The critical role of p38 Mitogen Activated Protein Kinase (MAPK)- alpha in pulmonary hypertension:

Linking inflammation with pulmonary vascular remodelling

A thesis presented by

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Abstract

Background: The p38 Mitogen Activated Protein Kinase (MAPK) system is increasingly recognised as an important inflammatory pathway in systemic vascular disease but its role in pulmonary vascular disease is unclear. Indeed, Inflammation is becoming increasingly recognised as driving the process of pulmonary vascular remodelling. Previous *in vitro* studies suggest p38MAPKα is critical in the proliferation of pulmonary artery fibroblasts, an important step in the pathogenesis of pulmonary vascular remodelling. In this study the role of the p38MAPK pathway was investigated in both *in vitro* and *in vivo* models of pulmonary hypertension and human disease.

Aims: To investigate the role that the pro-inflammatory pathway mediated by p38MAPK and the alpha isoform in particular, might have in pulmonary hypertension and whether manipulation might offer a mechanism for reversal of pulmonary vascular remodelling.

Methods and results: Pharmacological inhibition of p38MAPKα in both chronic hypoxic and monocrotaline rodent models of pulmonary hypertension *prevented* and *reversed* the pulmonary hypertensive phenotype. Furthermore by using a novel and clinically available p38MAPKα antagonist, reversal of pulmonary hypertension was obtained in both experimental models. Increased expression of phosphorylated p38MAPK and p38MAPKα was observed in the pulmonary vasculature from patients with idiopathic pulmonary arterial hypertension, suggesting a role for activation of this pathway in pulmonary vascular remodelling. A reduction of IL-6 levels in both serum and lung tissue was found in the drug treated animals, suggesting a link between p38MAPK and the inflammatory pathway in pulmonary hypertension. Furthermore a reduction in the amount of soluble collagen was also observed in the drug treated animals. In vitro work has shown that the pulmonary artery fibroblast is an important source of both inflammatory mediators and collagen, released through a p38MAPK dependent system, and that this cell may be essential in the propagation of vascular remodelling.

Conclusions: This study suggests that the p38 MAPKα pathway plays a pathogenic role in both human disease and rodent models of pulmonary hypertension potentially mediated through IL-6. Selective inhibition of this pathway may provide a novel therapeutic approach that targets both remodelling and inflammatory pathways in pulmonary vascular disease.

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List of Accompanying Material

Work from this thesis has been presented at many national and international meetings and published in abstract form.

Inflammatory profiling of adventitial fibroblasts in pulmonary hypertension
Thorax 2010;65:A88

p38MAPK : An important pathway in the pathobiology of pulmonary hypertension and pulmonary vascular remodelling
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Inhibition of p38MAPK both prevents and reverses experimental pulmonary hypertension
Scottish Society of Experimental Medicine 2011 : spoken presentation

p38MAPK is essential for development of pulmonary vascular remodelling in pulmonary hypertension: effects mediated through an IL-6 dependent pathway
Association of Physicians of the UK and Ireland 2014 : spoken presentation

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature……..

Printed name……..

Definitions/Abbreviations

α-SMA	Alpha smooth muscle actin
ADP	Adenine diphosphate
ANOVA	analysis of variance
ASK-1	Apoptosis signalling kinase-1
ATF-2	Activating Transcription Factor-2
ATP	Adenine Triphosphate
BCA	Bicinchoninic acid
BMP	Bone morphogenetic protein
BMPR2	BMP type 2 receptor
BSA	Bovine serum albumin
cAMP	Cyclic adenine monophosphate
cGMP	Cyclic guanine monophosphate
CH	Chronic hypoxia
CHPAF	Chronic hypoxic PAF
CML	Chronic myelogenous leukaemia
cond med	conditioned media
COPD	Chronic obstructive pulmonary disease
CO_2	Carbon dioxide

cpm	counts per million
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DMEM	Dulbeccos modified Eagle's medium
DMSO	Dimethyl sulphoxide
DPX	Di-n-butylPhthalate in xylene
EC	Endothelial cell
ECL	Enhanced chemiluminescence
ELISA	Enzyme linked immunosorbent assay
eNOS/NOS3	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cells
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
FGF	Fibroblast growth factor
GDP	Guanine diphosphate
HCl	Hydrochloric acid
HE	Haematoxylin & Eosin

HIF	Hypoxia inducible factor
HPAH	Heritable PAH
HPV	Hypoxic pulmonary vascoconstriction
HRP	Horseradish peroxidase
IL-6	Interleukin-6
IHC	Immunohistochemistry
ILD	Interstitial lung disease
IP_3	Inositol Trisphosphate
i.p.	intra-peritoneal
IPAH	Idiopathic PAH
JNK	c-Jun N-terminal kinase
kDa	kiloDalton
K_v	Voltage gated Potassium channel
LV	Left ventricle
MAPK	Mitogen-activated protein kinase
MCT	Monocrotaline
MKK	MAPK kinase 6
mPAP	mean pulmonary artery pressure
MPO	myeloperoxidase

miR/miRNA	microRNA
mRNA	Messenger ribonucleic acid
NaOH	Sodium Hydroxide
NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear Factor kappaB
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
OD	Optical density
p38 inhib	p38MAPK inhibitor
PAF	Pulmonary artery fibroblast
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline / Tween
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PH	Pulmonary hypertension

PVR	Pulmonary vascular resistance
Rho	Ras Homolog family
RIPA	Radioimmunoprecipitation assay (buffer)
RPAF	Rat pulmonary artery fibroblast
ROS	Reactive oxygen species
RV	Right ventricle
RVH	Right ventricular hypertrophy
RVSP	Right ventricular systolic pressure
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl sulphate polyacrylamide gel electrophoresis
SERT	Serotonin transporter
SFM	Serum free media
sGC	soluble guanylate cyclase
siRNA	small interfering ribonucleic acid
SMC	Smooth muscle cell
SPVU	Scottish Pulmonary Vascular Unit
STAT	Signal transducer and activator of transcription
TAK-1	TGF-β activated kinase 1
TCA	Trichloroacetic acid

TGF-β	Transforming growth factor-β
TIMP-1	Tissue inhibitor of metalloproteinases-1
TRAF	TNF receptor-associated factor
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
WHO	World Health Organisation
WT	Wild type

1 Introduction

1.1 Pulmonary Hypertension

This thesis focuses on a condition called pulmonary hypertension (PH). This introductory chapter will briefly describe the definition and clinical classification of pulmonary hypertension (PH), the recent developments in elucidating the underlying pathogenesis of this condition and the current clinical unmet need that remains for these patients. It will summarise many of the new and exciting areas of understanding and development in pulmonary vascular biology and contextualise the research direction undertaken in this thesis.

1.1.1 Definition of pulmonary hypertension

Pulmonary hypertension is a clinical description rather than a diagnosis. It is based upon a haemodynamic measurement taken at right heart catheterisation, where the mean pulmonary artery pressure (mPAP) is obtained. PH is defined as a persistent elevation of the mPAP of greater than 25mmHg at rest(1). However this only describes a patient as having pulmonary hypertension but does not provide a diagnosis as to the aetiology of the PH, of which there are a wide variety of clinical causes.

1.1.2 Clinical classification and investigation of pulmonary hypertension

In response to an outbreak of pulmonary hypertension secondary to the appetite suppressant Aminorex, the World Health Organisation established a forum of international experts on PH in order to foster further clinical recognition and research into the field. This has continued every 5 years and the last world congress held in Nice, France in 2013, has provided state of the art reviews in the clinical and pathological aspects of the condition(2).

The current clinical classification divides the causes of pulmonary hypertension into 5 broad categories as shown in Table 1-1(3). From this it is clear that both pulmonary (Group 3) and cardiac (Group 2) conditions can result in elevated pulmonary pressures. However the focus of this thesis is on the conditions seen in group 1 (pulmonary arterial hypertension or PAH), group 4 (chronic

thromboembolic pulmonary hypertension or CTEPH) and group 5 (miscellaneous). These conditions are the groups currently shown to achieve some benefit from disease-targeted therapies. These conditions require the further haemodynamic definition of having normal left heart function (defined as pulmonary artery wedge pressure less than 15mmHg) and an elevated pulmonary vascular resistance (defined as greater than 3 Wood units)(1). This again is measured at the time of right heart catheterisation and excludes the pressure rise to be the result of passive elevation due to venous backflow from a high left atrial pressure, or as a consequence of a high cardiac output state.

Updated Classification of Pulmonary Hypertension
1.Pulmonary arterial hypertension 1.1 Idiopathic PAH 1.2 Heritable PAH 1.2.1 BMPR2 1.2.2 ALK-1,ENG,SMAD9,CAV1,KCNK3 1.2.3 Unknown 1.3 Drug and toxin induced 1.4 Associated with: 1.4.1 Connective tissue disease 1.4.2 HIV infection 1.4.3 Portal hypertension 1.4.4 Congenital heart diseases 1.4.5 Schistosomiasis 1' Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis 1'' Persistent pulmonary hypertension of the newborn (PPHN)
2. Pulmonary hypertension due to left heart disease 2.1 Left ventricular systolic dysfunction 2.2 Left ventricular diastolic dysfunction 2.3 Valvular disease 2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
3. Pulmonary hypertension due to lung diseases and/or hypoxia 3.1 Chronic obstructive pulmonary disease 3.2 Interstitial lung disease 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern 3.4 Sleep-disordered breathing 3.5 Alveolar hypoventilation disorders 3.6 Chronic exposure to high altitude 3.7 Developmental lung disease
4. Chronic thromboembolic pulmonary hypertension (CTEPH)
5. Pulmonary hypertension with unclear multifactorial mechanisms 5.1 Haematologic disorders: chronic haemolytic anaemia, myeloproliferative disorders, splenectomy 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders 5.4 Others: tumoural obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

Table 1-1 Clinical classification of pulmonary hypertension

Since accurate diagnosis is critical in order to establish appropriate treatment, it is essential that patients undergo investigation by specialists in pulmonary vascular medicine. Patients can present with a wide variety of symptoms such as syncope (collapse) and exertional breathlessness. The clinical signs can be quite subtle and a high index of clinical suspicion is often needed. Investigations include echocardiography, pulmonary function tests, computed tomography scanning and cardiac magnetic resonance imaging but despite the increasing ease of access to diagnostic tests the median time from onset of symptoms to diagnosis has not changed over the years.

1.1.3 Epidemiology of pulmonary arterial hypertension

PAH is a rare disease with the incidence ranging between 1-3 per million adults(4). This has been recorded by a number of registries across the globe which have studied the PAH population in their countries, including the UK, France and the US(5-7). The original registry which characterised the PAH population was the National Institutes of Health (NIH) registry recorded in the USA in 1981, but newer registries have now recorded the PAH population in the modern era(8). The comparisons between these registries have shown significant changes.

For example, it is worthy of noting the changing demographics of the population of PAH. The mean age at diagnosis in the NIH cohort was 36 years compared to the modern registries of between 50-65 years of age. Perhaps more importantly is the effect that current clinical treatments have had on the survival of patients with PAH. Survival at one year has improved from 68% in the NIH registry to now over 90% in the UK registry(5). Indeed median survival in the US database has gone from 2.8 years to 7 years(9). There is little doubt that this is the result of novel drug treatments that have stemmed from the greater understanding of the pathogenesis of pulmonary hypertension.

1.1.4 Natural history of pulmonary arterial hypertension

PAH is a relentless condition, which results in the progressive narrowing of the pulmonary blood vessels. This leads to a rise in the pulmonary(10) arterial pressure with increased resistance to blood flow across the pulmonary vascular

bed. In addition the stiffening of larger more proximal pulmonary vessels leads to reduced compliance and interruption of the ventriculo-arterial coupling. These factors lead to increased RV afterload and eventually the right ventricle, which is responsible for the cardiac output into the pulmonary circulation, starts to dilate and then fails. This leads to right heart failure and premature death(11). The aim of physicians treating this condition is to prevent this from happening and current therapies, although delaying this right heart dysfunction, have not yet led to a cure.

1.1.5 Current treatments for pulmonary arterial hypertension

The last 20 years or so have seen a dramatic improvement in the treatment of PAH. There are currently three classes of drug treatments which are licensed in Europe and the USA for the treatment of PAH: phosphodiesterase (PDE) 5 inhibitors, endothelin receptor antagonists (ETRA) and prostanoids. These therapies have been shown in multiple clinical trials to improve the functional characteristics of patients with PAH and now are well established in the treatment algorithm(12). Despite this, only two clinical studies have actually demonstrated a clear survival benefit in treatment (epoprostenol and macitentan) (13,14).

The current belief is that these drugs act principally through a pulmonary vasodilatory mechanism with relaxation of smooth muscle cells. Although this may improve the blood flow (and hence symptoms) through the pulmonary vascular bed, it does not seem to alter the underlying pathological process and unfortunately this condition of PAH still progresses to eventual premature death.

It is therefore clear that there is still a large unmet clinical need for the treatment of these patients. Current treatments do not offer a cure and many are associated with side effects and inconvenient routes of administration. For these reasons further translational biomedical research in PH is essential.

However, in order to address these issues and develop a cure, the pathology and pathogenesis of PAH must first be understood.

1.2 Pathology of pulmonary hypertension

As previously described, in pulmonary hypertension there are a number of different aetiologies. However in terms of the pathological appearances there are a number of shared features. This section will outline the pathology of pulmonary hypertension with predominant focus on pulmonary arterial hypertension. Following this, the current understanding on the molecular and cellular mechanisms responsible for the disease process will be examined.

However in order to recognise what is abnormal, the normal must be first understood.

1.2.1 The normal pulmonary circulation

The normal pulmonary circulation is a low pressure, high capacitance and high flow system which accommodates the entire venous return of the systemic circulation. This allows it to cope with substantial increases in flow and cardiac output, for example during exercise, without dramatically increasing pressure. In order to allow this, the pulmonary vessels are thin walled and compliant. In addition, there is under-recruitment in the pulmonary vasculature such that not all vessels are perfused under normal circumstances at rest. This allows the right ventricle to be thinner and not have to generate such high pressures as compared to the systemic left ventricle. There are 15 divisions of pulmonary arteries between the main pulmonary artery and the capillary network(15).

A normal pulmonary artery consists of 3 cellular layers separated by elastic laminae, although the content of these layers differs the further distal and smaller the artery or arteriole.

1. **Intima**: This is the innermost layer and consists of endothelial cells and the basement membrane

2. **Internal elastic lamina**: composed of thin elastin fibres

3. **Media**: This is the middle layer and comprises of smooth muscle cells and extracellular matrix

4. **External elastic lamina**: composed of thin elastin fibres

5. **Adventitia**: containing fibroblasts, extracellular matrix and vasa vasorum

In the proximal vessels (division order 15-13) the medial smooth muscle layer is thinner and the adventitia contains the vasa vasorum, which are blood vessels derived from the systemic bronchial circulation. The medium sized resistance arteries (division order 12-4) have thicker smooth muscle layers while the smaller vessels and arterioles lack a muscular layer. Usually vessels with a diameter of below 100μm lack a smooth muscle layer(16).

These then feed into a capillary network which are under 10μm in diameter and form an intimate contact with the alveolar epithelial cell and allows gas exchange to occur.

The normal pulmonary circulation has a low resistance across it in order to allow high flows. This is termed the pulmonary vascular resistance (PVR) and is calculated by the relationship between pressure and flow (PVR= pulmonary artery pressure/ cardiac output). Normal values are less than 3 Wood units and are determined by relationship to both diameter and length of blood vessel and the viscosity of blood. Under normal situations, the length and viscosity of the system is constant such that the PVR is principally related to the fourth power of the pulmonary arterial radius (Poiseuille's Law). In the normal circulation most of this is determined by the small and medium sized vessels(17). Thus a relatively small change in diameter of these vessels can have profound effects on the pulmonary resistance.

1.2.2 The abnormal pulmonary circulation in pulmonary hypertension: General introduction

The basic abnormal principles underlying the development of pulmonary hypertension are felt to be vasoconstriction, pulmonary vascular remodelling, loss of pulmonary vessels (vascular pruning) and pulmonary vascular thrombosis. These all occur to varying degrees depending on the underlying aetiology of the pulmonary hypertension but the hallmarks of all forms of PH are recognised as sustained vasoconstriction and vascular remodelling. For example in group 4

chronic thromboembolic pulmonary hypertension (CTEPH) patients, the thrombotic component is a key feature while in group 1 PAH this is less so and is only observed in very small vessels(18). In group 3 patients with hypoxic lung disease, there is a large contribution from hypoxic pulmonary vasoconstriction, but there is also rarefaction and distortion of the architecture of the pulmonary vascular bed, for example as a result of cigarette smoke and emphysema.

1.2.3 The abnormal pulmonary circulation in pulmonary hypertension: pulmonary vasoconstriction

There is little doubt that pulmonary vasoconstriction plays an important part in the pathophysiology of pulmonary hypertension. The detail of hypoxic pulmonary vasoconstriction is dealt with more fully in section 1.3.2.6 and 1.2.4.1.2.

The rise in intracellular calcium in the pulmonary artery smooth muscle cells (PASMC), (via calcium flux through channels or release from sarcoplasmic reticulum), leads to interaction with calmodulin and myosin light chain kinase (MLCK). This then phosphorylates MLC which brings about actin myofilaments to contract. MLC phosphatase is an enzyme which removes the phosphate from MLC and leads to vasodilation, hence providing a close regulatory feedback.

More recently RhoA and Rho-kinase have been implicated in the process of control of vasoconstriction, especially in hypoxia. Rho kinase inhibits MLC phosphatase which leads to a prolonged sustained vasoconstriction. Rho kinase can be activated by a number of factors which have been implicated in the imbalance between vasoconstrictors and vasodilators. These include endothelin, serotonin and thromboxane A_2. Rho kinase inhibitors such as fasudil, have been shown in animal models to reduce the pulmonary pressures in the MCT and sugen/hypoxia animal models(19). Indeed in human patients with PAH, fasudil has been shown to reduce the pulmonary vascular resistance(20).

As explained later, the calcium channels are involved in PASMC contractile responses. In the context of hypoxic pulmonary vasoconstriction, activation of the channel TRPC6 by Diacylglycerol has been implicated in the early responses to hypoxia(21,22).

However the debate as to the degree of importance that vasoconstriction plays in relation to structural cellular changes remains(23). This is particularly important since most of the therapies now available target vasoconstriction rather than addressing the underlying structural abnormalities. There is no doubt that there exists an important role for vasoconstriction, but current therapies have not in general been shown to have a mortality benefit. Therefore it could be argued that the treatments are improving symptoms and slowing down deterioration of the patient's condition, but are not addressing the underlying pathological process.

In addition there is a strong overlap between the molecular mechanisms, which control vascular cell contraction, and proliferation, such that dysregulation of the pathways can result in both vasoconstriction and pulmonary vascular remodelling. For example Rho kinase inhibition can result in regression of pulmonary vascular remodelling in serotonin transporter over expressing mice by inhibiting pulmonary vascular fibroblast proliferation(24).

Therefore, although vasoconstriction is important, the common theme, present in all forms of PH and felt to represent the most important underlying biological mechanism for the progression of pulmonary hypertension is that of pulmonary vascular remodelling.

1.2.4 The abnormal pulmonary circulation in pulmonary hypertension: pulmonary vascular remodelling

This pathobiological process is characterised by pulmonary vascular cellular proliferation, cellular hypertrophy, cellular migration, increased extracellular matrix deposition and inflammation. One of the key features is the appearance of smooth muscle containing cells within the small arterioles in the vasculature, so called distal muscularisation. These features can occur throughout the vessel wall and result in progressive narrowing of the lumen with increased resistance to flow. In addition abnormal endothelial proliferation and function are felt to be responsible for one of the pathognomonic features of PAH pathology, the plexiform lesion. This is a complex vascular structure characterised by

endothelial and myofibroblast lined vascular channels, which can lead to obstruction of the vascular lumen(25).

It should be remembered however that pulmonary vascular remodelling can be a normal response to normal physiological stimuli, and that aberrant signalling or pathological stimuli may be responsible for the persistent maladaptive response seen in PAH(26). The physiological remodelling process can help to strengthen vessel walls in times of increased pulmonary vascular pressure and flow, for example living at altitude in a hypoxic environment. This is reversible and adaptive. In PAH this process is maladaptive and leads to an inappropriate increase in pulmonary vascular remodelling with impaired RV function.

Pulmonary vascular remodelling is a complex multifactorial process and the underlying molecular mechanisms which initiate and drive the process are still not fully elucidated. This justifies on going research in this field.

1.2.4.1 Cellular changes in pulmonary vascular remodelling

The cellular changes associated with pulmonary vascular remodelling involve more than just the cells intrinsically linked to the pulmonary vessel. Indeed new exciting research has demonstrated the involvement of circulating fibrocytes and endothelial progenitor cells in this process and suggested an increasing role for inflammatory cells in the initiation and propagation.

1.2.4.1.1 Pulmonary artery fibroblast

The pulmonary artery fibroblast (PAF) is the principle residential cell of the adventitial layer. For many years it has been understudied in the pathobiology of pulmonary hypertension and thought simply to provide mechanical strength to the vessel wall through production of extracellular matrix. However there is abundant evidence to suggest that this cell is not simply a passenger in pulmonary vascular remodelling but actually plays a role in both the initiation and development of this process. Indeed the fibroblast could be the first cell that responds to the initiating stimuli that leads to pulmonary hypertension and act in the adventitia to organise the development of pulmonary vascular remodelling from ‘outside in’: an extraluminal detection, processing and control system with the PAF orchestrating this(27).

The earliest pathological features observed in pulmonary vascular remodelling in response to chronic hypoxia in animals are adventitial thickening and increased PAF proliferation(28). Furthermore the adventitial fibroblast proliferates faster than any other vascular cells in response to hypoxia(29). These features are also observed in other models of pulmonary hypertension (although not as prominently) and in patients with Idiopathic pulmonary arterial hypertension(30). These findings would suggest the importance of the adventitia in remodelling. Evidence from the systemic literature supports this in that in systemic hypertension and endothelial injury the adventitia is also seen to increase as one of the earliest features(31).

The adventitial PAF cells however are a heterogenous population with various subsets identified(32). This has also been observed within the alveolar lung fibroblast and indeed the systemic adventitial fibroblast. Indeed fibroblasts can exhibit different organ and tissue site-specific gene expression patterns. The different potential sources of these fibroblasts may be responsible for the heterogeneity. It is felt that the adventitial fibroblast can be derived from one of three places: primary mesenchymal tissue, local epithelial-mesenchymal transition (EMT) and bone marrow derived circulating precursors, such as fibrocytes. Fibrocytes are circulating mesenchymal and haemopoietic derived cells which can secrete collagen and differentiate into myofibroblasts(33). These cells have been found to be increased in PAH patients and also to circulate into the pulmonary adventitia through the vasa vasorum(33-35). In addition depletion of these cells can lead to reduced pulmonary vascular remodelling and prostanoids can reduce the recruitment of the cells to the vascular wall(36).

The resident adventitial fibroblast can be activated and differentiate into myofibroblasts in response to a number of stimulants including hypoxia, growth factors and cytokines such as IL-6(37-39).

Wherever the source of the PAF, these cells can undergo activation and transformation into myofibroblasts that display the characteristic appearance of alpha-smooth muscle actin (α-SMA) expression and have contractile functions. These cells can migrate through the vascular wall and lead to distal muscularisation of previously non-muscularised vessels(40-42).

The extracellular matrix has been shown to be important in vascular remodelling and the fibroblast is the principal vascular cell which determines this. The PAF can increase the deposition of extracellular matrix proteins such as elastin, collagen, fibronectin, osteopontin and Tenascin-C. This can lead to a reorganisation of the fibrillar proteins in the vessel wall and increased stiffness and hence reduced compliance. Furthermore these can lead to direct stimulation of smooth muscle cells, activation of PAF and allow migration of cells through the adventitia and media. Importantly, the inhibition of serine elastases can lead to reversal of experimental pulmonary hypertension through changes in smooth muscle cell proliferation and extracellular matrix deposition(43,44). Associated with this is the increased release by the activated fibroblast of matrix metalloproteinases (MMP). This is matched by a reduction in the release of the tissue inhibitors of metalloproteinases (TIMP), which act to halt MMP activity. This combination can lead to further breakdown of the extracellular matrix and ease the migration of myofibroblasts through into the media of the vessel(45,46).

The role of inflammation in the pathobiology of pulmonary hypertension is more fully discussed later. However it is important to be aware that the adventitial fibroblast is now considered an integral part of the innate immune response and can initiate pro-inflammatory signalling in the adventitia(47-49). It can release a number of chemokines and cytokines that can lead to recruitment and activation of inflammatory cells. The fibroblast has also been shown to be capable of releasing key mitogens such as ET-1 and TGF-β. These can have direct stimulatory effects on smooth muscle proliferation and differentiation of myofibroblasts(50).

In summary the pulmonary artery fibroblast is now recognised as an important cell in the development of pulmonary vascular remodelling for the following reasons.

- It is one of the first vascular cells to proliferate in response to stimuli such as hypoxia,

- it increases the deposition of extracellular matrix proteins

- it can differentiate into a myofibroblast which can migrate into the media of non-muscularised vessels

- it can release pro-inflammatory and mitogenic stimuli

1.2.4.1.2 Pulmonary artery smooth muscle cell

In pulmonary vascular remodelling there is marked medial hypertrophy. This results from pulmonary artery smooth muscle cell (PASMC) proliferation with expansion of the media and the muscularisation of the small pre-capillary arterioles. There is also increased matrix deposition and infiltration of myofibroblasts from the adventitial layer(51).

There also exists a balance between the apoptosis of PASMC and their proliferation and in remodelling it is suggested that the cells undergo a phenotypic change and become apoptosis resistant and pro-proliferative. In support of this are observations that PASMC from patients with PAH have an increased resistance to apoptotic stimuli such as Bone morphogenetic protein (BMP)-2 and -7 compared to normal PASMC(52).

A key factor underlying the PASMC proliferation is membrane depolarisation which can allow influx of calcium and subsequent contraction, migration and proliferation. Potassium (K_v) and calcium channels affect the homeostasis of the membrane potential. Experimental studies have shown that chronic hypoxia downregulates K_v1.5 and 2.1 channels and that by augmenting these channels in PASMC *in vitro* and *in vivo* proliferation can be stopped and apoptosis facilitated(53). This suggests remodelling can be reversed with manipulation of the K_v channels. In addition reduced levels of K_v 1.5 have been found to be reduced in the PASMC from IPAH patients(54).

The calcium influx into PASMC can be controlled by voltage-gated calcium channels and calcium permeable non-selective cation channels, for example transient receptor potential (TRPC) proteins. Experiments using inhibitors for the voltage gated channels suggest that these do not play an important role in mediating the calcium influx in PASMC, and that most of the control is mediated

by the TRPC. Furthermore, there is increased expression of TRPC in patients with PAH(55).

In chronic hypoxia, mediated by Hypoxia inducible factor-1α (HIF-1α), there is upregulation of the TRPC 1/6 which can then lead to increased calcium influx and PASMC contraction and proliferation. Decreasing the function of these channels can prevent cell proliferation. The calcium influx can activate various transcription factors such as Nuclear Factor of activated T-cells (NFAT) which in turn has been shown to reduce expression of K_v channels and prevent PASMC apoptosis(56). Calcium influx can also affect expression of Aquaporin-1 which is necessary to allow cell movement and migration(57).

There are a number of factors which can induce PASMC proliferation and these are discussed more fully later in this introduction.

1.2.4.1.3 Pulmonary artery endothelial cell

The pulmonary artery endothelial cell (PAEC) forms the endothelium which acts to protect vessel integrity, tone, coagulation and cellular proliferation. It can be exposed to damage from many factors such as toxins, sheer stress and inflammation. Indeed it has been suggested that some of the earliest pathogenic mechanisms in pulmonary vascular remodelling occur in the endothelial cell.

Endothelial proliferation with neointimal changes is one of the hallmarks of vascular remodelling. However the intima can also undergo fibrosis with increased extracellular matrix collagen and invasion from myo-fibroblast cells that further contribute to the neointimal thickening. However differentiation of endothelial cells into smooth muscle like cells (so called endothelial-mesenchymal transformation) can also occur(58).

PAEC can control cell proliferation and tone by release of various mediators. These include vasodilators such as prostacyclin and Nitric oxide (NO), and vasoconstrictors, for example endothelin-1 (ET-1) and Thromboxane. In pulmonary vascular disease there is an alteration in the balance between these agents, favouring vasoconstriction(59). ET-1 can also result in increased permeability to the lungs by disrupting the integrity of the endothelium and this

could result in the flow of circulating cells and growth factors into the vascular wall(60).

One of the pathognomonic features of PAH is the plexiform lesion. Although not seen in all cases of PAH, when it is observed the diagnosis is secured(61). These are complex vascular structures which are often found downstream of occluded vessels at the points of vessel bifurcation. It is postulated that the initial event in plexiform lesion production is an increased rate of apoptosis by endothelial cells. This leads to the emergence of an apoptosis resistant clone which proliferates to form the vascular lesion(62). This has been suggested due to the observation that the endothelial cells in the plexiform lesion are monoclonal. Observation of increased levels of vascular endothelial growth factor (VEGF), survivin and HIF suggest these play a role in the formation along with increased perivascular inflammatory cells(63,64).

Recent focus has been on the part that endothelial progenitor cells might play in the biology. These are believed to be bone marrow derived circulating stem cells which can undergo differentiation into endothelial cells. As noted previously loss of blood vessels in the peripheral circulation (vascular pruning) occurs and early endothelial progenitor cells (EPC) may be able to reform these vessels. Studies using EPC's transfected with eNOS have reversed PH in animal models of established disease(65,66). However the EPC's may also have adverse effects on the vessels if they are dysfunctional. Therefore it is not entirely clear the extents to which EPC's are beneficial or detrimental in the biology of PH(67). There is ongoing interest in this area and the results of a preliminary clinical trial assessing safety of administering autologous cultured eNOS-transduced mononuclear cells in IPAH patients are eagerly awaited (NCT00469027).

1.2.4.2 Molecular and cellular signalling in pulmonary vascular remodelling

The last 20 years or so has seen an explosion in the understanding of the pathobiology of pulmonary hypertension. Key effector molecules have been identified that by targeting and either interrupting or promoting can lead to alterations in the disease. Indeed some of these discoveries have led to clinically available drugs.

1.2.4.2.1 Endothelin

This was first identified in 1988 as a potent 21 amino-acid vasoconstrictor peptide secreted from endothelial cells(68). There are three identified isoforms but ET-1 is the principal one involved in PH. Endothelin is synthesised as a large prepro-ET which then undergoes cleavage to pro-ET. These are then modified by endothelin converting enzymes to produce the 3 isoforms(68).

ET-1 has diverse effects which are mediated by G-protein coupled ET_A and ET_B receptors(69). These are linked to Gq-protein which links to phospholipase C and produces Inositol Trisphosphate (IP_3) as a secondary messenger. This allows calcium efflux from the sarcoplasmic reticulum and cell contraction. In the pulmonary vasculature, ET-1 is secreted mainly by the endothelial cell and it then acts on the PASMC which expresses both receptors. The endothelial cell only expresses the ET_B receptor and it is felt that this acts to stimulate a negative feedback loop by reducing ET-1 production and stimulating NO production. ET-1 in addition to powerful vasoconstriction has also been found to act as a potent mitogen, especially for PASMC(70).

Increased production of ET-1 has been found in both animal models and patients with PAH. A mouse model with ET_B receptor deficiency demonstrates exaggerated pulmonary pressures in hypoxia. Endothelin blockade in animal models led to prevention and reversal of the PH phenotype, findings which have been translated into the clinical arena. Although not a cure, ET blockade in humans has led to dramatic improvements in exercise capacity and quality of life.

ET-1 has other effects however, which are pertinent to the development of pulmonary vascular remodelling. ET-1 can increase the proliferation and migration of PAF's and furthermore can stimulate neovascularisation of the adventitia with formation of vasa vasorum networks. It is recognised that hypoxia can act as a stimulus to cells to release ET-1, again identifying the PAF as an important cell.

1.2.4.2.2 Nitric oxide pathway

Nitric oxide is important in pulmonary hypertension and it is interesting to note that clinically it can be used to determine prognosis in PAH. Patients in whom the administration of inhaled NO at right heart catheterisation leads to a fall in the mPAP and reduction in pulmonary vascular resistance have a much better outcome compared to non-responders. The exact mechanism for this differentiation is unclear, but provides a useful prognostic marker(71). However it is clear that disruption of the NO pathway plays a role in all types of PAH.

Nitric oxide (NO) is a diffusible gas which is produced by nitric oxide synthases (NOS1-3) mainly in endothelial cells through the conversion of L-Arginine to L-Citrulline and NO. NO has diverse physiological effects and can act as an autocrine and paracrine signal resulting in smooth muscle relaxation, bacterial killing, platelet acativation and neurotransmission. NOS3 (or eNOS) is the main source of NO generation in the pulmonary circulation and is located in endothelial cells(72).

The NO, once generated, can act within the cell or diffuse to adjacent cells, usually PASMC. It activates soluble guanylate cyclase (sGC) through interaction with the heme-iron of the molecule which increases the activity of the enzyme and leads to production of the second messenger cyclic guanosine monophosphate (cGMP). This molecule mediates its effects via action on cGMP-dependent kinases, -ion channels and –phosphodiesterases. NO in PASMC inhibits cell proliferation, DNA synthesis, cell migration and collagen production. The principle effect of NO, perhaps, is to lead to pulmonary vascular dilatation and it is proposed to have a selective action on this circulation because it is rapidly scavenged by haemoglobin. The effect of cGMP is terminated by the action of phosphodiesterase type 5 (PDE5) which hydrolyse the molecule(72).

Various genetic and pharmacological interventions have demonstrated the importance of the NO system in the maintenance and control of the pulmonary vasculature. Genetic knockouts of mice (for example eNOS and GTP-cyclohydrase-1, a key enzyme in BH4 synthesis) have been implicated in the development of elevated pulmonary pressures(73). In addition knockout of one allele of DDAH1 (dimethylarginine dimethylaminohydrolase) which is an enzyme

involved in the degradation of an endogenous NOS inhibitor called ADMA has demonstrated an increase in the right ventricular systolic pressure and thickened walls of pulmonary arterioles in mice exposed to hypoxia(74). Endogenous NOS3 inhibitors such as asymmetric dimethylarginines (ADMA) are found to be elevated in animal models of PH and in serum of patients with PAH. In fact serum levels of ADMA are found to correlate with disease severity and outcome. In normal humans the use of specific NOS antagonists have demonstrated an increase in resting pulmonary vascular resistance(75). Thus it seems clear that NO plays a pivotal role in the homeostasis of pulmonary vascular tone.

Evidence also exists of the importance of lack of NO in the pulmonary system as playing an aetiological role in pulmonary hypertension. In animal models there is documented reduction in cGMP activity in the pulmonary vasculature and increasing NO levels in animals can also ameliorate the rise in pulmonary artery pressure(76,77). In humans, studies have shown a lower level of NO in patients with pulmonary hypertension. Patients with idiopathic pulmonary hypertension have lower exhaled breath levels of NO and decreased levels of eNOS have been seen in pulmonary vessels from explanted lungs in IPAH patients, although plexiform lesions do seem to have higher expressed levels(78). At altitude the degree in which dwellers are able to maintain or increase their NO levels have been shown to be important in the development of pulmonary hypertension(79). In addition levels of ADMA are higher in patients with IPAH and these are potent NOS inhibitors and may play a role in the elevated pressures(80).

Evidence has also shown that in the disease state homeostatic mechanisms attempt to utilise the NO pathway in order to normalise the pulmonary pressures. For example in patients with IPAH there are higher levels of the naturetic peptides such as BNP and ANP which are released from the stressed right ventricle and can activate the sGC pathway. This relates to both ANP and BNP acting on membrane bound type 1 guanylate cyclase receptors(81).

These findings described above have provided the rationale for utilising the NO pathway in treating pulmonary hypertension.

1.2.4.2.2.1 Phosphodiesterase biology

The control of cGMP signalling is by the action of cGMP dependent phosphodiesterases (PDE) which act to hydrolyse the second messengers. There are 11 PDE families which have been identified currently and they differ in terms of tissue distribution, the messengers which control them and their resultant actions. PDE1, PDE5 and PDE3/4 seem to be particularly involved in the pulmonary circulation. In particular PDE5 inhibitors sildenafil and Tadalafil have made substantial improvements in the treatment of patients with PAH.

PDE5 is the enzyme which has been the main focus of research and manipulation in terms of the NO pathway in PAH. Similar to other PDE's the enzyme has a conserved catalytic domain at the C-terminus which acts to bind and hydrolyse cGMP. The regulatory domains are found at the N-terminus and contain so-called GAF domains which bind cGMP and result in a 10-fold increase in activation of the enzyme(82). This is an example of positive feedback where the actual substrate of the enzyme can actually activate it. Furthermore the downstream signalling effector of cGMP, PKG, can also phosphorylate a serine residue on the GAF domain and lead to activation of the enzymatic activity.

The gene for PDE5 allows the generation of 3 splice variants called PDE5A1-3. PDE5 is expressed in most tissues although large levels are identified in vascular smooth muscle and the lung, however there are differences with the splice variants with the PDE5A3 expression being limited to smooth muscle. Importantly the level of PDE5 was found to be greater in lung homogenates and laser microdissected pulmonary vessels in patients with PAH. The promoter region of the PDE5 gene also contains cyclic nucleotide responsive binding sites which can allow upregulation of the transcription of the enzyme(72).

The rationale for using PDE5 inhibitors has been established in animal models of PAH, namely the monocrotaline and chronic hypoxic rat model. In isolated rat lungs they inhibit the vasoconstriction induced by hypoxia and thromboxane antagonism(83,84). In both animal models chronic sildenafil administration has both attenuated the development of pulmonary hypertension and also been used

to partially reverse the pathology when used as a treatment strategy(85,86). Furthermore in vitro studies have shown that the cGMP pathway can inhibit cellular proliferation in distal pulmonary artery smooth muscle cells and this can be effected by use of PDE5 blockers such as sildenafil. Evidence of beneficial effects of combination therapy of the PDE5 inhibitors with either prostanoids or endothelin receptor antagonists have also shown been demonstrated(86,87).

Initial work on the cardiomyocyte showed that there was very little PDE5 expressed under normal basal conditions and enzyme activity is low(88). Indeed some evidence suggests repression of PDE5 expression during embryonic development(89). However more recently studies have suggested that this might not be the case and that PDE5 may actually have a physiological role to play(90). Excitingly, in patients with pulmonary hypertension and right ventricular hypertrophy, PDE5 is expressed in higher levels compared to normal hearts in humans and rats(91). Furthermore the inhibition of PDE5 led to elevated cGMP levels and cAMP levels which resulted in a net effect of improving right ventricular function suggesting a direct effect on the ventricle and therefore a therapeutic role in patients with RV dysfunction(91). It has also been demonstrated that sildenafil can increase the cardiac output compared to inhaled nitric oxide suggesting a direct effect on the RV contractility with sildenafil(92).

1.2.4.2.2.2 Soluble Guanylate Cyclase (sGC) biology

The main target for diffusible NO is the soluble guanylate cyclase (sGC) and can be activated by nanomolar concentrations of NO. These are widely expressed enzymes which when activated convert GTP into the second messenger cyclic GMP (cGMP). These are heterodimers with 2 subunits – α and β, of which the $\alpha1\beta2$ and $\alpha2\beta1$ are most well characterised(93). The smaller β subunit contains an evolutionary conserved amino-terminal haem-binding domain which is critical for the sensing of NO(94). However the $\alpha1$ subunit has been shown to be of particular importance in limiting the degree of pulmonary vascular remodelling seen in mice who are deficient in this subunit(95).

Of further importance to the functioning of the enzyme is that the haem moiety requires a reduced ferrous Fe^{2+} state in order to sense and bind the NO; indeed

removal of the ferrous state removes the ability of NO to activate the enzyme. Currently it is felt that the ferrous iron interacts with the four nitrogens of the haem group and a histidine residue on the β-subunit. The binding of NO forms a hexacoordinated unstable complex which leads to cleavage of the haem-histidine bond and this acts as a key molecular switch with conformational change which activates the enzyme. Thus the redox state of the cell is an important predictor of the action of NO on the sGC. If there is oxidative stress in the cell with production of free radicals (O_2^- or peroxynitrite) then this can lead to an NO-insensitive sGC and interruption of the normal NO cellular effects such as vasorelaxation(96). As a direct consequence of this, agents which increase NO levels will still not be able to stimulate the sGC.

As explained above the functioning of the soluble guanylate cyclase enzyme in response to nitric oxide is dependent on the presence of a reduced ferrous moiety on the heme group in the enzyme. Thus in conditions in which oxidative stress exists such as pulmonary hypertension the heme group can be oxidised and become unresponsive to the NO. Novel dugs have been developed and are now entering clinical practice which can stimulate the sGC independent of NO. There are two defined groups – the haem dependent sGC stimulators who require the heme moiety to be in a reduced state and the haem independent sGC activators which can still activate the enzyme independent of the redox state of the enzyme.

The sGC stimulators, of which there are now a number, act by stimulating the sGC in its reduced heme state and also have potent synergistic effects in the presence of NO. They can be viewed as NO sensitisers in that the sGC produces a greater cGMP response for any given concentration of NO when in the presence of the sGC stimulator. These agents have also been tested in animal models of PAH and shown that the right ventricular systolic pressure and the vascular remodelling can be reduced(97-99). Furthermore *in vitro* testing has shown that they also have anti-proliferative effects on vascular smooth muscle and reduce the secretion of matrix metalloproteinases(100). This may add an anti-mitogenic and anti-matrix remodelling dimension to these agents. Work is ongoing in this area. A recent study using larger than clinically relevant doses has demonstrated

an inhibitory effect on PDE5 which tanatlisingly may suggest a further mechanism by which these agents increase the cGMP levels(101).

Riociguat is the first of the sGC stimulators to have now entered clinical practice and it has been shown in two clinical trials to be efficacious in both group 1 and 4 PAH(102,103).

1.2.4.2.3 prostacyclin

Studies in the early 1990's suggested that there was an imbalance between prostacyclins which are vasodilators and thromboxanes which are potent vasoconstrictors. PAEC from patients with PAH have low levels of prostacyclin synthase and overexpression of this enzyme in animal models prevents the development of PH. Hypoxia can inhibit the production of PGI_2 by PAEC.

Prostacyclin (PGI_2) is an arachidonic acid derivative from cyclooxygenase action and is produced principally in the endothelial cell in the pulmonary circulation. Prostacyclin acts through the G-protein coupled IP receptor and signals through cyclic adenosine monophosphate (cAMP) and results in pulmonary vasodilation, inhibition of platelet aggregation and inhibition of vascular cell proliferation, at least in experimental PH(104). Use of prostanoids in experimental animal models has shown that there is improvement and reduced proliferation(105,106).

The above findings led to the treatment of PAH using prostaglandin analogues. Indeed intravenous treatment has been shown to improve survival and is still considered to be the gold standard therapy in this condition(14).

1.2.4.2.4 Genetic mutations

There is a clear genetic predisposition to PAH(107). The knowledge base associated with this is increasing. The most well recognised mutation is in the Bone morphogenetic protein receptor 2 (BMPR2) signalling pathway. The BMP signalling pathway is critical for the normal embryological development, with regulation of cell proliferation and cell death, and the BMPR2 receptor is a member of the TGF-β superfamily(108).

In 70% of patients with Heritable PAH and 10-20% of patients with IPAH there is an associated mutation in the BMPR2 receptor(109,110). Most mutations are loss of function mutations resulting in premature stop codons but some also exist in promoter regions. Interestingly those IPAH patients without a BMPR2 mutation also have lower levels of BMPR2 compared to normal patients suggesting that other mechanisms may exist for the knockdown of BMPR2 in patients with PAH. Furthermore, being a female with a BMPR2 mutation puts you at a higher risk of developing PAH compared to males indicating a possible interaction between BMPR2 and sex hormones(111). Supporting this theory is that the sex difference in pulmonary hypertension is not observed pre-puberty. In contrast, despite the mutation being present, a low penetrance for the gene means that as many as 80% of people who have a BMPR2 mutation will not go on to develop PAH(112).

The signalling cascade is complex for BMP. The ligands bind to the receptors and the canonical pathway allows action via Smad1/5/8 affecting ID genes. However signalling can also occur through PI3K and the MAPK pathways including p38, Jnk and Erk(113-115). There may also be suggestions that ET-1 levels can be increased as a consequence of abnormal BMPR2 signalling(116).

A BMPR2 heterozygote (+/-) knockout mouse model of PAH has been generated which exhibits reduced Smad1/5/8 signalling in pulmonary artery smooth muscle cells(117). This has a mild pulmonary hypertensive phenotype with distal muscularisation but lack any right ventricular hypertrophy. They also require a second hit, such as hypoxia, in order to develop significant pulmonary hypertension.

Further evidence suggesting that the TGF-β receptors are involved in the pathogenesis of PAH come from the recognition that patients with Hereditary Haemorrhagic Telangiectasia (HHT) are at risk of developing PAH. Mutations in endoglin or ALK-1 in patients with HHT have been associated with development of PAH and indeed a heterozygous ALK-1 knockout mouse seems to have a spontaneous pulmonary hypertensive phenotype(118-120).

There are now other genetic mutations which have been identified in families with PAH, some linked to BMP signalling such as Smad4 and Smad8(121). These also include caveolin-1 which is essential for the formation of caveolae(122).

These are lipid rafts in the cell membrane surface which can act as depots for the location of many cell surface receptors including G-protein coupled ones and downstream NO signalling(123,124). However caveolin-1 can also directly regulate Smad signalling(125). Experimental evidence shows that in caveolin knockout mice there is actually upregulation of eNOS and this can lead to pulmonary vascular remodelling through tyrosine nitration(123). Furthermore caveolin-1 levels have been shown to be reduced in lungs from patients with IPAH(126).

A more recently identified mutation is in the KCKN3 potassium channel which leads to loss of function and alteration in the cellular potassium current and can affect smooth muscle cell function(127).

It is suggested that the presence of a monogenic mutation is not enough on its own to lead to the development of pulmonary hypertension, and a 'second hit' may be required(128).

1.2.4.3 Epigenetic control

The pro-proliferative phenotype seen in vascular cells in pulmonary vascular remodelling persist when the cells are grown ex-vivo. This could be explained by a genomic imprinting such as controlled by epigenetic factors(129). The control of vascular cell function by microRNA is discussed later but recent evidence has shown both DNA methylation and histone acetylation to be important. Use of histone deacetylase inhibitors (HDAC) such as valproic acid, have shown mixed results with favourable outcomes in the chronic hypoxic model, but worsens right ventricular function in other models(130,131). DNA methylation of CpG islands controls the downregulation of superoxide dismutase seen in the fawn-hooded rat which can develop a spontaneous pulmonary hypertensive phenotype. Reversal of this methylation by inhibiting the DNA methyltransferase can reverse this(132).

As with all finely tuned cellular systems, in order to target this effectively, specific inhibitors will need to be developed.

1.2.4.4 DNA transcription factors

The recognition that abnormal signalling in the nucleus and upregulation of pro-proliferative and anti-apoptotic genes, has fostered the theory that some underlying events driving PAH can be compared to neoplasia. Hence the move to adopt anti-proliferative strategies for pulmonary vascular remodelling. However for many years it was felt that targeting central downstream effector molecules was not advisable because it would lead to multiple side effects and inhibition of other key processes. However it is now clear that because of redundancy in many receptor kinase signalling pathways blocking upstream signals may not lead to inhibition of the downstream pathway. Therefore targeting key final downstream targets such as transcription factors may be possible. There have been a number of ones identified and a brief overview is detailed here.

1.2.4.4.1 Notch signalling

This is a key regulator of embryonic development and of smooth muscle cell differentiation and development(133,134). Notch3 has been shown to be upregulated in lung tissue from patients with PAH and the amount correlates with the severity of the disease(135). Notch3$^{-/-}$ animals are protected from hypoxia induced pulmonary hypertension and inhibition of Notch3 in wild type chronic hypoxic mice reverses the PH phenotype(135). Jagged-1, an inhibitor of notch signalling, has been shown to prolong the survival in MCT rats suggesting beneficial effect of blocking Notch signalling(136). This all suggests that Notch signalling is important in pulmonary vascular remodelling.

1.2.4.4.2 Wnt/B-Catenin signalling

These are classical signalling pathways which are necessary for normal vascular embryological development. It has been shown that increased Wnt signalling can occur in the vessels of PAH patients and that the Wnt and Beta-catenin pathways are critical for both PAEC and PASMC survival(137). The presence of wnt leads to the prevention of GSK-3β targeting the Beta-catenin for proteosomal degradation(138). This leads to accumulation of beta-catenin and translocation to the nucleus which in turn can switch on genes involved in cell differentiation and proliferation(139). Indeed it is very important for angiogenesis(140,141) and has been implicated in endothelial cell survival and BMP signalling(142).

1.2.4.4.3 Nuclear Factor of activated T cells (NFAT)

NFAT is a key activator of T cells but it also seems to be activated in PASMC derived from PAH patients and is seen to be activated in lung tissue from patients. In addition ET-1 can activate NFAT and *in vivo* administration of cyclosporine, a NFAT inactivator, can lead to improvements in MCT-induced and chronic hypoxic pulmonary hypertension(56,143). NFAT also reduces K-channel activity and leads to smooth muscle cell proliferation(56).

1.2.4.4.4 Peroxisome proliferator-activated receptor (PPAR)

These are ligand-activated transcription factors which are intimately involved in processes as diverse as lipogenesis to inflammation and angiogenesis(144,145). These are transcription factors that can act downstream of BMPR2 signalling(146,147). BMP-2 can act via PPAR-γ to inhibit cell proliferation and rosiglitazone, a PPAR-γ agonist, can reduce RV hypertrophy in chronic hypoxic and MCT animals(148-150). The levels of PPAR-γ are reduced in the plexiform lesions and in the vascular lesions observed in the sugen hypoxia model(151). PPAR-γ activation also results in reduction of ET-1 and ADMA production, factors which aid vasoconstriction in pulmonary hypertension(152,153).

1.2.4.4.5 Signal Transducer and Activator of Transcription (STAT)-3

STAT-3 is a transcription factor activated by phosphorylation by a number of signals including cytokines (for example IL-6), mechanical stress (through integrin receptors), growth factors (PDGF,VEGF), and vasoconstrictors (ET-1)(154,155). STAT-3 can then promote downstream signalling which sustains cellular proliferation and anti-apoptosis signals. It can lead to the following.

