demonstrated that sorafenib – a multi-kinase inhibitor/PDGF B and VEGF inhibitor –

inhibits hypoxia-mediated pulmonary arterial remodelling in a rat model (Moreno-Vinasco *et al*, 2008). There is a body of literature – both experimental and from clinical practice – that demonstrates that multi-kinase inhibitors are well tolerated and effective in modifying pulmonary arterial remodelling (Ghofrani *et al*, 2005; Patterson *et al*, 2006).

6.5.3. small interfering RNA (siRNA)

The discovery of post-translational gene silencing by RNA molecules in plants as a strategy against viral infection in the late 90's (Hamilton and Baulcombe, 1999) and the subsequent discovery that small nucleotide sequences (21-23 nucleotide in length) in double-strand conformation efficiently result in targeted post-translational gene silencing in man (Elbashir *et al*, 2001), has resulted in interest in post-translational gene silencing as a therapeutic strategy. There are a wide variety of Biotech companies – for example *Dharmacon* - who are able to engineer specific siRNA for targeted genes which can be used experimentally to generate knockdown models both *in vitro* and *in vivo*. For example Teichert-Kuliszewska *et al* (2006) used siRNA to BMPRII in order to create a BMPRII knockdown model while investigating the effects of BMPR2 deficiency in human circulating EPC. These investigators were able to demonstrate a 50% reduction in BMPR2 expression in transfected cells following use of siRNA to BMPRII.

siRNAs to p38 MAPK are commercially available and have been used experimentally to assess effective delivery to bronchial epithelial cells in a murine model (Moshos *et al*, 2007). These investigators found that intra-tracheal delivery of anti-p38 MAPK siRNA conjugated to cholesterol, TAT (48-60) (transactivator of transcription)

originally isolated from HIV TAT, and Penetratin (isolated from the insect *Antennapedia*) resulted in 30 – 45% knock down of p38 MAPK expression in macrophages and EC within the murine bronchial tree that was maximal at 6 hours post delivery. The effectiveness was short-lived however. In addition these investigators also noted that TAT (48-60) and Penetratin independently resulted in generalised non-specific alterations in gene expression between 12 and 24 hours post delivery. The conjugation of p38 MAPK siRNA to cholesterol appeared to increase the duration of effectiveness.

Issues concerning siRNAs as both an experimental and therapeutic strategy centre on the mode of delivery to the cell, the duration of their effectiveness and worries concerning their ability to activate host cell mediated immunity. siRNAs are relatively large anionic molecules which need an effective delivery system to enable them to cross the cell membrane. Cholesterol molecules and so-called cationic cell penetrating peptides such as TAT (48-60) have been investigated as possible agents that might cargo siRNA into the cell. As noted above, initial experimental work suggests that both TAT (48-60) and Penetratin may result in gene expression modification independent of their ability to transport siRNA, a finding that may limit their use. Concerns regarding the potential immunogenicity were proven to be well founded in a murine model as Penetratin was also noted to activate the innate immune system via its action on Toll like receptor (TLR) 3-7 and 8 (Moschos *et al,* 2007). Concerns have also been expressed in the literature regarding the lack of demonstrated effectiveness in clinically relevant routes of delivery – for example oral or intravenous delivery systems – and the mechanisms by which siRNA is rapidly cleared from the plasma is not yet fully understood. It does appear however that

topical delivery systems are more effective and better tolerated: for example Moschos *et al* (2007) demonstrated effective reduction of p38 MAPK in bronchial epithelial cells via an endotracheal delivery system. siRNAs are an attractive experimental tool in the investigation of disordered p38 MAPK, BMPR2 and Smad signalling pathways in pulmonary hypertension, however they may also represent a potential therapeutic strategy.

6.6. Conclusion

In summary, in this work we have demonstrated that human pulmonary arterial fibroblasts proliferate to acute hypoxic exposure whereas human systemic arterial fibroblasts do not. We have shown that the proliferative response to acute hypoxic exposure witnessed in human pulmonary arterial fibroblasts is dependent on activation of p38 MAPK which is biphasic (at t = 6 and 16 hours) with the proliferative response being dependent on the activity peak at t = 16 hours and being congruent with the activation of HIF1α. These findings concur with those from our own laboratory using adult rat and bovine models, they also concur with work from other laboratories. In addition we have demonstrated that acute hypoxic exposure results in Smad 1, 5 and 8 activation in human systemic arterial fibroblasts, a signalling pathway that is generally held to be growth inhibitory in cells of mesenteric origin. The activation of a growth inhibitory pathway to acute hypoxia in human systemic arterial fibroblasts appears to be a novel finding.

As previously discussed, there is an extensive body of literature that documents cross-talk between BMP/BMPR2/Smad and p38 MAPK signalling pathways and between

p38 MAPK and HIF1α activation in a variety of experimental models. p38 MAPK certainly appears to be central to pulmonary arterial fibroblast proliferation to hypoxia, whether acute or chronic, which suggests that it might represent an attractive therapeutic strategy against pulmonary arterial remodelling associated with hypoxic lung disease. It would be of interest to establish whether p38 MAPK activation was also as central to dysfunctional pulmonary vascular cell proliferation in BMPRII mutation-related FPAH and IPH. p38 MAPK inhibition (either directly by enzyme inhibition or indirectly by post-translational gene silencing) would be a novel therapeutic strategy for pulmonary arterial hypertension.

Appendix

Consent documents for experimental work.

DEPARTMENT OF CARDIOTHORACIC SURGERY

CONSENT FORM FOR PERMISSION TO USE RESECTED LUNG TISSUE FOR RESEARCH PURPOSES IN PATIENTS UNDERGOING LUNG OPERATIONS

As it will have been explained to you, you are about to undergo an operation to remove all, or part of, your lung. Doctors in the Pathology Department will perform a number of tests on the tissue that is removed during the operation. This will allow your surgeon to give you a diagnosis and prognosis and guide you if any other treatment is required. Once they have completed their examination, the tissue is normally discarded.

Because we are constantly engaged in research ourselves and in collaboration with others, we are seeking your permission to use some of this tissue that is normally discarded. This would be used in a number of ongoing projects designed to investigate cancer and in detailing the anatomy & physiology of the blood vessels within the lung.

If you wish your resected tissues to be used in this way, the following guarantees will be given:

- No tissue will be removed apart from what is deemed necessary for your operation as decided by your surgeon.
- Sampling of tissue will not prejudice in any way the results of your operation or affect the ability of the pathologist to produce an accurate report.
- Samples used for research will be removed from tissue that would normally be discarded.
- In cases of cancer research, small samples may be frozen and stored to allow tests to be done at a future date.

Should you not wish tissues to be removed, this will not affect your treatment in any way.

Details of the current research projects and the project leaders are to be found below. The ultimate responsibility rests however with your surgeon to whom all queries should be addressed.

The pharmacology of endogenous vasoactive factors in human pulmonary arteries.
Supervisor: Professor M MacLean, University of Glasgow

Molecular markers of chemosensitivity in non-small cell lung cancer.
Supervisor: Professor R Brown, Beatson Institute

The physiology of human pulmonary vasculature.
Supervisor: Dr. AJ Peacock, Western Infirmary, Glasgow.

These projects have been approved by the Research & Ethics Committee for North Glasgow Hospitals University Trust.

CONSENT

I, _____ consent to the use of any tissues removed from me during my lung operation to be used for the purposes of research. I have read the notes above and understand them. I appreciate that under certain circumstance it may be necessary to store some tissue samples for analysis at a later date.

Patient signature: _____ Date: _____

Surgeon's name: _____

Surgeon's signature: _____ Date: _____

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