- increased expression of Pim-1 which is an activator of NFAT(156). This can activate NFAT

- activation of Kruppel-like Factor 5 which increases surviving production which is an anti-apoptotic signal(157)

- reduction in eNOS production(158)

As can be seen there are a number of key upstream pathways that feed into the STAT-3 pathway and may be a target for future drug development(159).

1.2.4.5 Purinergic signalling

Extracellular adenosine tri-phosphate (ATP) has been shown to have pro-angiogenic and pro-inflammatory effects(160). Many pathophysiological processes which can play a role in PH such as inflammation, hypoxia, mechanical shear stress, can lead to the release of ATP from a variety of cells suggesting ATP as a 'danger signal' and some studies have shown that adventitial fibroblasts exposed to hypoxia can release ATP which can in turn result in fibroblast proliferation and migration(161,162). Moreover ATP can cause proliferation and migration of fibrocytes and smooth muscle cells(163). These effects are mediated by P2Y receptors on fibroblasts and this all suggests a potential role for purinergic signalling in the biology of PH(164).

1.2.4.6 Summary

It is evident that the biology of pulmonary vascular remodelling is complex and perturbations in a myriad of cellular and molecular pathways seem to be involved. In addition many of the molecules and factors which initiate the process have been identified.

1.3 Pathogenesis of pulmonary hypertension

The last two decades of pulmonary vascular research has revealed a wide number of factors and events which are now known to be involved in vascular cell proliferation and remodelling. Most of these discoveries have been through the investigation of experimental pulmonary hypertension using *in vitro* and *in vivo* models. These are now explored in the next section.

1.3.1 Cellular and animal models of pulmonary hypertension

In order to study and understand human disease the underlying pathological processes have to be explored. This is not always possible in humans. Indeed since the modern treatment era patients are not undergoing as many lung transplants for PAH and this reduces the availability of tissue for study.

Therefore cellular and animal models are often used with the caveat that these do not always mirror human disease completely. Due to this fact there are usually a number of models for the same disease. This is true in pulmonary hypertension, although the chronic hypoxic and monocrotaline models are perhaps the most well recognised.

A debate on the merits and flaws of individual models is beyond the remit of this thesis. However it is clear that no one model reproduces the human disease faithfully. This perhaps reflects the multi-functionality of pathways which are dysregulated in the development of human disease. Indeed the animal models, which recapitulate PAH the closest, have a number of 'hits' rather than just one.

1.3.1.1 Acute and Chronic Hypoxic model

The use of hypoxia in studying pulmonary hypertension and pulmonary vascular remodelling comes from the observation that humans who reside at altitude have evidence of pulmonary hypertension and remodelling with right ventricular hypertrophy. The advantage of hypoxia as a stimulus to the study of PH is that this is a well-recognised cause of actual PH in humans and does not involve the administration of toxins/drugs which may also induce other unexpected and unwanted effects.

For cellular models, acute normobaric hypoxia is used where the fraction of oxygen in the normal air environment is reduced (from 21% to around 10%) for a period of 24-48 hours(165).

For animal models a chronic hypoxic exposure is used in which the fraction of oxygen in the air is maintained but the overall atmospheric pressure is reduced, so called hypobaric hypoxia. This is what residents at altitude are exposed to. The animals are exposed to this environment and as shown later in the thesis after 14 days the animals are shown to have increased pulmonary pressures, right ventricular hypertrophy, muscularisation of the smaller vessels and loss of vessels. However complex plexiform lesions are not seen in this animal model(165-167).

The most commonly used animals are either rats or mice, however it should be noted that there are differences between the degree of pulmonary vascular remodelling seen within and between species(168). In mice for example there is haemodynamic evidence of PH but the histological features of remodelling are not very dramatic. This is in contrast to the rat which has extensive remodelling. However even within the rat there are differences between the species(169,170). In addition the younger the animal the more extensive the remodelling, as noted especially with newborn calves(171,172). This may reflect the differences in the relative abundance in growth factors present in these animals.

In rats, when the animals have been removed from the hypoxic environment, the haemodynamics, the remodelling and right ventricular hypertrophy (RVH) can return to baseline, studies have shown that there can be persistent loss of vessels as far out as 8 weeks after recovery(173). Therefore the model is not fully reversible. In addition the animals do not die in the chamber unlike many other potential animal models of PH, therefore it is likely that this is a more moderate model of PH. Nevertheless it is the model which most closely resembles human disease in terms of initiation and allows close study of the pathophysiology of PH(174).

1.3.1.2 Monocrotaline toxin

The monocrotaline (MCT) toxin model is commonly used and is said to represent the PAH pathology more closely than the chronic hypoxic model. Monocrotaline is a pyrrolizidine alkaloid which is derived from the plant seeds of *Crotalaria spectabilis* species(175). The MCT delivered is a pro-drug and undergoes dehydrogenation in the liver to form the active pyrrole form by cytochrome P-450 CYP3A(176). However this agent is quite a toxic pro-inflammatory drug and it has widespread effects on other organs such as inducing a myocarditis in the heart and a disease state similar to hepatic veno-occlusive disease in the liver(177,178).

In the pulmonary circulation there is extensive remodelling and RVH. This results from endothelial cell disruption possibly as a direct toxic effect but also as a result of inflammatory infiltration. There is loss of essential membrane proteins

such as caveolin-1 which has also been shown more recently to be a genetic determinant of PH(124).

Traditionally the drug is administered as a once off subcutaneous injection with dose of between 40-60 mg/kg body weight. After 14 days there is evidence of pulmonary hypertension and remodelling which continues to develop and eventually leads to death in the animal. There are no complex vascular lesions seen in this model(174).

1.3.1.3 SU5416 / Hypoxia model

This model involves the exposure of a rat (or more recently mouse) to 3 weeks of chronic hypoxia and the VEGF receptor-2 (VEGFR-2) inhibitor Sugen-5416(179,180). The VEGFR-2 is essential for the function of endothelium and the presence of the inhibitor leads to endothelial apoptosis. In hypoxia however an apoptosis resistant endothelial cell clone is felt to develop and result in the formation of complex plexiform like lesions. This is a more severe model of PH and one that has similar complex vascular lesions seen to that in IPAH. It also may represent a more realistic model of PAH due to the double hit hypothesis, principally hypoxia and the VEGFR inhibition.

Other models which have been shown to develop pulmonary vascular remodelling but are not discussed further are Vasoactive intestinal peptide knockout model, Apolipoprotein-E knockout model, Angiopoietin-1 overexpression model and S100A4/Mts-1 model(181-184).

The complexity of these models simply underlines the notion that pulmonary vascular remodelling is a heterogenous process which results from a diverse number of stimulants. This perhaps also implies the importance of focussing on downstream pathways which provide a final integration for these many signals and would allow a more targeted approach to inhibition of the cellular responses.

1.3.2 Pulmonary vascular remodelling: Initiating factors

There are a plethora of proteins, peptides, purines which have been found in the last decade to play a part in the development of PH. A brief overview of the key ones is now presented.

1.3.2.1 Growth factors

These are proteins which interact with cell bound receptors and elicit biological responses leading to cell proliferation and differentiation. Important growth factors that have been implicated in pulmonary vascular remodelling include platelet-derived growth factor (PDGF), basic fibroblast growth factor (β-FGF or FGF-2), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF).

1.3.2.1.1 Platelet derived growth factor (PDGF)

Evidence from laser capture micro-dissected pulmonary arteries from IPAH patients undergoing lung transplantation shows that PDGF and its tyrosine-kinase receptors (PDGFR-α and PDGFR-β) are upregulated compared to normal lungs. This is also seen in animal models.(185). Furthermore serum levels of PDGF-B were found to be elevated across the pulmonary circulation in PAH patients compared to normal. PDGFR-β has been shown to be involved in smooth muscle proliferation and is a strong mitogenic stimuli(186). Schermuly et al went further and showed that inhibition of the PDGF receptor using a Tyrosine Kinase inhibitor (TKI), could reverse the pulmonary hypertensive phenotype in two animal models of PH(187).

Clinical translation of these findings however has proved not to be as successful. Despite an initial favourable case report, clinical randomised trials have not shown these agents to be very efficacious(188,189). In fact more recently another TKI called Dasatinib has actually been linked to causing PAH(190). This is despite animal work suggesting Dasatinib could be beneficial and simply underscores the complex nature of intracellular signalling(191).

1.3.2.1.2 Fibroblast growth factor (FGF)-2

This member of a family of heparin binding proteins has been shown to be upregulated in PASMC in response to hypoxia and sheer stress(192,193). Izikki et al has shown that FGF-2 can cause hyperplasia of PASMC and levels are increased in PAH models and in human disease(194).

1.3.2.1.3 Epidermal growth factor (EGF)

EGF is a potent inducer of proliferation and migration of PASMC. Moreover transgenic overexpression of TGF-α which utilises EGFR as its receptor led to pulmonary vascular remodelling(195). An EGFR inhibitor has been shown to improve survival in an animal model of PH(196).

1.3.2.1.4 Vascular endothelial growth factor (VEGF)

The role of VEGF in the pathogenesis of PH is not clear. It certainly is expressed in high levels in the lungs and is one of the key growth factors for endothelial cells and development of the lung as an organ(197). However it has been shown to be capable of both improving and causing pulmonary hypertension. In hypoxic models of PH levels of VEGF were found to be elevated and adenoviral transfer of VEGF to chronic hypoxic rats led to attenuation of right ventricular hypertrophy(198). This was also seen in monocrotaline treated animals although the basal levels of VEGF were actually found to be decreased in that model(199).

However when chronic hypoxia and VEGF receptor-2 inhibition are combined together in a rat there is dramatic development of pulmonary hypertension with the appearance of complex vascular lesions akin to plexiform lesions(180). In addition high levels of VEGF are expressed on human plexiform lesions(200,201). Hence the true role of VEGF in PH biology is as yet unclear.

1.3.2.2 Bone morphogenetic protein (BMP) signalling

The signalling through BMP is complex and tightly regulated and published data suggests that dysregulation in this system is important in the development of pulmonary vascular remodelling. The BMP receptors are part of the TGF-β superfamily and these consist of type 1 and type 2 receptors(202). The TGF-β

ligands signal through heterotetrameric complexes of these receptors of which there are five type 2 and seven type 1 subtypes. The BMP ligands individually signal through a complex of Activin receptor-like kinase type 1/2/3/6 (type 1 receptors) and BMPR2 or activin receptor type IIA or IIB (type 2 receptors).

The type 2 receptors are constitutively active serine/threonine kinases which when in complex with the type 1 receptors after ligand binding, can phosphorylate the inactive type 1 kinase domain. This leads to recruitment and phosphorylation of the receptor-Smads 1/5/8 which are the principle intracellular signalling molecules. These smads then form a complex with a common mediator Smad4 and co-locate to the nucleus(203). These then interact with a group of proteins called Id (or Inhibitors of differentiation) 1-3. As suggested these act to inhibit cellular differentiation and can promote cellular proliferation. The Id proteins have a helix-loop-helix motif which can bind to transcription factors and sequester the transcription factor and inhibit their binding to DNA(204).

Most interest has focussed on BMP2,4 and 9 as being key pathogenic signalling molecules. Experiments *in vitro* on endothelial and smooth muscle cells have shown the signalling path can cause cell survival, proliferation, migration and production. BMP9 in particular has been implicated in ET-1 production. However differential effects of BMP on PASMC have been found, depending on the location of the vessel(205).

It is important to be aware that BMP do not only signal through the above pathway. There is an alternative Smad-independent route characterised by activation of the MAPK pathways. When a BMPR2 mutation exists as is seen in the case of HPAH, the BMP ligand signals through activation of TGF-β activated kinase-1 (TAK-1)(206). This is normally bound to BMPR2 and is quiescent but when a mutation is present the TAK-1 becomes free and can become phosphorylated and active in the presence of BMP. The TAK-1 is a member of the MAPK activation pathway and can lead to phosphorylation and activation of p38 mitogen activated protein kinase (MAPK). This has been shown in BMPR2 mutation-containing PASMC to result in increased cellular proliferation and provides an important link between the MAPK and the BMP signalling pathways(207).Further interaction between BMP signalling and p38MAPK exists.

TAB1 (TAK-1-binding protein) can interact with p38MAPK-α and induce autophosphorylation and activation(208).

Some recent research has suggested that replacement of the BMPR2 protein through gene transfer, induction of BMP signalling or increased protein export from the stressed endoplasmic reticulum could rescue the PH phenotype(209-211). These discoveries may prove of therapeutic value in patients with BMPR2 mutations.

1.3.2.3 Serotonin

The suggestion that serotonin is involved in the pathogenesis arrived with the anorexigen associated PAH outbreak in Europe in the 1960's. These agents are believed to have been involved in reversing the normal serotonin uptake system via the serotonin transporter system (SERT) and this leads to increased serum serotonin(212). Serum serotonin is actually normally quite low and most circulating serotonin is stored in platelets. Although in patients with PAH circulating levels of serotonin have been found to be elevated, more recent findings have suggested this may not be the case(213,214). However there has been the finding that the total platelet content of serotonin is reduced in patients with PAH.

Serotonin is a recognised pulmonary arterial vasoconstrictor but can also induce cellular proliferation including PASMC and fibroblasts(215). The serotonin transporter is increased in vascular cells in hypoxia and in patients with IPAH and can lead to elevated intracellular serotonin levels. Maclean et al have shown that a SERT-overexpressing mouse model develops exaggerated pulmonary vascular remodelling in response to hypoxia(216). Indeed in PASMC derived form patients with IPAH, elevated levels of SERT have been observed(217). Furthermore gene polymorphisms in the SERT gene have been associated with the development of PAH while mice lacking the SERT gene have a blunted response to hypoxia(218-220).

More recently the interaction between serotonin and the female sex hormones oestrogen and its metabolising pathway have been implicated in the pathogenesis of PAH(221). This stems from the recognition that male mice

overexpressing SERT do not get PH whereas the female counterparts do. The serotonin hypothesis is one possible explanation for the observed female preponderance in the PAH registries.

1.3.2.4 mIR biology and pulmonary hypertension

Although not examined in this thesis, mention is required of a blossoming area of research in pulmonary vascular disease. miRNA are small, non coding RNA strands which are between 19 - 24 amino acids in length. These have areas of specificity in them called 'seed' regions which bind to defined mRNA and lead to mRNA degradation of translational repression. This leads to changes in the genetic control of the cell (reviewed in (222)).

Each miRNA can target a number of mRNA which encode key regulatory proteins such as transcription factors and consequently can have an effect on a whole number of genes. Perhaps one of the best characterised mIR pathways is miR-204. This was identified to be significantly down regulated in PASMC from patients with IPAH. When Mir-204 was inhibited in PASMC in vitro it lead to a pro-prolifertaive and anti-apoptotic phenotype. Mechanistically miR-204 was found to repress Src activity which upregulates STAT-3 and inhibition of the miR-204 led to increased activity of STAT-3. In animal models of PH, miR-204 was found to be downregulated and the phenotype could be reversed by using a miR-204 expressing vector delivered intratracheally to rats(223).

However the plethora of miR targets which are being identified would tend to suggest that such a simple straightforward linear pathway connecting one miR to pulmonary vascaular remodelling is not likely, and debate exists as to whether the mIR's will become suitable therapeutic targets.

1.3.2.5 Reactive Oxygen Species

Reactive oxygen species (ROS) are chemically reactive molecules formed as by-products from oxygen metabolism which can interact with a number of compounds and lead to effects on cell structure and signalling, including DNA damage. The adventitia and fibroblasts are an important source of ROS producing them through the action of NADPH oxidase (isoforms NOX2 and 4 are most important in the fibroblast)(224). ROS and superoxide can affect

proliferation of cells via direct modification of transcription factors such as Nuclear Factor-κB (NF-κB), Nrf-2, BMPR2 and MAP Kinases(225). Superoxide can also rapidly oxidise NO to form peroxynitrite which has been shown to induce proliferation of PASMC and PAEC by a variety of mechanisms, leading to uncoupling of the NO system(226). In addition superoxide dismutase 3 (SOD3) has been shown to be important for pulmonary vascular remodelling(227). SOD3 overexpression can protect against pulmonary hypertension. A recent link between SOD3 and BMPR2 signalling in the release of IL-6 has also been shown(228).

1.3.2.6 Pulmonary Vascular remodelling: Hypoxia, HIF and metabolic plasticity

Hypoxia is one of the commonest factors resulting in pulmonary hypertension across the world. Indeed it is estimated that as many as 140 million people live 2500m above sea level and are at risk of PH. In addition there seems to be differences between human populations about how they respond to hypoxia at altitude for example(229). Hypoxia is a term which means that there is an insufficient oxygen supply to a cell(230). This can be a result of a global reduction in the supply of oxygen (for example at altitude with a reduced $Fi0_2$) or at a more specific site (such as a narrowed blood vessel). The effect that hypoxia has on cells can vary depending on the type of cell and the location of the cell, and differs if the exposure is acute or chronic.

Acute hypoxia in the systemic circulation leads to vasodilation and an increase in supply of oxygen. In contrast the pulmonary circulation constricts on exposure to alveolar hypoxia, termed hypoxic pulmonary vasoconstriction. The rationale for this is believed to be a result of embryogenesis where the hypoxic environment *in utero* allows blood to bypass the pulmonary circulation. In adulthood the existence of HPV perhaps allows shunting of blood away from areas of poorly ventilated lung and this prevents ventilation-perfusion mismatch (VQ mismatch).

The mechanisms underlying this HPV are as yet not clearly identified. In the PASMC, hypoxia results in production of reactive oxygen species and calcium release from the sarcoplasmic reticulum, and the influx of extracellular calcium via the opening of cationic channels(21,231,232). This increase in calcium and

concomitant closure of potassium channels ($K_v1.5$) leads to membrane depolarisation and cell contraction. The adjacent endothelial cells are important modulators in the process and the release of ET-1 and reduction in eNOS can stimulate further vasoconstriction.

In chronic hypoxia there is a modified process with changes in the pulmonary vascular cells leading to pulmonary vascular remodelling as already described(233). It is important to note that the hypoxia inducible factor (HIF) pathway is key to many of these chronic changes. HIF is a heterodimer consisting of a constitutively expressed beta subunit and an inducible alpha unit. Under normoxic conditions proline hydroxylation in the oxygen-dependent degradation domain of the α-subunit leads to targeting for proteosomal degradation involving the von Hippel-Lindau protein complex. In hypoxic times, this does not occur and allows for stabilisation of the α-subunit, which combines with the β-unit to allow it to bind to hypoxic-responsive elements (HRE) on various target gene promoters(234). In fact it is estimated as many as 2% of the endothelial cell genome can be regulated by HIF(235).

The development of heterozygote mice ($HIF1a^{+/-}$) with loss of function in HIF-1α has led to an understanding of HIF importance. They are no different to normoxic counterparts but when placed in hypoxia the HIF heterozygote is protected from the development of a pulmonary hypertensive phenotype. In addition, HIF1α increases ET-1 and ET-1 also provides a positive feedback loop by activating HIF1α in PASMC(234).

HIF is also implicated in the metabolic reprogramming of PAH cells. This emerging paradigm in pulmonary vascular remodelling is where the vascular cells adopt an aerobic glycolysis path for energy metabolism as opposed to the normal mitochondrial mechanism. HIF drives the increase in glycolytic genes responsible for this shift and reduces the numbers of mitochondria seen in both PASMC and PAEC(236). Increased metabolic activity in the right ventricle and lungs in animals and humans with PAH has been detected by ^{18}Flouro-deoxyglucose and PET scanning. The use of dichloroacetate which targets the glycolytic pathway has shown benefit in animal models and is being trialled in patients (NCT01083524)(237,238).

The identification of miRNA which are specifically upregulated by hypoxia has identified a further mechanism by which HIF can regulate multiple signalling pathways. These 'hypoxamirs' have been shown to have pleiotropic effects on a wide number of genes which are involved in proliferation, differentiation, apoptosis, inflammation, angiogenesis and fibrosis(239). Further research is required however to fully understand if these agents are wholly responsible for the dysregulated gene expression in PAH or simply reflect further upstream abnormal processes which are mediated through the miRNA(240).

1.3.2.7 Pulmonary vascular remodelling: The importance of Inflammation and autoimmunity

There is increasing evidence that supports the notion that inflammation is important as an initiating factor in PH but also as a driver of continued pulmonary vascular remodelling. It is recognised that autoimmune conditions are more common in women and this occurs in PAH. There is a firm link between PAH and certain autoimmune inflammatory conditions such as systemic sclerosis, systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD). Furthermore the development of PAH in inflammatory and infectious conditions such as HIV infection, Hepatitis C, Castlemans disease and plasma cell dyscrasia's (POEMS syndrome) point to inflammation as being important(241).

Tuder et al, in 1994, were among the first to suggest the importance of inflammation and the immune system in the development of PAH when they noticed the appearance of inflammatory foci akin to germinal centres, adjacent to the plexiform lesion in lungs from patients with PAH(62). This has been confirmed in a repeat pathological study in the modern era with patients who have been receiving disease-targeted therapy. *In vitro* data and animal studies have suggested that the current clinical therapies possess immunomodulatory properties but despite receiving the medication evidence of increased immune activity remained in the lung specimens(61). This suggests that current treatment strategies are not anti-inflammatory.

There have been a number of studies which have shown that many adaptive immune cells are dysregulated in the pathology of PAH, including regulatory T cells (T-regs) and B-cells. However there is also evidence that the innate

immune response is abnormal with impaired Mast cells, macrophages, dendritic cells, and Natural Killer (NK) cell function(242-246). Indeed athymic nude rats that lack T-regs develop severe pulmonary hypertension with monocrotaline and SUGEN even in normoxia, suggesting an immunomodulatory role for these cells(247,248).

Various pro-inflammatory mediators such as the eicosanoids leukotrienes (LTB_4), TNF family members and GM-CSF have been implicated in the biology of pulmonary vascular remodelling. LTB_4 has been shown to cause smooth muscle contraction and proliferation and inhibition of it can reverse experimental animal models of pulmonary hypertension(249). TRAIL is a TNF ligand and has been found to be upregulated in patients with PAH(250). GM-CSF mobilises stem cell progenitors to areas of vascular wall damage and can also recruit macrophages. Treatment of animals with GM-CSF can induce pulmonary hypertension(251). These are simply examples of some non-cytokine mediators of inflammation which seem to be involved in remodelling.

The presence of circulating auto-antibodies to both endothelial and fibroblast cells in patients with IPAH also suggests inflammation as important. Indeed anti-endothelial cell antibodies have been shown to activate endothelial cells and can induce apoptosis, features that are believed to play an initial role in the development of pulmonary vascular remodelling(252,253).

Importantly there has been the identification of anti-fibroblast antibodies in patients with IPAH and systemic sclerosis. These can target cellular proteins involved in the activity of the cytoskeleton and cell contraction and cellular energy pathways(254). Furthermore these antibodies have been shown in systemic sclerosis to activate fibroblasts and lead to a pro-inflammatory phenotype and increase in the cell matrix turnover with increased matrix metalloproteinase (MMP)-1 activity. As previously discussed this is important in pulmonary vascular remodelling in allowing migration of myofibroblasts. It has also been shown that passive transfer of identified autoantibodies from rats with PH into normal rats, led to the development of pulmonary vascular remodelling in those animals(244). This is strongly supporting inflammation in the development of PH.

The immune cells which are located in the pulmonary vasculature and bronchus-associated lymphoid tissue act to produce cytokines and chemokines. Evidence for these having a pathogenic role comes from in vitro, in vivo and human work with increased levels being demonstrated in the serum and lung tissue of patients with PAH and include IL-1,-6-8-10-13 and TNF-α(255). Indeed in experimental animal models antagonism of certain interleukins can prevent PH(256). Perhaps Interleukin-6 is best understood in this way.

IL-6 is a pleiotropic cytokine which can be produced by a number of immune and non-immune cells. It activates through a cis- and trans-pathway involving either its cognate membrane bound receptor or through a complex with a soluble form, sIL-6R. These then form a dimer with a membrane protein gp130 and this leads to downstream activation of STAT-3(257).

With reference to pulmonary vascular disease there are a number of important experimental findings implicating IL-6 in the pathobiology. Increased levels of IL-6 have been reported in the serum of patients with IPAH and systemic sclerosis and have correlated with prognosis(258,259). In addition in POEMS syndrome with PAH, elevated levels of IL-6 have been found. In one patient with PAH and systemic sclerosis and another with PAH and adult onset Stills disease, the use of the immunosuppressive anti-IL6 agent Toclizumab led to the reversal of the PAH(260,261). Elevated levels of IL-6 have been found in patients with COPD who also have PH compared to COPD alone, and the levels correlated with the mPAP. In addition, the GG polymorphism in the IL-6 gene has been associated in COPD with higher pulmonary artery pressures(262). Finally patients with portopulmonary hypertension were shown to have elevated IL-6 levels compared to control cirrhotic patients. It is thus clear that there is strong evidence linking IL-6 in humans to PAH.

In animal models of PH such as chronic hypoxia and MCT, elevated levels of IL-6 have been found. In chronic hypoxic model, Savale et al found IL-6 knockout mice to be protected from development of PH(263). Steiner et al developed a transgenic mouse model which overexpressed IL-6 in the lungs(264). This model demonstrated evidence of pulmonary hypertension and pulmonary vascular remodelling at rest. These features were exaggerated upon exposure to chronic hypoxia with the appearance of some neointimal angio-proliferative obliterative

lesions. PH was also induced when wild-type mice were given supraphysiological doses of IL-6(265). However it should be noted that in some models of inflammatory pulmonary vascular disease, IL-6 has been found to be protective. This has been reported in the Schistosomiasis model of PH(266).

Mechanistically IL-6 has a number of potential routes to resulting in pulmonary vascular remodelling. IL-6 can cause smooth muscle cell proliferation and increases the matrix metalloproteinase-9 which can regulate cell attachment and promote migration of cells. Intriguingly IL-6 can also cause upregulation of the miRNA-17/92 cluster which has an effect to reduce the translation of BMPR2 mRNA. This may be a factor accounting for observed reduction in BMPR2 levels in cells from patients with PAH but without a BMPR2 gene mutation. In addition it has been shown that reducing BMPR2 function in PASMC can lead to increased IL-6(267).

Although the above cytokines are undoubtedly important in PH, recent evidence also suggests that chemokines are involved in the pathogenesis. Chemokines are chemotactic cytokines which act principally to direct cells to areas of inflammation. Chemokines such as Fractalkine, CXCL10, CCL2 and chemokine receptors such as CXCR7 have been implicated in the development of PH in animal models(268-272).

Immunosuppression using dexamethasone in animal models of PAH has been shown to be of benefit(273). However despite this and the evidence presented above, so far anti-inflammatory and immunosuppressive treatments in patients with PAH has been disappointing. Apart from a few conditions such as SLE and MCTD, immunosuppression has not been shown to be efficacious in patients with IPAH or Systemic sclerosis(274,275). This suggests that the correct target of inflammation has not yet been identified.

In summary, from the above discussion, it is clear that inflammation is a critical player in the onset of pulmonary vascular remodelling and that targeting inflammation provides a valid avenue for future research and potential treatment. Indeed one of the best characterised pro-inflammatory pathways which has been identified is mediated by the MAPK family of kinases.

1.3.3 Pulmonary vascular remodelling: the role of protein kinases

Cells require an ability to detect and immediately respond to extracellular stimuli. The cell needs to identify signals on the cell surface, transmit the information downstream and orchestrate a response. This requires a complex signal transduction mechanism which has evolved through time. One of the most recognised mechanisms for cellular responses is the post-translational modification of proteins by phosphorylation. Indeed eukaryotic cells possess a large number of these vital protein kinases which facilitate this process with the term kinome being used to describe the total population.

The importance of these kinases is emphasised by the fact that abnormalities in some kinase signalling has been directly linked to the aetiology of certain human diseases. Perhaps the best understood is that of the genetic translocation yielding the *bcr-abl* tyrosine kinase abnormality linked to chronic myelogenous leukaemia(276). This constitutively active tyrosine kinase can be inhibited by specific inhibitors and has revolutionised the treatment and survival of CML. Therefore identification and inhibition of aberrant kinase signalling pathways is important in the treatment of disease.

1.3.3.1 Overview of the human kinome

The human kinome was first described in 2002 and the term coined by Manning et al. It describes over 500 human protein kinases and constitutes 2% of the genome in many eukaryotic organisms(277). There are eight main groupings which are divided according to the protein sequences of their catalytic domains. We will discuss three main groupings: tyrosine kinase, serine/threonine kinases and G-protein complex kinases as these are perhaps the predominant cellular signalling pathways in the biology of PAH(278).

1.3.3.2 Tyrosine kinase signalling

These are membrane bound receptors with intrinsic tyrosine phosphorylation activity. When a ligand binds to the extracellular domain, it induces receptor dimerization and autophosphorylation of the intracellular domain tyrosine residues(279). This now forms sites for docking of molecules with Src homology 2(SH2) components and activates downstream pathways such as Ras, Janus

kinases and PI3K/Akt(280). This then ultimately leads to an effect on genetic transcription. There are approximately 20 identified families and as previously discussed some are implicated in pulmonary hypertension, namely PDGF, EGF, VEGF and FGF(281).

1.3.3.3 G-protein coupled signalling

The G-protein coupled receptors are receptors which have seven transmembrane domains and are linked via an intracellular domain to G-proteins. These consist of heterotrimeric subunits (α,β and γ) which in the inactive state bind GDP but when activated through ligand binding to the receptor and conformational change can bind GTP. This then allows the diffusion the Gα unit to interact with its second messaging system. This is either adenylyl cyclase, phospholipase-C or rho(282). In pulmonary hypertension, examples of the G-protein receptors implicated are endothelin and prostacyclin.

1.3.3.4 Serine / Threonine kinase signalling

The serine / threonine kinases are enzymes which act to phosphorylate serine at threonine residues on target proteins. The TGF-β superfamily work through this mechanism. The type 2 receptor binds the ligand and then dimerises with the type 1 receptor. This leads to phosphorylation and activation of the type-1 receptor, leading to receptor-Smad phosphorylation(283). In terms of relevance to pulmonary hypertension the BMP system utilises this form of receptor.

However another important kinase system is that of the Mitogen activated protein kinase pathways, and mounting evidence from our group suggests these are important intracellular kinases in cellular responses to stimuli which can induce PH.

1.3.4 The biology of mitogen-activated protein kinase (MAPK) signalling

The MAPK signalling pathway is recognised as one of the key intracellular pathways and biological processing systems allowing cellular responses(284). These are serine/threonine kinases and have been characterised in mammals

into seven groups(285). The so-called conventional MAPK are the best studied and characterised and fall into four distinct subgroups within the MAPK family.

1. Extracellular signal -regulated kinases (Erk) family 1/2
2. c-jun-N-terminal kinases (Jnk) family 1/2/3
3. ERK5
4. p38 MAPK family (α,β,γ,δ)

The remaining members are described as atypical MAPK's as they do not share many characteristics of the above members. Indeed they do not share the standard MAPK activation pathway consisting of three evolutionary conserved kinases acting sequentially on each other. This is a key feature of the conventional MAPK members(286).

The MAPK has an immediate upstream MAPK-kinase (MAPKK) which in turn has a MAPKK-kinase (MAPKKK). The general cascade of signalling for the MAPK family is shown in Figure 1-1. The MAPKKK is activated and phosphorylated by interaction with cellular signalling mechanisms such as Ras/Rho and G-proteins, which then leads to phosphorylation of the MAPKK. This activates the MAPK by dual phosphorylation on a well-conserved Threonine-X-Tyrosine motif which is found in the activation loop of the kinase subdomain VIII. The MAPK then phosphorylate serine or threonine residues on target proteins which have a Proline residue following the sequence: they are Proline-directed kinases. One of the principle groups which the MAPK can activate is the MAPK-activated protein kinases (MAPKAPK) which can then amplify the signal cascade by targeting further proteins not directly phosphorylated by the MAPK(284).

Substrate specificity for the MAPK is conferred by the proline specific sequence, the use of scaffolding proteins which allows compartmentalisation of the signalling path, and the use of specific interaction docking sites between MAPK and its substrate. The MAPK are usually located in the cytosol but after activation they can also translocate into the nucleus to allow targeting of transcription factors(287).

The MAPK family is key to many cellular responses to extracellular stimuli including hypoxia and mitogens. It is an evolutionary conserved mechanism by which cells can be alerted to environmental danger signals. Of the 7 groups, the p38MAPK family has been described as the principle component of the cellular stress reaction(288). Since pulmonary hypertension can be induced by so many 'stressful' stimuli it was felt that this would be a logical and rational target to explore the hypothesis of the role of p38MAPK in PH. Hence the reason our laboratory has focussed on the study of this in pulmonary hypertension.

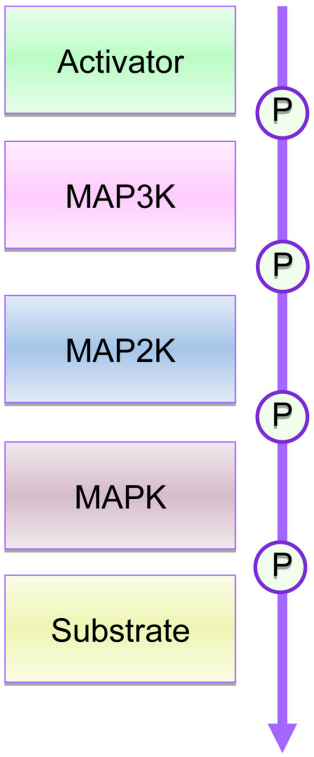

Figure 1-1 The signalling cascade template for the MAPK family

1.3.5 The role of p38MAPK

The p38MAPK family was first identified as a rapidly tyrosine phosphorylated 38kDa protein in response to lipopolysaccharide (LPS), with the tyrosine phosphorylation occurring on a conserved motif of Thr-Gly-Tyr (TGY)(289). This

was then identified as p38MAPK and was identified as a homologue of the key yeast osmotic-responsive protein Hog1 in *Saccharomyces cerevisiae*, underlining its conserved importance in nature. Indeed there are similar p38MAPK proteins in fly, frog and worm. This was identified as the alpha isomer, p38MAPKα, and its documentation was soon followed by the discovery of 3 other family members, p38-β, p38-γ and p38-δ.(290)

The family members share approximately 60% homology with each other and 40% with the other MAPK families. They are encoded by different genes: p38-α (MAPK14), p38-β(MAPK11), p38-γ(MAPK12) and p38-δ (MAPK13). The members differ in their tissue expression and mechanisms of activation. The p38-α and –β are ubiquitously expressed in cells (although p38MAPKα is greater than β) and the p38-γ and –δ are more tissue restricted (for example p38-γ in skeletal muscle and p38-δ in endocrine glands). The p38MAPKα is felt to be the most critical as MAPK14$^{-/-}$ genetic knockout mice are not viable and die during organogenesis due to a defect in placental development(291). Despite this there is actually a limited degree of redundancy provided within the family members as murine knockouts in the other members are viable and show no clear phenotypic abnormality(292).

The p38MAPK family are also characterised by the inhibition of activity provided by the pyridinyl-imidazole drugs and the cellular activation of MAPKAPK-2(293). The pyridinyl-imidazole agents were first used to identify and probe the signalling mechanism of p38MAPKα as it has the greatest selectivity for the alpha isoform, although it can inhibit the other members too. These drugs acting on the alpha isoform were initially identified as anti-inflammatory agents as they inhibited the production of pro-inflammatory cytokines. This immediately suggested the importance of p38MAPKα in the inflammatory process.

The p38MAPKα isoform is the best defined of the group members and since it has the most widespread expression and is important in driving inflammation and the response to varied extracellular stimuli, the next part of the introduction will focus on the biology of this isoform. Reviews on the other isoforms have recently been published, but they are outwith the focus of this thesis.

1.3.5.1 The structure of p38MAPK-alpha

The structure of p38MAPK has been characterised by x-ray crystallography. This reveals a similar structure to other protein kinases and other members of the MAPK family. The structure involves 2 domains (N- and C-terminal lobes) separated by a deep groove wherein various substrates could interact. The N-terminal domain acts to bind the ATP whereas the C-terminal lobe has the catalytic activity(294).

The initiation of enzymatic activity by p38MAPK occurs through phosphorylation of the activation loop or phosphorylation lip. This is a Thr-Gly-Tyr sequence which in the unphosphorylated state orientates itself and adjacent residues to block the peptide binding site. Phosphorylation of the targeted Threonine and Tyrosine residues leads to a conformational change which then exposes the peptide binding site.

In addition the active catalytic site of p38MAPK is misaligned in the non-phosphorylated state by 3-5 Angstroms which allows the enzyme to be inactive. This key site involves a conserved Asp-Phe-Gly (DFG) motif which is essential in determining the catalytic activity(295). Phosphorylation of the activation loop allows the conformational change to occur and bring the key catalytic residues of the active loop into closer proximity.

1.3.5.2 The signalling cascade of the p38MAPK-alpha isoform

There are a number of stimuli which can filter through to activate the p38MAPK pathway through canonical or non-canonical pahways. These include, growth factors, cytokines, lymphocytic cell interaction and environmental signals such as UV light or oxidative stress and signal through their respective cell surface receptors(296,297). Figure 1-2 shows a general outline of p38MAPK signalling and it is recognised that most stimuli signal through the canonical pathway which is now described.

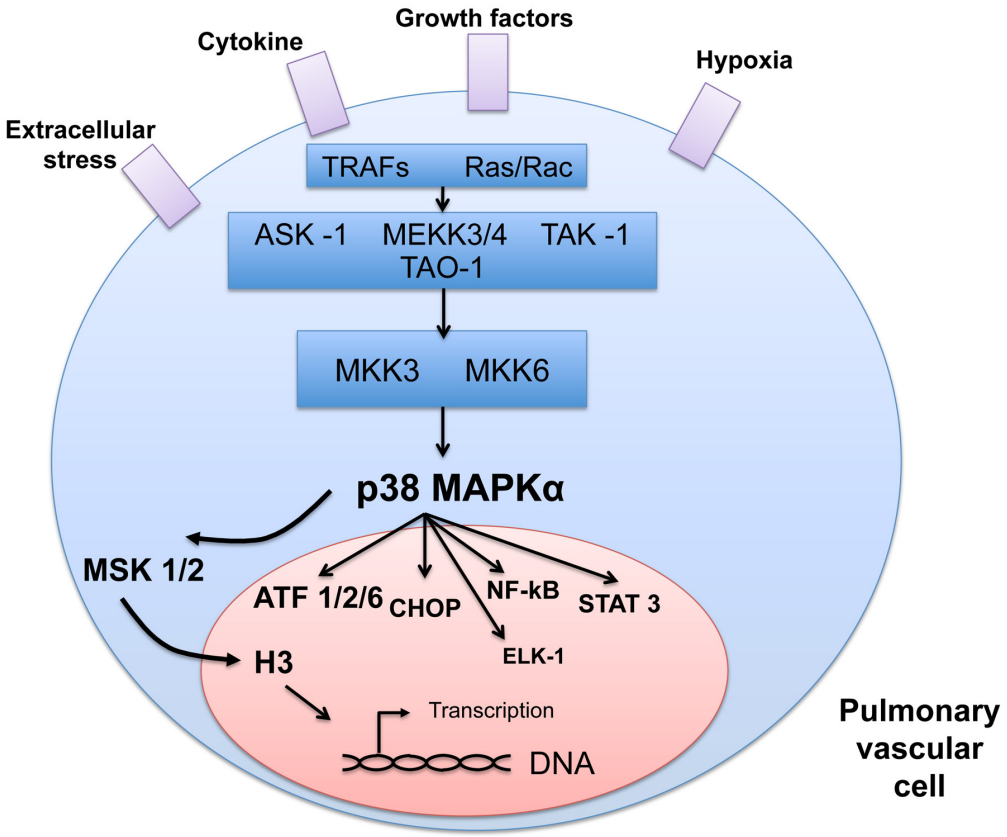

Figure 1-2 Outline of key intracellular pathways for p38MAPK

The first point in the signalling chain is to filter the cell surface receptor activation to the MAPKKK. This is complex and differs according to the stimulus. For example in the TGF-β cytokine receptors TAK-1 is the principle MAPKKK which is activated by the interaction of TNF-receptor-associated-factor (TRAF)-6 with the cell surface receptor. TRAF-6 is a E3 ubiquitin ligase and acts as a scaffold protein which allows the interaction of the TAK-1 and TRAF-6(298). This activates the TAK-1 which can occur independently of activation of the usual Smad signalling path associated with TGF-β superfamily(206).

The GTP-binding protein families Rho/Rac/Cdc42 can also interact between G-protein coupled receptors and various MAPKKK such as MEKK-1. Our group has previously shown that in the pulmonary artery fibroblast the Rac-1 GTP signalling

protein is important in the cellular response to hypoxia (unpublished work, Carlin 2009).

There are a number of MAPKKK, including ASK-1 and TAK-1, which are comprehensively listed in the table 1-2. Many of these proteins can activate both p38MAPK and JNK, however differential activation is seen and is probably conferred by cell type and compartmentalisation of the signalling cascade.

MAPKKK			
Apoptosis-signal-regulating kinase (ASK)-1	Dual-leucine-zipper-bearing kinase (DLK)-1	Transforming growth Factor(TGF)-b Activated kinase (TAK)-1	Thousand-and-one amino acid (TAO)-1 and 2
Mixed-lineage kinase (MLK)-3	MAPK/ERK kinase kinase (MEKK)-3 and 4	Leucine zipper and sterile α-motif kinase (ZAK)-1	TAK-1 Binding protein (TAB)-1

Table 1-2 Upstream activators of p38MAPK cascade

The MAPKKK's target downstream MAPKK's by phosphorylation of conserved serine/threonine residues in the activation loop of the MAPKK's. The main MAPKK's that activate p38MAPK are MKK3 and MKK6, although MKK4 has also been shown to be involved to a lesser extent. Moreover MKK6 can activate all p38 isoforms although MKK3 is more selective for p38-α,-γ.-δ isoforms(299). These are immediately upstream of p38MAPK and act as dual kinases which target the TGY motif in the activation loop.

This phosphorylation leads to a conformational change in the activation loop which then allows the ATP binding catalytic site to become open and interact with the substrates. Substrate specificity is conferred by the presence of docking domains which are separate from the serine/threonine targets on the substrates.

The interaction between substrate and kinase also depends on scaffolding proteins which act to physically link the molecules together to allow phosphorylation. An example is the OSM (osmosensing scaffold for MEKK3) which allows Rac, MEKK3 and MKK3 to complex with p38MAPKalpha(300).

There are a multitude of MAPK targets which include protein kinases, transcription factors and cytosolic proteins. The effects of p38MAPKα are broad and include regulation of gene transcription through chromatin modification, activation of transcription factors, regulation of protein degradation, mRNA stability, cell migration, cytoskeletal function and apoptosis(206).

The immediate downstream targets that allow amplification of the p38MAPKα signal are MAPKAPK-2 and -3 (also known as MK2 and MK3). These can have effects on transcription factors such as CREB, key translational promoters (eEF2K) and proteins such as Argonaute and heat shock protein (hsp27)(301,302).

As a result of interactions with downstream proteins, the p38MAPK pathway can cross-talk with a number of other vital intracellular pathways as shown in Figure 1-3. This interplay between pathways is important as it allows the fine-tuning between biological signals. For example activation of the p38MAPK path leads to deactivation of the ERK pathway via direct interaction between molecules and also increased activity of PP2A, a protein phosphatase which inactivates ERK(303). A similar structure is reported for the JNK pathway.

Clearly, given the fact that p38MAPKα can control so many processes means that control of both activation and deactivation is of prime importance. The length of activation of p38MAPKα is critical as this determines the signal intensity and the ultimate effect. Again, there are a multitude of control processes. Perhaps the most important is the dephosphorylation of the kinases through the action of single protein phosphatases (protein phosphatase 2A or 2C) and dual phosphatases (MAPK phosphatases or MKP 1,4,5,7)(304). These phosphatases can also be transcriptionally upregulated by similar stimuli which activate the p38MAPKα, thus providing a negative feedback control loop.

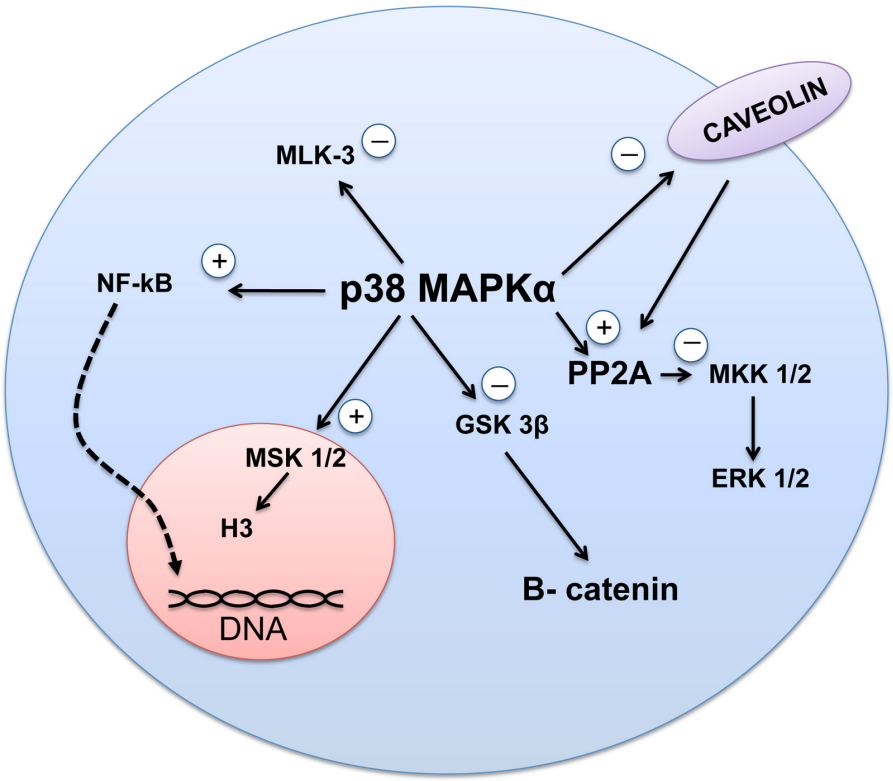

Figure 1-3 Cross-talk between p38MAPK and other intracellular signalling pathways

1.3.5.3 The role of p38MAPK and the alpha isoform in cardio-respiratory disease

The p38MAPK pathway is involved in the inflammatory driven pathogenesis of a number of diseases such as Crohns disease and rheumatoid arthritis(305,306). In particular the synovial and articular destruction observed in RA has been reduced in animal models using p38MAPK inhibitors.

More recently the activity of p38MAPK in the pathogenesis of cardio-respiratory diseases have been investigated. In chronic obstructive pulmonary disease (COPD) increased staining for phosphorylated-p38MAPK has been observed in bronchial epithelium in alveolar walls and macrophages. In animal models of asthma, using allergen induced airways hyperresponsiveness, upregulation of p38MAPK is seen and inhibition can reduce the inflammation and reduce vascular leak via VEGF. Conditional p38MAPKα allelic knockout in lung epithelial cells leads to dysregulation of stem cell growth and can become sensitised to tumourigenesis(307).

In a number of cardiovascular diseases, the p38MAPK pathway has been shown to be of importance. In atherosclerosis the pro-inflammatory signals produced by p38MAPKα (IL-1, TNF-α) can mediate the development of atherosclerotic plaques. In addition low density lipoprotein (LDL), a key biological stimulus to atherosclerosis, can activate p38MAPK. In systemic hypertension generation of reactive oxygen species has been linked to p38MAPK and results in reduced nitric oxide availability(308). This leads to smooth muscle contraction. Our own group has also observed this in the pulmonary circulation(309). In animal models of myocardial damage there is increased p38MAPK activity which favours the upregulation of pro-fibrotic genes and inhibition can result in the reduction of infarct size.

As a consequence of these findings, p38MAPK inhibitors were developed which could be used in patients.

1.3.5.4 The development of p38MAPK inhibitors

The first drugs to be identified that target the p38MAPK group were the pyridinyl-imidazole drugs with the prototype being SB203580. These drugs were initially developed as anti-inflammatory agents and they act by binding competitively to the ATP binding site. Although said to have targets on other non-MAPK proteins, these agents at low dose have most specificity for p38MAPK alpha, then beta. They do not inhibit the p38-γ or p38-δ due to substitutions in amino acid residues in the binding sites such that there is allosteric inhibition to binding.

There are now a number of p38MAPK inhibitors which can be used. The prototype identified was SB203580 and that this binds into the ATP binding pocket in the p38MAPK-alpha structure(310). The pyridine ring nitrogen allows a bond with the methionine residue and stabilises the structure within the pocket.

A novel agent and more specific inhibitor for p38MAPK –alpha is PH-797804. This agent is contrasted with SB203580 in Table 2-3, in terms of kinase specificity and structure. However the mechanism of inhibition of p38MAPK is very similar, namely ATP binding inhibition. However the specificity for the alpha isoform is afforded to it by two mechanisms(311). There is a bidentate hydrogen bonding between the PH-797804 pyridinone carbonyl oxygen and Gly-110 and Met-109 which requires a peptide bond flip only seen when the glycine residue is present (the TXXXG motif). In addition the 2,4-difluorophenyl group binds into a lipohilic pocket in the alpha isoform which is gated by Thr-106, where it is shielded from solvent. Taken together these should lead to increased specificity.

Initial developments of more specific p38MAPKα inhibitors were met with undesirable side effects such as abnormal liver function and skin rashes. This was felt to be a result of poor drug selectivity and off target effects on other kinases. Therefore many of the development programmes began to focus on improving drug structure, specificity and tolerability with the result that there are now a number of new more specific p38MAPKα inhibitors available for clinical trials.

Pharmaceutical companies such as Pfizer and Glaxo-smithkline (GSK) have developed agents that are being actively used in clinical trials of COPD and vascular diseases with good initial results in proof of concept and tolerability studies(312-314).

The fact that there are now these drugs available which can safely target p38MAPKα and seem to be better tolerated than historical drugs, justifies further research into the p38MAPK pathway and its role in various inflammatory related disease processes.

1.3.6 Pulmonary vascular remodelling: The interaction of hypoxia, p38MAPK and pulmonary artery cells

It is clear from the previous discussions in this introduction that the pulmonary artery fibroblast is important, if not critical, in the development of pulmonary vascular remodelling. In addition, hypoxia is a significant cause of pulmonary hypertension across the globe with one of the earliest pulmonary vascular responses being the development of marked adventitial thickening. The p38MAPK pathway is a vital intracellular signalling system which can integrate extracellular stress into a defined cellular response. Therefore it seemed logical to investigate the activity of p38MAPK signalling in the pulmonary artery fibroblast in response to hypoxia and our group has previously published the results of these studies.

The key results identified, which set the context for the work in this thesis, are outlined as follows.

- The exposure of PAF to acute hypoxia leads to increased cellular proliferation.

This has been shown to occur in the absence of other mitogenic stimuli and the proliferation is more sustained and faster compared to PASMC or PAEC(29). In addition the effect has been reproduced in other laboratories and it is conserved across species (rat, bovine, human)(315,316).

- PAF from chronic hypoxic animals have a phenotypic switch making them constitutively hyperproliferative.

Fibroblasts isolated from chronic hypoxic animals but grown in normoxia demonstrate an increased response to mitogens compared to normal PAF from normoxic animals(32,317,318). Other groups have shown that these cells also have different secretory profiles in terms of chemokines, cytokines and mitogens(48). This suggests that these cells have undergone a ‘phenotypic’ change in that there is loss of the normal control mechanisms which renders the cells quiescent unless activated. The CHPAFs are now constitutively active.

- Acute hypoxic proliferation of PAF and chronic hypoxic phenotypic switch is mediated through a p38MAPK dependent mechanism.

The proliferation in acute hypoxia was abrogated by incubation with SB203580, a p38MAPKα inhibitor(318). Although ERK phosphorylation is also observed and can be involved in cell proliferation, there was no effect on the hypoxic proliferation seen when UO126, a specific ERK inhibitor, was added. In the chronic hypoxic PAFs there is constitutive activation of the p38MAPK pathway with high levels of phosphorylated p38MAPK. Furthermore this pro-proliferative capacity can be blocked by the administration of p38MAPK inhibitors(317). This suggests an important role for p38MAPK in the control of cellular proliferation and activation of these cells.

- The p38MAPKα and -γ isoforms are actively phosphorylated in the fibroblast in response to hypoxia.

Importantly only the p38MAPKα and -γ isoforms have been shown to be actively phosphorylated in the fibroblast in response to hypoxia(319). This suggests cellular and p38MAPK isoform specificity for this process.

- Hypoxic induced proliferation of PAF is pulmonary circulation specific and not observed in the systemic circulation.

This is critical. The proliferation of PAF in response to hypoxia is not seen in systemic vasculature derived fibroblasts(320). Moreover the p38MAPK pathway is not activated in the systemic fibroblasts in response to acute hypoxia or indeed when isolated from chronically hypoxic animals(317). It emphasises that the p38MAPK pathway and the pulmonary fibroblast respond to certain stimuli in a different manner compared to the systemic circulation.

- Increased p38MAPK activity in endothelial cells can reduce endothelial function and NO generation

Our group has shown that the hypoxic pulmonary artery increases p38MAPK activity and this leads to an increase in superoxide generation and a reduction in NO. This leads to pulmonary vasoconstriction and increases the resistance across

the vascular bed. Inhibition of p38MAPK leads to reversal of these features and restores endothelial function(309). This agrees with previous findings that suggested p38MAPK is involved in acute hypoxic pulmonary vasoconstriction but further indicates the importance of the process in chronic hypoxic vasoconstriction.

- Hypoxic pulmonary artery fibroblasts can stimulate smooth muscle cell proliferation

It is known that hypoxic PAF can release mitogenic stimuli, which can have a paracrine effect on adjacent smooth muscle cells, and lead to increased proliferation of the PASMC(321). Our group has shown this to be blocked by pre-treatment of the fibroblast with SB203580 indicating that p38MAPK is involved in the release of the mediators(322).

- Activated pulmonary artery fibroblasts can release collagen

The PAFs in response to hypoxia or other stimuli can result in increased secretion of collagen. This can increase the vessel wall and results in increased stiffening and reduced compliance of the pulmonary circulation(323).

In terms of intracellular processes there is a number of potential areas of cross-talk between p38MAPK and other key signalling pathways which can offer a link between p38MAPK and development of PH.

- p38MAPK positively regulates NF-κB pathway

p38MAPK can interact and stimulate the NF-κB pathway at a couple of points. NF-κb is a pro-inflammatory pathway and can upregulate a number of cytokine genes. One control by p38MAPK is by epigenetic control whereby phosphorylation of a serine at H3 histone can lead to chromatin conformational change and lead to exposure of NF-κB promoters for cytokines such as IL-8(324). This effect is thought to be mediated by MSK-1 rather than a direct p38MAPK phosphorylation. In addition p38MAPK can activate the p65 subunit of NF-κB by phosphorylation of IKK-β which then allows NF-κB to upregulate transcription of genes such as IL-6(325,326).

- p38MAPK can activate the beta-catenin system

Normally GSK-3β is controlled by Akt phosphorylation, however p38MAPK can also phosphorylate this enzyme and therefore inactivate it(327). This leads to inhibition of beta-catenin degradation and accumulation in the nucleus resulting in an increase in gene transcription in a pro-proliferative phenotype.

- p38MAPK can stabilise HIF-1α levels

Our group has previously shown a link between the HIF-1α and p38MAPK activation in fibroblasts(319). In hypoxia there is increase in HIF-1α which when repeated in the presence of a p38MAPK inhibitor is attenuated. This suggests an interaction between p38MAPK and HIF, either by stabilisation of HIF with p38MAPK, or p38MAPK works upstream of HIF(328,329). Either way p38MAPK via HIF seems to be involved in the up-regulation of hypoxic responsive element (HRE) genes.

- p38MAPK is involved in pulmonary vascular constriction

There is evidence from our group and others of p38MAPK mediated pulmonary vascular constriction(309,330). Vasoconstriction is an important component of the phenotype of pulmonary hypertension.

- p38MAPK is linked to increased generation of reactive oxygen species

Our group has shown that in pulmonary artery rings taken from chronically hypoxic animals, there is increased levels of superoxide observed. When these were treated with a p38MAPK inhibitor, the levels of superoxide reduced substantially(309). A p38MAPK stimulator (anisomycin) was also found to increase the generation of ROS in arterial rings. Another study has shown that in systemic vasculature, adventitia derived hydrogen peroxide (H_2O_2) activates p38MAPK pathway to cause smooth muscle contraction and vasoconstriction(331). These facts suggest an important role for p38MAPK in response to ROS in the vasculature.

- p38MAPK can induce a positive feedback on itself via caveolin and PI3K/Akt pathways

P38MAPK can be inactivated by the action of Akt and protein PP2A. This activation of Akt is dependent on an interaction between caveolin-1 and PP2A and p38MAPK can inhibit this interaction, thus negatively affecting the Akt pathway. This would lead to a positive feedback on p38MAPK activity(332). In addition it is possible that caveolin-1, mutations which have been identified in families with PAH, could exert the same stimulatory effect on p38MAPK activity. Importantly myoblast differentiation is also dependent on the dual action of the PI3K/Akt pathway and p38MAPK activity and our group has previously shown that in hypoxia Inositol Trisphosphate (IP_3) is increased in pulmonary artery fibroblasts, linking PI3K, hypoxia and p38MAPK(320).

- p38MAPK signalling is important in BMPR2 mutations

It has previously been discussed that BMP and p38MAPK signalling are linked and that in PASMC with a BMPR2 mutation and defective Smad signalling, p38MAPK signalling becomes active. This leads to a pro-proliferative phenotype. In transfected cell lines with vectors expressing BMPR2 mutations, Morrell et al have shown that this can lead to constitutive activation of p38MAPK(207,333).

- p38MAPK can be anti-apoptotic

The effect which p38MAPK has on the apoptotic pathway differs depending on the cell type. It has been shown to work upstream or downstream of caspases which are the key destructive enzymes in the apoptosis sequence(334,335). Apoptosis in patients with PAH has been shown to be suppressed in PASMC and BMP-4 has been shown to be responsible for this in peripheral PASMC. This effect is mediated by p38MAPK activity, independent of Smad-1(207).

Taken together it is clear that the p38MAPK pathway is important in the biology of many key processes which can lead to pulmonary vascular remodelling and pulmonary hypertension. However p38MAPK signalling is ubiquitous, complex and

pleiotropic and inhibition of this pathway may prove to have serious off-target effects. However the effects in the fibroblast have been shown, at least in hypoxia, to be circulation specific and this may provide a degree of specificity to the pulmonary circulation when proposing to target the p38MAPK pathway. Furthermore in the fibroblast there is isoform specificity which again could allow for more focussed targeting on the alpha isoform and avoid inhibitory effects on the other isoforms.

The fibroblast can respond to many different stimuli and may be one of the first cells to react to stimuli such as hypoxia which can lead to pulmonary hypertension. Evidence shows that the p38MAPK pathway is important in this cell for proliferation and release of mitogens which can stimulate adjacent PASMC, although what those mitogens are is unclear. The role of inflammation in the aetiology of pulmonary hypertension is now recognised. Fibroblasts in general are known to be able to release pro-inflammatory chemokines and cytokines. The p38MAPK pathway in other cells, such as neutrophils, has been shown to be pro-inflammatory and in rheumatoid arthritis, the use of p38MAPK inhibitors has been suggested as an anti-inflammatory treatment.

From these observations it is evident that p38MAPK and the pulmonary artery fibroblast can act together to induce pulmonary vascular remodelling.

1.3.7 Summary

Pulmonary hypertension results principally from pulmonary vasoconstriction and vascular remodelling. In particular the development of pulmonary vascular remodelling is a multifaceted process which involves intricate biological signalling processes leading to abnormal cellular proliferation and progressive narrowing of pulmonary arteries and arterioles. Current strategies to treat patients seem to focus on the vasoconstrictive aspect and act mainly as pulmonary vasodilators. Indeed the underlying structural pathological changes are not felt to be altered despite the modern era of disease targeted therapies.

This leads to the inevitable conclusion that unless we target pulmonary vascular remodelling using anti-proliferative strategies the vasculopathy will continue and pulmonary arterial hypertension will remain incurable. This thesis examines

whether a novel strategy of targeting a common downstream pathway in the biology of pulmonary vascular remodelling can lead to anti-remodelling effects and reverse the pulmonary hypertensive phenotype.

1.4 Hypothesis and Aims

The hypothesis of this body of work is that the p38MAPK pathway, and in particular the alpha isoform, is a critical pro-inflammatory signalling cascade which drives pulmonary vascular remodelling and the modulation of this pathway can prevent and reverse pulmonary hypertension.

The aims identified are:

1. To demonstrate increased activity of the p38MAPK pathway in animal models of pulmonary hypertension

2. To identify the importance of the p38MAPKα isoform in the process of pulmonary vascular remodelling and if inhibition of this isoform with selective inhibitors would have a beneficial effect on animal models of PH

3. To examine a cellular and molecular mechanistic link between p38MAPK inflammatory signalling and pulmonary vascular remodelling which explains the observations

4. To examine the expression of the p38MAPK pathway in the lungs of patients with PAH.

This thesis will demonstrate a key biological link between the pro-inflammatory p38MAPK pathway and pulmonary vascular remodelling through *in vitro* and *in vivo* work and provide some preliminary confirmatory evidence of dysregulation in human disease. In summary this study provides a novel connection between the role of inflammation in pulmonary hypertension and pulmonary vascular remodelling mediated by p38MAPKα.

2 Material and Methods

2.1 Reagents and drugs

All general chemicals were of analar grade. Tissue culture plasticware was obtained from Greiner Labortechnik Ltd (Gloucestershire,UK), Costar (Uk) and Corning Incorporated (NY,USA).

2.1.1 Solutions

Reagent	Composition
Phosphate buffered Saline (PBS)	8g NaCl, 1.16g Na2HPO4, 0.2g KH2PO4 in 1litre distilled water, pH adjusted to 7.4
Cell culture medium	Dulbecco's Modified Eagles Medium (DMEM), 10 or 20% fetal calf serum (FCS), 100iu penicillin/streptomycin/ml, 2mM L-glutamine (obtained from Invitrogen,UK and Sigma,UK)
Wash buffer	0.05% Tween-20 in PBS or TBS
Reagent/Assay buffer	1% BSA (Fraction V) in PBS pH7.2-7.4
Stop Solution	2N H_2SO_4
Substrate solution	1:1 mixture of H_2O_2 and TMB (Tetramethylbenzidine) (obtained from Pierce)
Lysis buffer	RIPA buffer (Thermo scientific) containing protease and phosphatase inhibitors (Calbiochem)

Table 2-1 Details of commonly used experimental reagents
All reagents were obtained from Sigma unless otherwise stated.

2.1.2 Drugs and cytokines

Drug	Action	Supplier	Diluent
Monocrotaline	Endothelial injury to pulmonary circulation	Sigma	1M HCl to dissolve, then neutralised with equal volume of 1M NaOH
SB203580	p38MAPK inhibitor	Calbiochem Selleck chemicals	DMSO
PHA-797804	p38MAPKα inhibitor	Pfizer	DMSO
Recombinant IL-6	Cytokine	R&D systems	Sterile distilled water

Table 2-2 Details of commonly used drugs and reagents

Inhibitor	SB203580	PH-797804
IC_{50}	50nM	26nM
Ki	21nM	3.9nM
Mechanism	Competitive inhibition	Competitive inhibition
Chemistry	4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole	3-bromo-4-[(2,4-difluorophenyl)methoxy]-6-methyl-2-oxo-1(2H)-pyridinyl]-N,4-dimethyl-, (_)-(9CI)
Kinase profiles	Can inhibit beta isoform at 10μM Has some activity against GSK-3β, Raf and LCK	4 times more specific for alpha versus beta isoform. No other kinases inhibited >200uM concentration.

Table 2-3 Comparison of the p38MAPK inhibitors used in thesis
Refs: (311,336,337)

2.1.3 Chemicals

[methyl-^{3}H] Thymidine stock (1mCi/ml) was obtained from Amersham (UK).

Dimethylsulfoxide (DMSO) from Fischer scientific.

2.1.4 Antibodies

Antibody	Company
P38 MAPK total	Cell signaling
P38 MAPK phosphorylated(Thr180/Tyr182)	Cell signaling
P38 MAPK alpha	Cell signaling
Beta-actin	Abcam

Alpha-smooth muscle actin	Abcam
ATF-2 phosphorylated	Cell signaling
Rat anti-IL-6	R&D systems
STAT-3 total	Cell signaling
STAT-3 phosphorylated(Y705)	Cell signaling
Vimentin (R28)	Cell signaling
Anti-rabbit IgG, HRP-linked	Cell signaling
Rabbit (DA1E) IgG isotype control	Cell signalling
Texas red Goat IgG anti-rabbit	Vector laboratories

Table 2-4 Details of type and source of antibodies used

2.2 *In vitro* methods

2.2.1 Primary cell culture

The principal cells utilised in this PhD were either pulmonary artery fibroblasts or smooth muscle cells. These were derived from rat. The explant techniques to grow primary cells have been well established in the laboratory and previously published.

To minimise chances of contamination all procedures involving making up of solutions or changing media were performed in sterile conditions using a clean Microflow laminar flow hood (model number M25121/1). Non-sterile equipment was sterilised using a Prestige Medical 'classic 210' autoclave or filtered through a 0.2μm pore filter.

2.2.1.1 Primary adventitial fibroblast culture

The main pulmonary artery was dissected out from the rat under microscopic control (Zeiss STEMI SV-11).

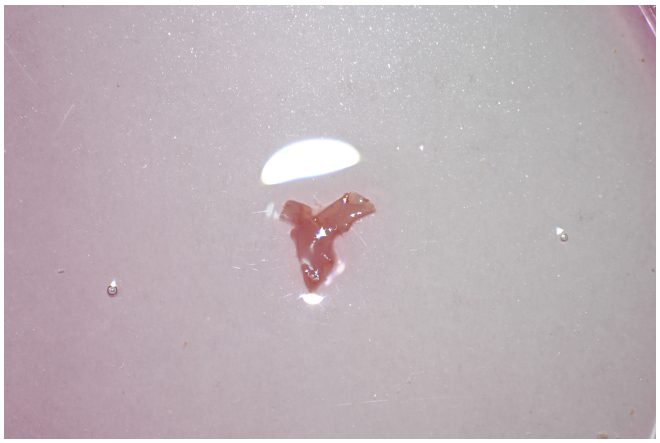

Figure 2-1 Light microscopic appearance of dissected rat main pulmonary artery

This was then cleaned of adherent adipose tissue and the artery cut longitudinally and opened into a flat sheet. The endothelial and muscular layers were removed by a mixture of dissection and abrasion, leaving only the adventitial layer. This was then dissected into 5mm^2 pieces and these were then placed carefully on the base of a 25cm^3 tissue flask. These were then covered with culture media containing 20% FCS. The flask was placed in a humidified atmosphere with 21%O_2 and 5%CO_2 at a controlled temperature of 37^{o}C (Galaxy-R hypoxic chamber). Within 7 days cells grow out from the explant in a monolayer.

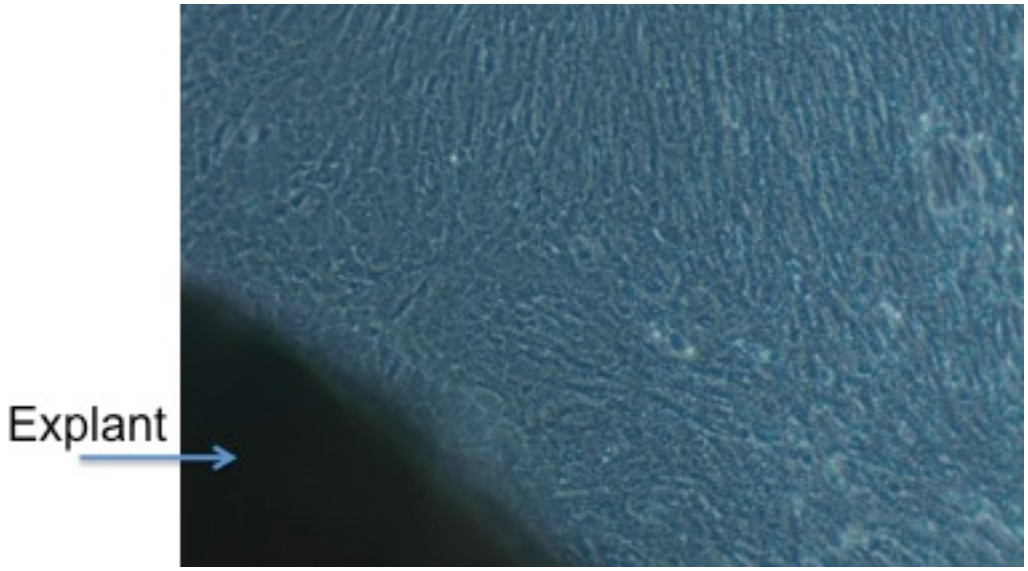

Figure 2-2 Emergence of fibroblast cells from explant of pulmonary artery

Once 50% of the flask was covered with emerging cells, the explants were tryspsinised off and aspirated. The cells were suspended in culture medium and then pipetted into a new T-25 flask and allowed to settle. After a further 7 days the cells were trypsinised and grown in a 75cm^3 tissue flask (T-75).

Cells were confirmed to be true fibroblasts by:

1. Typical appearance and morphology in cell culture
2. Absence of smooth muscle α−actin and eNOS in western blot
3. Positive for Vimentin

Experiments were performed on cells in passage 2-5.

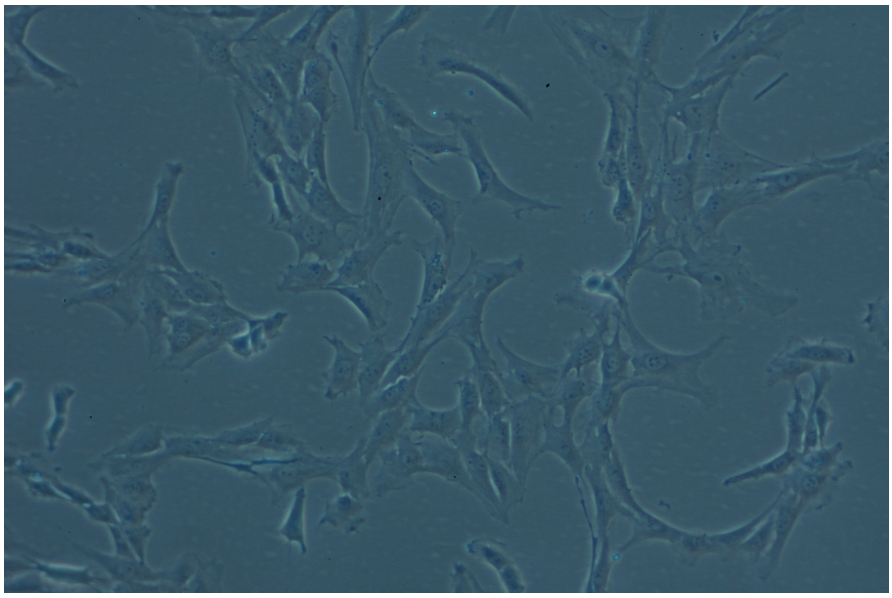

Figure 2-3 Typical appearance of pulmonary artery fibroblasts

2.2.1.2 Primary smooth muscle cell culture

The main pulmonary artery from Sprague-Dawley rats were isolated and under dissecting microscope (Zeiss) the excess adipose tissue removed.

The artery was then placed in a solution containing Hanks balanced salt solution (HBSS) containing 1.5mg collagenase and incubated at 37^{o}C for 20 minutes. The adventitia and the endothelium were then stripped off using fine forceps and

under a dissecting microscope. The tissue was then left for 24 hours in DMEM containing 20% FBS. Then further incubation with 1.5mg collagenase and 0.5mg elastase in 2ml of HBSS at 37°C for 30 mins. The tissue was triturated through a 20G needle 3-4 times and then the mixture was resuspended in 20 ml of DMEM with 20%FBS. The cells were centrifuged and the pellet resuspended a further 2 times.

The cells were then suspended in a T-25 cell culture flask and allowed to adhere for 48 hours before the media was changed. Cell purity was monitiored by observation of typical cell culture morphology and also staining positive for smooth muscle α–actin.

2.2.1.3 Routine cell culture

Cells were grown in 75cm^3 flasks with cell culture medium as outlined previously. The cells were kept in a humidified atmosphere of 5% CO_2 in air (FiO_2 of 0.21) in a Galaxy 170-R incubator (Wolf laboratories, York, UK). Cells were grown to 90% confluency prior to passage.

For cell passage, the culture medium was initially removed and cells were washed with serum free DMEM. Then the cells were washed with 2ml of trypsin solution and left for 10seconds. This was then aspirated and the cells incubated at 37°C for 2-5 mins or until the cells were observed under light microscopy to detach from the culture flask surface. The flask was gently agitated to help cell dislodgement. The trypsin was inactivated by the addition of 10 mls of DMEM with 10% FBS. Then the solution was diluted 1 in 10 by addition of 1ml of the cell suspension into a new flask containing 9 ml of DMEM/FBS. Cells were also plated out in 6-, 12- or 24- well culture plates ready for further experimentation.

2.2.1.4 Preparation of pulmonary artery fibroblast conditioned media

Pulmonary artery fibroblasts were grown in 24 well plates and at 70% confluency were quieseced in serum free medium for 24 hours. Then the cells were replaced with fresh 500ul of serum free media and placed in either normoxic or hypoxic environment depending on the experiment. In addition the media could have various investigational compounds added in, for example Interleukin-6.

After 24-48 hours of incubation the media was removed and stored at -80°C until needed.

2.2.1.5 Cell storage

Confluent cells at early passage were trypsinised and then resuspended in cell culture medium and then centrifuged at 1000 g for 10 minutes in order to develop a cell pellet. The pellet was then resuspended in 1ml of cryopreservation medium (DMEM containing 10% Dimethylsulphoxide (DMSO) and 10% FCS). This was then placed in a crypotube and cooled to 4°C for 2 hours then -20°C for 6 hours and then -80°C overnight. Then it was placed in liquid nitrogen.

For thawing of the cells the cryotubes were placed in 37°C water bath and rapidly thawed. Then the cells were resuspended in 9mls of cell culture medium and dispensed into a cell culture flask. After the cells had adhered the medium was changed.

2.2.2 Acute hypoxic environment

The Galaxy R incubator allows control of the $Fi0_2$ by allowing the supplementation of the atmosphere with nitrogen. This allows control of the internal oxygen levels to between 0-21% whilst maintaining CO_2 at 5%. This is achieved by using nitrogen cylinders connected to a Pneuchange automatic gas cylinder change over unit (NTC, Woulton, Liverpool, UK) which allows fresh supply of nitrogen on demand.

Previous measurements using oxygen sensors and pH probes have demonstrated that over 48 hours of maintenance of cells in this environment achieves a PO_2 of between 15mmHg (2kPa) and 35mmHg (4kPa) and a stable physiological pH. Controls in normoxia were obtained by using a second incubator at normoxic atmospheric conditions.

2.2.3 Determination of cell proliferation and viability

2.2.3.1 [H^3] Thymidine Proliferation Assay

This allows a determination of cellular proliferation by the incorporation of [^{3}H]-Thymidine into DNA during cell division.

Cells are seeded at density of $5X10^3$ – $1X10^4$ cell per well in a 24 well plate. This allowed an appropriate density to let the cells grow up to 60% confluency prior to any experiments. The total volume per well was 500μl. The cells were quiesced by replacement of the 500ml cell culture medium by serum free medium. Then the cells were stimulated by the addition of various investigational products and incubated for 24-48 hours depending on the experiment. Again this could be done in normoxia or hypoxia depending on the experiment.

Dilute [^{3}H]-Thymidine was prepared by adding 10μl of stock solution (1mCi/ml) to 2.5ml of serum free DMEM. For the final 4 -6 hours of the experiment, 25μl of the dilute [^{3}H]-Thymidine was added into the well.

To end the experiment the reaction was stopped by washing the cells twice with ice cold PBS. The proteins were precipitated using 5% Trichloroacetic acid (TCA) and then the lipid fractions solubilised by washing twice with 100% ethanol. The resultant precipitants were then re-dissolved in 0.3M NaOH and left for 30 minutes. For each solution 500ml was added per well.

The solutions were aspirated from the wells and added to 1ml eppendorf tubes and made up to 1ml with Ecoscint XR scintillation solution (Ecoscint, Atlanta, Georgia,USA). The vials were agitated and then left to settle overnight. The resultant radioactivity was expressed as either disintegrations per minute (DPM) or counts per minute (CPM) as measured by scintillation counter.

2.2.3.2 Cell counting and cell viability

Cells were seeded in appropriate wells and experiments performed. At the end of experiments the cells were briefly exposed to 400μl of trypsin solution (0.05% trypsin/ 0.02% EDTA) and once detached the cells were resuspended in 1ml of

PBS. 10μl of the cell suspension was then mixed with 10ul of trypan blue in a 1:1 dilution. Then 10μl of the mixture is gently pipetted out under the cover slip on a Neubauer Haemocytometer. The cells which take up the trypan blue are non-viable. The total cells and non-viable cells are counted and the percentage of viable cells can be calculated.

To determine the total number of cells in the original solution the total number of cells are counted and under the light microscope at x10 power. The following calculation is then used:

Total number of cells in original solution=

$$\text{dilution factor}(2) \times \frac{\text{no of counted cells in 4 squares}}{4} \times 10000$$

In order to use SB203580 in experiments *in vitro* it needs to be dissolved in DMSO. In order to ensure that the DMSO was not toxic to the cells control experiments checking the viability of the cell culture with reducing doses of DMSO were performed. Cell viability was assessed by checking daily under light microscopy and using trypan blue. We found that the cells were not affected as long as the final concentration of DMSO was below 0.1% as shown below.

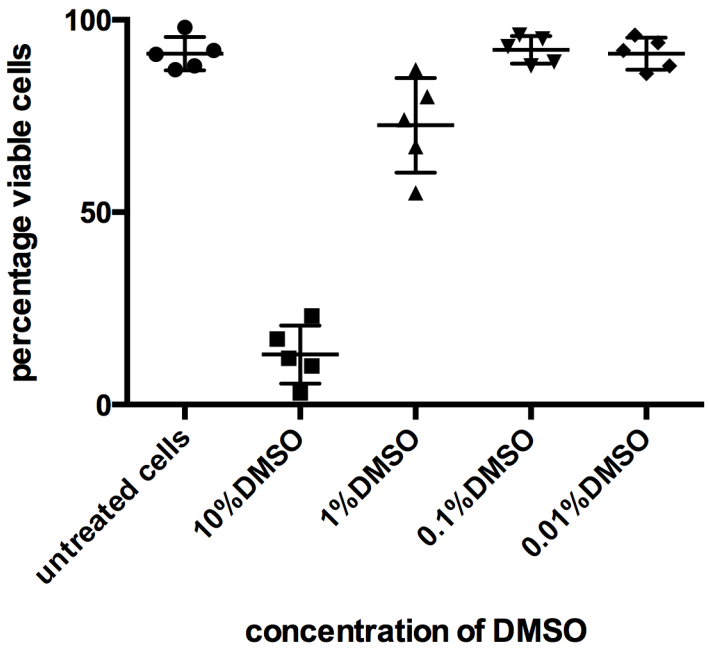

Figure 2-4 Cell viability with DMSO concentrations
Cell viability was assessed using trypan blue and scored as a total number of viable cells compared to non-viable cells. Data represents mean + SD.

2.2.4 Cell immunofluorescence and Confocal microscopy

The general protocol used will be outlined here.

The cells were plated out in 100μl density of approx. $5x10^6$cells/ml. The cells were allowed to settle overnight and grow for 24-48 hours. Then they were washed twice using PBS and then fixed with 3.7% formaldehyde for 10 minutes at room temperature. Then they were washed again with PBS.

The cells were then blocked with PBS containing BSA and 0.3% Triton-X for 60 mins. Then the primary antibody was added to the cells for 1 hour. Then the cells were washed again twice with PBS. The fluorochrome conjugated secondary antibody was added and allowed to incubate for 30-60 minutes in the dark. The cells were washed three times in PBS and mounted with Vectashield mounting stain with DAPI and a coverslip.

The cells were left in the dark overnight at 4^oC. Then using a Zeiss Confocal microscope the immunofluorescence was detected.

2.2.5 Cell migration

The migratory ability of the pulmonary artery fibroblast was measured under various experimental conditions. The principle involves using a 8um pore polycarbonate membrane inserts to separate the well into 2 distinct chambers. The migratory cells can travel through the membrane and then be counted on the bottom of the membrane as per figure. This was a Cytoselect™ kit (Cell Biolabs, INC, San Diego,USA) and was carried out as per manual.

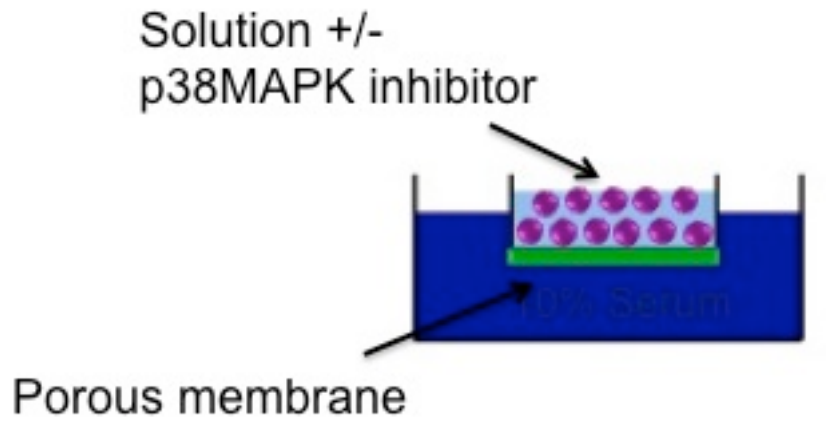

Figure 2-5 Migration assay

Briefly, a cell suspension of fibroblasts was prepared as before using trypsin and resuspended in serum free media aiming for a density of 0.5-1x10^6 cells/ml. The polycarbonate insert is placed in the well and the lower chamber filled with 500ul serum free media plus/minus chemoattractants. For hypoxia experiments there were no chemoattractants.

To the inside of each insert 300ul of cell suspension was added and the wells were incubated at 37^{o}C for 24 hours. The media from the inside of the insert was aspirated and then using a cotton bud the inside was scraped to remove any non-migratory cells. The insert was then moved to a new well that had 400ul of cell stain solution and was incubated for 10 minutes at room temperature. The inserts were then washed in distilled water and allowed to air dry.

The cells on the bottom of the membrane could be counted directly using light microscopy at high power. The inserts were then transferred to an empty well and 200ul of extraction solution added and incubated at room temperature for 10 minutes on an orbital shaker. Then 100ul of the stained solution was now transferred to a 96 well plate and placed in a Sunrise absorbance plate reader and the OD 560nm measured. This allows 2 separate ways to quantify cell migration.

2.2.6 Cytokine immunoassays

Enzyme linked immunosorbent assays (ELISA) were performed for cytokine quantification using paired antibodies and kits as specified in Table following the manufacturers instructions. Briefly, ELISA 96 well plates were coated with 50μl/well capture antibody, diluted in PBS, and incubated overnight at 4°C. All wash steps used appropriate wash buffer. Following 3 washes the plates were blocked for 1 hour at room temperature using 200-300μl of x1 assay diluent buffer in each well. The wells were then washed.

50-75μl of sample was added to the appropriate wells. For the standard curve a minimum of 7 serial dilutions of the standards in duplicate wells was performed for each plate using assay buffer. Two wells were used as blanks with assay buffer only in them. The plates were then incubated at room temperature for a minimum of 2 hours.

The plates were washed a minimum of 3 times and then 50μl of appropriately diluted detection antibody was added to each well and incubated for 1 hour at room temperature. Plates were washed and then HRP-streptavidin (in an appropriate dilution usually 1:1000) was added to each well and then left in the dark for 30-60mins.

A minimum of 6 washes were then performed and then 100μl of TMB substrate solution added to each well and left in dark to develop, with close periodic checking. The reaction was terminated using 100μl Stop buffer. The optical density (OD) was measured in a Sunrise absorbance plate reader at appropriate wavelength (usually 450nm wavelength) with correction set at 570nm.

Cytokine/chemokine	Supplier
IL-6	R&D, Duoset
Endothelin-1	Assay Designs
Rat Proteome Profiler (cytokine array panel A)	R&D systems
Rat Pro-collagen Type 3	Uscn Life sciences Inc.
Rat pro-collagen Type 1	Uscn Life Sciences Inc.

Table 2-5 Details of ELISA, cytokine array and supplier

The following table details the cytokines and chemokines identified in the above multiplex analysis:

Rat Proteome Profiler	CINC-1;CINC-2α/β;CINC-3;CNTF;Fractalkine;GM-CSF;sICAM-1;IFN-γ;IL-1α;IL-1β;IL-1ra;IL-2;IL-3;IL-4;IL-6;IL-10;IL-13IL-17;IP-10;LIX;L-selectin;MIG;MIP-1α;MIP-3α;RANTES(CCL5);CXCL7;TIMP-1;TNF-α;VEGF-A

Table 2-6 Details of detected factors of the rat proteome cytokine profiler

2.2.7 Quantification of ATP in conditioned media

ATP levels in conditioned media were quantified using a bioluminescent assay (Sigma). Briefly ATP is consumed and light emitted when firefly luciferase catalyses the oxidation of D-Luciferin using ATP. The light emitted is related to the concentration of ATP present in the sample. Standards of known ATP concentrations are generated by serial dilution and a standard curve generated. The samples of conditioned media from the cell culture are mixed with an ATP assay mix (containing luciferase, luciferin, $MgSO_4$, DTT,EDTA,BSA,tricine buffer salts) in a glass vial which can be measured in a luminometer. The luminometer integrates the amount of light produced over 6 second interval.

2.2.8 RNA isolation and quantitative polymerase chain (PCR) reaction

Cells were exposed to various conditions and once the experiments were completed then the cells were centrifuged until a pellet was obtained. RNA extraction was performed using Qiagen RNeasy minikits as per manufacturers instructions. Quantification of the RNA was achieved using Nanodrop 1000 machine and then 1ug of RNA used to make copy DNA (cDNA) by standard polymerase chain reaction (PCR) using Multiscribe reverse transcriptase kit (Applied Biosystems) following manufacturers instructions.

Real time quantitative PCR was performed in triplicate for each sample using SYBR Green, primer sets and ABI Prism 7900 Sequence Detection System instrument (Applied Biosystems). Relative gene expression was quantified by comparison of the ‘Ct’method relative to beta-actin as a house keeping gene. Primers were supplied by Integrated DNA Technologies.

Primer name	Primer sequence
Rat Beta actin	5'-GTAGCCATCCAGGCTGTGTTG-3' Forward 5'-TGCCAGTGGTACGACCAGAG-3' Reverse
Rat Interleukin-6	5'-AAGAGACTTCCAGCCAGTTGCC-3' Forward 5'-ACTGGTCTGTTGTGGGTGGTATC-3 Reverse
P38 MAPK alpha	5'-AACCTGTCCCCGGTGGGCTCG-3' Forward 5'-CGATGTCCCGTCTTTGTATGA-3' Reverse
Rat endothelin-1	5'-GGGATGGCCTCGGACCT-3' Forward 5'-CTCTCTGGATGATGCCACTCAG-3' Reverse

Table 2-7 Details of the primers used in qRT-PCR

2.2.9 siRNA knockdown

siRNA to p38MAPK-alpha (Thermo-scientific) was used to knockdown expression in primary cultured fibroblasts. The concentration required to allow greater than 90% cell viability and greater than 80% knockdown of target protein was determined by dose ranging experiments. Then the optimal concentration was transfected into the fibroblasts using lipofectamine (Invitrogen). The cells were left for 48-72 hours before experiments were carried out. Control cells received transfection agent only, negative control and scrambled siRNA. The cells then were stimulated in normoxia or hypoxia for a proliferation assay as previously outlined. Protein knockdown was established by western blot techniques.

Essentially the cells were plated in cell culture wells in antibiotic free medium. Then a stock solution of 5mM siRNA solution was diluted down 1:20 in serum free

media. This was then diluted further with lipofectamine and the mixture added to the wells and left for 48-72 hours to allow maximal effect on protein synthesis. Through a number of experiments the optimal dose of siRNA and lipofectamine was determined to allow maximal cell viability and protein knockdown. Cell viability was assessed by light microscopic appearances of the cells and by trypan blue dye testing.

2.2.10 Detection and analysis of proteins

2.2.10.1 Preparation of cell samples for protein analysis

The cells were grown on 6-well plates and experiments performed once cells reached 90% confluency. Once the experiment had been completed the cells were put on ice and washed with ice cold PBS twice. Lysis of cells was completed by addition of 50ul of Radioimmunoprecipitation assay buffer (RIPA buffer) to each well for 10 minutes. Cells were mechanically disrupted by using a cell scraper and the solution aspirated and stored in an eppendorf tube at -80^{o}C.

2.2.10.2 Preparation of tissue samples for protein analysis

Tissue samples such as lung or heart were also used for analysis of proteins. Tissue had been snap frozen by immersion into liquid nitrogen and then stored at -80^{o}C until ready for further analysis. Then the tissue was thawed on ice, and homogenised using a homogeniser (PowerGen 500, Fisher Scientific) in a 1:20 mix with T-PER protein extraction solution (Thermo scientific USA), containing phosphatase inhibitors and protease inhibitors (Calbiochem). This was then centrifuged and the supernatant removed and the protein concentration quantified using BCA protein assay kit (Pierce, USA).

2.2.10.3 Western blot procedure

2.2.10.3.1 Gel electrophoresis

SDS-sample buffer and reducing agent was added to an appropriate concentration of lysate in a 1:3 ratio. Samples were incubated at 80^{o}C for 5

minutes to denature the proteins and disrupt disulphide bonds. 20ul of sample was then loaded into Sodium Dodecyl sulphate polyacrylamide (SDS-PAGE) gels (Invitrogen). The gels were either 10% or 4-12% NuPage gels (Invitrogen). A molecular marker (See-blue Plus 2, Invitrogen) was loaded into one well to allow determination of the protein sizes. The gels were assembled into a Bio-Rad electrophoresis unit and running buffer added (Invitrogen). A voltage of 150V was applied and run for 1 hour or until the protein front neared the bottom of the gel.

2.2.10.3.2 Transfer to nitrocellulose membrane

Once the gel electrophoresis had completed, the gels were carefully removed and then the proteins were transferred to a nitrocellulose membrane using a Bio-Rad transfer cassette. A constant voltage of 30V was applied for 1 hour. Once finished the membrane was briefly coated with Ponceau red solution to ensure equal transfer of protein bands.

2.2.10.3.3 Immunoblotting

The nitrocellulose membranes were then washed in PBS/T or TBS/T (PBS or TBS containing 0.02% Tween-20). The membrane was then blocked using either 5% BSA in wash buffer or 10% non-fat milk (Marvel) depending on the antibody to be used. The membrane was agitated at room temperature for 1-2 hours. The blots were then incubated overnight at 4^0C in 5ml of 5% non-fat milk with an appropraiate dilution of the primary antibody, but usually 1:1000.

After this the blots were washed 3 times for a minimum of 20 minutes in wash buffer and then the blot was incubated with 5% non-fat milk in wash buffer with an appropriate dilution for the secondary antibody conjugated to horseradish peroxidase (HRP). This was for 1 hour and the blots were then washed.

To detect the protein bands the blots were incubated with ECL chemiluminescent solution (Amersham) for 1 minute. The membrane was then placed in a developing cassette and an X-ray film placed next to it for different

time periods of exposure. The film was then developed using a Kodak X-OMAT processor.

2.2.10.3.4 Re-probing

In certain experiments the same blot was used to look for different proteins. In this case the blot would be incubated with Stripping buffer (Restore™ Thermo scientific) for 30 minutes at 50°C with agitation. Then the blot was reprobed with secondary antibody to ensure that the primary antibody had been removed. If this was the case the blot was re-probed with a different primary as before.

2.2.10.3.5 Densitometry

Blot densitometry was analysed by using Image J (USA) and Adobe Photoimage or Bio-Rad software and scanner.

2.2.10.4 Detection and isolation of protein bands on SDS-PAGE gels

Bands to be identified on gels were stained using Novex Colloidal Blue Staining kit (Invitrogen). This identified bands on SDS-PAGE gels. Once the gels were identified then they could be isolated from the gel using sterile aseptic conditions in a laminar flow hood. Sterile scalpel blades were used to cut out the bands of interest and then placed in sterile eppendorf tubes. These were then analysed on a proteomics platform in the University of Dundee.

2.2.11 p38 MAPK activity assay

This was supplied as a kit from Cell Signalling. Briefly, equal amounts of homogenised lung tissue on ice from experimental animals were added to sepharose beads which had immobilised anti-phospho-p38MAPK antibody attached. The slurry was incubated overnight at 4°C with gentle agitation. Then an in-vitro kinase assay is performed using 1ug of ATF-2 as a substrate and ATP. The read out for this is on a western blot with staining for phospho-ATF-2. The staining intensity determines the concentration of ATF-2 that has been phosphorylated and hence reflects the amount of active p38MAPK in the initial sample.

2.2.12 Collagen assay

This allows the detection and quantification of soluble and recently synthesised collagen in tissues or cell supernatant. Supplied by Biocolor, Belfast,UK.

The lung is weighed, then dissected into pieces and washed in ice cold PBS on an orbital shaker. Then to release the acid insoluble collagen, the lung was incubated overnight at 4^{o}C with pepsin (Sigma) at a concentration of 0.1mg/ml of 0.5M acetic acid. The supernantant was then concentrated and centrifuged to leave a pellet in a low protein binding eppendorf. Then the Sircol dye (Sirius red in Picric acid) was added and agitated for 30minutes. Following various washes and centrifugation with acid-salt wash, the pellet was redissolved in NaOH to give a red colour. 100ul was then used to determine the absorbance at 570nm wavelength. Standards underwent the same procedure using blank solvent, 5, 10 and 15μg of known collagen stock.

2.2.13 Myeloperoxidase assay

A bijou containing 2ml of MPO buffer solution was weighed. Then a sample of lung tissue was added to the solution and the combined weight was recorded. The solution was homogenised and then sonicated for three 30 second periods at amplitude of 10μm. Then 1ml of solution was transferred into eppendorf tubes and spun down at 20000g for 4 mins. Then triplicate samples of 14μl was placed onto a standard 96 well ELISA plate.

The addition of 200μl of MPO reagent buffer then started the experiment. The reaction catalysed by MPO allowed detection with the ELISA plate reader. The results appear as mean change in OD sampled at time intervals and presented as mean change in OD/min. The results are normalised by weight by the equation MPO activity=(Rate of change*1x10^{6})/(mass of tissue(mg)*14).

MPO buffer for homogenising : 50mMol KH_2PO_4 with 0.5% HTAB in ddH20

Reagent buffer:

5ml MPO buffer in 45ml ddH20 with 8.4mg O-dianisidine and 10ul of 3%H_20_2

2.3 *In vivo* methods

Sprague-Dawley rats were used throughout in vivo experiments. These were obtained from stock in the animal house at the University of Strathclyde. All animals were kept in pathogen-free conditions in facilities managed by the biological services staff at the University of Strathclyde, under strict accordance with the guidelines laid out in the United Kingdom Home Office Animals (Scientific procedures) Act 1986. All procedures were performed under Project Licence number 60/3938.

2.3.1 Chronic hypoxic animal model

2.3.1.1 Chamber design

The chamber consists of two separate chambers and is made from transparent high resistance plexiglass. Air is continually removed by a vacuum pump to allow hypobaric hypoxia to develop. The pressure was reduced to 550mBar over 48 hours. Air constantly flows through the chamber to ensure that the moisture and CO_2 do not accumulate. Temperatures are maintained at a constant temperature. The chamber was housed in a special environmental room, which allowed temperature control at 21°C, humidity at 55% and a 12hour on/12 hour off light cycle. Animals and the apparatus are checked upon 5 times a day to ensure the animals are not distressed and the equipment is still working.

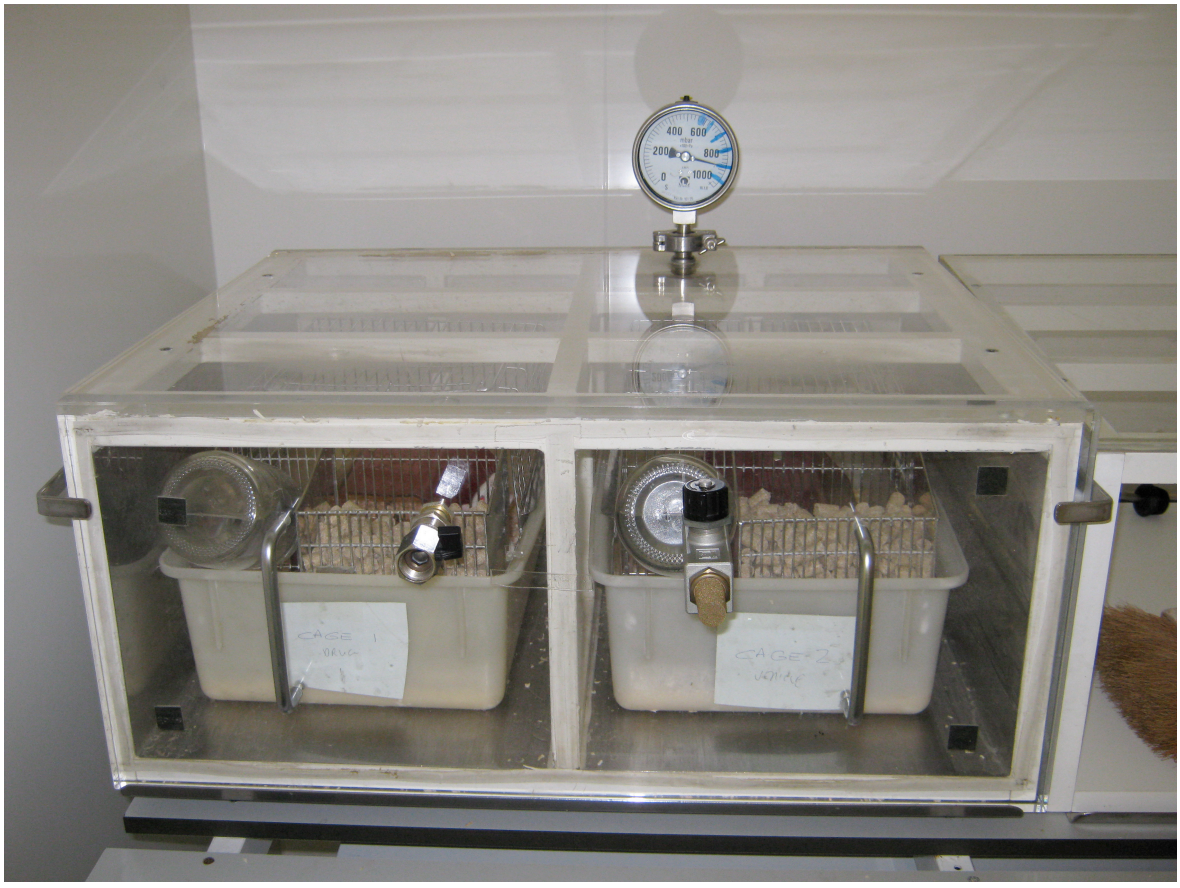

Figure 2-6 Hypobaric hypoxic chamber used for *in vivo* experiments

2.3.1.2 Induction and maintenance of pulmonary hypertension with chronic hypoxia

Up to 6 animals in 2 cages were placed in the chamber at any one time. Pressure was reduced over 48 hours to allow acclimatisation. After 14 days of chronic hypoxia exposure in the chamber the animals show evidence of pulmonary hypertension both with haemodynamic parameters and histology.

Those animals that required intra-peritoneal (i.p.) injections had the pressure in the chamber brought back to atmospheric over 1 hour. Then the animals were injected and the pressure re-established over a further 1 hour. The chamber was at atmospheric pressure for no longer than 25 minutes. This also allowed for cleaning of cage and replenishing food and water.

2.3.2 Monocrotaline animal model

Monocrotaline was dissolved in 1M HCl and then neutralised with a similar volume of 1M Sodium Hydroxide. Animals were then injected with 60mg/kg

monocrotaline subcutaneously in the scruff of the neck. Control animals received a similar volume of saline injected subcutaneously. After 2-3 weeks the animals show signs of pulmonary hypertension. The animals are housed in a special environmental room, which allowed temperature control at 21°C, humidity at 55% and a 12hour on/12 hour off light cycle. These animals are kept at atmospheric pressure throughout.

2.3.3 Intraperitoneal dosing

Injections were performed using sterile equipment and aseptic conditions. To facilitate intraperitoneal dosing an animal handler held the animals and then the right lower abdominal quadrant was identified and swabbed with a steristrip. The 25 Guage needle was then used to inject directly into the abdomen. The animals were closely observed for any adverse effects.

2.3.4 Assessment of pulmonary hypertension

2.3.4.1 Haemodynamics

To determine development of pulmonary hypertension the right ventricular systolic pressure (RVSP) was measured using a 21 Guage needle attached to a pressure transducer calibrated to zero. To allow this, animals were anaesthetised using a 1:1 mixture of hypnovel (midazolam) and hypnorm (Fentanyl/fluanisone) via a subcutaneous route at a dose of dose of 0.15ml per 100gm body weight. The animals were then secured on an in vivo table and tested for depth of anaesthesia. Then the airway was secured using a tracheal approach and inserting a small plastic tube into the trachea that is connected to a ventilator (TOPO ventilator, Kent Scientific). The animals were ventilated with an inspiratory pressure of 8-10 cmH_2O, a positive end-expiratory pressure of 3-5cmH_2O and a respiratory rate of 75-80 breaths per minute (tidal volume 1.2ml).

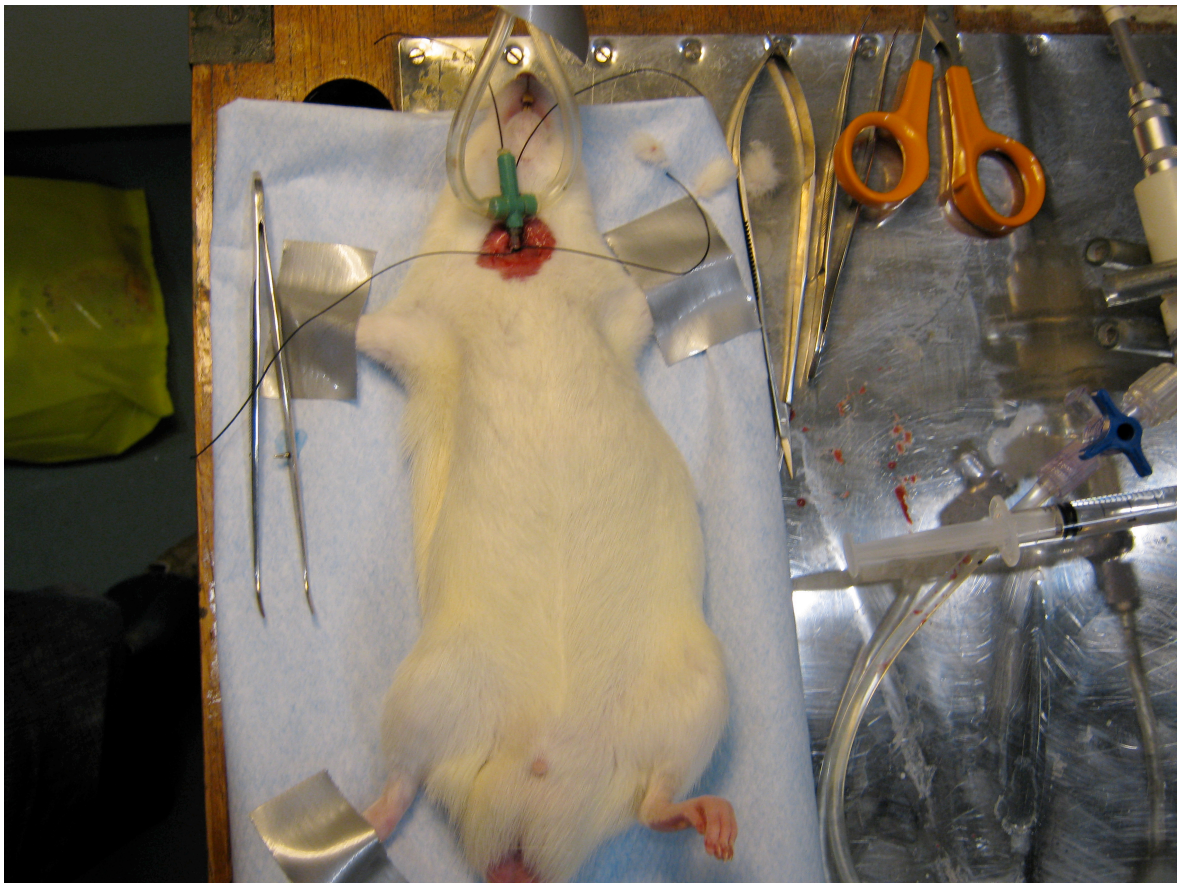

Figure 2-7 Set up for measurement of right ventricular systolic pressure with ventilated animal

Once deep enough the abdomen was opened and the 21 Guage needle advanced slowly into the thorax aiming for the right ventricle. Placement of the catheter in the right ventricle was established by direct visualisation and afterwards by post-mortem examination. The tracings were allowed to stabilise for 3 minutes. The recordings were made using Powerlab data acquisition system (AD instruments,UK) and analysed using LabChart v6 software. An average of 100 sequential beats were calculated to give the pressure readings. Normal RVSP was 15-25mmHg. The catheter and electrical system was calibrated using a mercury manometer.

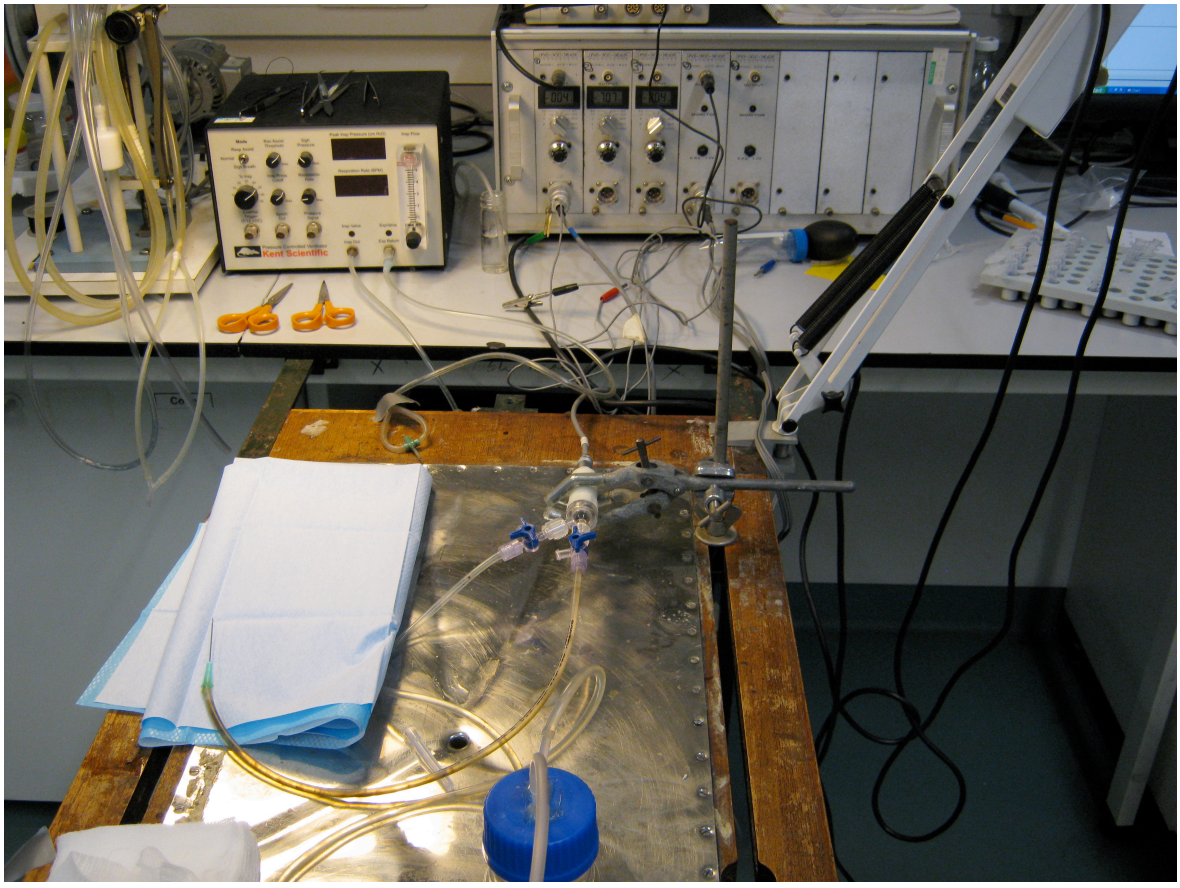

Figure 2-8 Pressure transducer equipment for pressure recordings

2.3.4.2 Right ventricular hypertrophy

The right ventricle (RV) was separated form the left ventricle (LV) and septum (S) using a dissecting microscope. The ventricles were then blotted and weighed. The degree of RV hypertrophy was determined by the ratio of the weight of the RV to the LV+S.

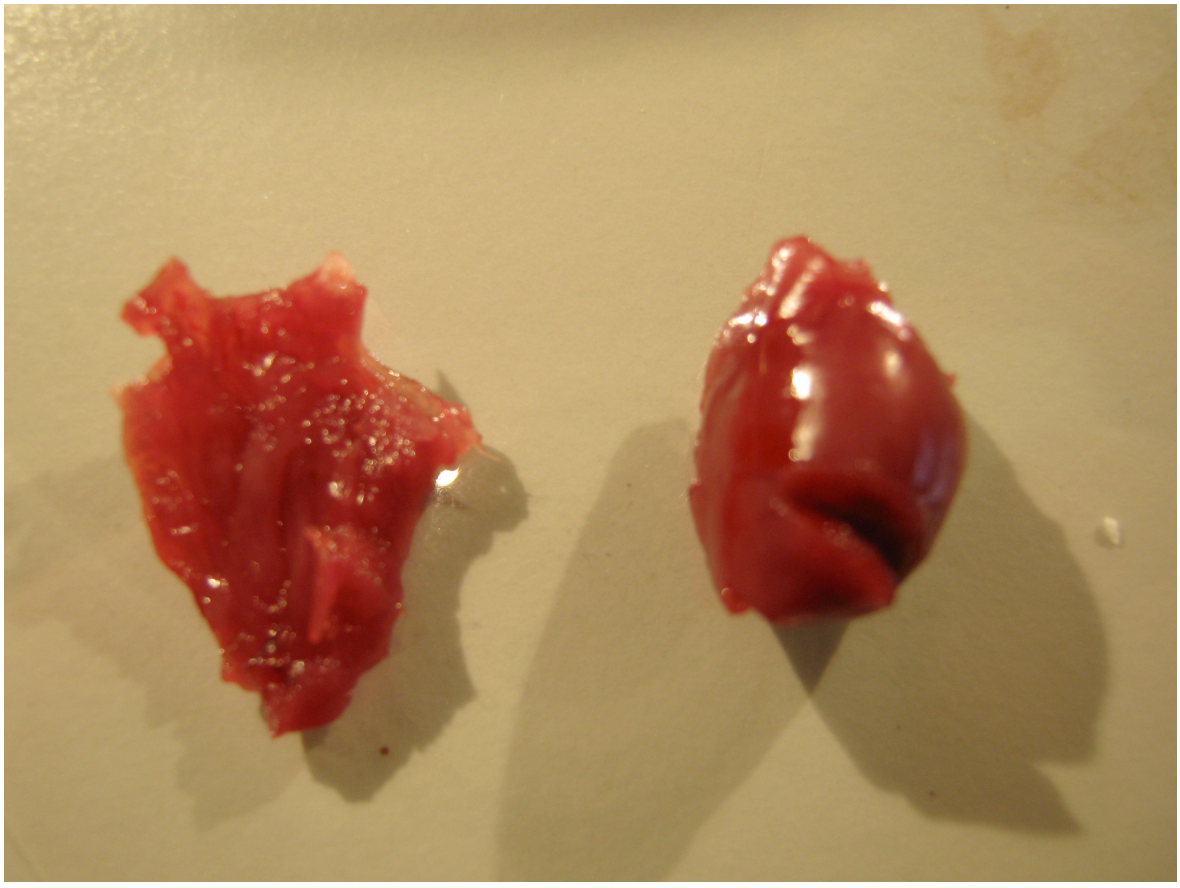

Figure 2-9 Right ventricular hypertrophy dissected from left ventricle + septum

2.3.4.3 Haematocrit

The haematocrit in the animals are determined to ensure adequate exposure to the chronic hypoxic environment, as in hypobaric hypoxia the haematocrit increases. At the time of sacrifice of the animal blood was aspirated in a heparinised syringe. This was then transferred into a capillary tube that had one end closed off using sealant. The tube was then centrifuged at 10000 rpm for 10 minutes to separate the blood into the plasma, buffy coat and red cell mass. The ratio of the length of the red cell mass to the total length of the separated blood gives the haematocrit.

2.3.4.4 Quantification of pulmonary vascular remodelling

Pulmonary vascular remodelling was quantified by assessing the degree of muscularisation present in smaller pulmonary arterial vessels with a diameter <80μm. Standard immunohistochemical techniques were used as described below with staining for alpha-smooth muscle actin. In each lung section pulmonary arteries were characterised as completely muscularised (with complete

occlusion or slit-like appearance of lumen), partially muscularised (with incomplete rings of smooth muscle) or non-muscularised (with no smooth muscle present). The number of vessels in each category were determined by counting the number in 5 random fields per slide and using 3 slides per animal. This identified around 60-80 arteries per animal .The individual assessor was blinded to the slides.

A second method of analysis was used in which all the muscularised vessels <100μm were counted and expressed as a percentage of the total number of vessels in the random fields. This represents another validated method for comparing the overall number of muscularised small pulmonary vessels.

2.3.5 Sample collection and processing

2.3.5.1 Serum collection

At the end of the experiment the animals were given terminal anaesthesia and then direct cardiac puncture using a 23G needle and 2.5ml syringe was performed. The blood aspirated was injected into a SST paediatric yellow tube and allowed to clot at room temperature. This was centrifuged at 13200rpm for 30 minutes and the serum collected and frozen at -20^{o}C until further analysis could be performed.

2.3.5.2 Lung collection

The lungs were collected using different methods depending on the downstream analysis planned.

For histology the left lung was collected and the main pulmonary artery cannulated and infused at a pressure of 20mmHg with buffered formalin to fix pulmonary vessels. Then the whole lung was placed in 5ml of 10% buffered formalin and left for 24-48hours to fix.

For protein analysis the left lung was removed and dissected into its lobes and then snap frozen in liquid nitrogen and stored at -80^{o}C.

2.3.5.3 Cardiac collection

The right and left ventricles were collected using different methods depending on the downstream analysis planned.

For histology the ventricles were collected and placed in a bijou containing 5ml of buffered formalin and left for 24-48hours to fix.

For protein analysis the heart was removed and dissected into its right and left ventricles and then snap frozen in liquid nitrogen and stored at -80^{o}C.

2.3.6 Histology

Lungs and cardiac chambers were collected in 10% buffered formalin as above. They were then embedded in paraffin using a Shandon citadel 1000 tissue processor (Thermo scientific) and submerged into paraffin blocks. These were then cut into 5um sections using a microtome and placed on histology slides (VWR). In order to facilitate staining the slides were deparaffinised and rehydrated using graded alcohol solutions (xylene, 100% ethanol, 70% ethanol and distilled water) as per standard protocol.

2.3.6.1 Haematoxylin and Eosin staining (H&E)

The slides were placed in Harris haematoxylin for 2 minutes and excess then washed off. The stain was enhanced using 1% acid/alcohol solution and Scots tap water. Then the slides were counterstained with eosin for 2 minutes and excess washed off. The sections were dehydrated with increasing concentrations of alcohol (70% ethanol, 100% ethanol, xylene). The tissue was covered with a drop of DPX (Di-n-butylphthalate in xylene) mountant (Sigma) and a coverslip adhered to it.

2.3.6.2 Trichrome Masson staining

The slides were deparaffinised and stained in haematoxylin for 2 minutes, then washed in tap water for 5 minutes. The slides were then stained with Masson's trichrome stain for 20 minutes. The slides were rinsed in 0.2% acetic acid and then washed in running tap water for 3 minutes. At the end the coverslide was applied with DPX.

2.3.6.3 Immunohistochemistry

This was performed in conjunction with the department of pathology, Southern General hospital, Glasgow.

The techniques used are briefly described. The system used was Leicabiosystems Bond Max machine. The slides, which had been prepared as above, were placed in slide holders in racks and placed in the machine. The antigen retrieval system used depended on the type of antibody used and the antigen to be identified. Baseline testing was performed to ensure optimal results.

Epitope Retrieval system 1: citrate based buffer and surfactant, pH 5.9-6.1 at room temperature.

Epitope Retrieval system 2: EDTA based buffer and surfactant, pH 9,0 at room temperature.

In general the antigens are exposed using the above methods and heated in the machine and then the following chemical steps are performed:

1. Endogenous peroxide: the specimen is incubated with H_2O_2 to quench any endogenous peroxide for 5 minutes.

2. Primary antibody: Incubation with the primary antibody directed against the antigen in question. Could be rabbit or mouse. Incubated for 30mins.

3. Post-primary antibody: This is a rabbit IgG which recognises anti-mouse antibody. Incubation was 8minutes.

4. Addition of polymer: This is a polymeric conjugate of IgG and HRP which allows the identification of rabbit antibodies. Incubation was 15 minutes.

5. DAB addition: The chromogen DAB is added and forms a brown appearance in the presence of the antigen. Incubation was 5 minutes.

6. Haematoxylin: allows identification of cell nuclei.

2.4 Data Analysis

For normally distributed values, responses were reported as Mean+/- SEM, and Students *t*-test was used. In certain circumstances mean +/- SD was used when a more descriptive approach to the data was needed. The n value refers to the number of animals involved per experimental procedure. In the cultured cell experiments the n values are more explicitly outlined in the figure legends. In general for each experiment I have performed triplicate replicates of each condition. Then the experiment has been performed on separate days with cells derived from at least 2 other animals. Where there were limited number of animals then the experiment was repeated using cells which were from different passages.

For multiple comparisons of means across different experimental groups analysis of variance (ANOVA) was performed with Bonferonni *post hoc* analysis. Values of $p<0.05$ were accepted as statistically significant. All graphical presentation and data analysis was performed using graphpad Prism v6 software, USA.

3 The pro-inflammatory p38MAPK pathway is essential in the *in vitro* cellular mechanism of pulmonary vascular remodelling

3.1 Introduction

As previously described in the general introduction, the adventitial layer of the pulmonary artery increases in size in pulmonary vascular remodelling. This is a mixture of increased deposition of extracellular matrix and the proliferation of cells including resident fibroblasts and infiltrating myofibroblasts from the media and from circulating cells, for example fibrocytes.

Previous work in our laboratory has shown the importance of the p38 MAPK pathway in the pulmonary artery fibroblast. In particular it is required for the increased proliferative response of the fibroblast to hypoxia and it seems to be important in mediating the release of factors from the fibroblast which can lead to increased proliferation of neighbouring smooth muscle cells. Further analysis has suggested that the isoform p38MAPKα is the critical component of the p38MAPK complex that mediates these effects in the fibroblast.

In addition it has been previously shown that the PAFs isolated from chronic hypoxic rats are constitutively hyperproliferative. They seem to have undergone a 'phenotypic switch' which makes them demonstrate increased proliferation in comparison to PAFs from normal animals even when grown in a normoxic environment.

We hypothesised that pro-inflammatory mediators were being released from the fibroblast and that by inhibiting the p38MAPK pathway this would reduce the stimulus to proliferation of adjacent smooth muscle cells. We analysed the mediators released by the fibroblasts and investigated the effect that the inhibition of p38MAPK would have on this. We also hypothesised that this would be true in pulmonary artery fibroblasts derived from different animal models of pulmonary hypertension and that this was a common theme in the pathobiology throughout experimental models.

3.2 Relevant Methods

3.2.1 Characterisation of the pulmonary artery fibroblast

As previously mentioned the fibroblast can be difficult to fully characterise as there is no unique specific cell marker. The absence of eNOS and α-SMA, and positivity for vimentin, as assessed using immunofluorescence with con-focal microscopy, were the criteria used.

It should be noted that as the fibroblasts became older in culture they started to undergo transformation into a myo-fibroblast type as shown by the expression of the α-SMA (Fig 3-1). However this did not occur until passage 7 and for our experiments the cells were used between passage 2-5. For this the α-SMA antibody was used at dilution of 1:400.

The general protocol used for immunofluorescence is outlined in chapter 2. The primary antibody used was Vimentin at dilution of 1:50 in PBS/BSA. The primary antibody was allowed to incubate for 1 hour at room temperature. The secondary antibody used was anti-rabbit conjugated with Texan red. This emits red light at wavelength of 543nm. The Zeiss confocal microscope was used with LSM software. This confirmed the cells were positive for the mesenchymal marker vimentin (Fig 3-2).

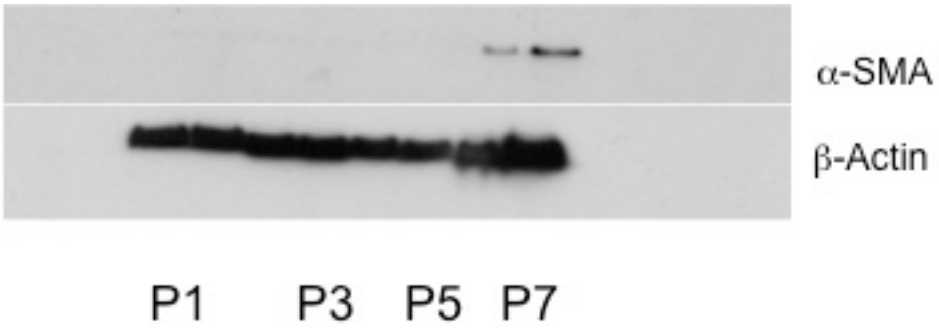

Figure 3-1 α-SMA appears after passage 6 in primary cultured fibroblasts *in vitro*
Pulmonary artery fibroblasts were grown in culture and passaged as previously described once cells were confluent. At passage 1,3,5&7 cell lysates were collected. Immunoblotting was performed for α-SMA in order to detect activation of fibroblast and differentiation into myofibroblast. Each passage is represented by 2 wells. Only after passage 6 was α-SMA detected. Therefore subsequent *in vitro* experiments were performed between passage 2-6. Immunoblot representative of 2 experiments.

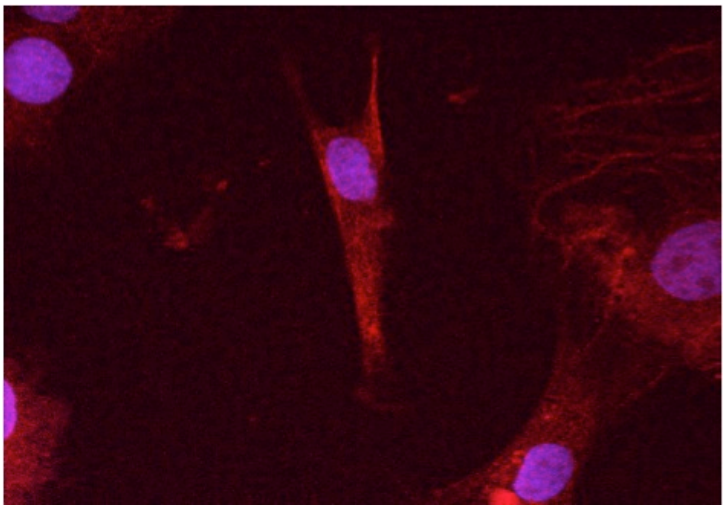

Figure 3-2 Immunofluorescence confocal microscopy confirms cultured cells to contain vimentin
Cultured cells were grown on culture specific wells and then stained for Vimentin, a mesenchymal marker. Using Texan-red conjugated antibody the cytoskeletal network of vimentin can be seen. Nucleii are stained with DAPI. Selected image representative of 2 separate experiments.

3.3 p38MAPK is important in pulmonary vascular remodelling

3.3.1 p38MAPK-alpha is critical for hypoxia induced proliferation of PAF

As previously discussed the proliferation of pulmonary artery fibroblasts is a key part of pulmonary vascular remodelling and has been linked in hypoxia to the p38MAPK pathway. To fully elucidate the isoform involved, specific siRNA against p38MAPK alpha was used as opposed to a pharmacological blocker, to demonstrate the importance of this isoform in the hypoxic proliferation of rat pulmonary artery fibroblasts.

Exposure of primary cultures of rat pulmonary artery fibroblasts to hypoxia for 24 hours led to increased phosphorylation of p38MAPK activity and increased proliferation (Fig 3-3B). The addition of siRNA led to a reduction in p38MAPK alpha, and reduction in phosphorylation of p38MAPK (Fig 3-3A). This corresponded to a significant reduction in the hypoxic driven proliferation of the fibroblast cells.

In addition, there was no effect on the p38 gamma (γ) isoform which is expressed at low levels in the pulmonary artery fibroblast. Knockdown of p38MAPKα protein in pulmonary artery fibroblasts was achieved as shown in figure 3-4.

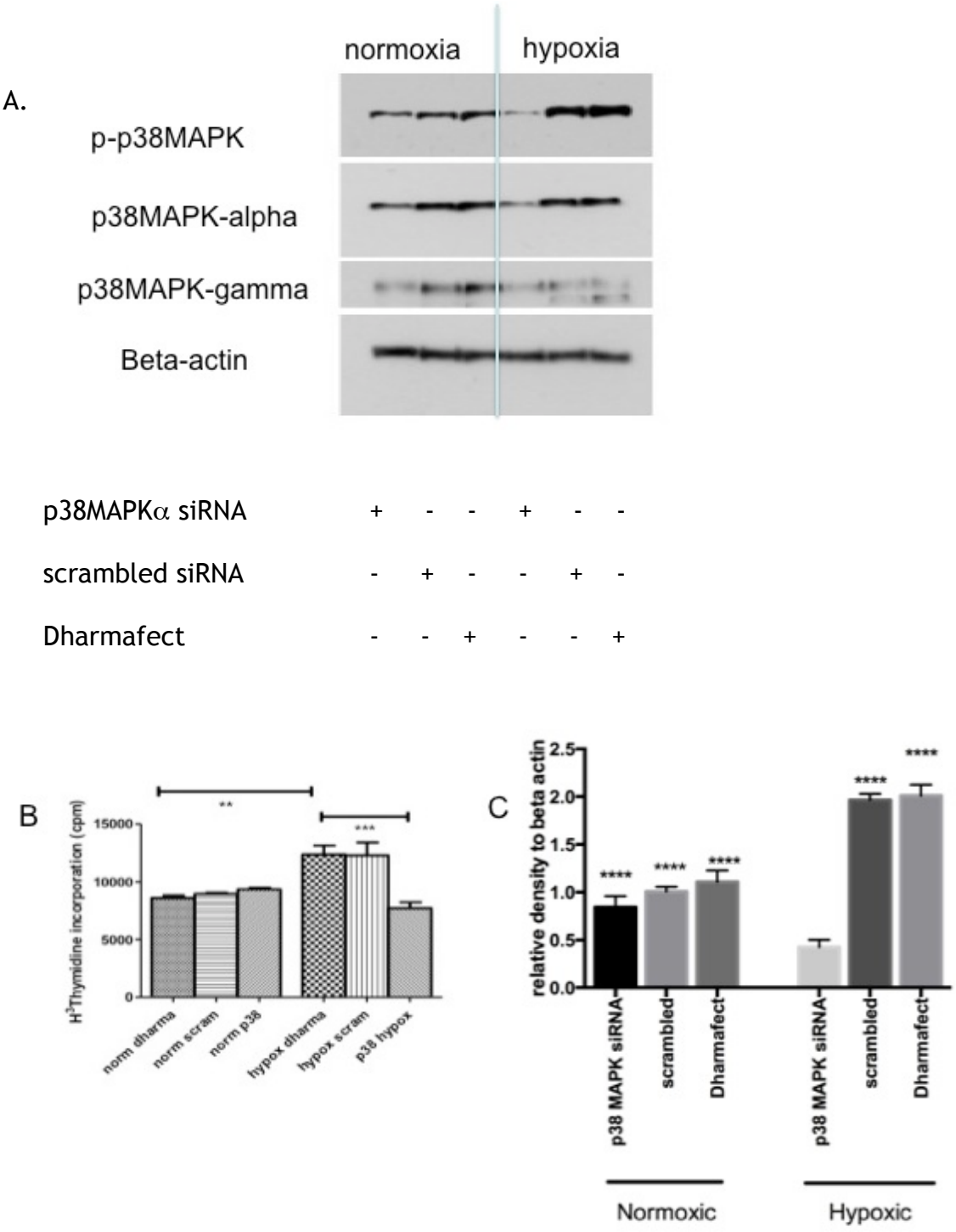

Figure 3-3 p38αsiRNA reduces hypoxic induced proliferation of PAF by reducing phosphorylation of p38MAPKα

PAF in early culture grown in antibiotic free media and then transfected with 100μl/well of p38-α siRNA, scrambled siRNA (control) or Dharmafect (transfection agent alone). (A) Immunoblotting confirms knockdown of p38-α and in hypoxia there is a clear reduction in the level of phosphorylated p38. This is mirrored with a reduction in the total p38MAPK and p38-α. There was no demonstrable effect on p38-γ. (B) The presence of p38-α siRNA, but not scrambled siRNA, reduced the proliferation of PAF when exposed to hypoxia. This confirms the importance of p38MAPK-α in the hypoxic mediated proliferation of PAF. Immunoblot and Thymidine assay representative of 3 experiments, using 2 different animals. Data shown as mean +SD. ** $p<0.005$, *** $p<0.001$. (C) is densitometry of phospho-p38MAPK blots. ****$P<0.0001$ relative to p38MAPK siRNA.

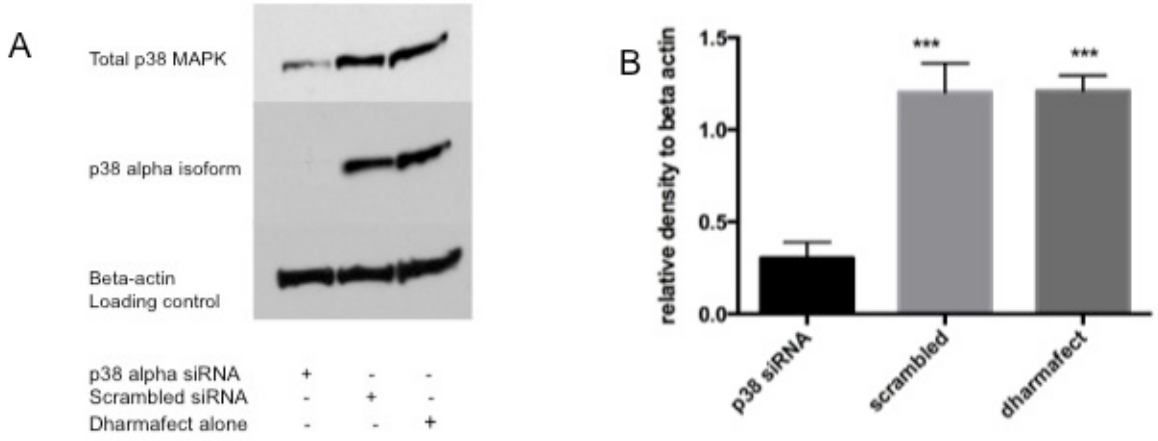

Figure 3-4 p38-α siRNA reduces total p38MAPK by reducing p38MAPKα isoform
This figure confirms the knockdown of p38MAPKα using siRNA for p38-α. The cells were transfected with increasing doses of siRNA until optimal knockdown and cell viability of >80% was achieved as shown by immunoblot. Cells were transfected and left for 24-48hrs with the siRNA and then the media was changed. Cells were grown for a further 48-72hrs to allow optimal time for protein knockdown. Immunoblot is representative of 3 experiments. (B) shows densitometry for the total p38MAPK blots.*** $P<0.001$ by ANOVA relative to p38MAPK siRNA.

3.3.2 Fibroblasts derived from monocrotaline animals have a p38 MAPK mediated pro-proliferative phenotype

Our group and others have previously shown that fibroblasts derived from the pulmonary artery of chronic hypoxic rats have an 'activated' phenotype with an increased basal proliferative response to serum. Our group has shown this to be p38MAPK dependent.

We initially wanted to explore the hypothesis that the same phenotype change would exist in PAF derived from monocrotaline animals as seen in the chronic hypoxic animals. In order to do this PAFs were taken from normoxic, chronic hypoxic and MCT animals and a proliferation assay was performed using different amounts of serum (Fig 3-5).

It can be seen that even when the cells are grown in serum free media the cells seem to have an increased proliferative response compared to normal cells, suggesting a pro-proliferative response. This is exaggerated when 1% serum is used although is not statistically different when 5% serum is used. This may reflect cell contact inhibition with this higher dose of serum.

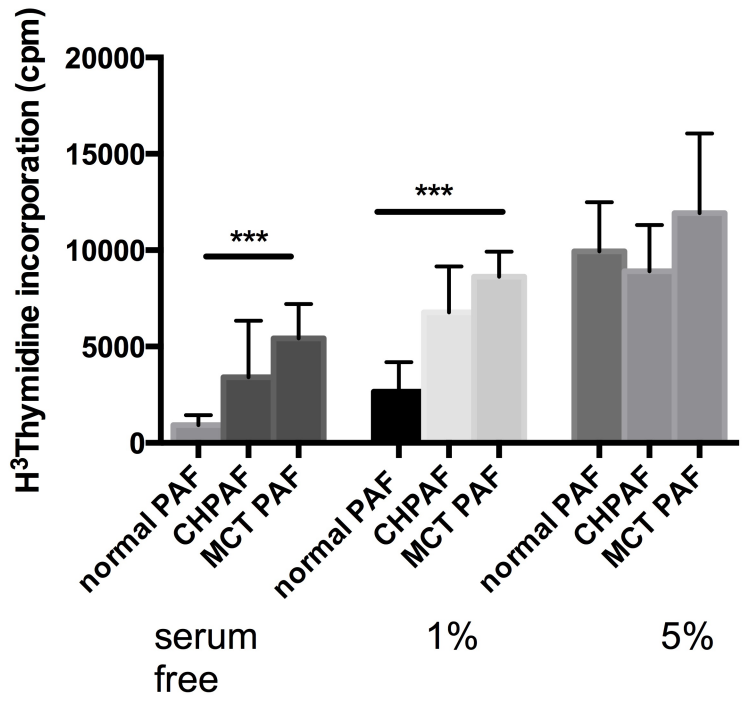

Figure 3-5 PAF from both chronic hypoxic and MCT animals have a pro-proliferative phenotype

Cells were isolated from the second order division of the pulmonary artery and grown in normoxic culture. The effect of serum stimulation was observed on these cells. At baseline with no serum stimulation the CH and MCT PAF had an increased proliferative rate compared to the PAF derived from normoxic wild type rats. This was also seen with low dose 1% serum stimulation. The effect was lost at 5% serum stimulation which may reflect cell contact inhibition. The Thymidine incorporation assay was used to assess the proliferation of the cells and the results presented are representative of 3 experiments with triplicate values in each experiment. Values are mean with SD. *** $p<0.001$.

We next examined by immunoblotting, the role that p38MAPK might have in this process. This showed that active phosphorylated p38MAPK was increased in the MCT fibroblasts under basal conditions compared to the normal cells, and was similar to the features previously observed in the CHPAF's (Fig 3-6). These findings indicate that the pulmonary artery fibroblasts that are derived from the MCT animals seem to have a pro-proliferative phenoytype. This seems to be identical to the CHPAF and would suggest that the fibroblast in different models of pulmonary hypertension have conserved responses to the initial stimuli which results in pulmonary hypertension. Clearly the increased proliferative phenotype can contribute to the adventitial thickening.

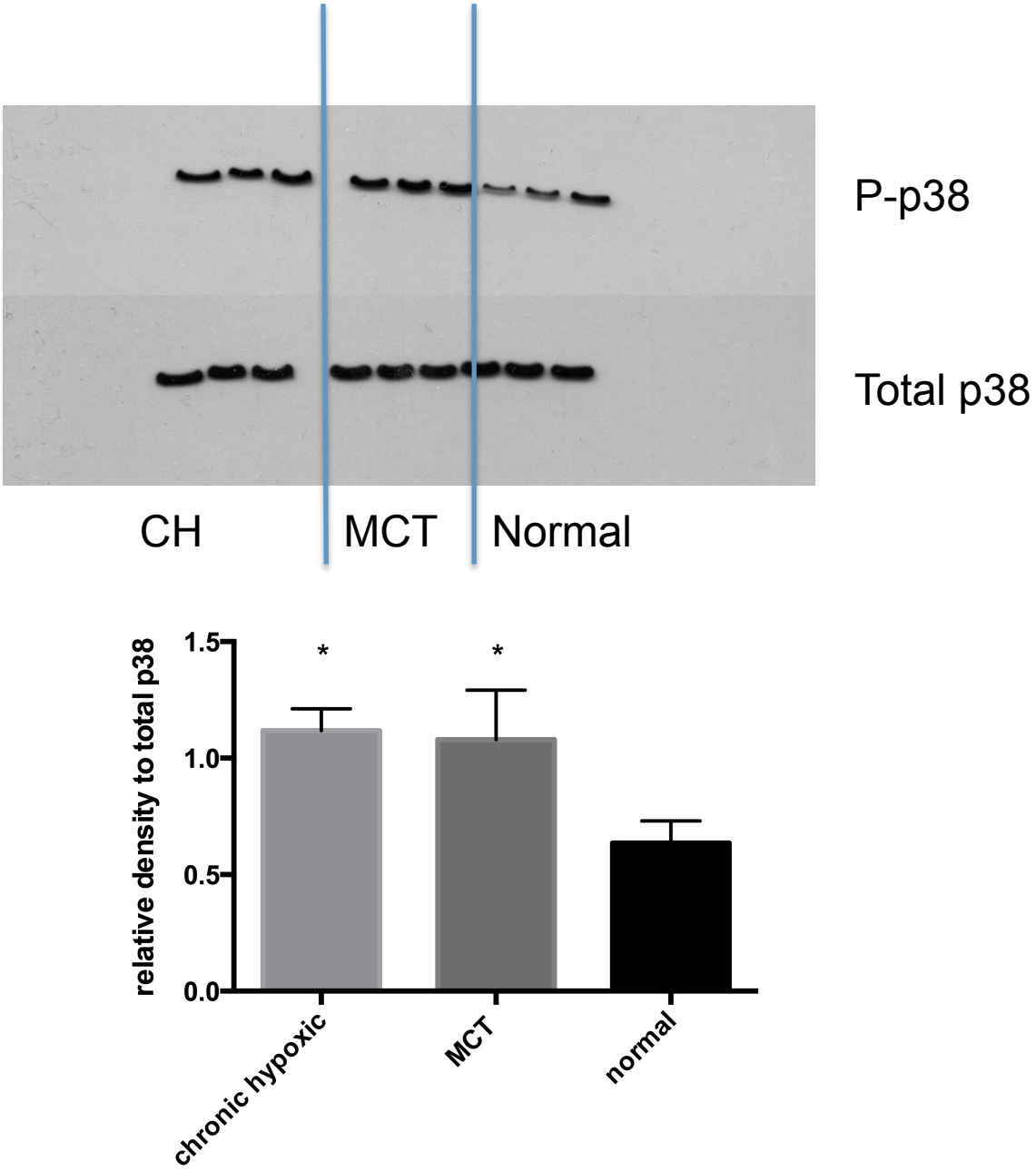

Figure 3-6 p38 MAPK activity is increased in PAF from both CH and MCT animals
This immunoblot shows that under normoxic conditions the PAF derived from both CH and MCT animals show a constitutive activation of p38MAPK with increased levels of p-p38 detected. Cells were from passage 3 and were quiescesed in serum free medium for 24 hours prior to harvest. Immunoblot for phospho-p38 and total p38MAPK was performed and representative of 2 experiments. Densitometry for repeat blots is shown. *p<0.05

3.3.3 Conditioned media, released via a p38MAPK dependent pathway from monocrotaline derived PAF, can stimulate smooth muscle cell proliferation

Previous experiments have shown that conditioned media from hypoxic PAFs can stimulate proliferation of smooth muscle cells. We hypothesised that the same would occur in a MCT-derived model of pulmonary hypertension.

Pulmonary artery fibroblasts were isolated from MCT animal models of pulmonary hypertension as previously described. Then the cells were plated into 24 well plates and conditioned media generated. Wells were incubated with either serum free media +/- SB203580 or 1% serum media +/- SB203580 for 24 hours and then the conditioned media collected. To determine the effect on smooth muscle cell proliferation, primary rat smooth muscle cells from normal rats were grown up in 24 well plates and a thymidine proliferation assay undertaken using the previously isolated conditioned media (Fig 3-7).

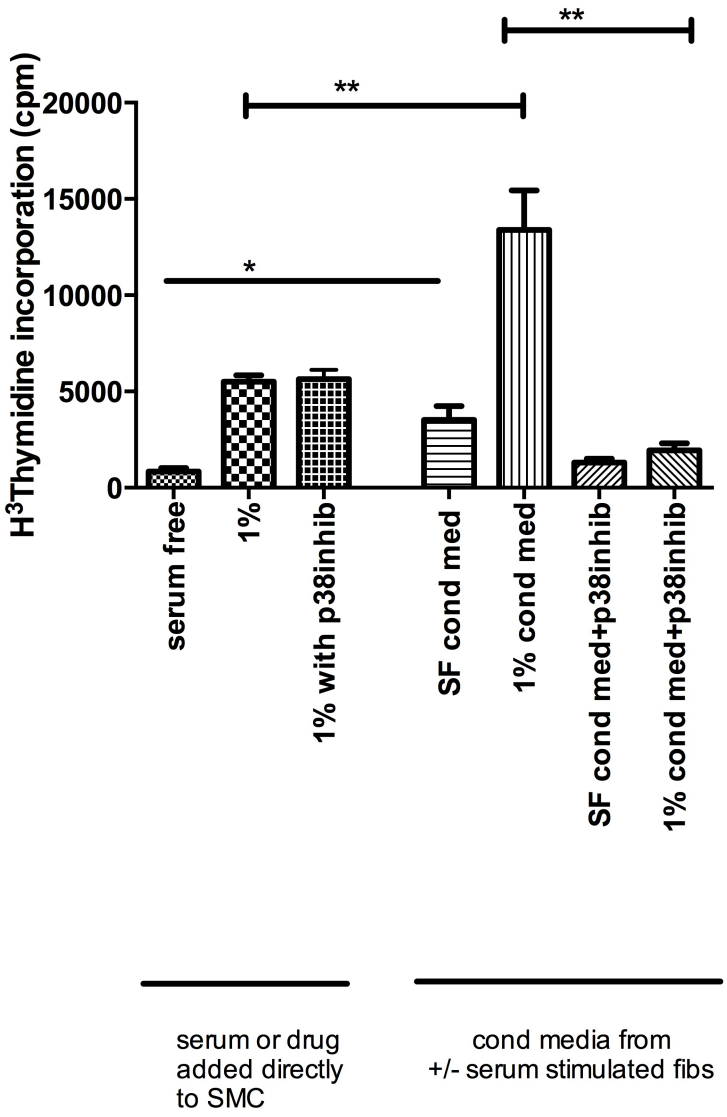

Figure 3-7 Conditioned media from MCT-derived PA fibroblasts stimulates smooth muscle cell proliferation

PAF derived from MCT animals were isolated and then quiescesed for 24 hours in serum free media. After 24 hours, cells were stimulated with either 1% serum or some remained in serum free media +/- SB203580 (p38 inhib). The conditioned media was aspirated from the wells after 24 hours and passed through a cell sieve to give conditioned media from serum free cells +/- SB203580 (SF cond med, SF cond med+p38 inhib) and 1% stimulated cells +/- SB203580 (1% cond med, 1% cond med+p38 inhib). The conditioned media was added to smooth muscle cells for 48 hours before a proliferation assay was performed. Both SF cond med and 1% cond med resulted in increased proliferation of PASMC while this effect was lost in the conditioned media from the PAF which had been co-incubated with SB203580. There was no effect of SB203580 directly on the PASMC. Values are mean + SD. Data shown from 3 experiments. * $p<0.05$ ** $p<0.005$

These results demonstrate that the conditioned media derived from the MCT PAFs can stimulate the proliferation of smooth muscle cells and that the release of these factors is p38MAPK dependent. This correlates nicely with similar findings from our laboratory using normal fibroblasts which have been exposed to acute hypoxia. It also further emphasises the suggestion that these fibroblasts have undergone a phenotypic switch in this model of pulmonary hypertension. This again is akin to previously published work looking at the fibroblasts derived from the chronic hypoxic animal model of pulmonary hypertension.

3.3.4 Conditioned media, released via a p38MAPK dependent pathway from chronic hypoxic derived PAF, can stimulate smooth muscle cell proliferation

Similar to the above experiments the effect that the conditioned media obtained from PAF derived from chronic hypoxic animals was investigated. It has been shown that the fibroblasts from these animals have undergone a phenotypic switch in which they have a pro-proliferative phenotype and have increased p38MAPK activity. It was hypothesised that the conditioned media would be able to induce the proliferation of smooth muscle cells.

Primary cell cultures of pulmonary artery fibroblasts from chronic hypoxic animals were obtained and grown in culture. The cells were incubated for 24 hours with either serum free media or 1% serum. The conditioned media was then collected. The experiment was also performed in the presence of a p38MAPK inhibitor SB203580.

The conditioned media that had been obtained from the fibroblast cells was then incubated with smooth muscle cells to observe the effect. An extra control of the p38 inhibitor directly added to the smooth cells was also used, (Fig 3-8).

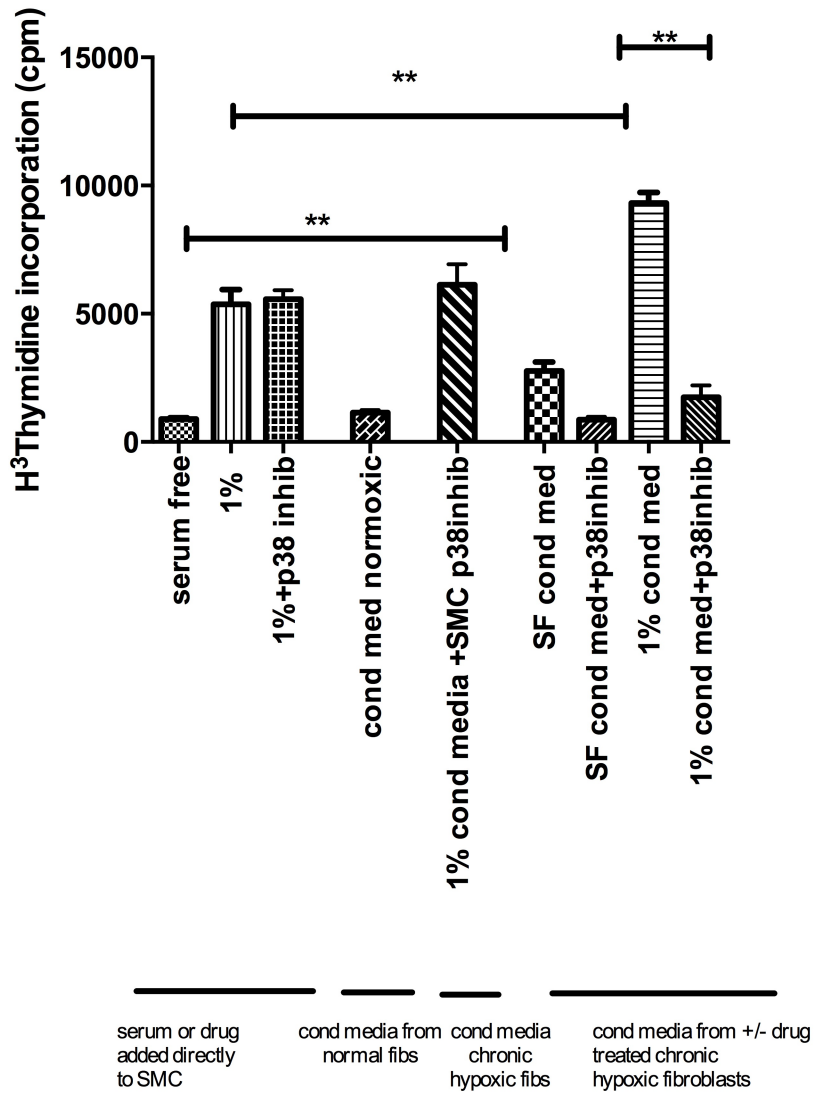

Figure 3-8 Conditioned media from chronic hypoxic-derived PA fibroblasts stimulates smooth muscle cell proliferation

PAF derived from CH animals were isolated and then quiescesed for 24 hours in serum free media. After 24 hours, cells were stimulated with either 1% serum or some remained in serum free media +/- SB203580 (p38 inhib). The conditioned media was aspirated from the wells after 24 hours and passed through a cell sieve to give conditioned media from serum free cells +/- SB203580 (SF cond med, SF cond med+p38 inhib) and 1% stimulated cells +/- SB203580 (1% cond med, 1% cond med+p38 inhib). The conditioned media was added to normal pulmonary artery smooth muscle cells for 48 hours before a proliferation assay was performed. Both SF cond med and 1% cond med resulted in increased proliferation of PASMC while this effect was lost in the conditioned media from the PAF which had been co-incubated with SB203580. There was no effect of SB203580 directly on the PASMC. Values are mean + SD. Data shown from 3 experiments. * $p<0.05$ ** $p<0.005$

The experiment showed that the conditioned media from the stimulated chronic hypoxic fibroblasts resulted in an increase in proliferation of smooth muscle cells over that of 1% serum alone. This effect was lost if the fibroblast was incubated with SB203580. However the effect was not stopped if the smooth muscle cells were stimulated with conditioned media and had the p38MAPK inhibitor directly added to the smooth muscle cells. This suggests that the effect on blockade of smooth muscle proliferation happens at the fibroblast stage in stopping the release of factors which seem to be important in the development of cellular proliferation.

3.4 Identification of mediators released by the PAF

Clearly the pulmonary artery fibroblast releases mediators which can lead to cellular proliferation. In order to try and identify the responsible mediators a number of experiments were carried out.

3.4.1 SDS-PAGE identification

The conditioned media isolated from hypoxic pulmonary artery fibroblasts was separated out on SDS-PAGE gels as outlined in general methods. When the conditioned media from the hypoxic culture was run on SDS-PAGE the resolution was poor indicating a substantial release of protein (Figure 3-9). It was hypothesised that the factors responsible for the induction of SMC proliferation were below molecular weight of below 30kDa. The proteins were filtered using Millipore Amicon ultra. This led to some improved resolution (Figure 3-10).

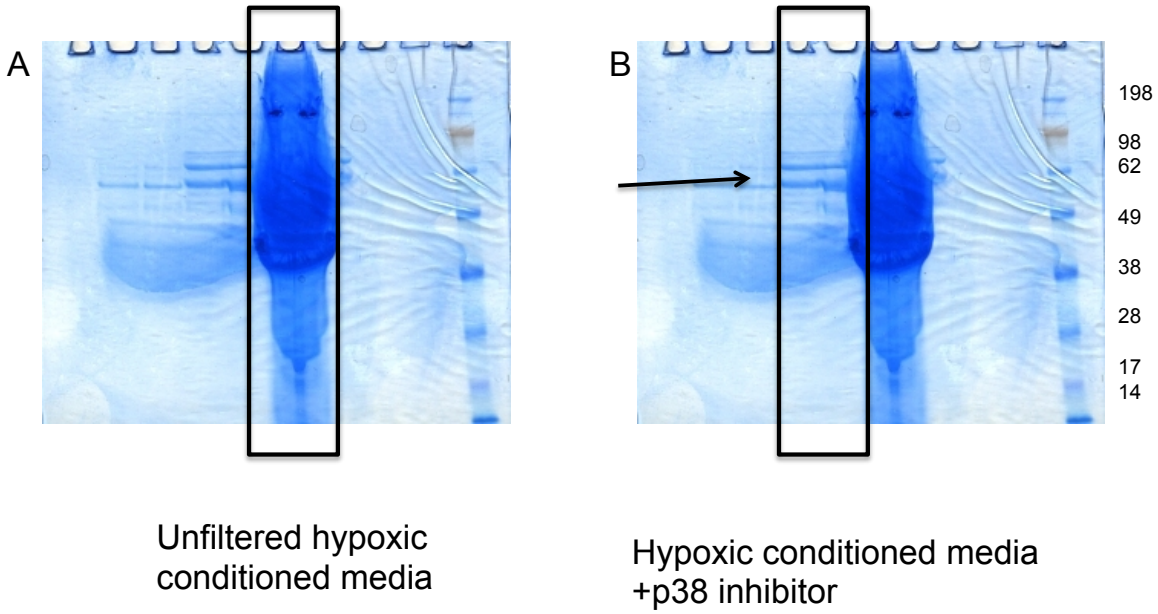

Figure 3-9 Conditioned media from hypoxic fibroblast +/- p38 inhibitor
The conditioned media was run on SDS_PAGE gel and there was a large amount of protein in the hypoxic lane (A) that was reduced after treatment of the cells with p38MAPK inhibitor (B).The arrow indicates the principal bands left after the administration of the inhibitor. Experiment repeated twice. Molecular weight markers indicated (kDa).

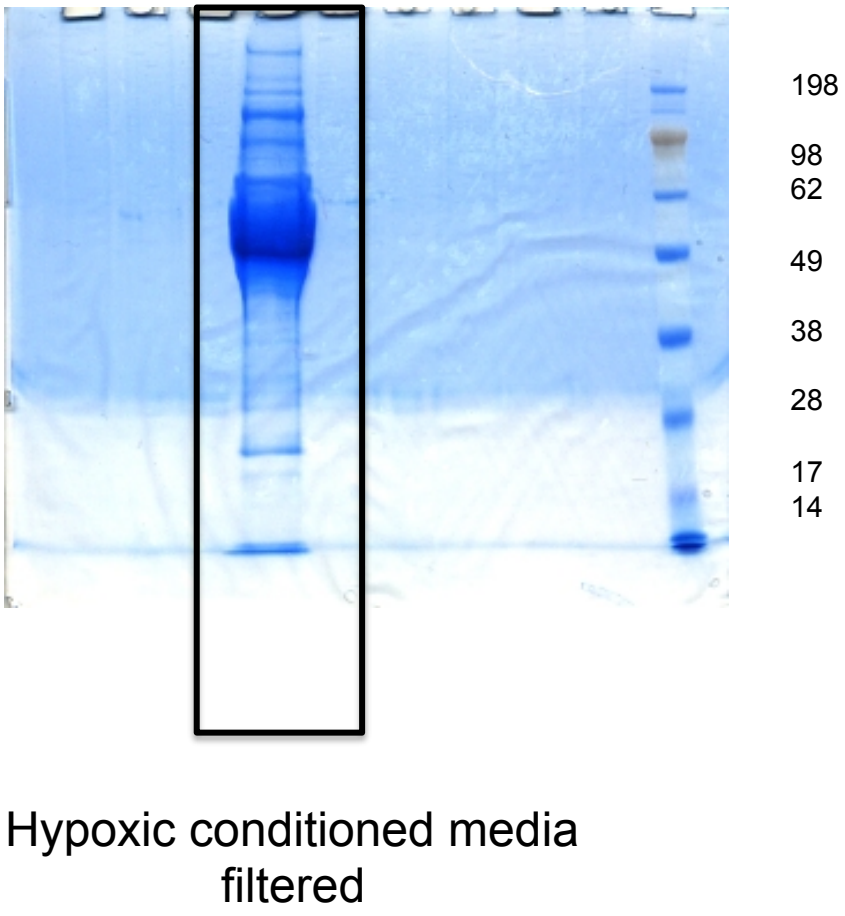

Figure 3-10 Filtered hypoxic conditioned medium
The molecular weight filtered medium resolved better on SDS-PAGE. Molecular weight markers shown (kDa).

The conclusion from these experiments was that there were a large number of proteins being released from the hypoxic fibroblasts.

3.4.2 Thermal energy abolishes the proliferative stimulus in the conditioned media

At an initial stage, in an attempt to try and clarify the nature of the molecules in the conditioned media which might be leading to the increased proliferative response, the solution was heated to denature the proteins and then tested to see if the proliferation still occurred (Fig 3-11). The heating of the solution led to the abolition of its proliferative stimulus, suggesting it was heat sensitive, for example a protein.

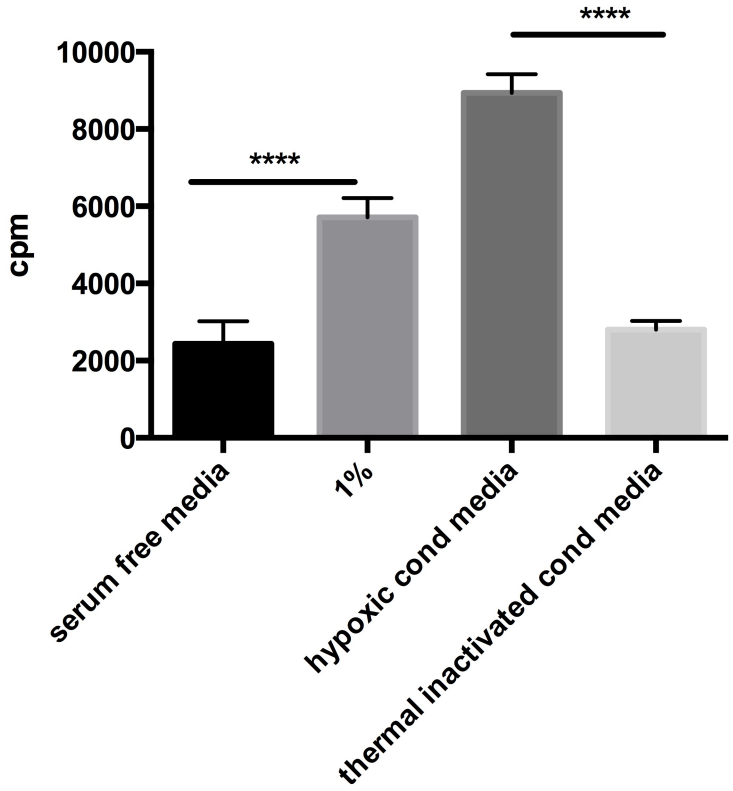

Figure 3-11 Thermal denaturation removes the proliferative stimulus from PAF conditioned media

Conditioned media taken from hypoxic fibroblasts was placed in a water bath at 80^{o}C for 10 minutes and then added to PASMC. The conditioned media was no longer able to stimulate proliferation of the PASMC. Data represents 2 experiments with mean values + SD. **** $p<0.0001$

3.4.3 ATP concentration is not increased in hypoxic PAF conditioned media

There is clear evidence in the literature that ATP can be released from cells as a 'danger' signal which can then lead to a cascade of adjacent pro-inflammatory events. To explore this further the conditioned media from hypoxic fibroblasts was collected and then analysed for ATP levels as per methods section. This showed that statistically there was no difference in the ATP concentrations identified between hypoxic and normoxic fibroblasts (Fig 3-12). This suggested that purine signalling was not a key factor in driving the smooth muscle cell proliferation observed in the co-culture experiments.

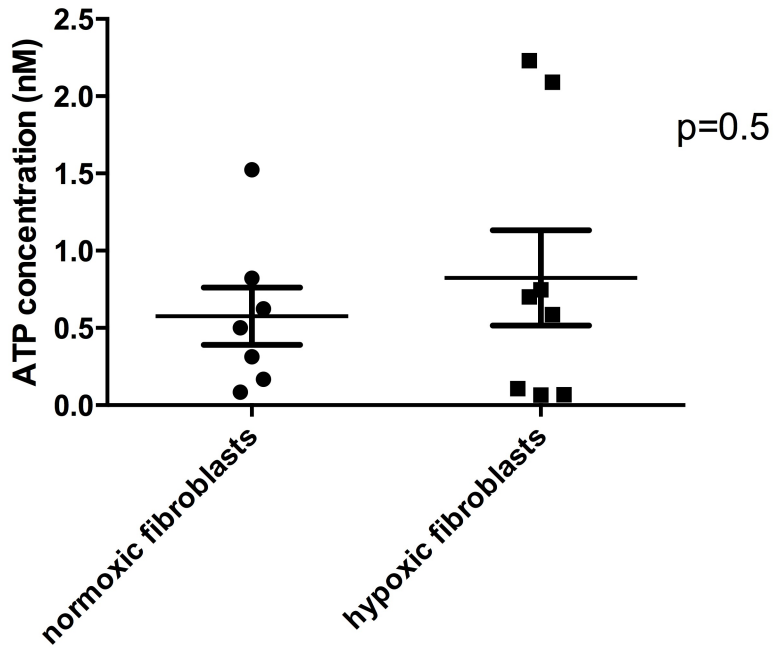

Figure 3-12 ATP release from hypoxic PAF s not significantly different from normoxic PAF
PAF cells were exposed to 48 hours of hypoxia or normoxia and the supernatant collected. This was then analysed using a bioluminescence assay for ATP activity. No difference was found between samples. Data represents individual values + SEM.

3.4.4 Endothelin-1 release is stimulated by hypoxia in the fibroblast

Previous work in our group has shown that the proliferation of smooth muscle cells by the conditioned media released from the hypoxic fibroblast could be inhibited by the action of Bosentan, an endothelin antagonist, acting on the smooth muscle cell. In order to investigate this further the conditioned media was analysed for levels of ET-1.

3.4.4.1 Hypoxic PAF release ET-1 and is reduced by p38MAPK inhibition

In the hypoxic PAF a time course of the release of ET-1 was observed. This showed that at 24 and 48 hours there was an increase in release of ET-1 in response to hypoxia. Furthermore when the experiment was repeated in the presence of SB203580, the ET-1 release was inhibited (Fig3-13).

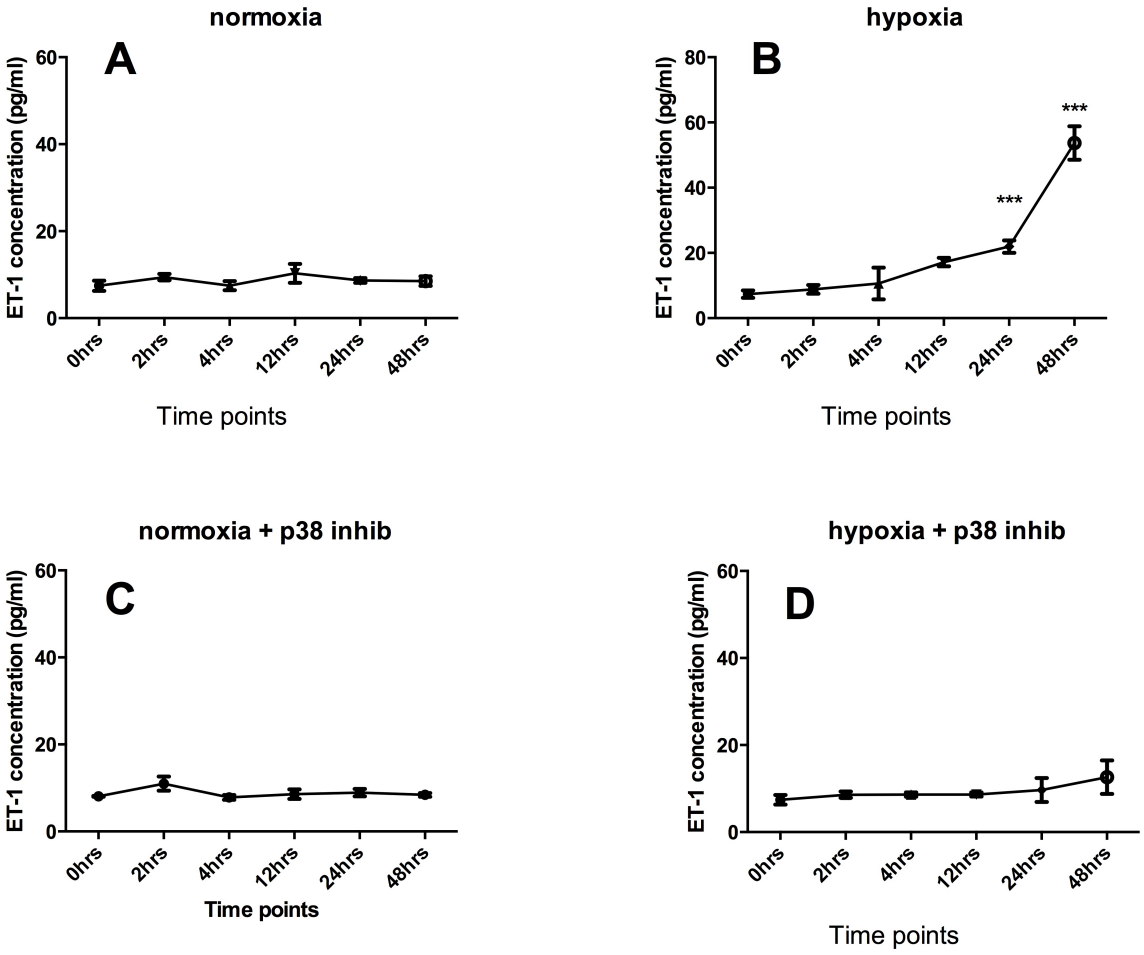

Figure 3-13 Hypoxia stimulates release of endothelin-1 from PAF
PAF in primary culture from normal rats were exposed to hypoxia or normoxia for 48 hours in the presence or absence of SB203580 (p38 inhib). The supernatant was collected at different time points and frozen immediately for later analysis. An ET-1 ELISA was performed on thawed supernatant and all samples were analysed together. Results are mean from triplicate samples with 3 separate animals. (A)normoxic fibroblasts, (B) hypoxic fibroblasts, (C) normoxic fibroblasts+p38 inhibitor, (D) hypoxic fibroblasts +p38 inhibitor. ***$p<0.005$ compared to 0hrs

3.4.4.2 ET-1 is released from chronic hypoxic- and MCT-derived fibroblasts

With low dose serum (1%) it was observed that the release of ET-1 from both chronic hypoxic and MCT derived PAF could be obtained. Levels were low but still significant. The release was also blocked by the use of SB203580. Exposure of the MCT PAF to hypoxia did not lead to any further increase in the release of ET-1 in comparison to low dose serum alone (Fig 3-14).

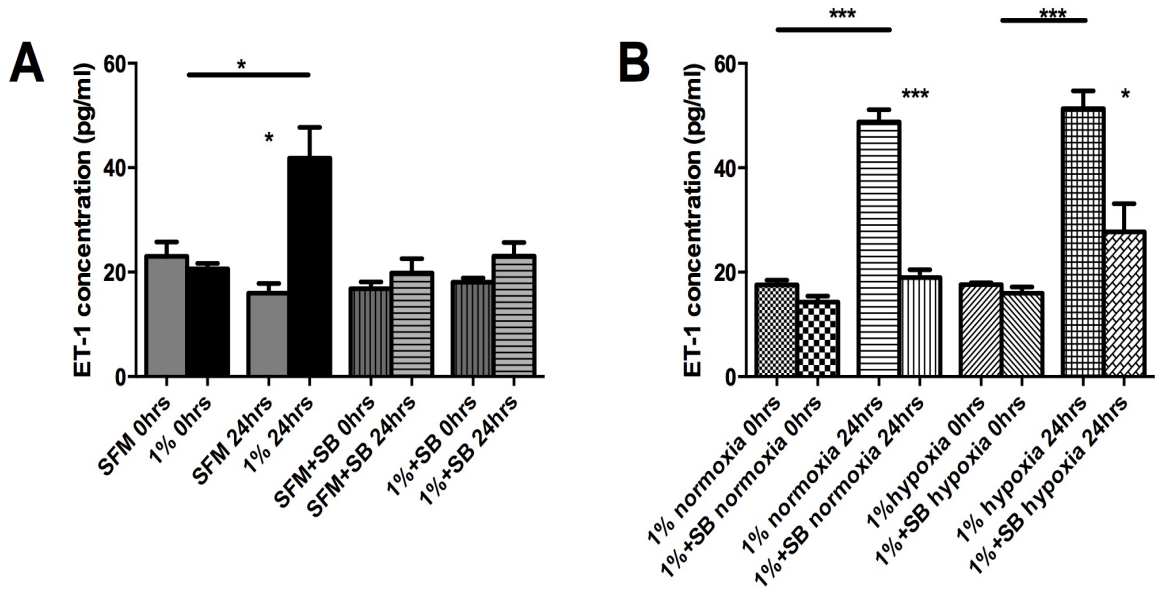

Figure 3-14 Low dose serum can stimulate release of ET-1 from both CH- and MCT-derived fibroblasts

PAF were grown from either CH (A) or MCT (B) animals and following 24 hours of growth arrest, were incubated with either serum free media or 1% media +/- SB203580. The supernatant was collected and analysed by ELISA. In the case of the MCT cells the experiment was also performed in hypoxia. Data represents mean values + SEM from 2 replicate experiments on cells from each animal; 3 different animals were completed. *p<0.05 ***p<0.005. SFM=serum free media;SB=SB203580;1%=1% serum

3.4.5 Cytokine and chemokine array screening

As our work has shown, p38MAPK is an important cellular pathway in the fibroblast. It has also been shown to be pro-inflammatory in other cells and therefore it was rational to investigate the role that this pathway might have in the release of pro-inflammatory mediators from the fibroblast.

In order to optimise the detection of potential mitogens a cytokine array screen was used. This can screen for 29 chemokines and cytokines in the conditioned media from the fibroblasts. This can be used as a preliminary screen to identify potential key inflammatory mediators which can then be further analysed using more standard quantitative ELISA technology. The total number of chemokines which can be identified is outlined in chapter 2.

Under basal state conditions the mediators identified as being released from the normoxic fibroblast were Vascular endothelial growth factor (VEGF-A) and tissue inhibitor of metalloproteinases-1 (TIMP-1). When the conditioned media from the hypoxic fibroblast was analysed there was a dramatic upregulation in a number of inflammatory mediators, as shown Figure 3-15.

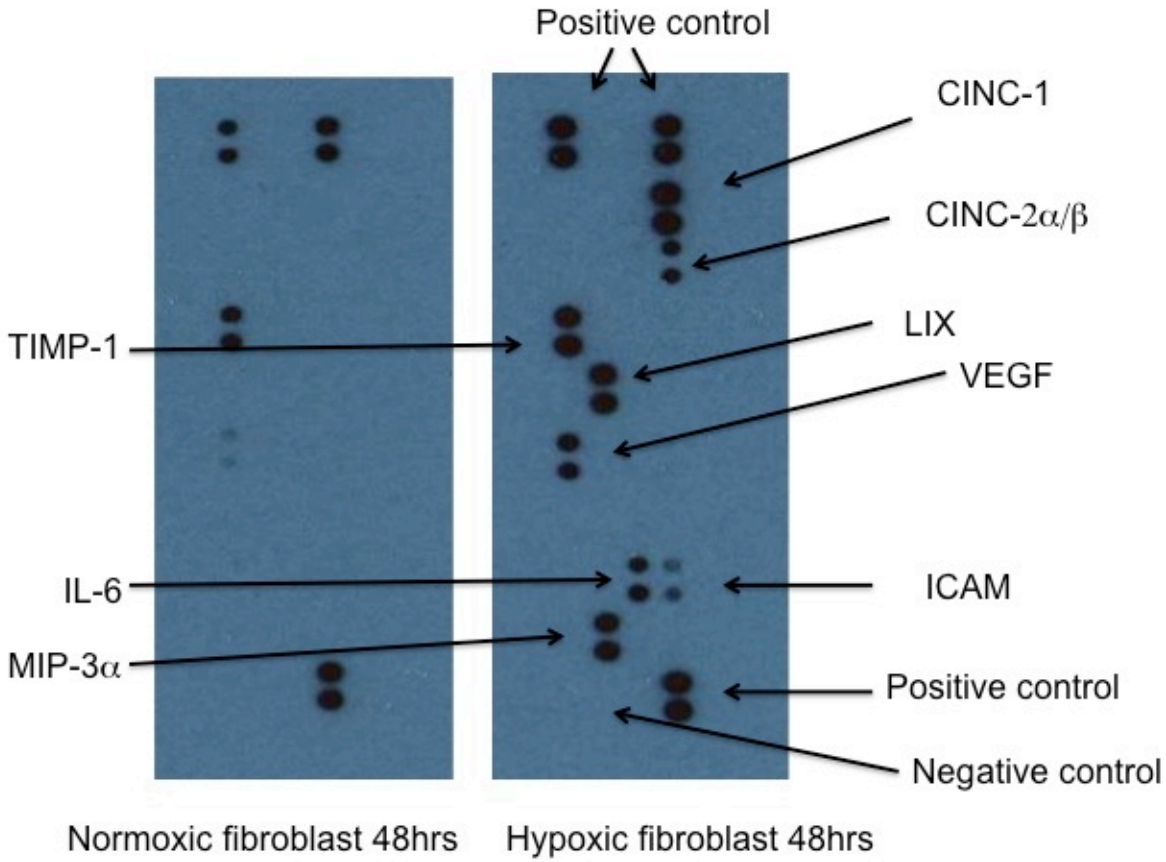

Figure 3-15 Hypoxia upregulates expression of cytokines and chemokines in the PAF
Supernatant from the fibroblasts were collected after 48 hours exposure to normoxia or hypoxia. This was then incubated overnight with the cytokine array nitrocellulose membrane at 4^0C and then developed. The length of exposure is the same for both membranes (1min). The marker for IL-6 is highlighted. Experiment repeated once.

The principle ones identified on the screen as being released de novo or upregulated from the hypoxic fibroblasts are displayed in the Table 1-1.

VEGF-A	CXCL1/CINC-1	ICAM-1	LIX
TIMP-1	CXCL3/CINC-2	IL-6	CCL20/MIP-3α

Table 3-1 Details of the upregulated cytokines detected from hypoxic PAF

The conditioned media used was isolated from pulmonary artery fibroblasts which were exposed to normoxia or hypoxia. This was repeated but in the presence or absence of the p38MAPKα inhibitor SB203580. As shown the p38MAPK inhibitor attenuates the release of the inflammatory profile and returns it back to that of an unstimulated PAF (Fig 3-16).

Of note IL-6 was strongly expressed on the cytokine array and as there was already substantial evidence that IL-6 may be involved in the pathobiology of pulmonary hypertension, this cytokine was investigated further.

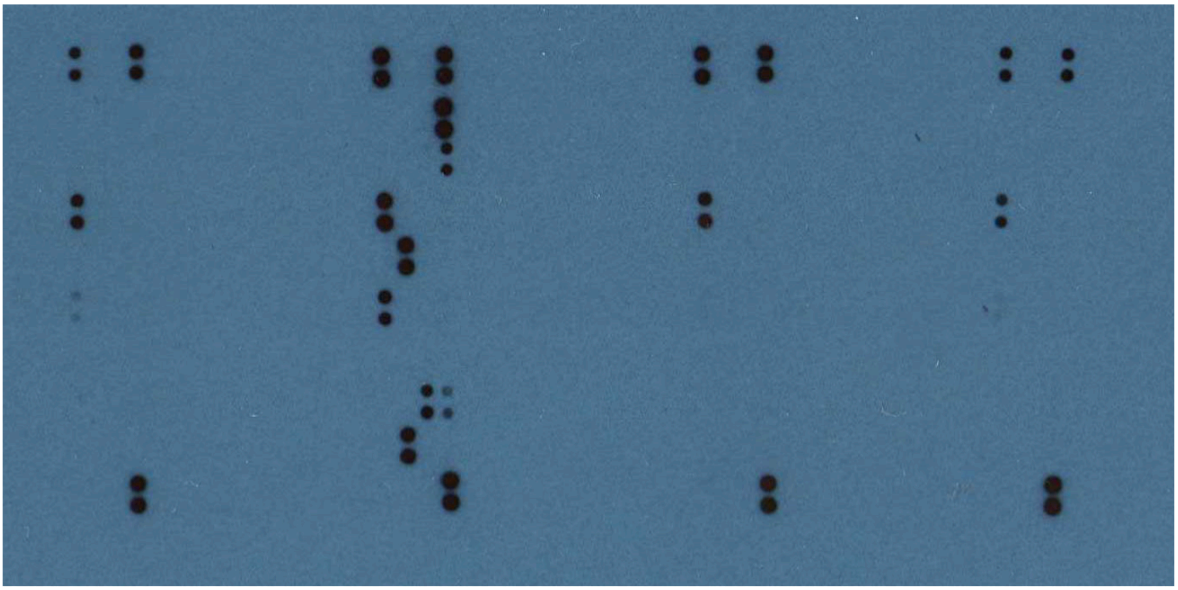

Figure 3-16 p38MAPK inhibition reverses the release of pro-inflammatory mediators from hypoxic PAF
PAF from normal animals were exposed to 48 hours normoxia or hypoxia +/- SB203580. The supernatant was collected and analysed using cytokine array. The above are representative of single samples from 3 different animals. Experiment repeated once.

3.4.6 Interleukin-6 is released by the hypoxic PAF and mediated by p38MAPKα

This identified Interleukin-6 (IL-6) as an important cytokine which seemed to be upregulated by hypoxia and therefore the biology of this cytokine and the fibroblast was analysed further. Quantitative ELISA was used to determine the time course and amount of the release and the effect SB203580 might have on this. The amount secreted by the PAF continued to increase over a time course with significant levels at both 24 and 48 hours after exposure of the cells to hypoxia. This release was blocked by the administration of SB203580 to the cells (Figure 3-17).

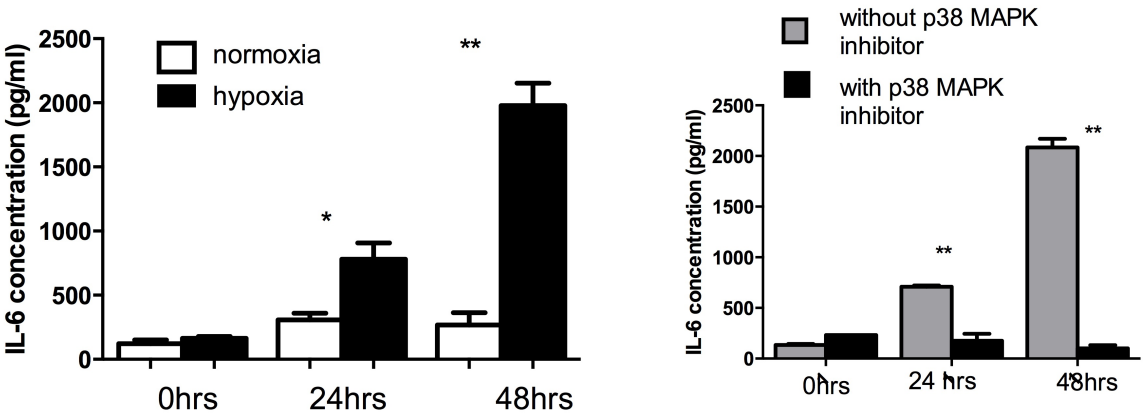

Figure 3-17 IL-6 release is significantly released after hypoxia and inhibited by p38MAPK blockade

Supernatant from normoxic and hypoxic PAF +/- SB203580 was analysed using a quantitative ELISA to determine actual amounts released. Cells were growth arrested in serum free media for 24 hours prior to hypoxic exposure. The values are mean + SEM values from triplicate wells for each sample and experiment repeated using cells from 3 animals. * $p < 0.05$ ** $p < 0.005$

A similar prevention of IL-6 release was observed by pre-treatment of the fibroblast with siRNA specific to the p38MAPK-alpha isomer and then hypoxic exposure (Fig 3-18).

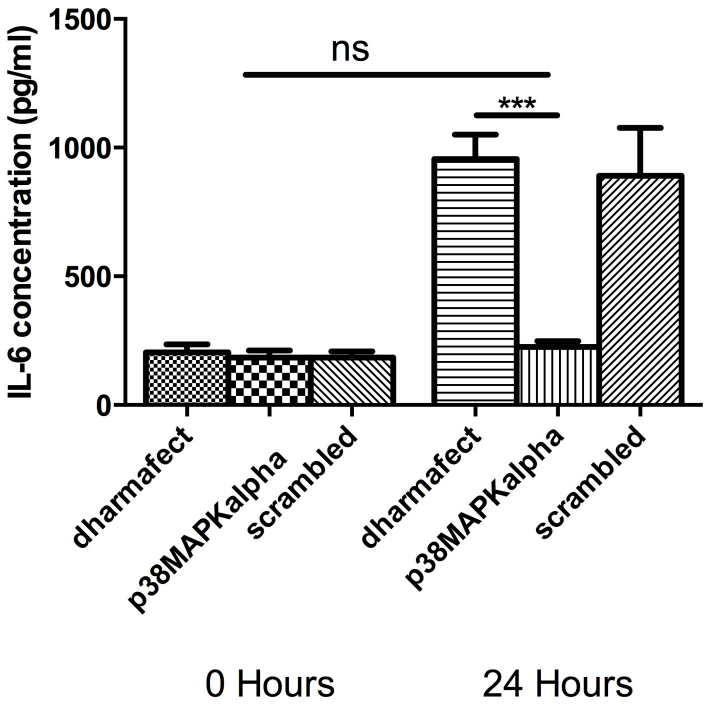

Figure 3-18 IL-6 secretion by hypoxic fibroblasts reduced using p38MAPK-α siRNA
Cells were exposed to p38-α siRNA, dharmafect alone or scrambled siRNA for 48 hours prior to exposure to hypoxia. The supernatant after a further 24 hours of hypoxia was removed and analysed for IL-6 by ELISA. Results are mean values + SEM and are of triplicate samples for 1 animal; repeated on 3 different animals. ***$p<0.005$

This was mirrored with a rise in the mRNA for IL-6 with maximal peak at 12 hours, as measured by qRT-PCR. The effect of p38MAPK inhibition was examined on the mRNA transcription of Il-6. From figure 3-19 it is seen that p38MAPK inhibition seems to reduce the transcription of Il-6 at a gene level.

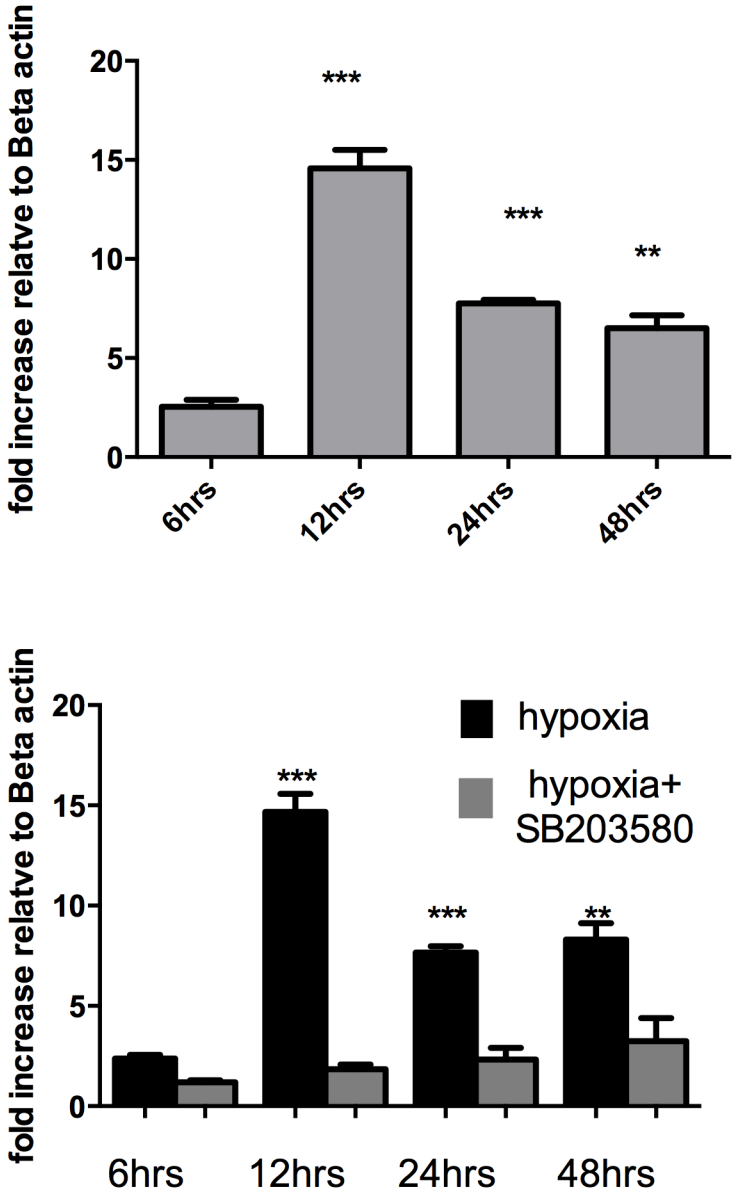

Figure 3-19 p38 MAPK inhibition reduces IL-6 mRNA transcription
PAF were exposed to hypoxia and then RNA isolated after 48 hours. The RNA was reverse transcribed into cDNA and then analysed using qRT-PCR. The mRNA increased and peaked at 12 hours of hypoxia (A). This was inhibited using SB203580 (B). Values represent ratio of increase of IL-6 gene mRNA relative to the housekeeping gene of β-actin and calculated using comparative Ct method. Values are mean + SEM and are representative of triplicate samples from 3 experiments using 3 different animals. **p<0.01, ***p<0.005

Since conditioned media from PAF isolated from MCT and chronic hypoxic animals also stimulated smooth muscle cell proliferation, we wanted to investigate if IL-6 was released from these cells.

3.4.7 IL-6 is released and modulated by p38MAPK in fibroblasts from MCT and chronic hypoxic animal models

IL-6 ELISA was performed on fibroblasts derived from both chronic hypoxic and MCT animals. These cells were exposed to low dose serum in order to stimulate them. We repeated the experiments in the presence of SB203580. The results are shown. In the CHPAF even at baseline there was an observed increase in IL-6 after just one hour suggesting that there is already an increased secretion of IL-6 at baseline (Figure 3-20). These effects were abrogated with SB203580.

In the MCT fibroblasts the levels of IL-6 were surprisingly lower than those observed in the CHPAFs. The explanation for this is unclear as indeed MCT is felt to be a stronger inflammatory stimulus. It is also interesting to note that the basal levels of IL-6 are higher in the MCT fibroblasts after 24 hours with no stimulation from serum (Fig 3-21). Again levels fall after p38MAPK inhibition.

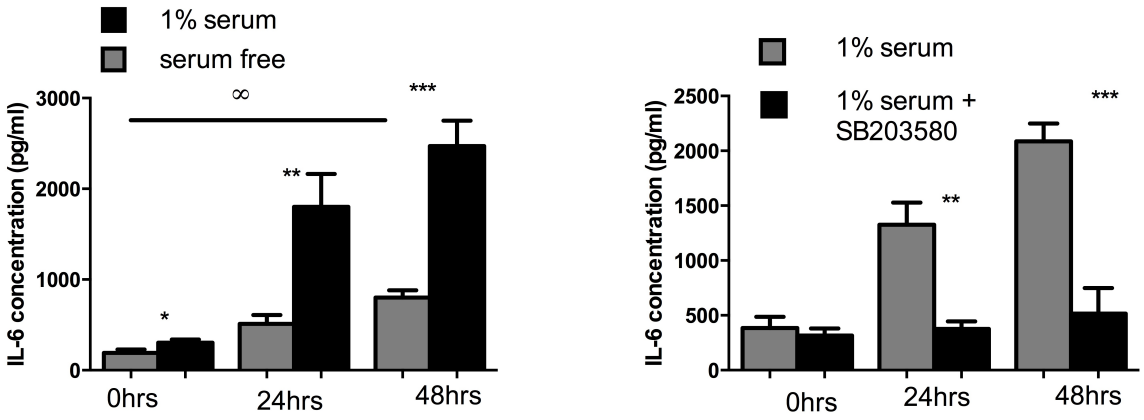

Figure 3-20 CH-PAFs release IL-6 in response to stimulation and this can be inhibited with p38 inhibition

PAFs isolated from chronic hypoxic animals were exposed to serum free media or low dose serum in normoxia. The supernatants were collected after 24 and 48 hours and analysed for IL-6 using ELISA. The experiment was repeated in presence of 1% serum +/- SB203580. Results are mean values +SD, representative of triplicate samples from 3 different animals. * $p<0.05$ ** $p<0.005$, *** $p<0.0001$, ∞ represents serum free 48hrs significantly different from 0hrs

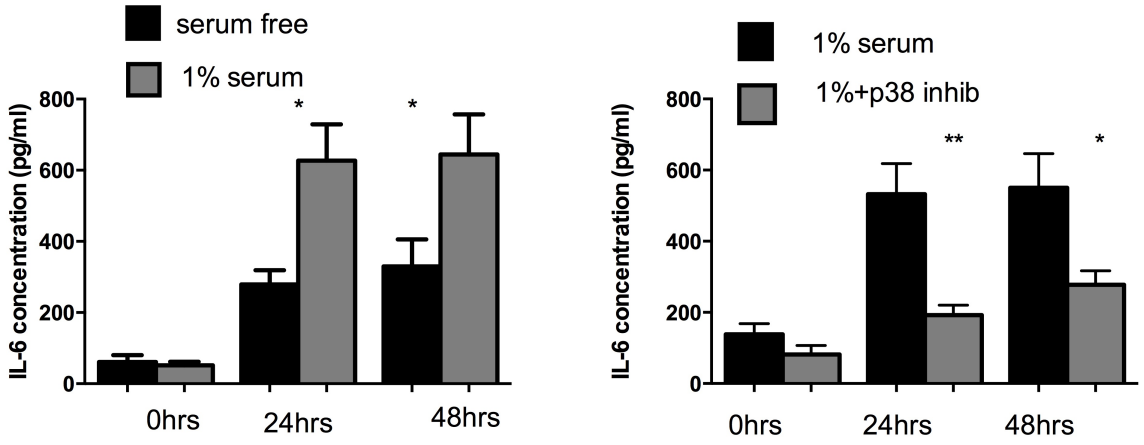

Figure 3-21 MCT-PAFs release IL-6 in response to stimulation and this can be inhibited with p38 inhibition

PAFs isolated from MCT animals were exposed to serum free media or low dose serum in normoxia. The supernatants were collected after 24 and 48 hours and analysed for IL-6 using ELISA. The experiment was repeated in presence of 1% serum +/- SB203580. Results are mean values +SEM, representative of triplicate samples from 3 different animals. * $p<0.05$ ** $p<0.005$

3.5 The potential importance of IL-6 in cellular pulmonary vascular remodelling

3.5.1 IL-6 stimulates the proliferation of pulmonary vascular fibroblast and smooth muscle cells

To test the importance of IL-6 in the pathobiology of vascular remodelling we examined the effect of IL-6 on rat primary fibroblasts and smooth muscle cells. Both the cis- and trans- activation pathways were tested in the PASMC to see if there was an additional effect mediated by either signalling pathway in particular. Therefore soluble IL-6 receptor (sIL-6R) was added along with Il-6 in an attempt to test the trans-pathway of activation. Thymidine assay and cell numbers showed an increased proliferative response to IL-6 by both cell types. There was no difference in the PAF between the IL-6 signalling pathways. In particular the addition of sIL-6R did not lead to increased proliferation of the cells. The increase in proliferation to IL-6 was also maintained in the hypoxic environment with the PAF (Fig 3-22).

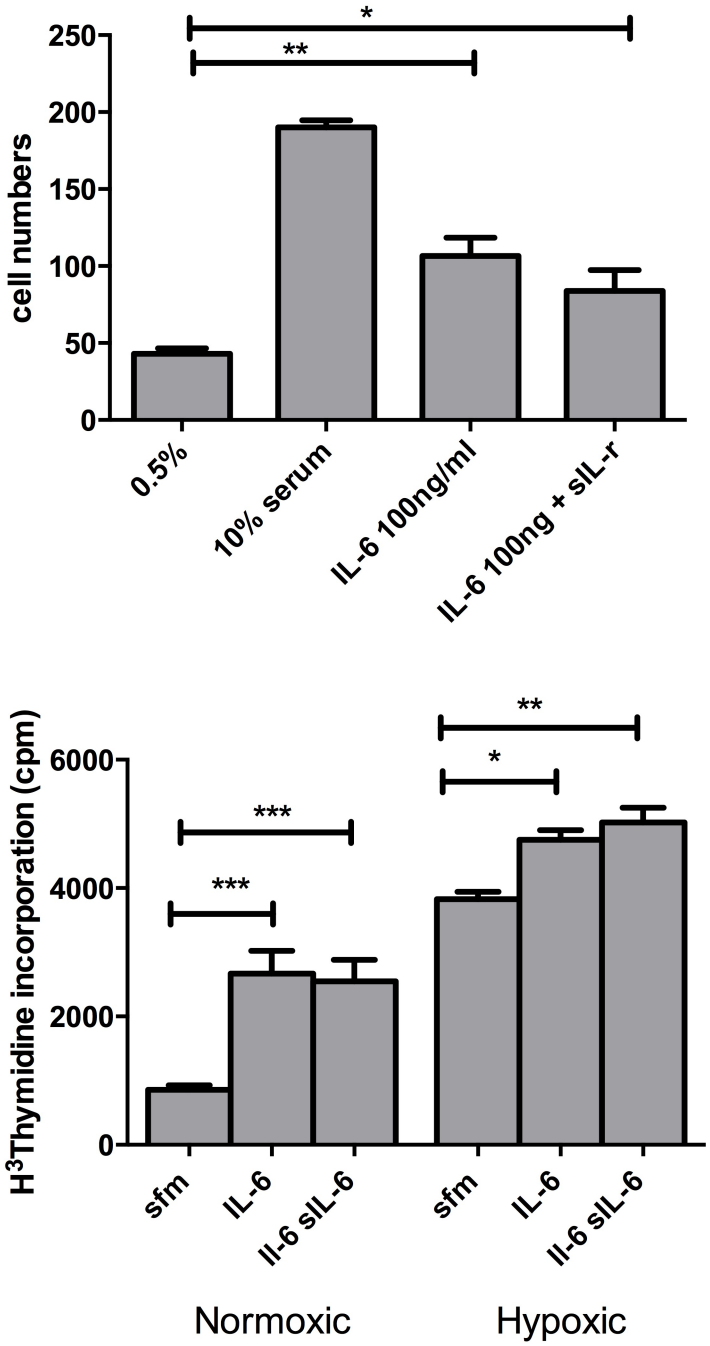

Figure 3-22 IL-6 leads to increased proliferation of PAF
PAF were isolated and incubated with IL-6 (100ng/ml) +/- soluble IL-6 receptor (sIL-6) and then using cell counting (top) and DNA synthesis (lower) as a marker of cell proliferation, the response was observed. Data are mean values + SEM and are representative of duplicate experiments performed on cells from 3 different animals. * $p<0.05$ ** $p<0.005$, *** $p<0.0001$

Il-6 led to increased proliferation of the pulmonary arterial derived smooth muscle cells (Fig 3-23). Immunoblotting from smooth muscle cells showed that IL-6 led to an increase in phosphorylation of STAT-3 which is the principle intracellular signalling pathway of IL-6 (Fig 3-24).

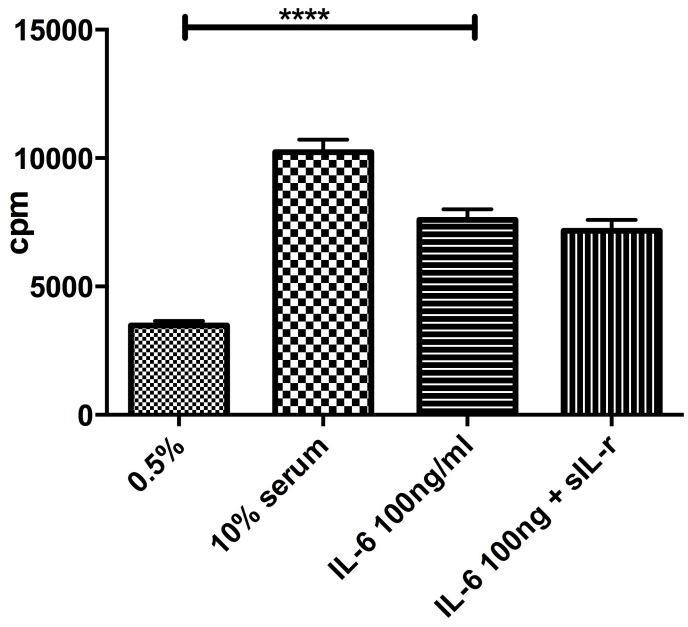

Figure 3-23 IL-6 results in increased proliferation of PASMC
PASMC were exposed to IL-6 IL-6 (100ng/ml) +/- soluble IL-6 receptor (sIL-r) and then using DNA synthesis as a marker of cell proliferation, the response was observed. Data are mean values + SD and are representative of duplicate experiments performed on cells from 3 different animals. **** $p<0.0001$

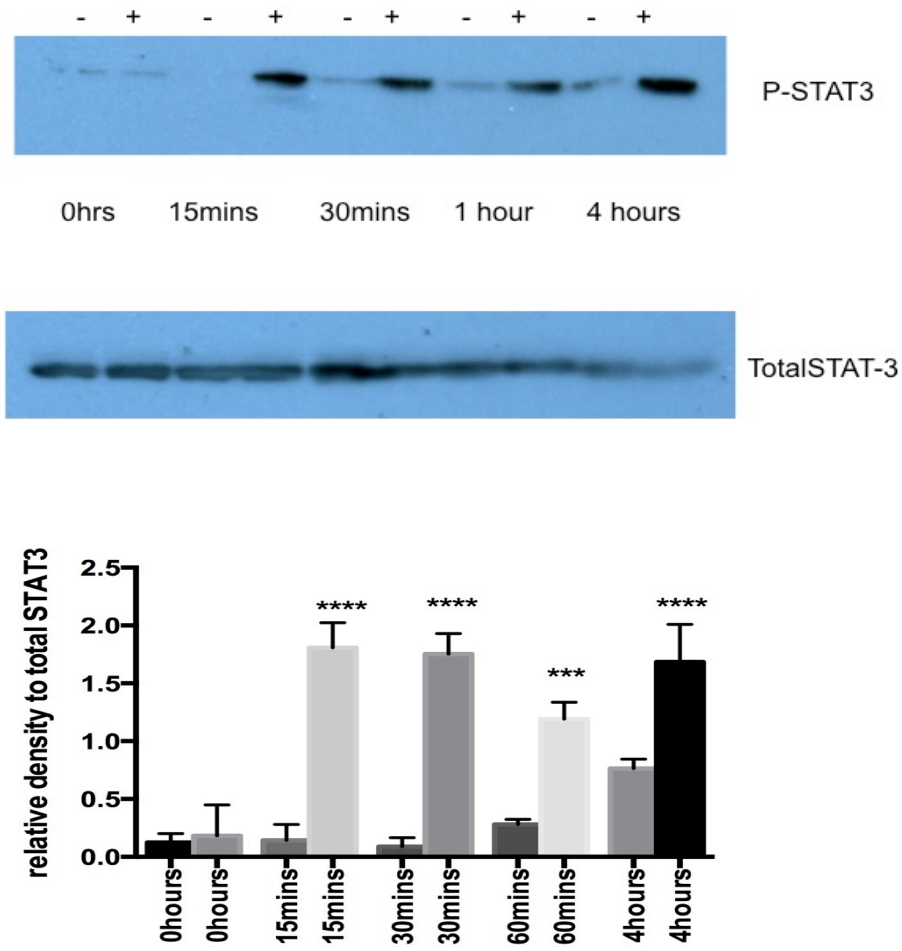

Figure 3-24 IL-6 increases phosphorylation of STAT-3 in a time dependent manner
PASMC were stimulated with 100ng/ml IL-6 (+) or without (-) and the protein harvested at baseline, 15mins, 30mins, 1 hour and 4 hours. The cell lysates were immunoblotted for phosphorylated STAT3 and total STAT3. Experiment was repeated 3 times, blots above are representative of those experiments. (B) shows densitometry from repeat blots. Values are mean + SD. ***p<0.01,****p<0.005

3.5.2 Anti-IL-6 blocks the proliferative response of pulmonary vascular cells to IL-6

Addition of anti-IL-6 antibody to pulmonary artery smooth muscle cells while being incubated with IL-6 led to an abolition of the IL-6 induced proliferation. This confirms that IL-6 can lead to proliferation of the smooth muscle cells. In an attempt to identify if IL-6 is the significant mediator of the hypoxic fibroblast conditioned media, anti-IL-6 was administered at the same time as the conditioned media (Fig 3-25).

As can be seen in the figure, the anti-IL-6 significantly reduces the effect of the conditioned media on the proliferation of the smooth muscle cells. However it does not completely remove it suggesting there may be other mediators which also contribute to the proliferative stimulus (Fig 3-26).

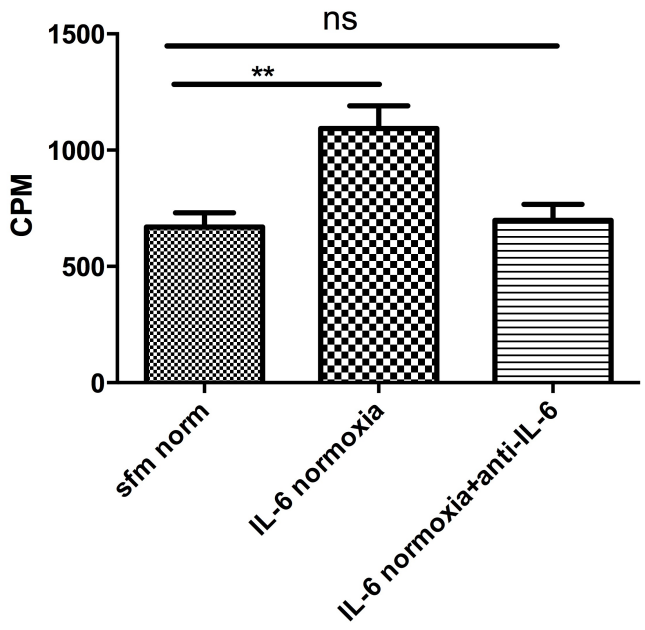

Figure 3-25 IL-6 is a potent mitogen for PASMC proliferation
PASMC were growth arrested for 24 hours and then incubated with serum free media, IL-6 or IL-6 and anti-IL-6 antibody. Thymidine assay was used to quantify DNA synthesis, a measure of cell proliferation. Results are plotted as counts per million. Values are mean + SD and represent mean of 3 experiments on cells from same animal. A total of 3 different animals were used. ** $p<0.01$

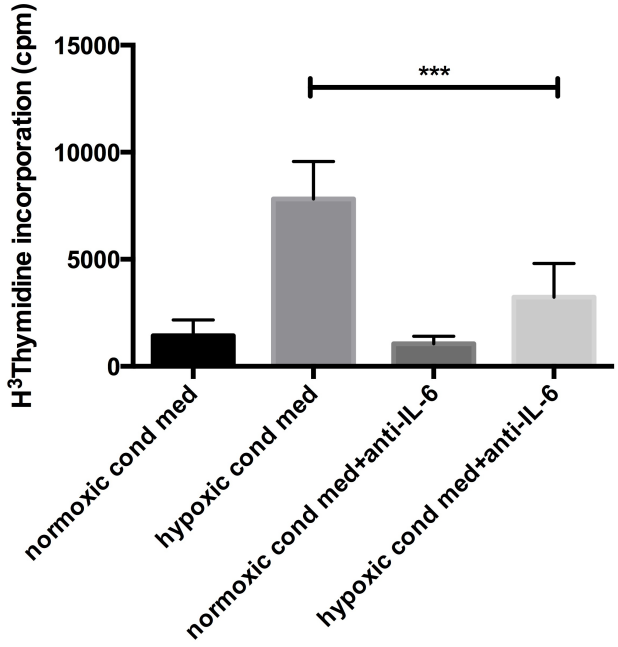

Figure 3-26 Anti-IL-6 reduces the proliferative stimulus on the PASMC from the PAF derived conditioned media

PASMC are quiescesed and then incubated with conditioned media derived from normoxic or hypoxic PAF. The hypoxic conditioned media induced PASMC proliferation. When both the conditioned media and anti-IL-6 were added to the PASMC there was an inhibition in the proliferative stimulus. Results are mean + SEM from 3 replicates from 3 different animals. *** $p<0.001$

3.5.3 Il-6 and hypoxia can stimulate the release of collagen from fibroblasts and is prevented by using SB203580

Pulmonary vascular remodelling involves both cellular proliferation and an increase in extracellular matrix deposition. The fibroblast is obviously an important cell type in facilitating the increase in ECM. To investigate this further the release of soluble collagen was measured after the PAF had been exposed to either hypoxia or IL-6 incubation for 24 hours. These stimuli both increased the amount of collagen released from the cells as shown in figure 3-27. This was inhibited by the co-administration of SB203580, suggesting p38MAPK as an important pathway in mediating this (Fig 3-28).

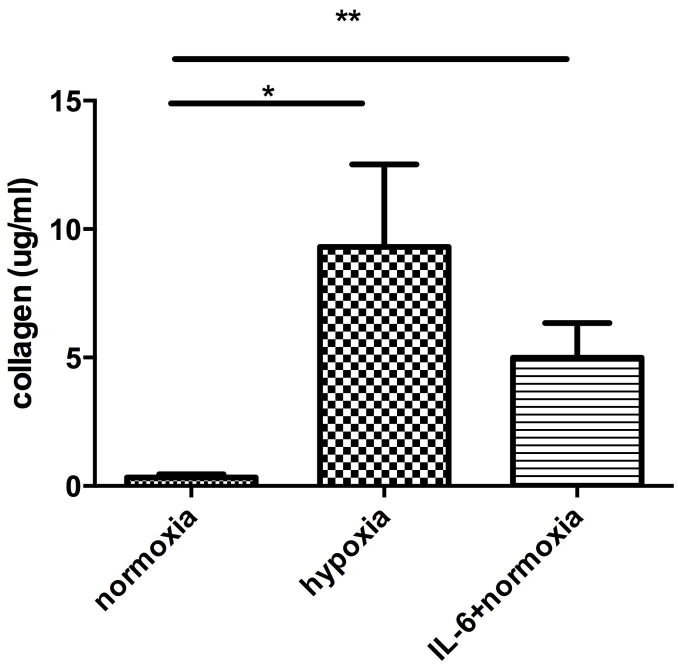

Figure 3-27 Hypoxia and IL-6 increase collagen production in fibroblasts
PAF from normal animals were isolated and stimulated in hypoxia or normoxia plus IL-6 (100ng/ml). The supernatant was collected and a collagen assay used to determine the amount of newly synthesised collagen released. Data are mean values + SEM and representative of 3 experiments for each animal; total of 2 animals used. * $p<0.05$, ** $p<0.005$

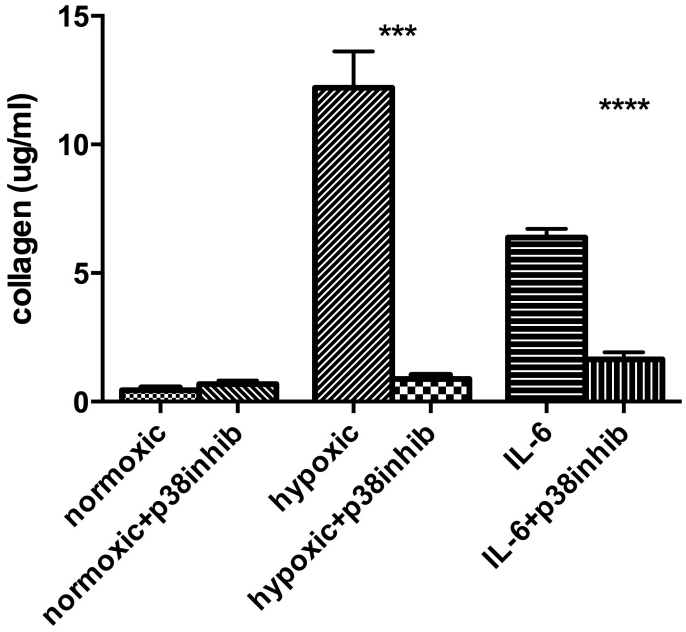

Figure 3-28 Inhibition of p38MAPK reduces the release of collagen from PAF
PAF were exposed to conditions of hypoxia or normoxia + IL-6 +/- SB203580. The data shown is representative of mean + SEM and duplicate experiments for each cell; PA from 3 animals in total were used. *** $p<0.005$ **** $p<0.0001$. p38inhib=p38MAPK inhibitor

3.5.4 Cell migration induced by hypoxia is controlled by p38MAPK

In pulmonary vascular remodelling there is the movement of cells between the layers of the vascular wall. This is especially noticed with fibrocytes and fibroblasts moving from the adventitia into the media layer and differentiating into myofibroblasts. Using a semi-permeable well insert, which allowed the migration of mobile cells through 8μm pores, fibroblast migration in response to hypoxia was measured (Figure 3-29). This showed that in hypoxia there was increased mobility of cells through the pores. This was determined by both cell number counting under light microscopy and also by spectrophotometry.

When the experiment was repeated in the presence of SB203580 the cell migration was reduced.

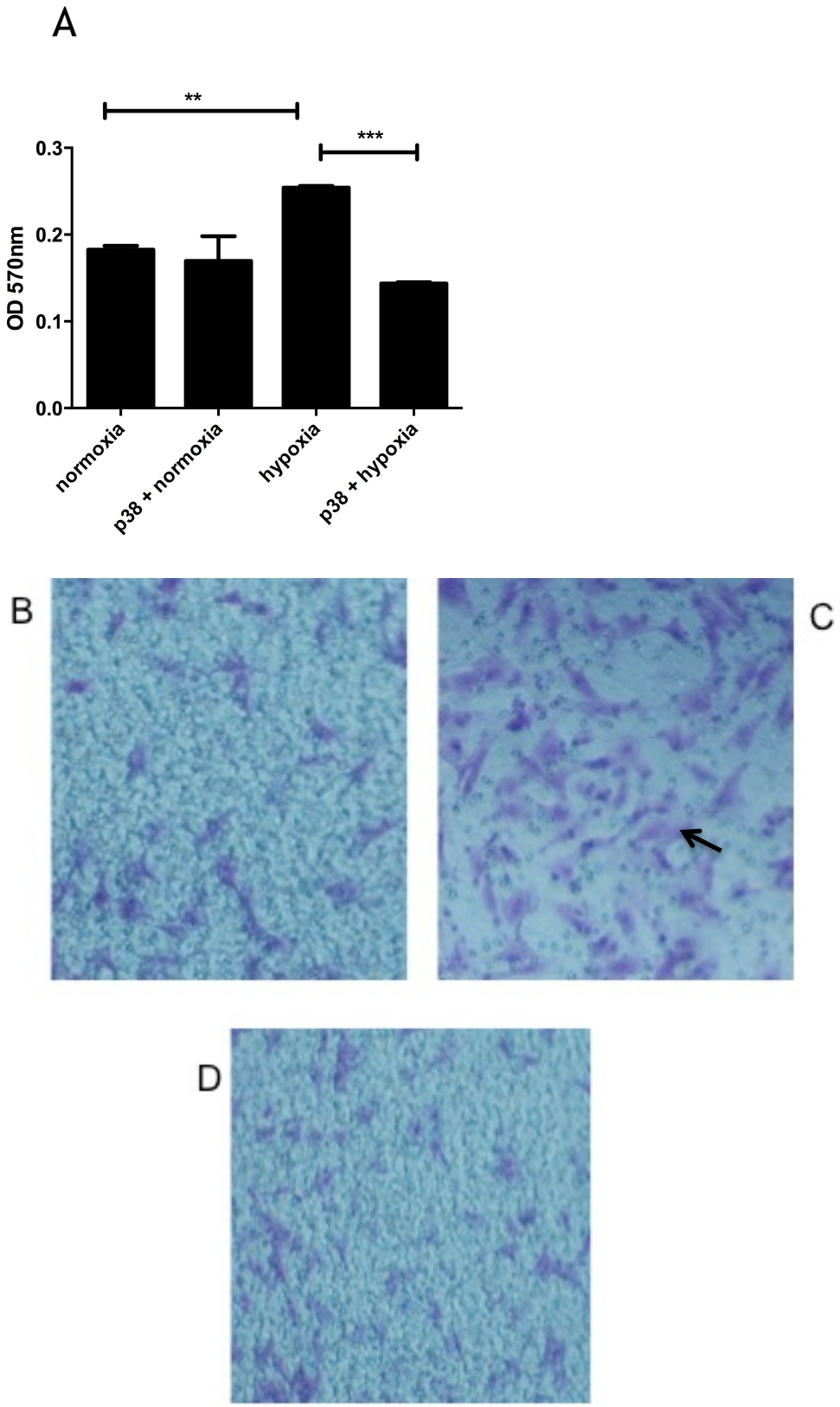

Figure 3-29 Hypoxia increases migration of PAF and is reduced by p38MAPK inhibition
Cells are plated out in known density ($1x10^6$) into the inserts containing the porous membranes. The inserts are placed into the wells with 0.1% serum +/- SB203580 and placed in normoxia or hypoxia and left for 24 hours. The cells, which have migrated onto the lower side of the insert, are isolated and quantified by means of a colorimetric assay (A). This shows that hypoxia can stimulate the movement of the PAF and this is mediated at least in part by p38MAPK. Visual representation is obtained by photographing the stained purple cells (arrow) on the underside of the insert (B=normoxia, C=hypoxia, D=hypoxia +p38inhib). Values representative of 3 experiments from different rats and duplicate for each experimental condition. ** $p<0.05$, *** $p<0.005$

3.6 Summary

This chapter has shown that PAF's releases factors which result in increased proliferation of adjacent smooth muscle cells. This is consistent in different cellular models, namely acute hypoxia, chronic hypoxic and monocrotaline derived fibroblasts. Interestingly both the unstimulated chronic hypoxic and monocrotaline derived fibroblasts seemed to release factors that could result in a basal increase in smooth muscle cell proliferation. The amount released was increased when the PAFs were stimulated with low dose serum at concentration of 1%. This clearly has implications for the development of pulmonary vascular remodelling. It also confirms the importance of the PAF in the biology of pulmonary hypertension in that no matter what the model used the fibroblast seems to have an altered phenotype which predisposes it to contributing to vascular remodelling.

In order to try and identify the factor or factors that were being released by these cells a logical approach was adopted. Initially thermal denaturation of the conditioned media was used to identify whether the mediator was a protein or some other material such as a lipid. Although a crude method the results suggested that a protein could be responsible for the effects.

Cytokine array techniques then identified a number of mediators which were released from the cell under hypoxic conditions. Of note, IL-6 was observed to be increased and there is growing interest in this cytokine as a key player in the pathogenesis of pulmonary vascular remodelling. Therefore this was focussed on. Under hypoxic conditions the PAF released a significant quantity of IL-6 and this was confirmed at protein and gene expression level. Importantly when this was done in the presence of a p38MAPK inhibitor, the IL-6 upregulation was prevented. This implicates p38MAPK as a critical part of the IL-6 regulation system in the PAF, and would correlate with findings in other cell types which show p38MAPK to have strong anti-inflammatory effects.

To confirm the importance of IL-6 in our model we examined its effects on smooth muscle cells and PAF. Published evidence has already shown that IL-6 is

a potent mitogen for the smooth muscle cell and our findings confirmed this. We also showed that IL-6 can act as a mitogen for the PAF and this might suggest an autocrine function for IL-6 in the proliferation of the PAF in pulmonary vascular remodelling. However IL-6 has additional effects on the fibroblast. This includes increased release of collagen, which can lead to increased extracellular matrix deposition, and reduced compliance of pulmonary arterial vessels. Again using a p38MAPK inhibitor inhibited the release of collagen. These findings again suggest an important role for both IL-6 and p38MAPK in the development of pulmonary hypertension.

Although IL-6 was identified in the conditioned media obtained from hypoxic PAF, it was unclear as to how significant IL-6 contributed to it's pro-proliferative effect. By concomitanly adding the conditioned media and anti-IL-6 antibody we showed that removing IL-6 reduced the proliferative effect by 60%. This suggests that IL-6 is a key component but not the only mediator present. Our findings have also shown that endothelin-1 is increased in the conditioned media and that together it is likely that these mediators also contribute to the proliferative effect. However importantly the blockade of p38MAPK also leads to reduction in the release of ET-1 from the fibroblast and therefore p38MAPK seems to have pleiotropic effects on release of mitogens from the PAF.

There is increasing recognition that PAF's, and indeed circulating fibrocytes, can migrate through the adventitial wall and undergo differentiation into a myofibroblast phenotype. These cells then become resident in the medial layer of the vessel wall and increase contractile responses but also increase extracellular matrix deposition. Using a motility assay data presented in this thesis has shown that hypoxia increases the cellular mobility of the PAF and that this seems to operate at least in part by a p38MAPK mechanism.

This would suggest p38MAPK as a logical target to try and prevent the cellular release of a number of initiating factors that promotes the pulmonary vascular remodelling. This is in contrast to the current approach in the clinical treatment of patients which involves preventing the end target effect of the mediators.

4 p38MAPK inhibition prevents and reverses pulmonary hypertension and pulmonary vascular remodelling in experimental animal models

4.1 Introduction

The previous chapter has shown the importance of p38MAPK signalling *in vitro* in the development of pulmonary vascular remodelling. It leads to the proliferation of the fibroblast, increases cellular migration and can stimulate the release of factors which lead to proliferation of adjacent smooth muscle cells. Moreover evidence presented previously identifies the alpha isoform as the important player in this. However, further evidence is required to establish the importance of this pathway in the pathobiology of pulmonary vascular remodelling. We investigated the role that p38MAPK and the alpha isoform might have in the development of *in vivo* models of pulmonary hypertension.

There are a number of animal models of pulmonary hypertension as previously discussed. The most recognised and best characterised of these are the chronic hypoxic and monocrotaline animal models. Therefore these models were developed in our laboratory and examined for evidence of p38MAPK activity. Perhaps more importantly the inhibition of the pathway was then studied to see if this resulted in both the prevention and reversal of the animal model phenotype of pulmonary hypertension.

In the prevention models of pulmonary hypertension the insult that induces the model of pulmonary hypertension (i.e. chronic hypoxia or monocrotaline) is given at the same time as the p38MAPK inhibitor in an attempt to prevent the development of pulmonary hypertension. Although this clearly gives an insight into the pathogenic mechanisms of pulmonary hypertension, it is not particularly reflective of human disease. Patients tend to present with established disease and therefore it is perhaps more clinically relevant to be able to reverse the established phenotype of pulmonary hypertension. Therefore the reversal strategy involved allowing the animals to develop disease and then try and reverse the phenotype with p38MAPK inhibition.

In this chapter evidence will be presented showing that p38MAPK is activated in animal models of pulmonary hypertension and that inhibition of this pathway in vivo can both prevent and reverse the pulmonary hypertensive phenotype.

4.2 Relevant techniques employed

Many of the general methods have already been outlined but more specific details are outlined here.

4.2.1 Drugs and antibodies

The p38 MAP kinase antagonist, SB203580 was obtained from Selleck chemicals and the dose used was 20mg/kg. The drug was dissolved in DMSO initially and made up to final volume with distilled sterile water. This was administered intraperitoneally (i.p.) once daily using 1ml syringe.

4.2.2 Animal models

(1) Chronic hypoxic model

Animals were maintained in a hypobaric chamber at ambient pressure of 550mmHg for up to 4 weeks, equivalent to an oxygen concentration of 10%. The chamber was depressurised initially over 2 days. To allow daily injections the chamber was brought back to atmospheric pressure over a 1 hour period, injections performed and then the chamber depressurised again with gradual reductions over 1 hour. The chamber was unsealed for a maximum of 30 minutes per day. Age and weight matched normoxic animals were housed in an adjacent perspex container but without depressurisation. Animals received food and water *ad libitum*. Animals body weights were measured twice weekly.

(2) Monocrotaline model

Monocrotaline was dissolved in 1M HCl and then neutralised with a similar volume of 1M Sodium Hydroxide. Animals were then injected with 60mg/kg monocrotaline subcutaneously in the scruff of the neck. Control animals received a similar volume of saline injected subcutaneously. The animals body weights were measured three times a week.

In order to test the treatment of p38MAPK in animal models the animals were divided up into the following groups:

(1) A *prevention* study for chronic hypoxia where the p38 MAP kinase antagonist SB203580 was administered at the start of the animals being exposed to the hypobaric chamber.

(2) A *reversal* study for chronic hypoxia where the animals were allowed to develop pulmonary hypertension for 2 weeks and were then treated with SB203580, a p38 MAP kinase antagonist.

(3) A *reversal* study for the monocrotaline treated animals with the p38 MAP kinase antagonist, SB203580.

Control chronic hypoxic and monocrotaline animals were injected with 0.9% saline.

4.2.3 Immunoblotting/Immunohistochemistry

The principle antibodies and dilutions used in these experiments were phospho-p38MAPK (1:500), total p38MAPK (1:500), p38MAPKα (1:400), Beta-actin (1:700). The degree of distal muscularisation was analysed by staining for alpha-smooth muscle actin (1:400).

4.3 Characterisation of *in vivo* animal models

To study the effects *in vivo* of p38MAPK two animal models were established: chronic hypoxic and monocrotaline.

4.3.1 Chronic hypoxic animal model

The methods of developing this animal model have already been outlined in a previous chapter. Confirmation of the development of the pulmonary hypertensive phenotype is presented here. Examples of the types of traces obtained from the haemodynamics are shown first (Figure 4-1).

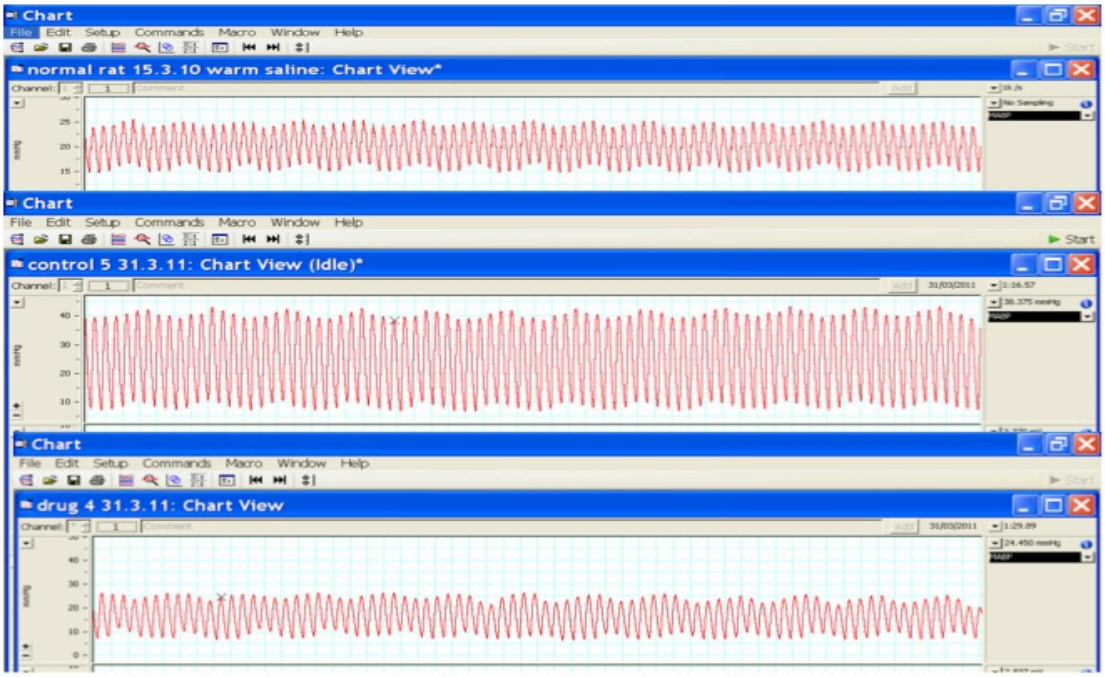

Figure 4-1 Right ventricular pressure tracings obtained from rats
The right ventricular pressure was measured using an 18G needle placed into the RV of anaesthetised animal. The pressure readings were obtained after a period of 1-2 minutes to allow the readings to settle. The mean pressure was calculated by averaging 5 sections of 100 beats. Data shown are examples of pressure readings from normal rat (top), hypoxic rat after 2 weeks (middle) and drug treated (SB203580) hypoxic rat (lower).

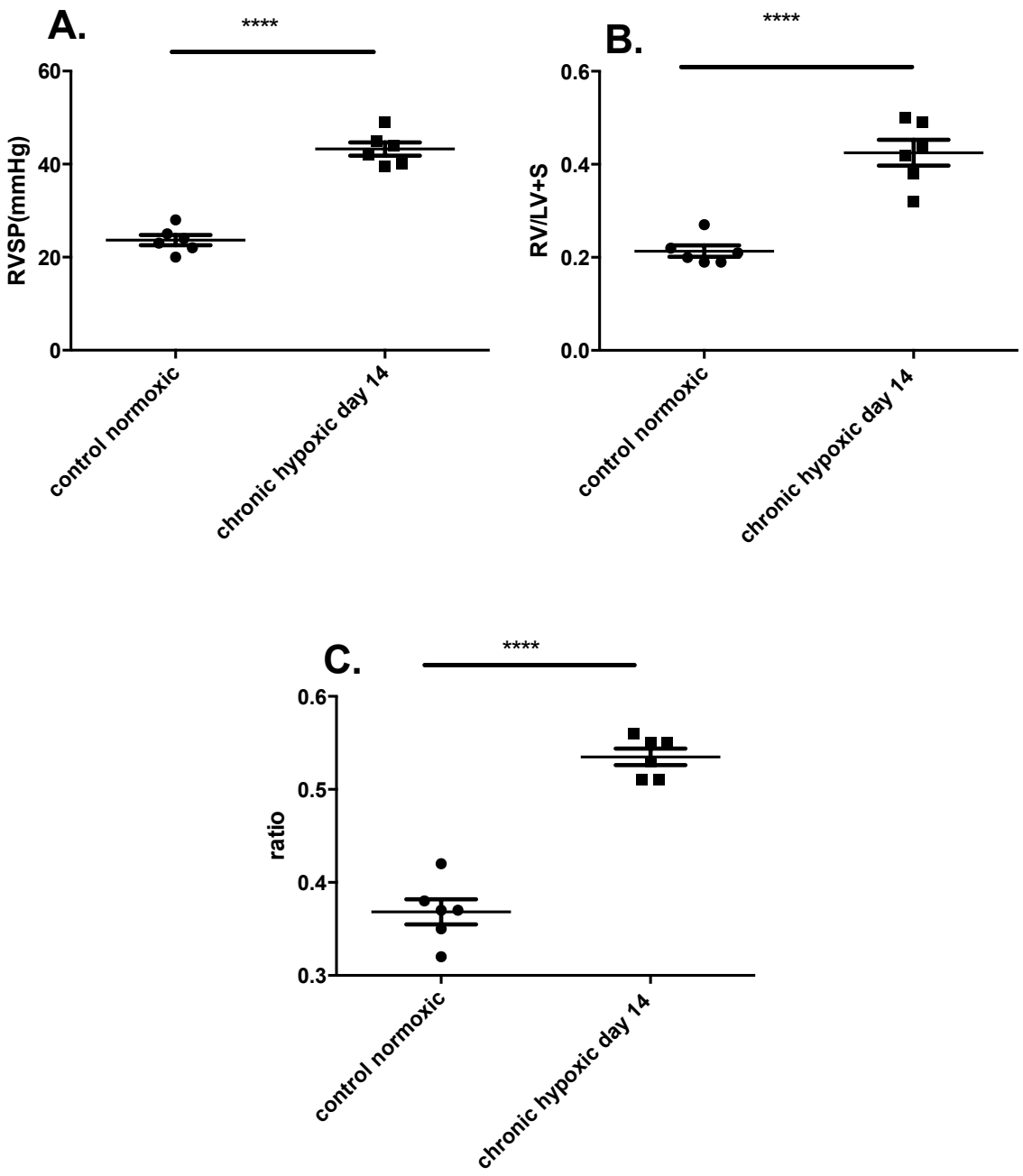

Figure 4-2 Exposure of rats to hypobaric hypoxia for 2 weeks results in pulmonary hypertension

Rats were placed in the hypobaric chamber for 2 weeks. Right ventricular systolic pressure (RVSP) (A), right ventricular hypertrophy as assessed by Fulton's index (B) and haematocrit (C) were all determined after 14 days. Data points indicate individual animals. Values are mean +SEM. **** $p < 0.0001$

As can be seen from the above results the animals that were exposed to chronic hypoxia for 2 weeks developed increased right ventricular systolic pressure and right ventricular hypertrophy (Figure 4-2). The haematocrit increased as expected to confirm exposure to hypoxia. There was clear remodelling of vessels observed under light microscopy (Figure 4-3).

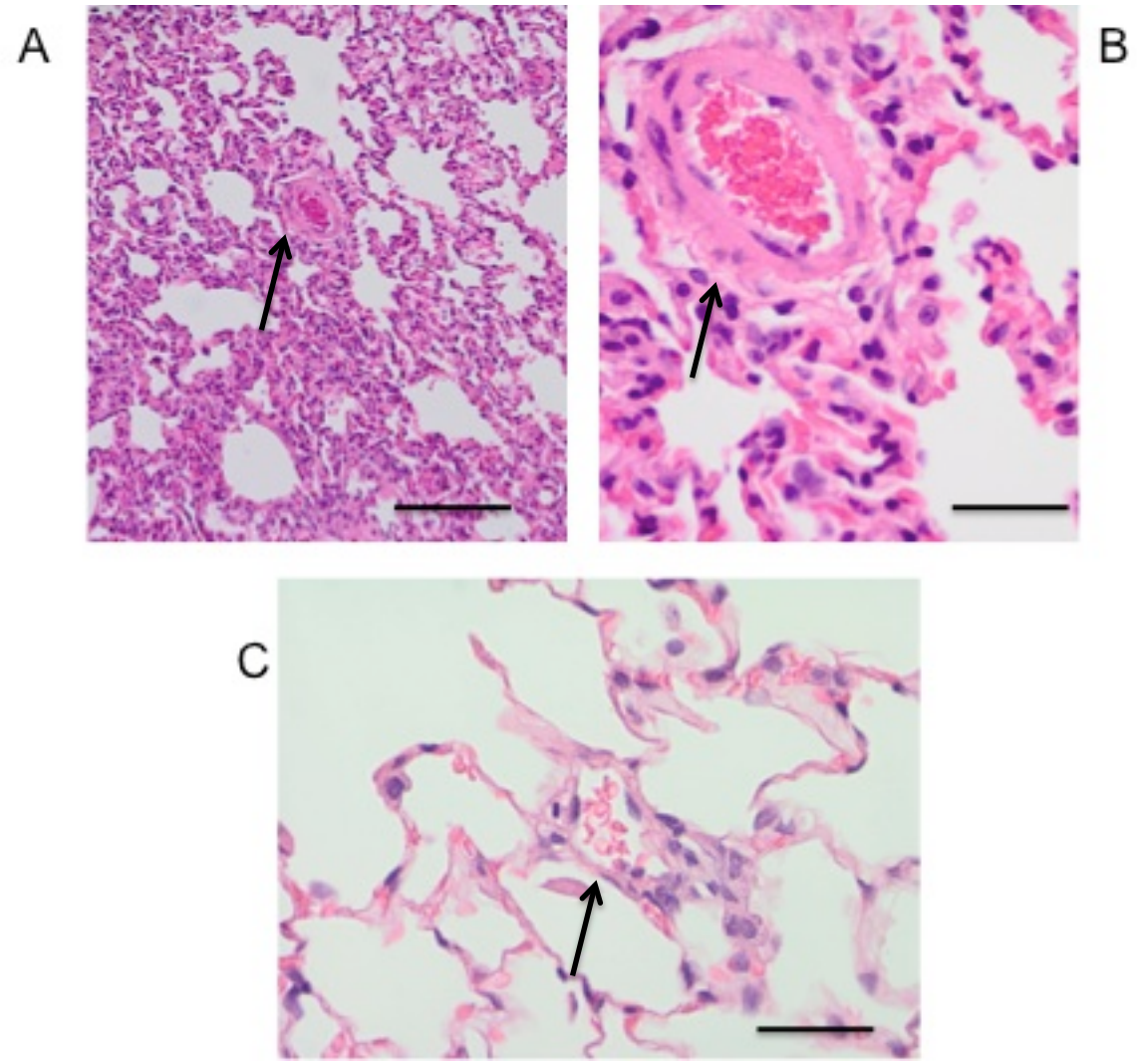

Figure 4-3 Appearance of chronic hypoxic and normal vessels
Both (A) and (B) show the appearance of lung from a chronic hypoxic animal at x10 and x40 respectively. (C) is a x40 power of a normal lung from a normoxic animal. Arrows indicate vessels. Bar represents 75μm at x40 and 150μm at x10.

This was also seen with muscularisation in the smaller vessels (Figure 4-4 and 4-5). A positive and negative control were used in all experiments as shown.

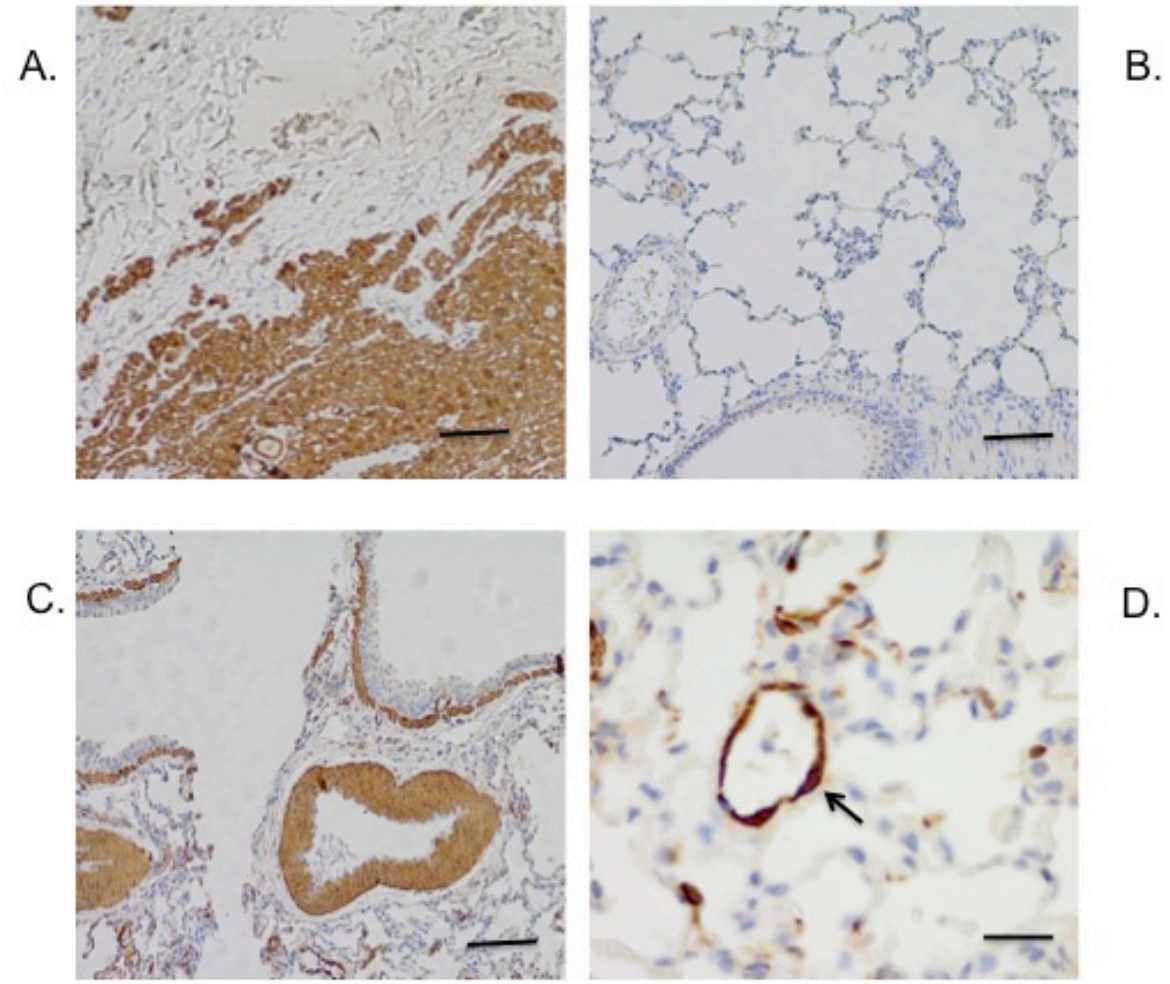

Figure 4-4 Staining for α-smooth muscle actin in lungs of chronic hypoxic rats
Rat lung sections were prepared in formalin and embedded in wax as outlined in chapter 2. Sections were cut at 5μm. Staining for α-SMA was performed using 1:400 dilution of anti-SMA antibody. Images shown are (A) positive control of placental smooth muscle, (B) isotype control, (C) larger pulmonary artery branch and (D) normal arteriole (arrow). Bar represents 150μm in A,B,C and 75μm in D. Images are X10 except for D which is X40.

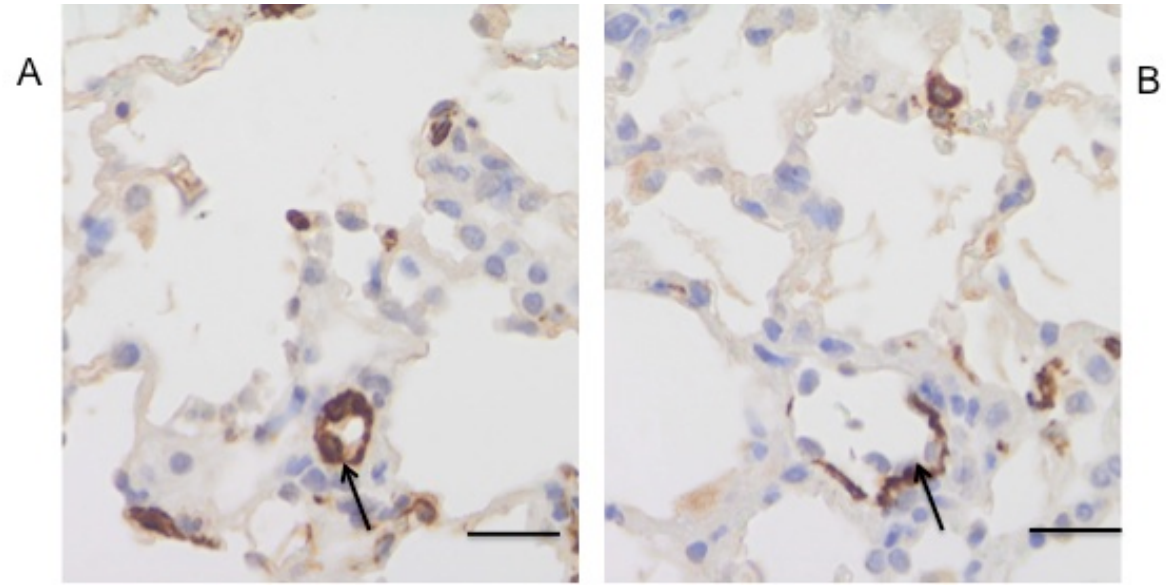

Figure 4-5 Muscularisation of small vessels in chronic hypoxic animals
Pictures shown are characteristic of (A) complete and (B) partial muscularisation. Staining for α-smooth muscle actin. Objective lens X40. Bar represents 75μm.

4.3.2 Monocrotaline animal model

The development of the monocrotaline model has been previously outlined.

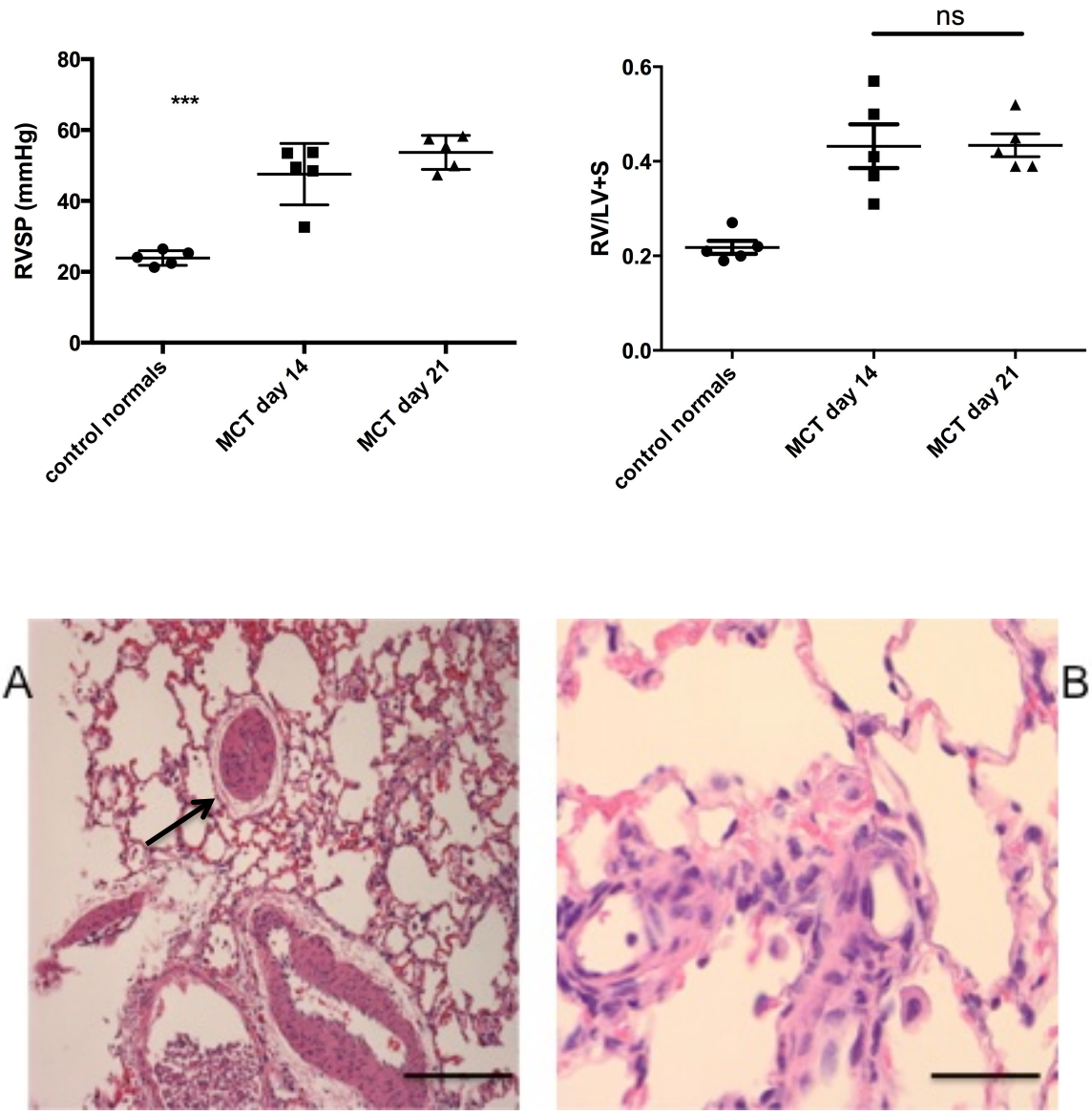

Figure 4-6 Characterisation of Monocrotaline induced PH animal models
Rats were injected subcutaneously with 60mg/kg of MCT. Haemodynamics were assessed at 14- and 21-days following injection. The right ventricular systolic pressure and degree of RVH is shown in top graphs. Each individual point is a single animal. Values are mean + SEM.

Histological analysis using H&E staining showing pulmonary vascular remodelling is shown in lower figure. Arrow indicates remodelled vessel. (A) X10 objective; (B) X40 objective. bar represents 75μm in (B) and 150μm in (A).

The above graphs demonstrate that at both 14-days and 21-days post MCT injection the animals develop pulmonary hypertension (Figure 4-6). Traditionally following 21 days from the injection of MCT, the animals are said to have developed pulmonary hypertension. In most of the literature, experiments on established pulmonary hypertension start after day 21 post-MCT exposure. However in our experience an unacceptable death rate of controls occurred at 28 days following MCT injection. However it is clear from the above data that after 14 days the MCT injected animals have developed pulmonary hypertension and that this phenotype is as robust as day 21. Furthermore other studies have used a similar time strategy and shown that animals at day-14 have evidence of established pulmonary hypertension.

4.4 p38 MAPK activity and expression is increased in animal models of pulmonary hypertension

The first strategy was to compare the baseline activity and protein expression levels present in normal animals and disease models of pulmonary hypertension.

4.4.1 Total p38MAPK activity is increased in animal models of pulmonary hypertension

The total activity of p38MAPK was assessed by using an *in vitro* kinase assay using phosphorylation of ATF-2 as a read out. This quantified the relative levels of active phosphorylated-p38 MAPK present in identical protein concentrations, (as determined by BCA assay), of lung homogenates between normal, chronic hypoxic and monocrotaline animals. It demonstrates a significant increase in the amount of activity in both chronic hypoxic and monocrotaline lungs compared to normal rat lung (Fig 4-7). This implicates higher activity levels of p38MAPK as being present in animal models of PH suggesting a potential role.

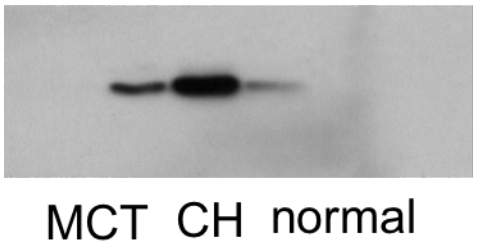

Figure 4-7 Increased p38MAPK activity in the lungs of both MCT and chronic hypoxic animals

Lungs from normal, chronic hypoxic and MCT animals were harvested and homogenised with a cocktail of phosphatase and kinase inhibitors. The protein concentration was quantified using BCA method. Equal concentrations were then used in a p38MAPK activity assay using immunoprecipitation and the phosphorylation of ATF-2 as a read out. Immunoblot shown is representative of 3 experiments using 3 different animals. MCT=monocrotaline rat lung; CH=chronic hypoxic rat lung;ATF-2 Activating Transcription Factor-2

4.4.2 phospho-p38 MAPK is increased in animal models of pulmonary hypertension

Next immunoblotting was used to determine the expression of phospho-p38 MAPK in lungs isolated from the animals. This demonstrated that there were increased levels of phosphorylated levels of p38 MAPK in both the monocrotaline and chronic hypoxic animals compared to normoxic animals (Fig 4-8). The total level of p38MAPK was not altered.

Taken together the above results suggest that the total p38MAPK activity is increased in both chronic hypoxic and monocrotaline animal models of pulmonary hypertension. We then wanted to identify if the alpha isoform was increased, which may explain the increased activity seen in these experiments.

The same is observed on immunohistochemistry (Figure 4-9). This shows increased activity of p38MAPK.

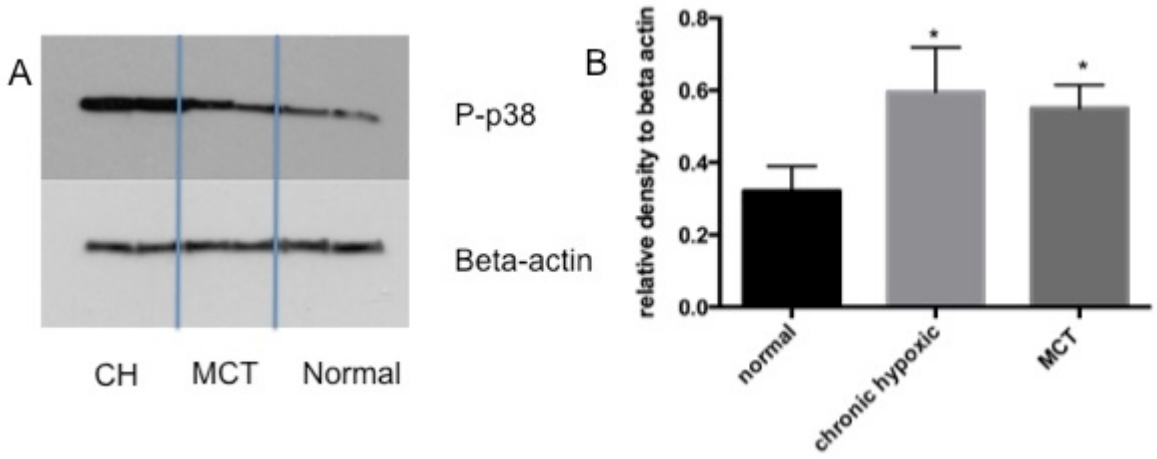

Figure 4-8 Increased phosphorylated-p38MAPK is found in lung homogenates of animal models of PH

(A) Lungs from normal, chronic hypoxic and MCT animals were harvested and homogenised with a cocktail of phosphatase and kinase inhibitors. The protein concentration was quantified using BCA method. Equal concentrations were then loaded on a gel and blotted for phosphorylated p38MAPK and beta-actin for loading control. Immunoblot shown is representative of 3 experiments using 3 different animals with each condition. MCT=monocrotaline rat lung; CH=chronic hypoxic rat lung. Densitometry is shown for remaining blots (B). *$p<0.05$

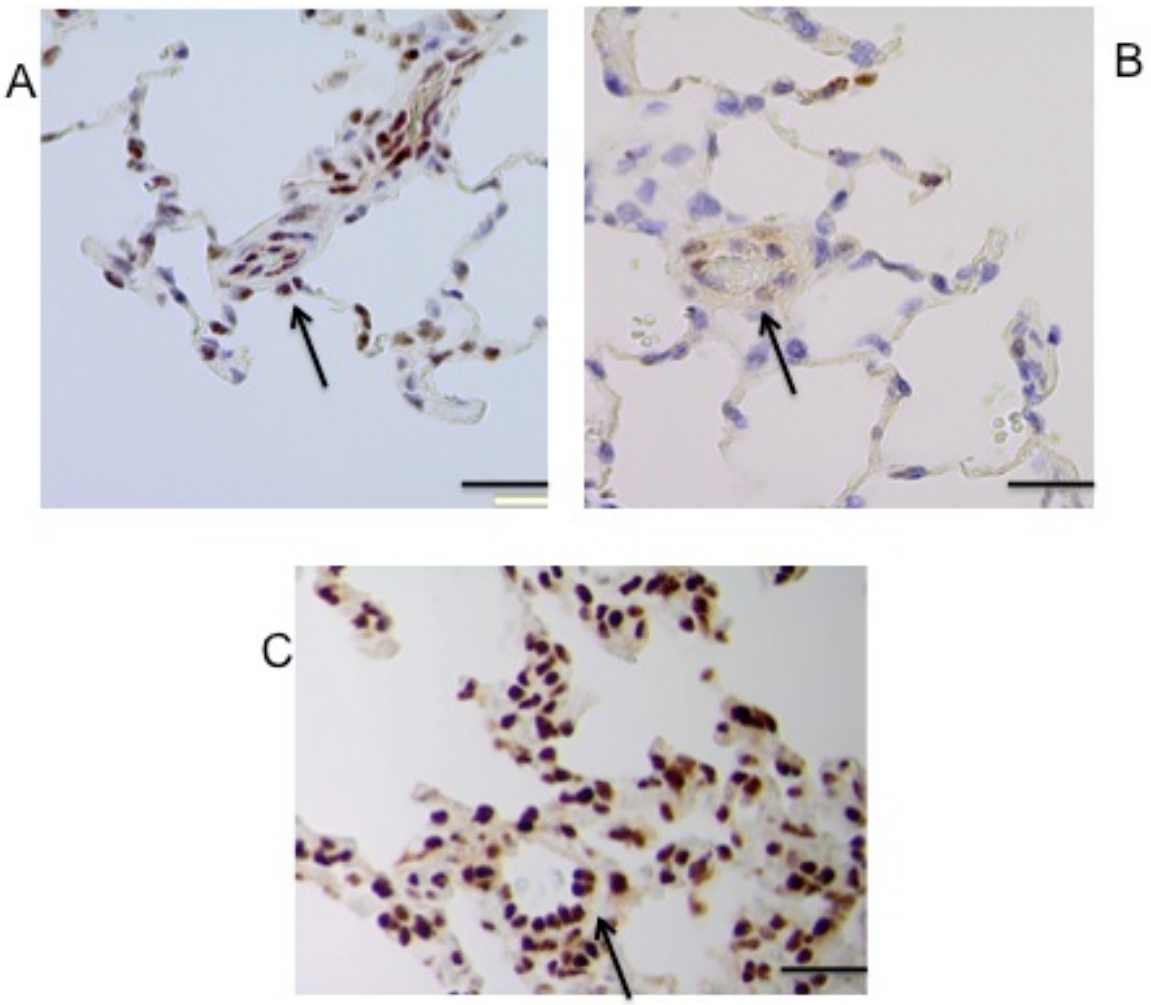

Figure 4-9 Increased staining for phospho-p38MAPK in MCT and chronic hypoxic lungs
Using IHC for phospho-p38MAPK (dilution1:300), (A) is chronic hypoxic animal, (B) is normal animal and (C) is MCT animal. X40 objective. Bar represents 75μM.

4.4.3 p38MAPKα protein levels are higher in animal models of pulmonary hypertension

Using immunoblotting techniques, the levels of p38MAPKα was observed to be higher in both monocrotaline and chronic hypoxic animal models (Figure 4-10). This was confirmed by densitometry.

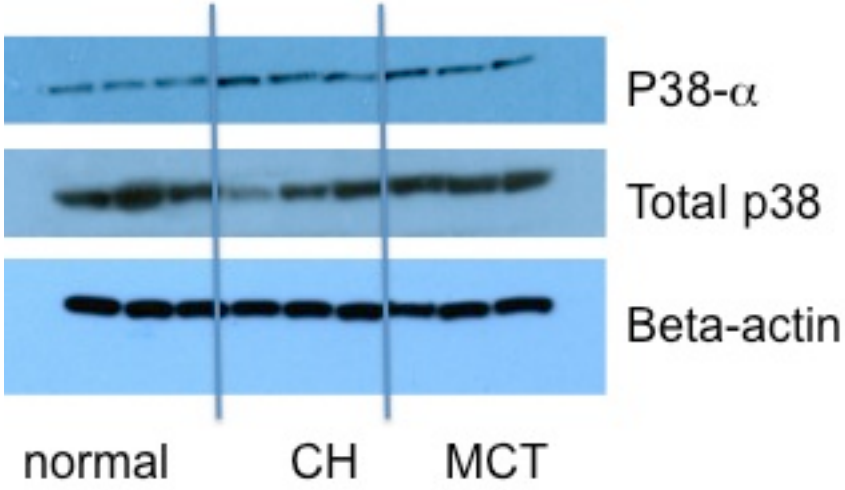

Figure 4-10 p38MAPK-α is increased in lung homogenates of animal models of PH
Lungs from normal, chronic hypoxic and MCT animals were harvested and homogenised with a cocktail of phosphatase and kinase inhibitors. The protein concentration was quantified using BCA method. Equal concentrations were then loaded on a gel and blotted for p38MAPK-α, total p38MAPK and beta-actin for loading control. There are 3 wells for each condition. Immunoblot shown is best representative of 3 experiments using 3 different animals with each condition. MCT=monocrotaline rat lung; CH=chronic hypoxic rat lung

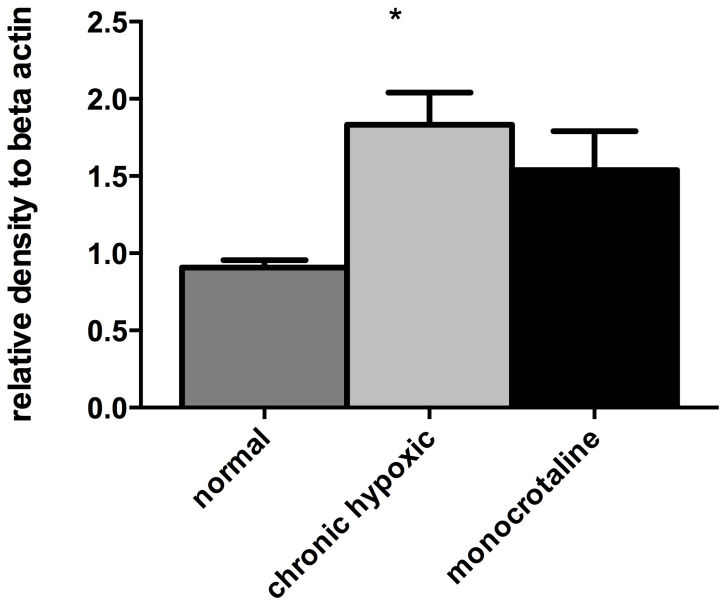

Figure 4-11 Densitometry of blots of p38MAPK-α lung homogenates
Blots from Figure 4-10 were analysed. Values are mean arbitrary values from 3 immunoblots expressed relative to the value for beta-actin.*P<0.05 by ANOVA.

Furthermore there were striking increases in the staining of p38MAPKα in lung sections from the animal models of pulmonary hypertension when compared to normal rat lungs (Figure 4-12).

These results suggest that p38MAPKα is an important isoform in the vasculature, at least in animal models of pulmonary hypertension.

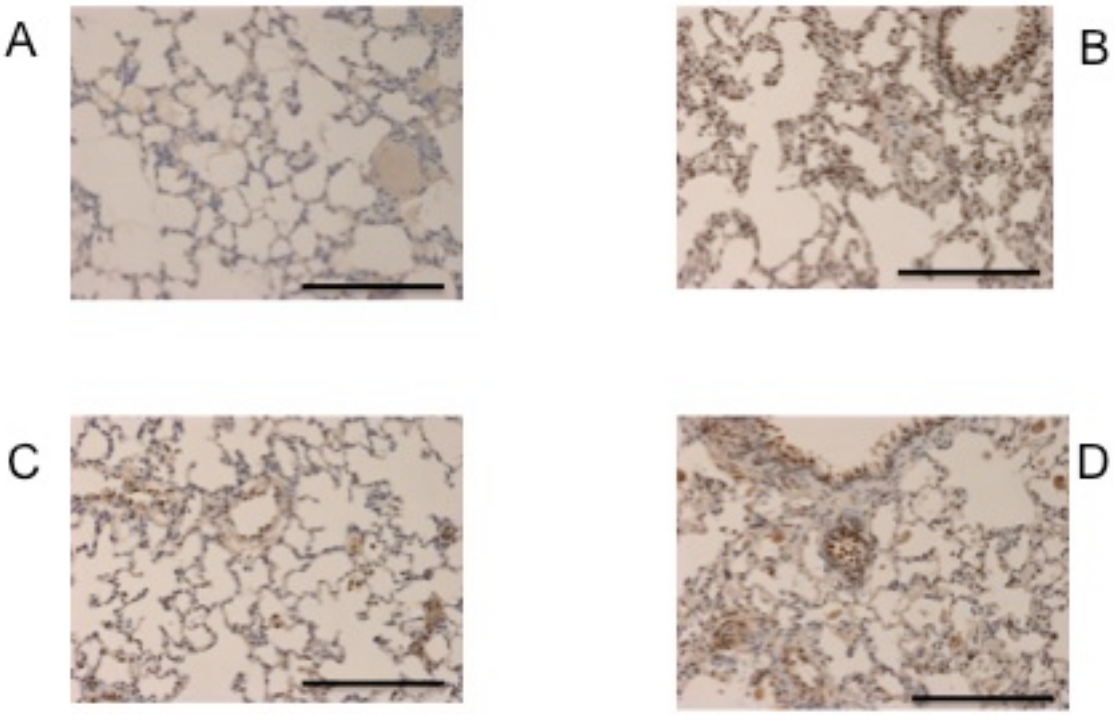

Figure 4-12 Increased staining of p38MAPK-α in lungs from animal models of PH
Lung sections (5μm) were prepared from normal (C), chronic hypoxic (B) and MCT (D) animals. Sections were stained for p38MAPK-alpha using 1:400 dilution. (A) is isotype control. magnification x20. Bar represents 150μm.

4.5 Administration of SB203580 *prevents* the development of chronic hypoxic induced pulmonary hypertension

To establish the importance of this increased p38 MAPK activity in the development of pulmonary hypertension *in vivo*, we administered a p38 MAPK specific pharmacological inhibitor (SB203580) to animals maintained in a hypoxic environment. This involved daily intraperitoneal (i.p.) injections of either vehicle (DMSO) or SB203580 to rats maintained in a chronic hypoxic chamber for 2 weeks. The right ventricular systolic pressure (RVSP) was significantly increased ($p<0.005$) in the vehicle treated animals but remained normal in the animals with the p38 MAPK inhibitor (Figure 4-13A). There was a similar increase in the haematocrit observed in both groups (Figure 4-13B).

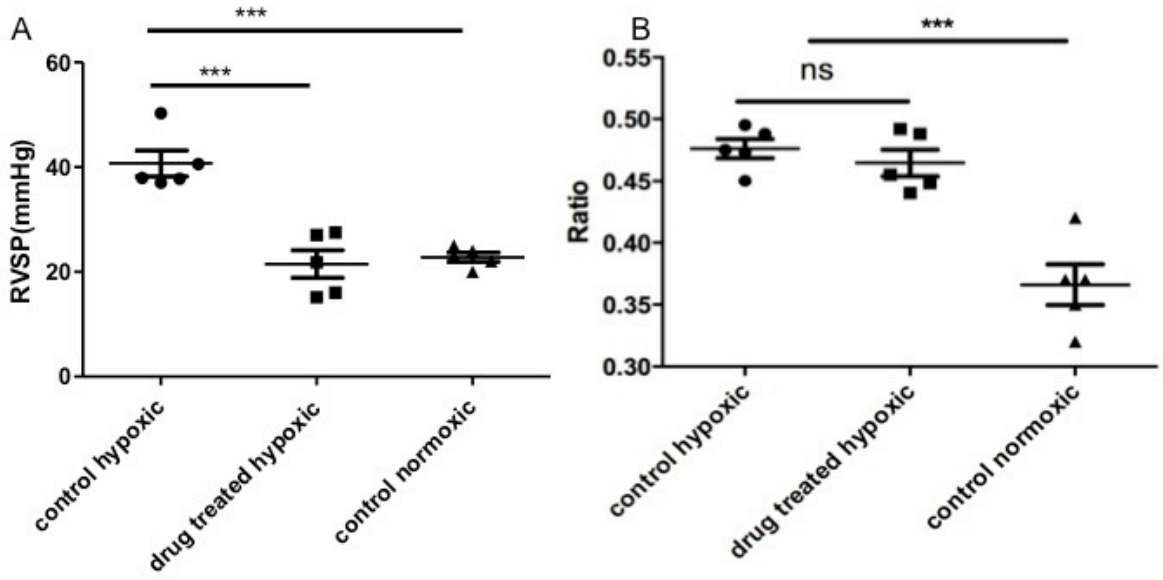

Figure 4-13 Pulmonary hypertension is prevented in chronic hypoxia by using a p38MAPK inhibitor

Animals were exposed to a hypobaric hypoxic environment for 2 weeks. Some animals received daily injections of SB203580. Haemodynamics (A) and haematocrit (B) were measured after 2 weeks. Data represent mean values + SD. Total animals n= 5-6 per group. ***p<0.005

The development of right ventricular hypertrophy as determined by the ratio of right ventricular to left ventricular plus septal weight was prevented in the drug treated group compared to the matched vehicle treated group. This was mirrored with an overall reduction in the total RV weight (Figure 4-14).

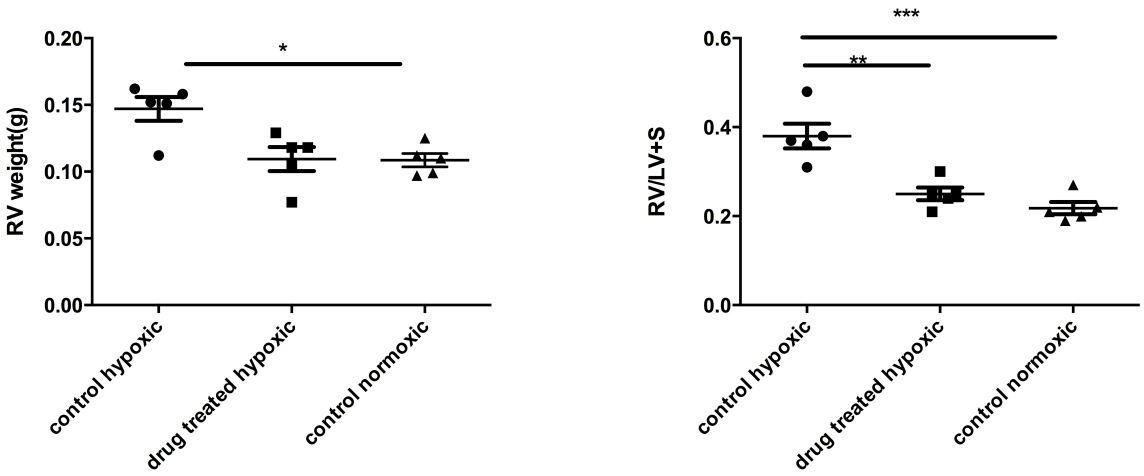

Figure 4-14 Right ventricular hypertrophy does not develop in p38MAPK-inhibited chronic hypoxic animals

Hearts were isolated from the animals and the RV dissected out from the LV and septum. The ventricles were dry blotted, then weighed and the ratio calculated (right graph). The total RV weight was plotted (left graph). Values are mean + SD. n=5 per group. *p<0.05,**p<0.01,***p<0.005

To assess the effect on pulmonary vascular remodelling the lungs were stained for alpha smooth muscle actin and the degree of muscularisation in vessels less than 80μm in diameter assessed (Figure 4-15). There was a significant increase in the percentage of fully muscularised vessels in the vehicle treated group compared to the normal animals. This increase was significantly less in the drug treated animal group indicating prevention of pulmonary vascular remodelling.

In addition when the total number of muscularised vessels were assessed in vessels of diameter <100μm, there was a significant reduction in the drug treated animals (Figure 4-16).

Therefore inhibiting p38 MAPK with SB203580 prevented the development of hypoxia induced pulmonary hypertension in this animal model.

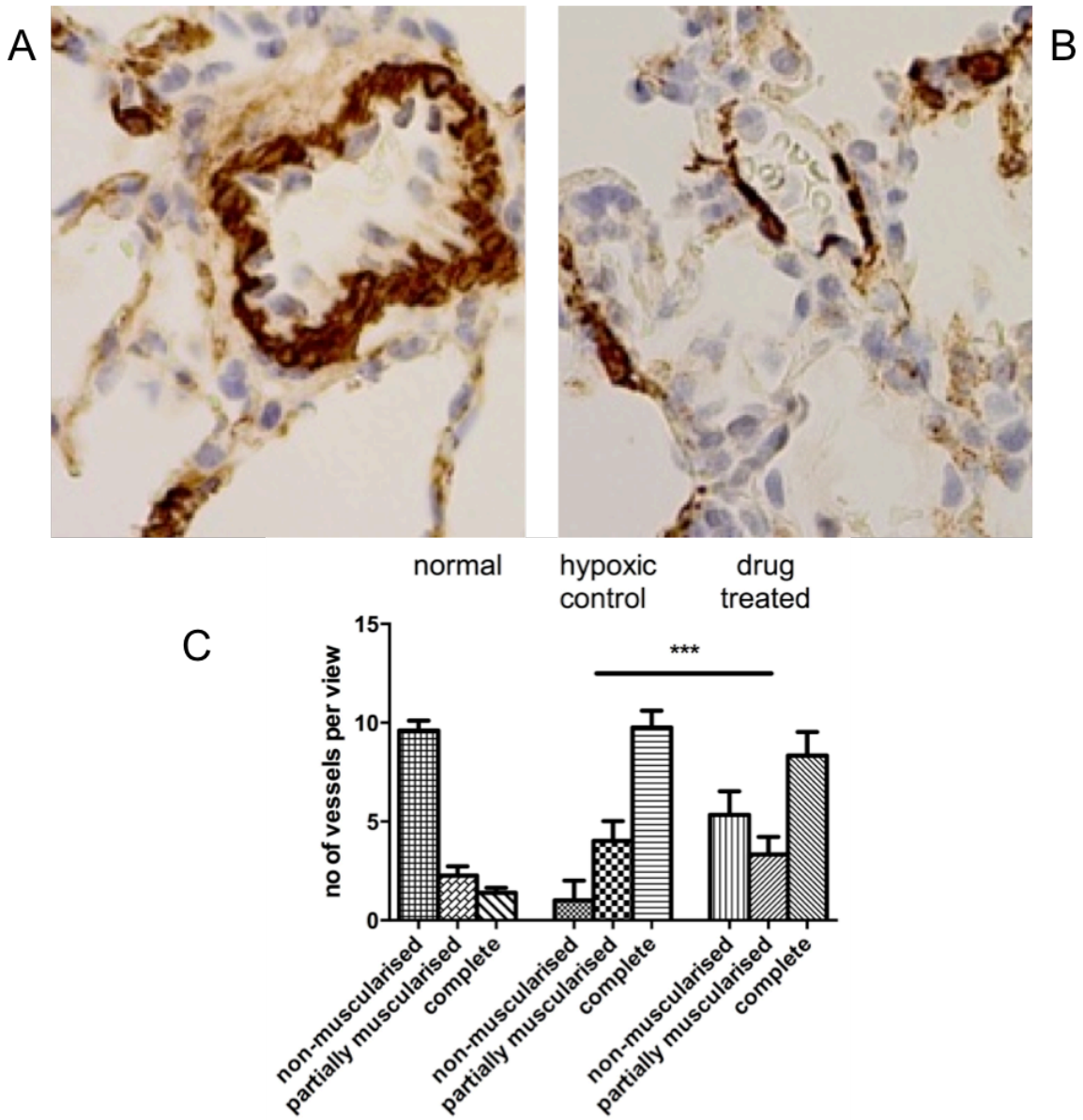

Figure 4-15 p38MAPK inhibition prevents pulmonary vascular remodelling in chronic hypoxia

Rats were exposed to hypoxia +/- SB203580. The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <80μm were analysed for degree of muscularisation. 5 random fields were analysed with 3 slides per animal. The vessels were categorised as completely, partially or non-muscularised. Groups analysed by ANOVA for overall change with post-test analysis. n=5 animals. ***p<0.001

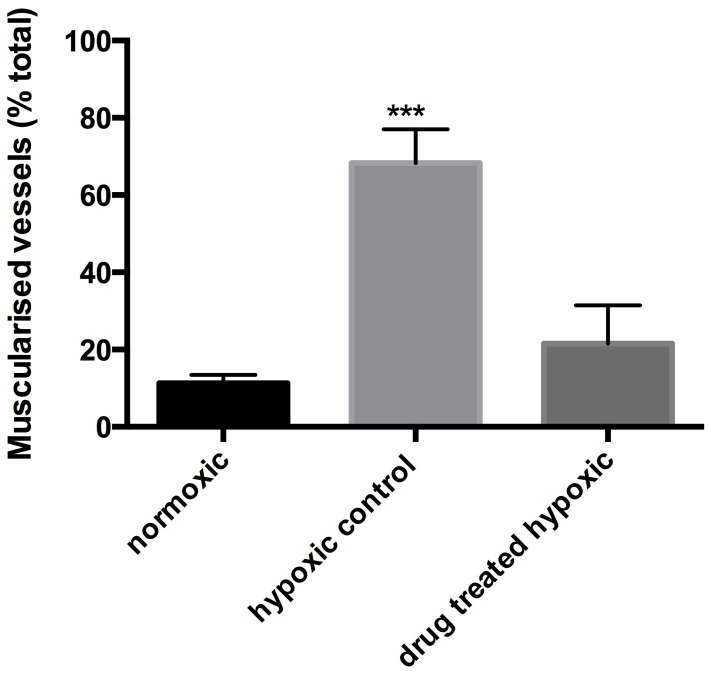

Figure 4-16 p38MAPK inhibition prevents the development of pulmonary vascular remodelling in chronic hypoxia

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <100μm were analysed for degree of muscularisation. 5-10 random fields (X40) were analysed with 3 slides per animal. The vessels were categorised as muscularised or non-muscularised and the percentage of muscularised vessels calculated. Groups analysed by ANOVA for overall change with post-test analysis. n=5 animals. ***p<0.001

4.6 Administration of SB203580 *reverses* chronic hypoxic induced pulmonary hypertension

However to give this true clinical usefulness, where patients present with established disease, an *in vivo* reversal strategy was needed. Therefore animals were exposed to chronic hypoxia for a period of 2 weeks and then treated with either daily i.p. injections of SB203580 or vehicle for a further 2 weeks.

There was a significant reduction in the RVSP in the drug treated group compared to that of vehicle treated at 4 weeks and control hypoxic animals after 2 weeks (Figure 4-17A). In fact the drug treated RVSP was almost fully reversed to that of normal animals. There were no significant differences detected in the increase in haematocrit between the hypoxic groups (Figure 4-17B).

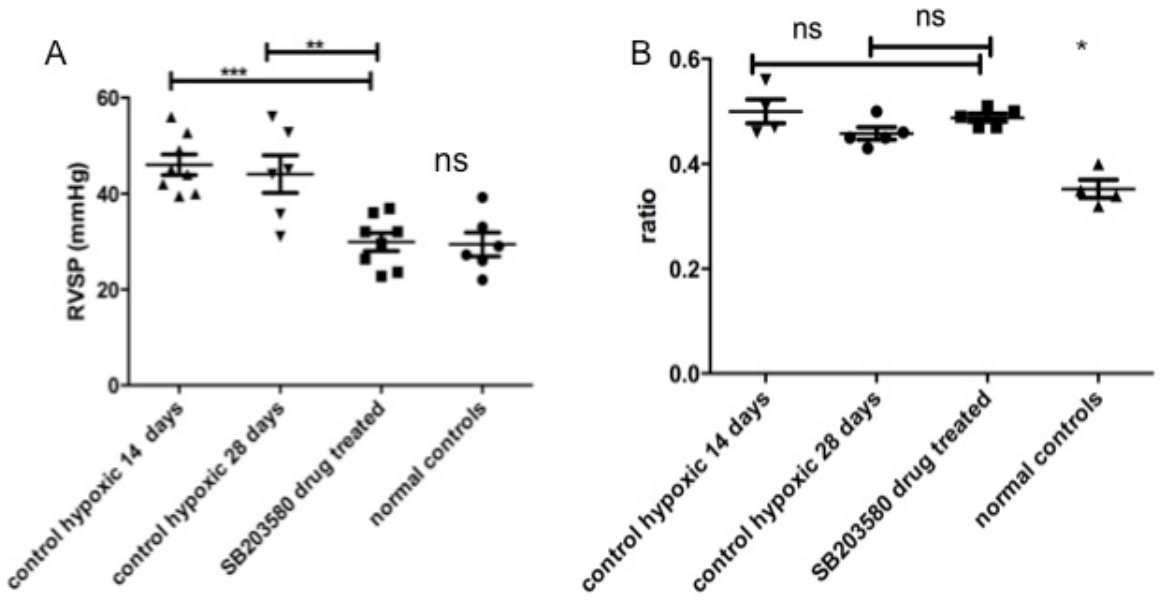

Figure 4-17 p38MAPK inhibition reverses the haemodynamics of pulmonary hypertension in chronic hypoxic animals

Animals were exposed to a hypobaric hypoxic environment for 2 weeks and then p38MAPK inhibition was commenced. Haemodynamics (A) and haematocrit (B) were measured after 4 weeks. Data represent mean values + SD. Total animals n= 5-6 per group. * $p<0.05$ for normal relative to all other conditions.

The degree of RVH was significantly reversed (by 29%) in the drug treated animals although it did not fully return to that of normal control animals (Figure 4-18). This was mirrored by an overall reduction in the total RV weight. The degree of pulmonary vascular remodelling was reduced in the drug treated animals compared to the control group untreated animals (Figure 4-19 and 4-20).

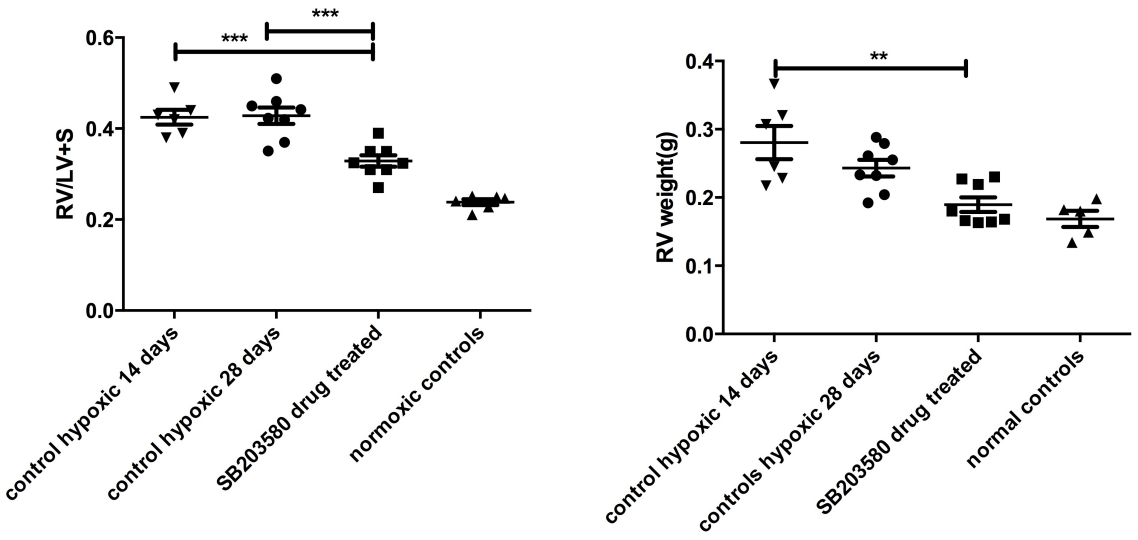

Figure 4-18 Right ventricular hypertrophy is reversed with p38MAPK blockade in chronic hypoxic animals

Hearts were isolated form the the animals and the RV dissected out from the LV and septum. The ventricles were dry blotted, then weighed and the ratio calculated (left graph). The total RV weight was plotted (right graph). values are mean + SD. n=6-7. **p<0.01, ***p<0.005

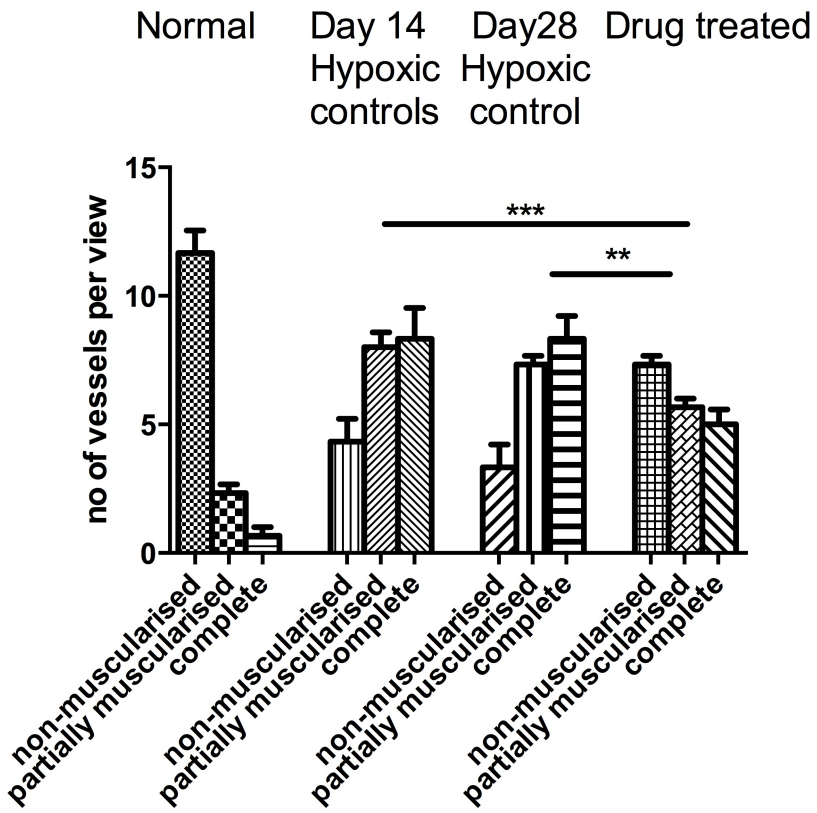

Figure 4-19 p38MAPK inhibition reverses pulmonary vascular remodelling in chronic hypoxic anmals

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <80μm were analysed for degree of muscularisation. 5-10 random fields were analysed with 3 slides per animal. The vessels were categorised as completely, partially or non-muscularied. Groups analysed by ANOVA for overall change with post-test analysis.n=6 animals. **p<0.01;***p<0.001

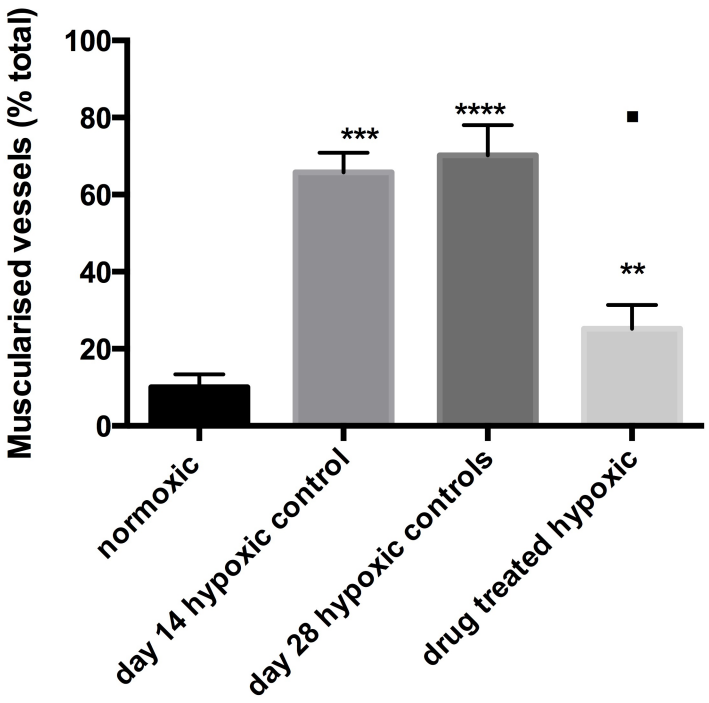

Figure 4-20 p38MAPK inhibition reverses pulmonary vascular remodelling in chronic hypoxic experimental animals

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <100μm were analysed for degree of muscularisation. 5-10 random fields (X40) were analysed with 3 slides per animal. The vessels were categorised as muscularised or non-muscularised and the percentage of muscularised vessels calculated. Groups analysed by ANOVA for overall change with post-test analysis. n=7 animals. ****p<0.0001; ■ p<0.05 for hypoxic drug treated vs day 14 hypoxic control.

The systemic blood pressure was unchanged between groups although the BP was significantly lower in the 28-day hypoxic group (Figure 4-21).

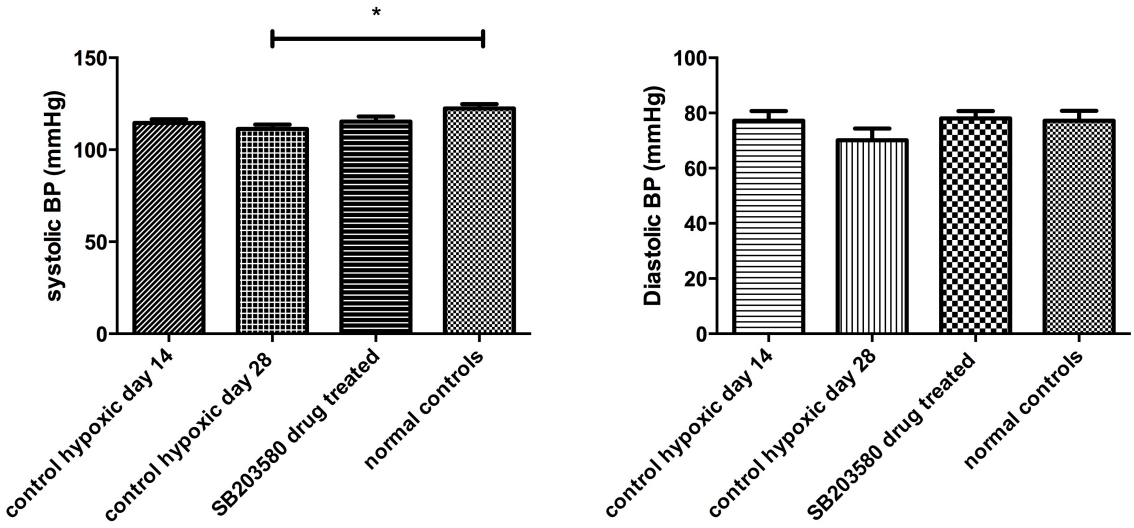

Figure 4-21 Systemic BP in chronic hypoxic treated animals is not affected by p38MAPK inhibition
Rat tail cuff BP was measured. Results are displayed as mean values + SD. * $p<0.05$

In order to confirm the knock down of the p38MAPK activity we looked at the lung levels of a downstream effector of p38MAPK. By immunoblotting of homogenised lung we compared phosphorylated-ATF-2 levels of control hypoxic and drug treated hypoxic animals (Figure 4-22). There is a reduction in the amount of phosphorylated ATF-2 in the drug treated animals confirming inhibition of p38MAPK activity.

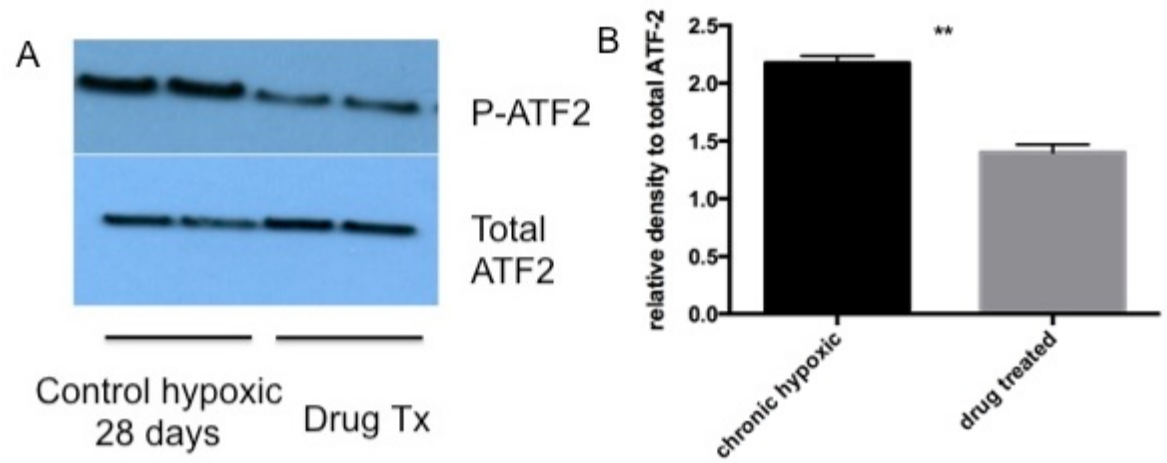

Figure 4-22 SB203580 reduces activity of p38MAPK as shown by reduced phosphorylation of ATF-2.
Lungs from chronic hypoxic control and drug treated hypoxic animals were isolated and homogenised. Blots were probed for phospho-ATF2. Representative blot from 2 experiments. **p<0.01

4.7 Administration of SB203580 *reverses* monocrotaline induced pulmonary hypertension

Two weeks after an injection of monocrotaline, there was an elevated RVSP and increased RVH consistent with the development of pulmonary hypertension in this group of animals. There was no difference between the RVSP rise seen in monocrotaline control animals at 14 or 28 days, although the degree of RVH was observed to be significantly more pronounced at 28 days (Figure 4-23).

After a 14-day time point these animals were injected daily with vehicle or SB203580 for a further 2 weeks. This led to a significant reduction in the RVSP compared to both 14- and 28-day time points in monocrotaline controls. There was a significant improvement in the degree of RVH observed in the drug treated animals which almost returned to normal.

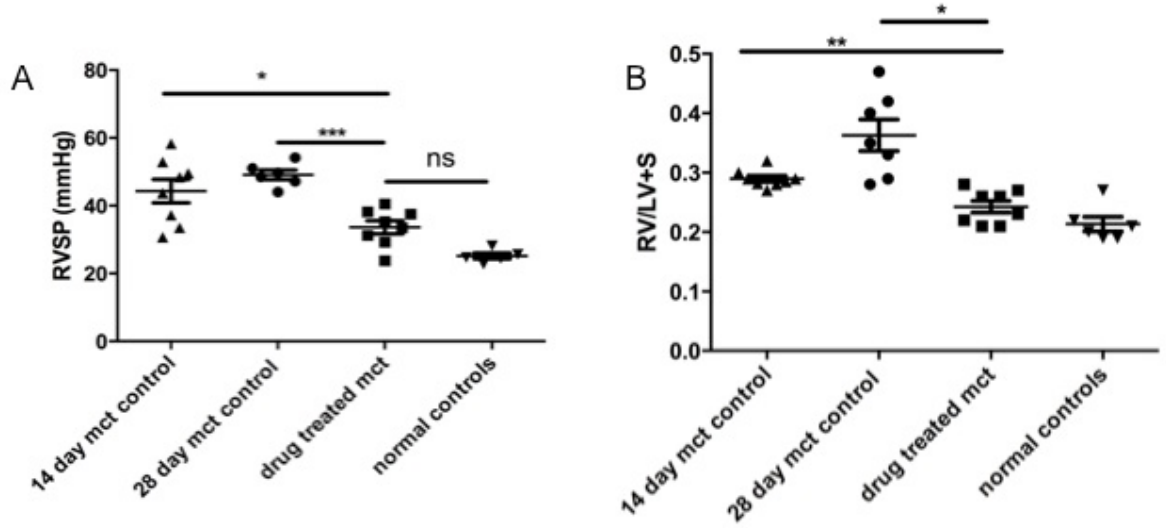

Figure 4-23 p38MAPK inhibition reverses the haemodynamic and RVH changes in MCT induced pulmonary hypertension

Animals were injected with MCT and after 2 weeks p38MAPK inhibition was commenced with daily injections. Haemodynamics (A) and RVH (B) were measured after 4 weeks. Data represent mean values + SD. Total animals n= 6-7 per group.

This was mirrored by a reduction in the degree of muscularisation in the small vessels in the drug treated animals (Figure 4-24 and 4-25).

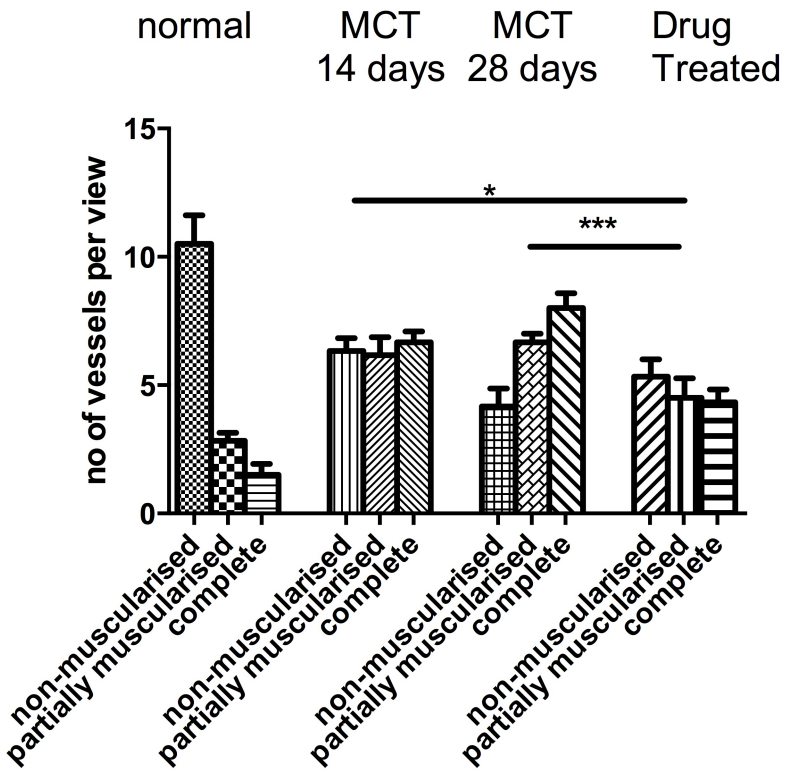

Figure 4-24 p38MAPK inhibition reverses pulmonary vascular remodelling in MCT animal model

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <80μm were analysed for degree of muscularisation. 5-10 random fields (X40) were analysed with 3 slides per animal. The vessels were categorised as completely, partially or non-muscularied. Groups analysed by ANOVA for overall change with post-test analysis. n=6 animals. *$p<0.05$; ***$p<0.001$.

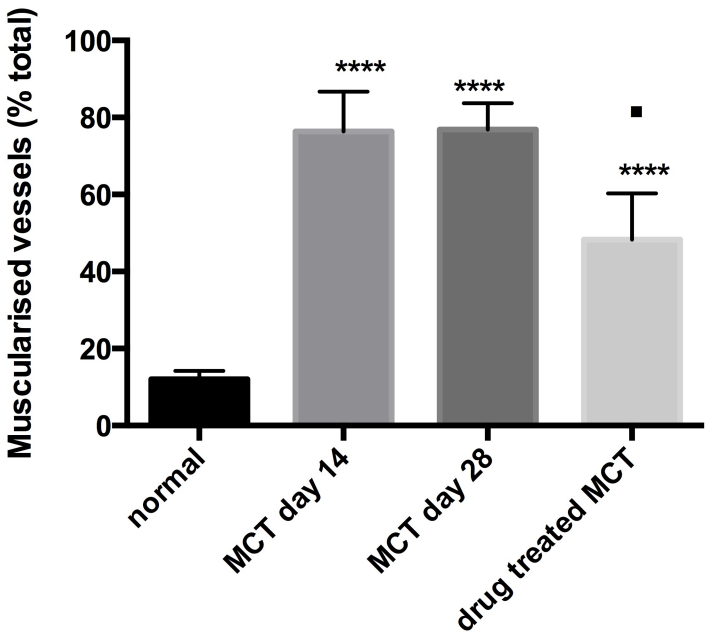

Figure 4-25 p38 MAPK inhibition reverses muscularisation in pulmonary vessels in MCT experimental animal

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <100μm were analysed for degree of muscularisation. 5-10 random fields (X40) were analysed with 3 slides per animal. The vessels were categorised as muscularised or non-muscularised and the percentage of muscularised vessels calculated. Groups analysed by ANOVA for overall change with post-test analysis. n=6 animals. ****$p<0.0001$; ■ $p<0.05$ for hypoxic drug treated vs day 14 MCT control.

There was no difference in the systemic blood pressure between the MCT groups (Figure 4-26).

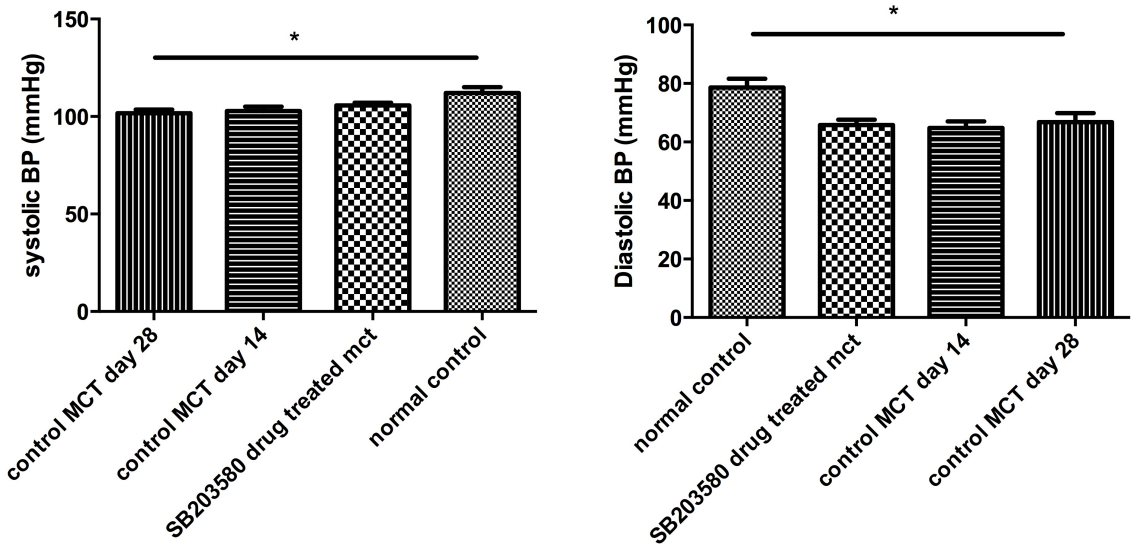

Figure 4-26 Systemic BP in MCT treated animals is not affected by p38MAPK inhibition
Rat tail cuff BP was measured. Drug treated and normal are at day 28. Results are displayed as mean values + SD. * $p<0.05$

The above experiments demonstrate that inhibition of p38MAPK can prevent and reverse the phenotype of pulmonary hypertension in animal models. The mechanisms that may underpin this were explored.

4.8 p38MAPK inhibition reduces IL-6 levels and signalling in the lung

From our preliminary experiments *in vitro* looking at the pulmonary artery fibroblast we could demonstrate that IL-6 was an important mediator of smooth muscle cell proliferation and pulmonary vascular remodelling. We therefore examined the levels of IL-6 in the above treated models of pulmonary hypertension.

In the *prevention* study of the chronic hypoxic associated pulmonary hypertension we found a reduction in IL-6 levels in the lungs of SB203580 drug treated animals compared to vehicle treated hypoxic controls (Figure 4-27 and 4-28).

Similarly in the *reversal* study using chronic hypoxic animals with SB203580, IL-6 levels were reduced in the lung and the amount of phospho-STAT3 signalling was reduced (Figure 4-29 and 4-30).

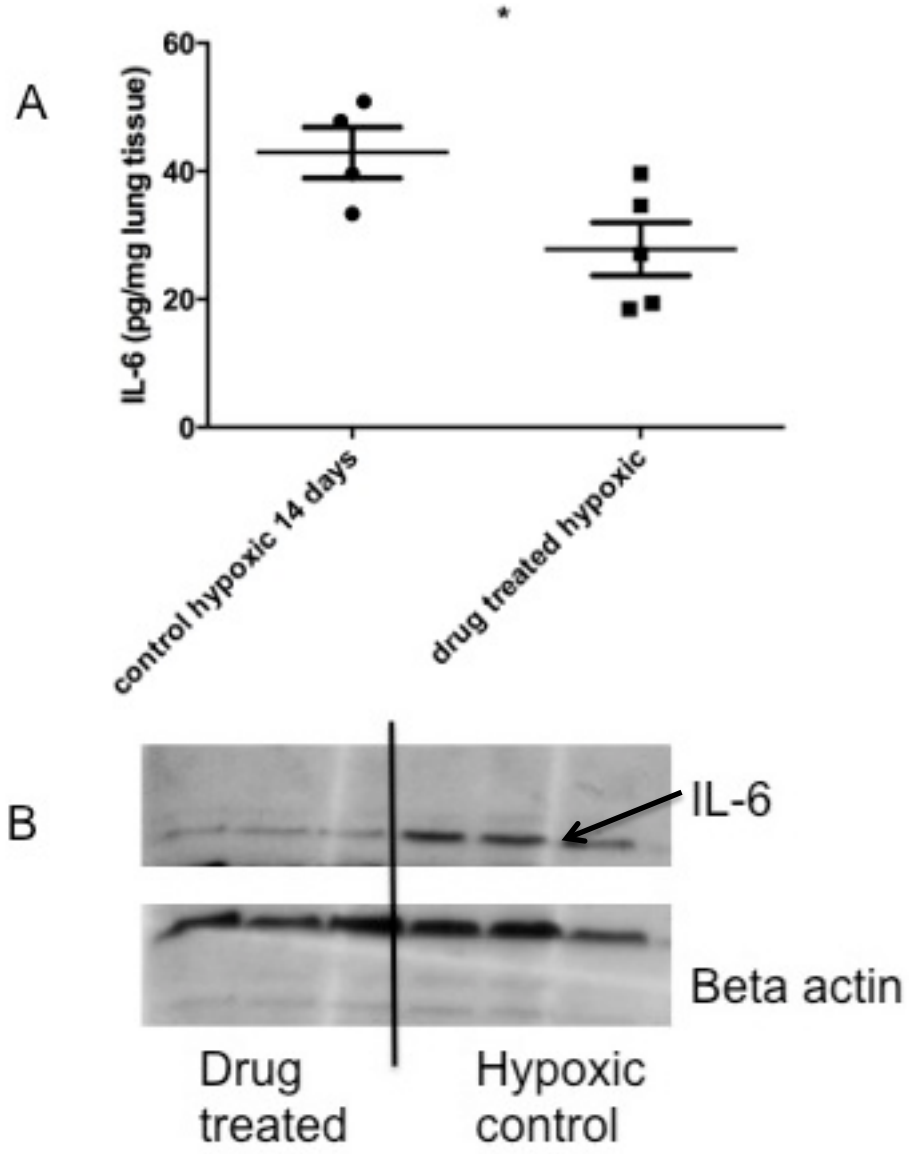

Figure 4-27 Reduced levels of whole lung IL-6 in prevention study with p38MAPK-inhibited chronic hypoxic animals

Lungs were isolated from chronic hypoxic animals and homogenised. The protein concentration was normalised by weight and protein concentration as per BCA method. (A) ELISA was used to analyse for IL-6 levels in the lung tissue. Data shown are mean values + SD from triplicate samples from 4/5 animals in each group. (B) shows immunoblot for IL-6 in lung homogenates. representative blot from 3 experiments. * $p<0.05$

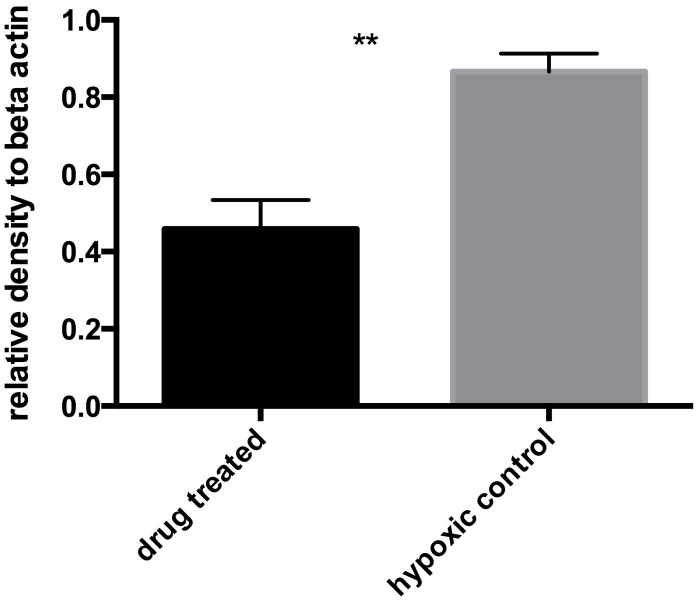

Figure 4-28 Densitometry of repeat immunoblots for Figure 4-27
**p<0.01

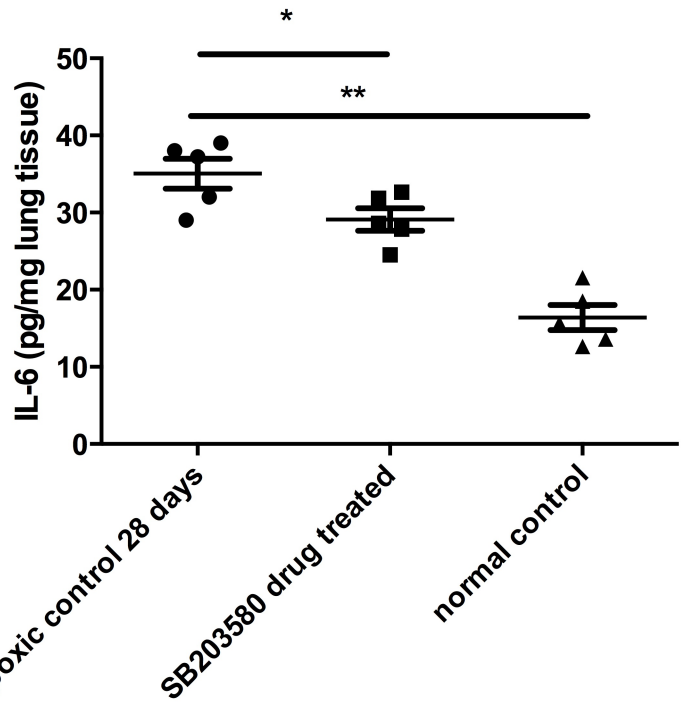

Figure 4-29 Reduced levels of whole lung IL-6 in reversal study with p38MAPK-inhibited chronic hypoxic animals

Lungs were isolated from normal and chronic hypoxic animals and homogenised. The protein concentration was normalised by weight and protein concentration as per BCA method. ELISA was used to analyse for IL-6 levels in the lung tissue. Drug treated and normal control are at 28 days. Data shown are mean values + SD from triplicate samples from 4/5 animals in each group. * $p<0.05$ ** $p<0.01$

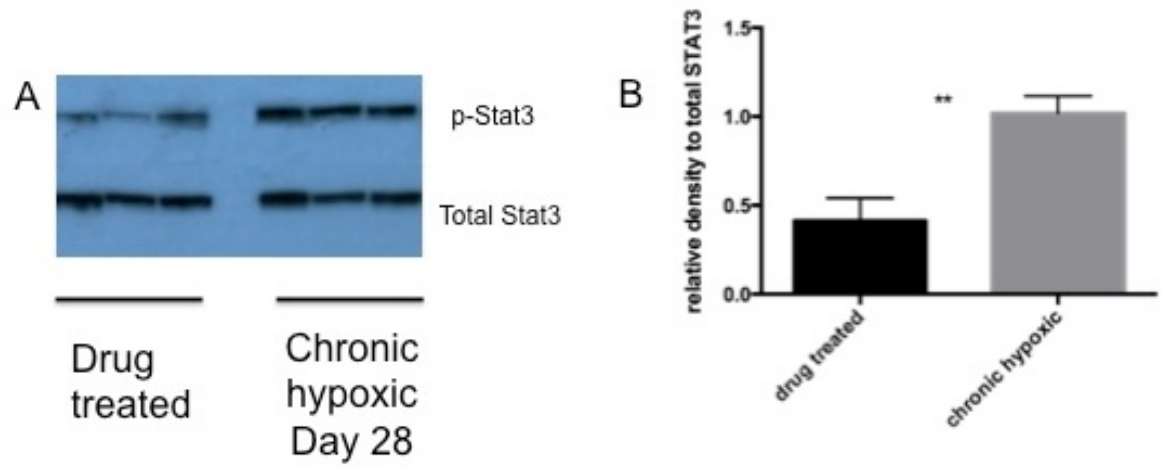

Figure 4-30 Reduced levels of STAT3 signalling in reversal study p38MAPK-inhibited chronic hypoxic animals
Lungs from chronic hypoxic 28 days controls and drug treated hypoxic animals were harvested and homogenised with a cocktail of phosphatase and kinase inhibitors. The protein concentration was quantified using BCA method. Equal concentrations were then loaded on a gel and blotted for phospho-STAT3 and total STAT3. Immunoblot shown is best representative of 3 experiments using lungs from 3 different animals with each condition. Densitometry is shown of other blots.

The same findings were observed in the monocrotaline animals with reduced IL-6 in the drug treated animals (Figure 4-31).

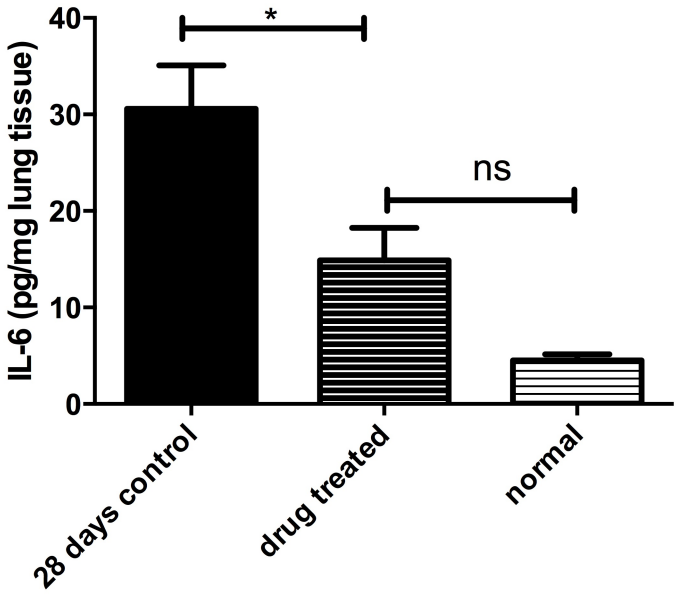

Figure 4-31 Reduced levels of whole lung IL-6 in reversal of PH in p38MAPK-inhibited MCT animals

Lungs were isolated from MCT and normal control animals and homogenised. The protein concentration was normalised by weight and protein concentration as per BCA method. ELISA was used to analyse for IL-6 levels in the lung tissue. Data shown are mean values + SD from triplicate samples from 4/5 animals in each group. * $p<0.05$, ns is not significant by ANOVA.

As previously discussed the source of IL-6 may be from a number of different cells, including the fibroblast. To investigate this further we analysed for neutrophil activity by measuring myeloperoxidase activity (figure 4-32). Neutrophils can be an important source of IL-6(338,339). However our experiments showed that there were no differences in the levels of myeloperoxidase activity between chronic hypoxic controls and normal animals, suggesting little difference between neutrophil influx between these animals. Interestingly, although it did not reach significance there was a trend to reduction in activity in the drug treated animals at 28 days. This suggests that p38MAPK inhibition may be able to reduce the neutrophil activity in these lungs, but further work in this area is needed.

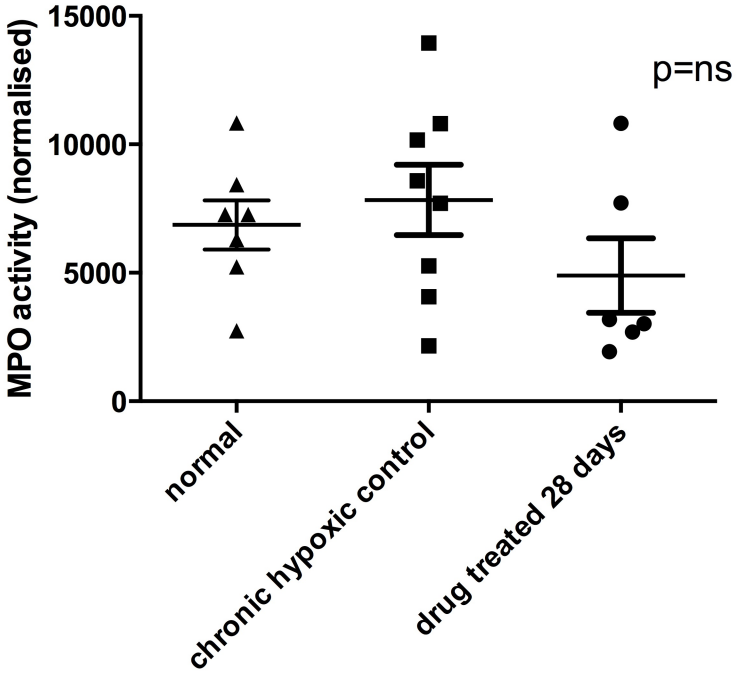

Figure 4-32 Myeloperoxidase (MPO) activity is not increased in chronic hypoxic animals
Whole lungs isolated from chronic hypoxic and driug treated animals were homogenised quantified by BCA method. Equal amounts were assayed for MPO activity using triplicate samples. Each data point represents a separate animal (total n=7-8 per group).

4.9 p38MAPK inhibition reduces soluble collagen production in animal models of pulmonary hypertension

As previously mentioned, *in vitro* experiments have suggested that IL-6 can lead to the increased release of collagen from the pulmonary artery fibroblast. This is felt to be important in pulmonary vascular remodelling. We looked at this in the *in vivo* experiments to see if this is altered *in vivo* after inhibition of p38MAPK. By using a soluble quantitative collagen assay we could demonstrate that the amount was lower in the lungs of the SB203580-treated animals. This was observed in both the prevention and reversal models (Figure 4-33 and Figure 4-34). This would suggest another possible mechanism by which p38MAPK and IL-6 are leading to pulmonary vascular remodelling. By inhibiting p38MAPK there is both a reduction in the release of IL-6 and potentially a direct inhibition of collagen synthesis.

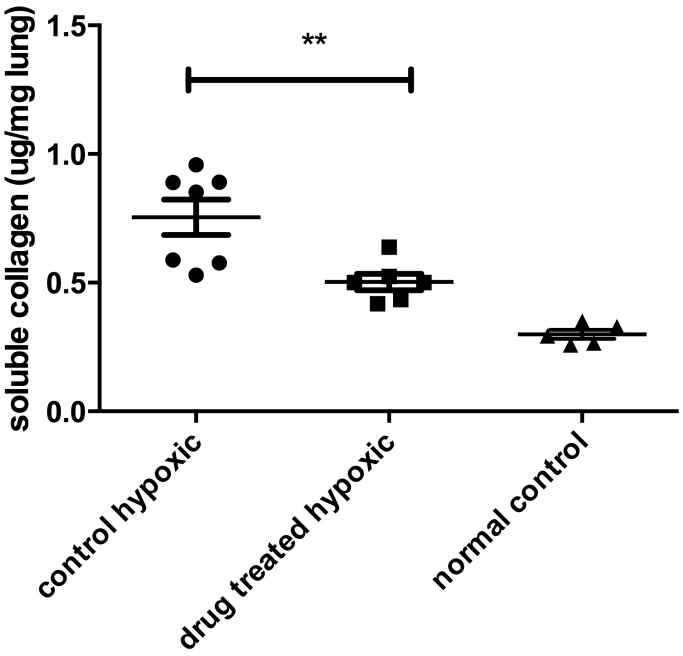

Figure 4-33 Collagen production is lower in lungs of p38MAPK inhibited animals in a prevention of PH strategy

Lungs from normal, chronic hypoxic and chronic hypoxic drug treated animal in a prevention strategy were isolated. Lung sections were dissected out and weighed. Collagen was extracted as per outlined in chapter 2. A concentration process was performed initially using overnight incubation with pepsin/acetic acid. Sircol dye was used to stain the collagen and the OD read of standard curve and samples. All animals are at day 14. Data presented represent the mean values +SD normalised for original lung weight. Total number of animals = 6-7 per condition. *p<0.05, **p<0.01

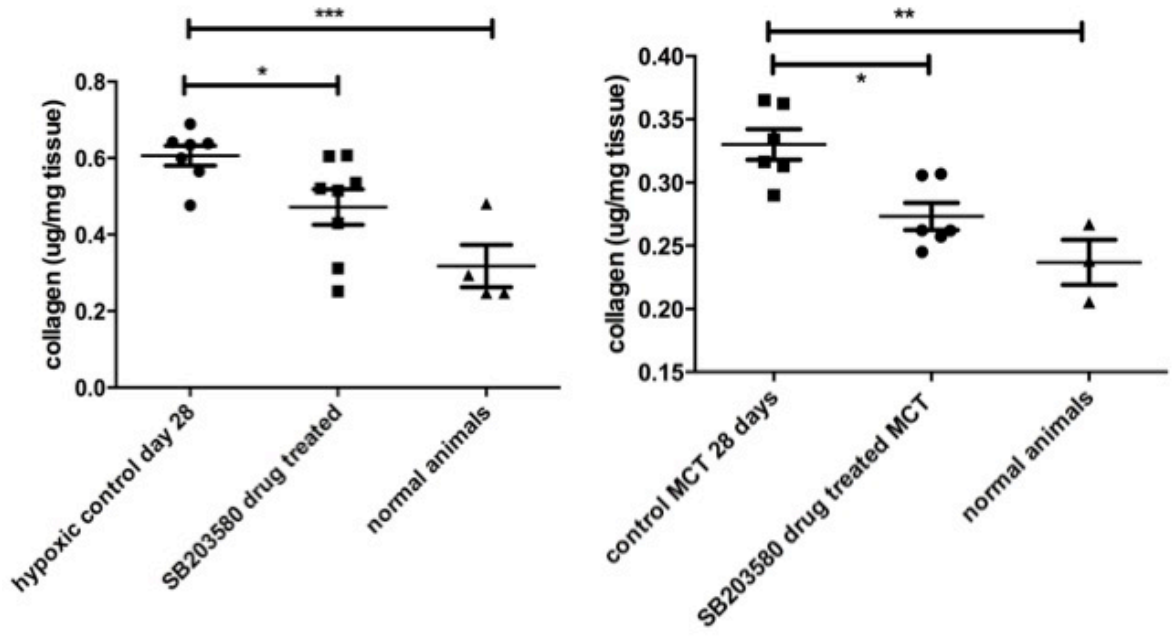

Figure 4-34 Collagen production is lower in the lungs of PH reversed, p38MAPK-inhibited animals

Lungs from normal, control hypoxic or MCT and drug treated hypoxic and MCT were removed. The drug treated animals had established PH and then were treated with p38MAPK inhibitor. Sections were dissected out and weighed. Collagen was extracted as per outlined in chapter 2. A concentration process was performed initially using overnight incubation with pepsin/acetic acid. Sircol dye was used to stain the collagen and the OD read of standard curve and samples. Drug treated and normal control were at day 28. Data presented represent the mean values +SD normalised for original lung weight. Total number of animals = 7-8 per condition. *$p<0.05$, **$p<0.01$, ***$p<0.005$

4.10 Summary

This chapter has shown that p38MAPK is important in the development of pulmonary hypertension phenotype *in vivo* and in pulmonary vascular remodelling. There is increased activity of p38MAPK in the lungs of both chronic hypoxic and monocrotaline animal models. In addition there is increased expression of the active phosphorylated-p38MAPK in histological slides of lungs from both these animals and using immunoblotting techniques. Using a specific p38MAPKalpha antibody there was an increased staining of this isoform in the lungs of the *in vivo* animal models. These suggest that p38MAPK is active in the disease phenotypes of pulmonary hypertension and that the alpha isoform has a differential expression.

To elucidate the importance of these findings and whether indeed the manipulation of the p38MAPK signalling pathway would have an effect on pulmonary hypertension, inhibition of the pathway *in vivo* was undertaken. A prevention study looking at blocking p38MAPKalpha in monocrotaline animals has previously been reported in the literature as showing a beneficial effect. The animals that received the p38MAPK inhibitor at the same time as administration of the MCT did not develop pulmonary hypertension. This chapter has shown that in another model of pulmonary hypertension, namely the chronic hypoxic model, administration of SB203580 can prevent the development of pulmonary hypertension. This confirms in two models that p38MAPK is involved in the pathobiology of pulmonary hypertension.

Prevention studies are important in determining key biological pathways which may be involved in the pathogenesis of pulmonary hypertension. However they are of limited use clinically as patients present to the physician with already established disease. Therefore to investigate this in more detail, reversal studies are needed. In these *in vivo* experiments the animals are allowed to develop pulmonary hypertension for 2 weeks and then the p38MAPK inhibitor is given to

see if the disease can be reversed. This would obviously be much more clinically relevant.

By administering the inhibitor to two different models of pulmonary hypertension this study shows that after 2 weeks of treatment there was normalisation in the pulmonary haemodynamics and the right ventricular hypertrophy. In addition there was an observed improvement in the degree of pulmonary vascular remodelling as measured by the degree of small vessel muscularisation.

To understand the mechanisms involved in this and to build on the *in vitro* data already generated and presented in the previous chapter, the role of IL-6 and collagen was analysed. This demonstrated that in lung tissue from the *in vivo* models, IL-6 levels were reduced in the drug treated animals in comparison to the untreated diseased controls. This corresponded with reduced STAT-3 signalling. This could potentially explain less cellular proliferation and improved vascular remodelling. In addition the levels of newly formed collagen was reduced in the drug treated animals again linking with an improvement in the pulmonary vascular remodelling.

Although we describe here the suggestion that the p38 MAPK pathway and the pulmonary artery fibroblast plays an important role in the development of pulmonary hypertension, the targeting of the p38 MAPK pathway *in vivo* may actually also involve other vascular cells. There is evidence from our own group that the endothelium in hypoxia associated pulmonary hypertension is associated with impaired relaxation and reduced nitric oxide production which can be reversed by inhibition of p38 MAPK(309). Hence part of the vasoconstrictive element of pulmonary hypertension may be mediated by p38 MAPK activity in the endothelium. However our data presented here suggest that p38MAPK blockade has a more important anti-remodelling effect rather than only relaxing the pulmonary vasoconstriction.

In addition, many pro-inflammatory cells such as neutrophils, macrophages and monocytes use p38 MAPK as a key signalling pathway leading to the generation and release of cytokines and chemokines. However our initial investigation into neutrophil activity did not suggest that there was increased numbers of

neutrophils and hence it is unlikely that the IL-6 increase noted comes from these cells. It was also interesting to note the trend towards a reduction in neutrophil activity in the p38MAPK inhibitor treated animals. It is known that p38MAPK is a survival signal in neutrophils and that inhibition of this can lead to increased caspase-3 activity and apoptosis(340). Our findings would agree with this and that inhibition of p38MAPK may act to stimulate apoptosis. In addition in chapter 3 we have provided evidence that the fibroblast is an important source of chemokines which are essential for neutrophil recruitment. In hypoxia, early influx of neutrophils is a feature and as adventitial fibroblast activity is increased early in the hypoxic process, the two could be linked. However further work to look at the role of p38MAPK inhibition in neutrophils and other inflammatory cells in pulmonary hypertension will examine this.

This chapter highlights important evidence that p38MAPK is involved in the development of pulmonary vascular remodelling and that by inhibiting this overactive pathway *in vivo* the pulmonary hypertensive phenotype can be reversed. This is of huge clinical relevance. Intriguingly the alpha isoform seems to be more involved *in vivo* which would tie in with the *in vitro* data suggesting this as an important isoform.

However one criticism of the study might be that although SB203580 is relatively selective for the p38MAPKα isomer it does have effects on other enzymes. This is true but in a study examining the effect on various kinases, SB203580 had the most effect on the p38-α isoform with only 9% activity remaining after 1μM dose(341).

To therefore further characterise the involvement of the p38MAPKα isoform, a more specific inhibitor was obtained and studied *in vivo*, as outlined in the next chapter.

5 The selective inhibition of p38MAPK-alpha reverses pulmonary vascular remodelling *in vivo*

5.1 Introduction

From the previous chapter it is clear that p38MAPK has an important role to play in the *in vivo* development of both pulmonary vascular remodelling and pulmonary hypertension. In addition inhibition of this pathway leads to both prevention and reversal of the phenotype. Potential mechanisms include reducing the inflammatory cytokine signalling such as IL-6 and inhibiting collagen deposition in the lung.

However we have shown that the p38MAPK alpha isoform seems to be critically involved in this mechanism, both at a cellular and *in vivo* level. To further characterise the importance of the alpha isoform we wished to employ a novel and more alpha isoform specific isoform. Collaboration with Pfizer, UK, was established as they had developed an agent called PH-797804 which has already been shown in the literature to have increased specificity for the alpha isoform. This has been previously shown to be extremely effective in animal models of rheumatoid arthritis and has been shown to be more specific for the alpha isoform of the p38MAPK family with a 6.9 fold increase in specificity over the p38 MAPK-beta isoform compared to existing inhibitor(336). Furthermore it has just been used in a clinical trial of patients with chronic obstructive pulmonary disease whereby it demonstrated improvements over placebo in lung function parameters and dyspnoea and was well tolerated(312). This allowed us to use a drug with the potential to be translated from animal work to use in the clinical treatment of patients with pulmonary hypertension.

This chapter employs the novel agent PH-797804 in two *in vivo* animal models of pulmonary hypertension.

I therefore hypothesised that the p38MAPKα isoform is a key signalling pathway in the pathobiology of pulmonary hypertension and by using a more specific p38MAPKα inhibitor would allow reversal of the disease process and minimise off target effects on other p38MAPK isoforms.

5.2 Relevant Techniques Employed

The methods used were very similar to those in chapter 4. Only differences are highlighted here.

5.2.1 Drug

The drug PH-797804 was kindly supplied in powder form by Pfizer,UK. This was made up in 0.5% methylcellulose (Sigma) and 0.025% Tween (Sigma). The final dose was 3mg/kg which has been shown to fall within the safe toxicological dose. To ensure that the plasma levels were adequate we used supra-ED80 doses which according to predicted pharmacokinetics would allow once daily dosing via intraperitoneal route (Figure 5-1).

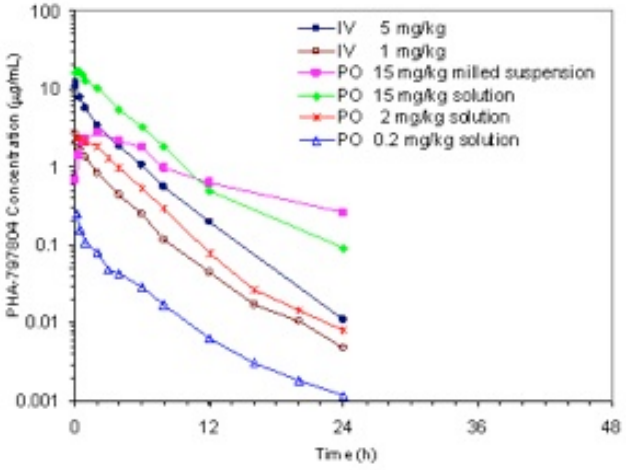

Figure 5-1 Pharmacokinetics of PHA-797804
The pharmacokinetics of varying doses and routes of PHA-797804 are shown.

5.2.2 Animal models

Both chronic hypoxic and monocrotaline animal models were used. The strategy utilised was to investigate the effectiveness of the more selective agent in the reversal models. Therefore the disease was allowed to become established for 2 weeks and then the animals had the drug administered i.p. daily.

Control animals were injected with methylcellulose/Tween suspension.

5.3 Administration of PH-797804 reverses chronic hypoxic induced pulmonary hypertension

Similar to the previous animal study with SB203580 we found a significant reduction in the RVSP in the drug treated animals after 2 weeks of treatment (Figure 5-2). This was accompanied by a similar marked reduction in the degree of RVH compared to both 14- and 28-day time points (Figure5-2).

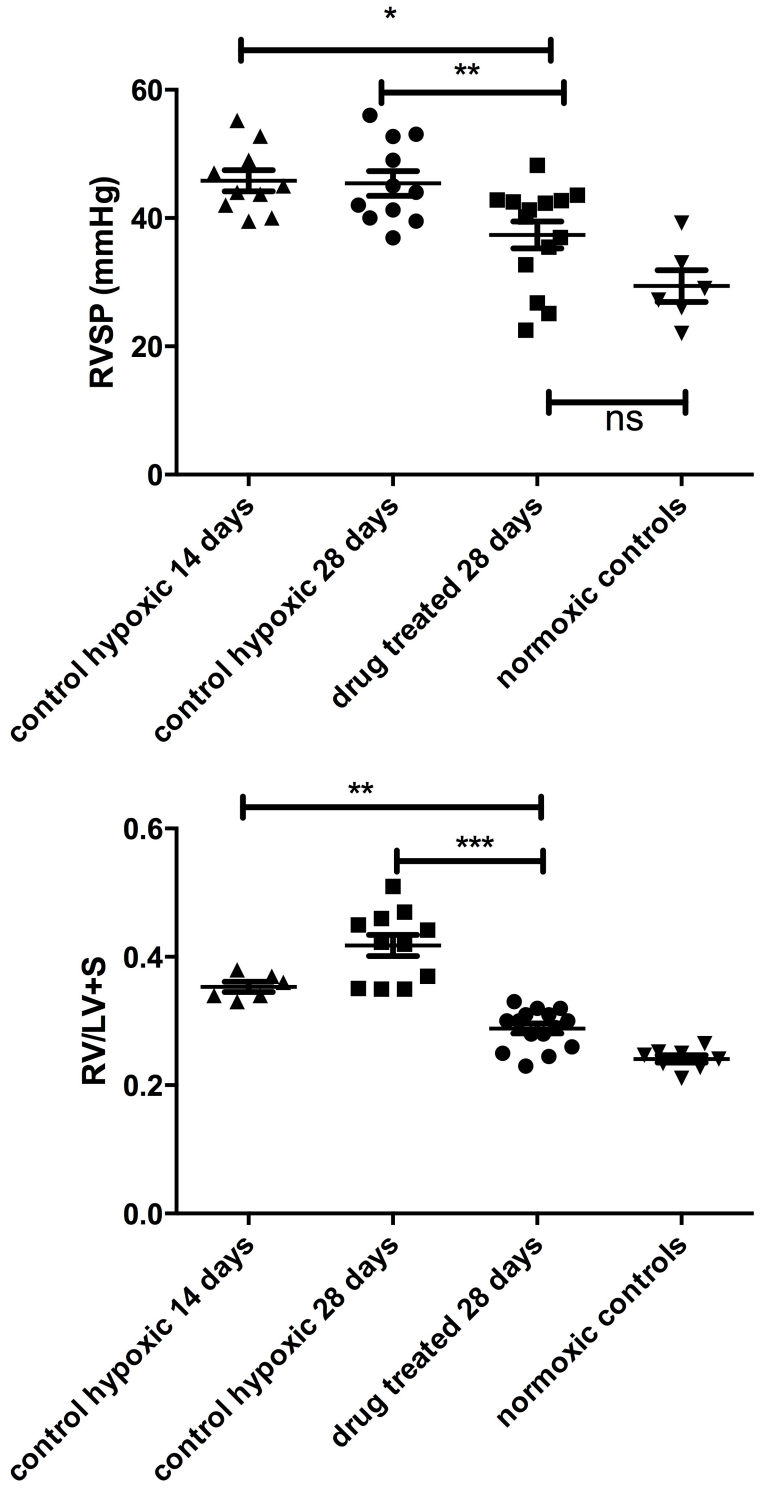

Figure 5-2 p38MAPK inhibition with PH-797804 reverses the haemodynamic changes and RVH seen in chronic hypoxic pulmonary hypertension
Animals were exposed to chronic hypoxia and after 2 weeks p38MAPK inhibition was commenced with daily injections. Haemodynamics (top) and RVH (lower) were measured after 4 weeks. Data represent mean values + SD. Total animals n= 11-12 per group.**p<0.01, ***p<0.005

The haematocrit did not show any significant difference between treated and untreated animals (Figure 5-3). There was a clear difference with the normoxic animals.

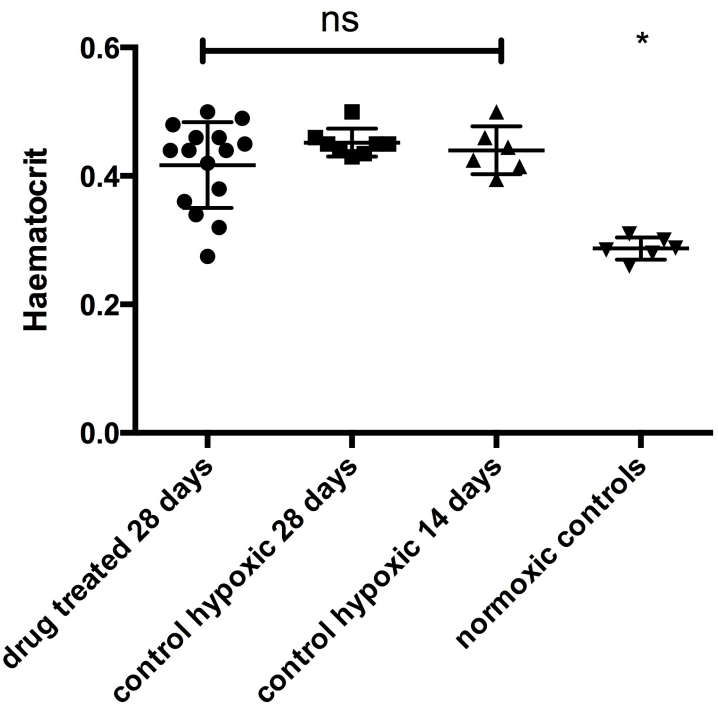

Figure 5-3 Haematocrit was unchanged with p38MAPK inhibition in chronic hypoxia
* $p<0.05$ of normoxic control relative to all other conditions.

The pulmonary vascular muscularisation was attenuated in the drug treated group compared to the 28-day animal untreated group (Figure 5-4). Importantly there was a significant difference in the number of completely muscularised vessels between the drug treated and the day 14 hypoxic controls suggesting true reversal of the remodelling process by that stage. In addition there was a significant difference in the total number of muscularised vessels as expressed as a percentage of the total vessels, again suggesting improvement in the remodelling process (Figure 5-4 and Figure 5-5).

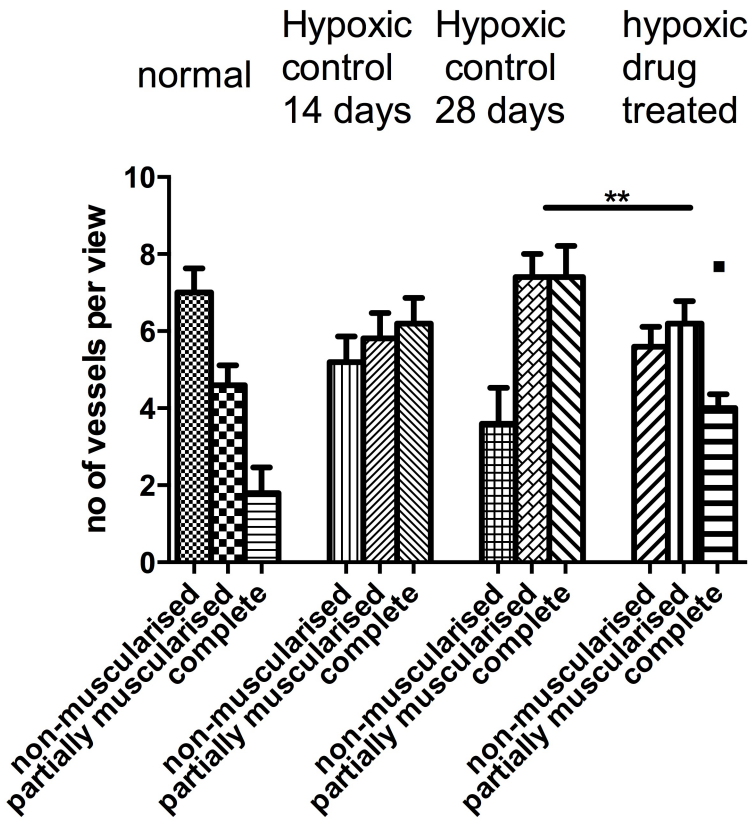

Figure 5-4 PH-797804 attenuates development of muscularisation in pulmonary vessels in chronic hypoxic experimental model

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <80μm were analysed for degree of muscularisation. 5-10 random fields (X40) were analysed with 3 slides per animal. The vessels were categorised as completely, partially or non-muscularised. Groups analysed by ANOVA for overall change with post-test analysis. n=10 animals. **p<0.01; ■ p<0.05 for complete muscularised group in drug treated vs day 14 hypoxic control.

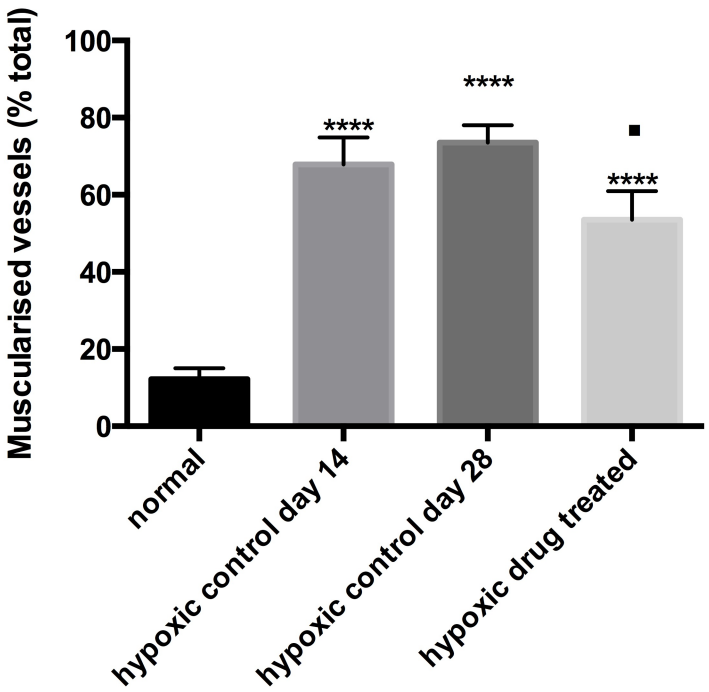

Figure 5-5 PH-797804 reverses muscularisation in pulmonary vessels of chronic hypoxic animal model

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <100μm were analysed for degree of muscularisation. 5-10 random fields (X40) were analysed with 3 slides per animal. The vessels were categorised as muscularised or non-muscularised and the percentage of muscularised vessels calculated. Groups analysed by ANOVA for overall change with post-test analysis. n=10 animals. ****$p<0.0001$; ■ $p<0.05$ for hypoxic drug treated vs day 14 hypoxic control.

There was no significant difference in the systemic BP in the drug treated animals before and after the experiment (Figure 5-6). In addition there was no difference between the control hypoxic and drug treated animals (Figure 5-7).

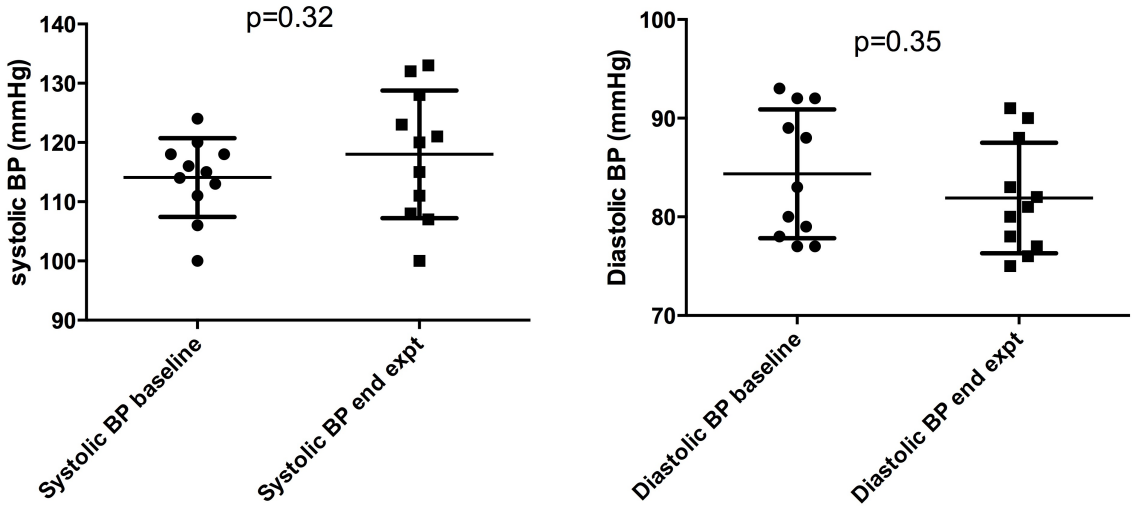

Figure 5-6 Systemic blood pressure was unchanged between start and end of experiment
Rat tail cuff BP is compared between start and end of experiment showing no difference between groups. individual data points are individual BP readings. total animals n=11

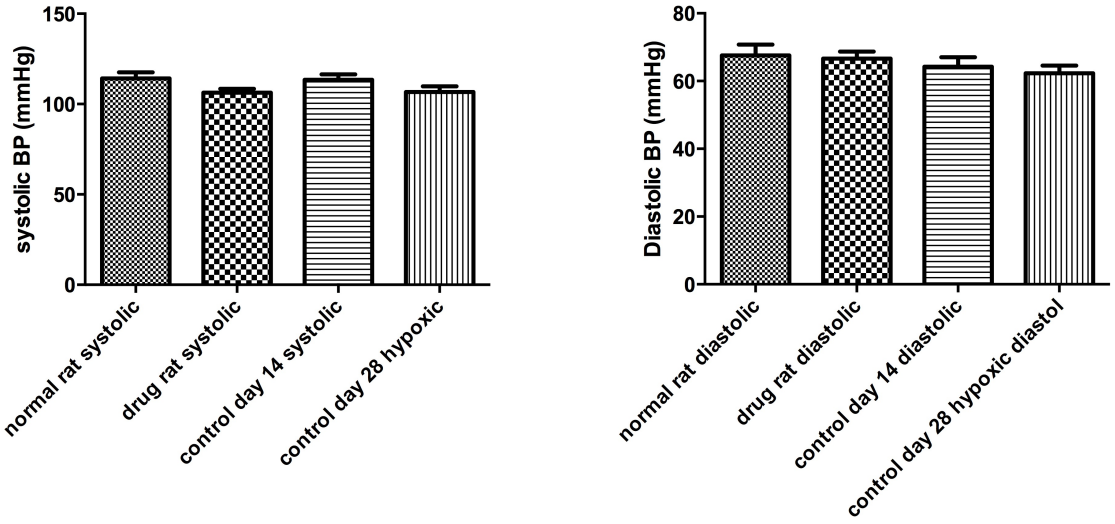

Figure 5-7 Systemic blood pressure was unaffected by PH-797804
Rat tail cuff BP was measured. Results are displayed as mean values + SD.

5.4 Administration of PH-797804 reverses monocrotaline induced pulmonary hypertension

PH-797804 was shown to significantly improve the RVSP in animals with established monocrotaline induced pulmonary hypertension (Figure 5-8). There was an improvement in the RVH in the drug treated animals compared to the 28-day time point but it was similar to that of the 14 day time point (Figure 5-8).

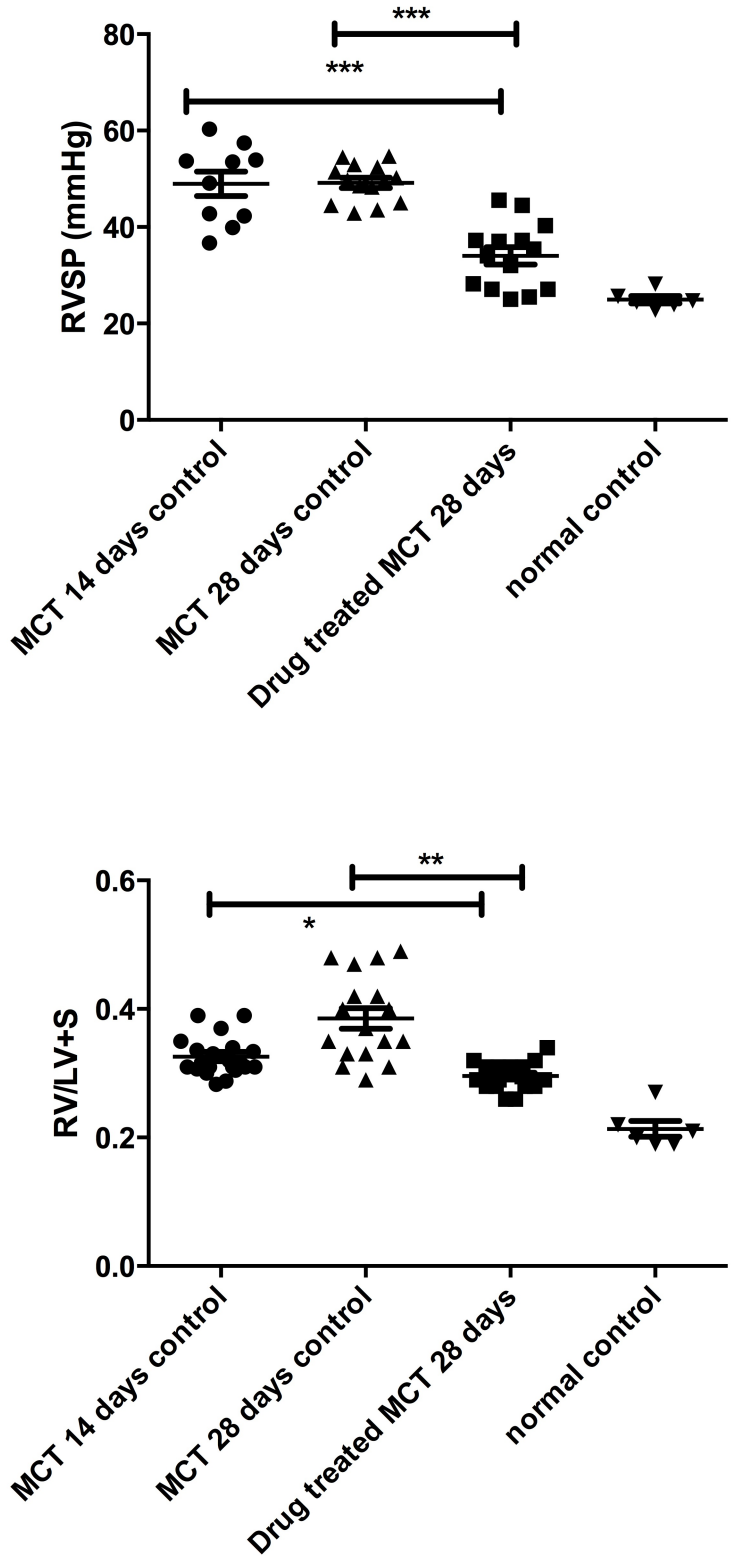

Figure 5-8 p38MAPK inhibition with PH-797804 reverses haemodynamic changes and right ventricular hypertrophy in MCT animal model of PH

Animals were injected with MCT and after 2 weeks p38MAPK inhibition was commenced with daily injections. Haemodynamics (A) and RVH (B) were measured after 4 weeks. Data represent mean values + SD. Total animals n= 14-15 per group.*p<0.05, **p<0.01, ***p<0.005

There was a significant attenuation of progression in the degree of muscularisation of the vessels in the drug treated animals compared to the day 28 untreated animals (Figure 5-9). A trend was suggested of reduction of muscularisation in the treated group compared to the day 14 untreated animals but this did not reach significance ($p=0.09$). However when the percentage of muscularised vessels was calculated and expressed as percentage of the total number of vessels, this demonstrated that there was a significant reduction in the overall degree of muscularisation (Figure 5-10).

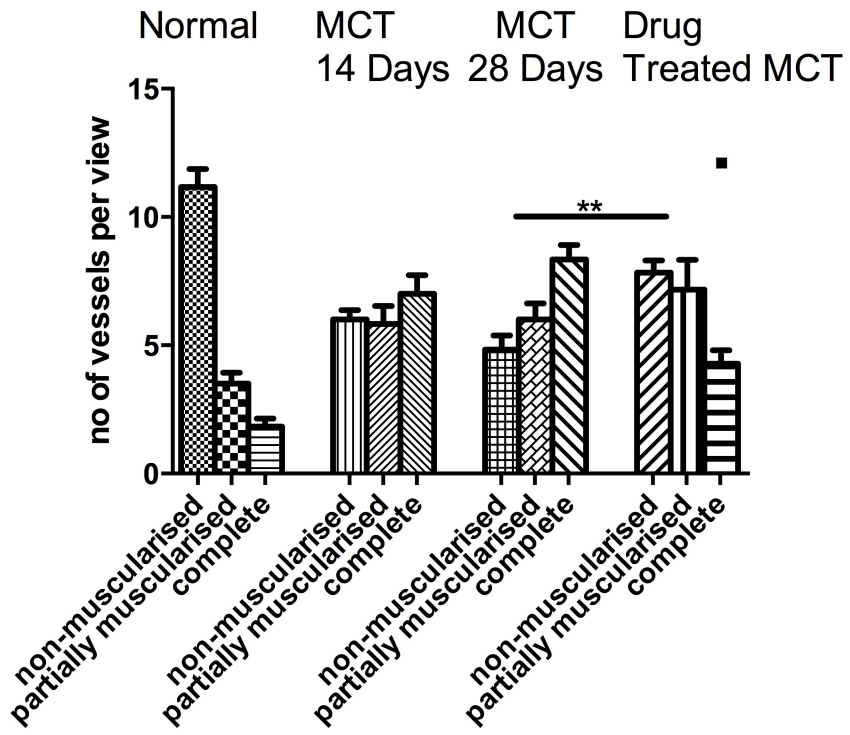

Figure 5-9 PH-797804 attenuates development of muscularisation in pulmonary vessels in MCT experimental model

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <80μm were analysed for degree of muscularisation. 5-10 random fields (X40) were analysed with 3 slides per animal. The vessels were categorised as completely, partially or non-muscularised. Groups analysed by ANOVA for overall change with post-test analysis. n=10 animals. **p<0.01; ■ p<0.05 for complete muscularised group in drug treated vs day 14 hypoxic control.

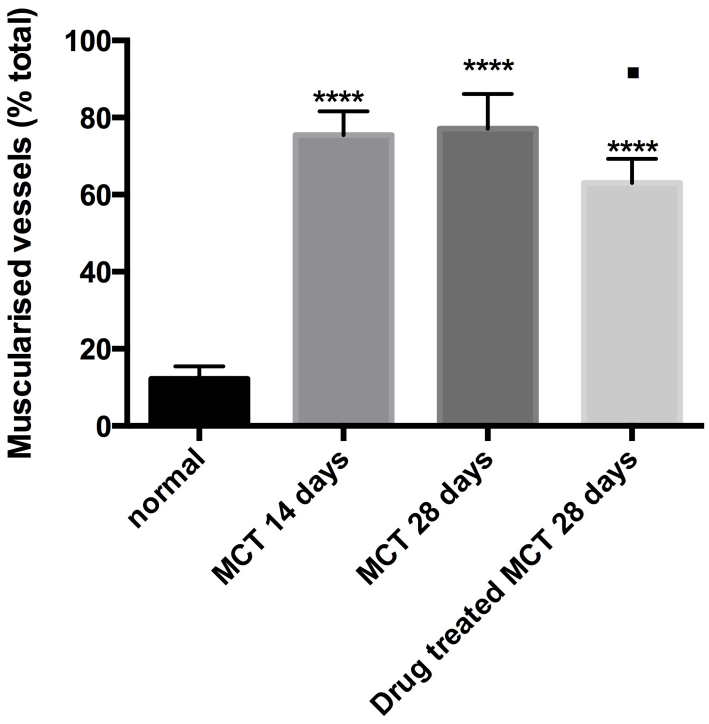

Figure 5-10 PH-797804 reverses total muscularisation of pulmonary vessels in MCT experimental animal model

The lungs were removed after experiment and sections (5μm) cut. These were stained with α-smooth muscle actin and the vessels <100μm were analysed for degree of muscularisation. 5-10 random fields (X40) were analysed with 3 slides per animal. The vessels were categorised as muscularised or non-muscularised and the percentage of muscularised vessels calculated. Groups analysed by ANOVA for overall change with post-test analysis. n=10 animals. ****$p<0.0001$; ■ $p<0.05$ for drug treated vs day 14 MCT control.

There was no significant difference in the systemic BP between the control MCT animals and the drug treated animals (Figure 5-11).

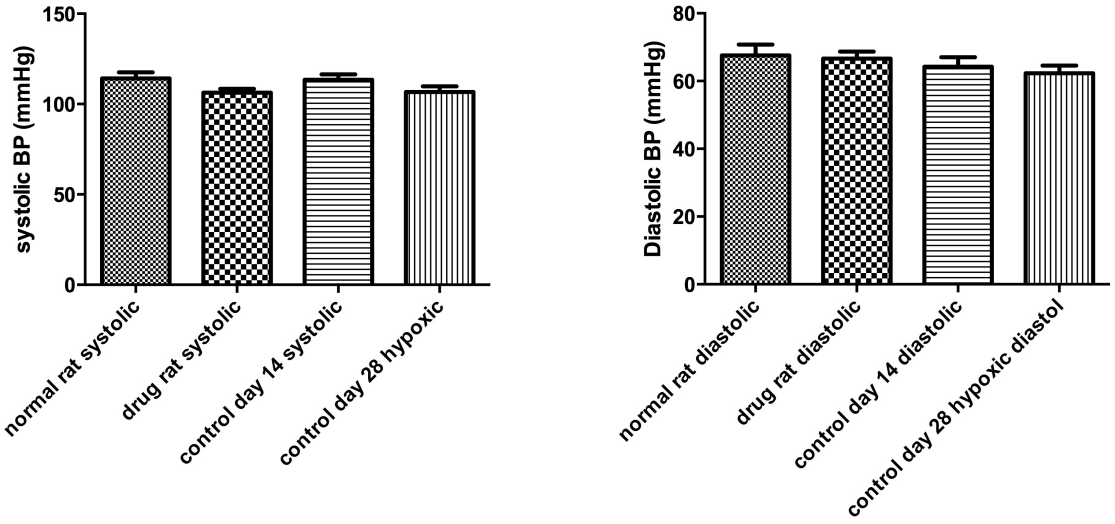

Figure 5-11 Systemic blood pressure was unaffected by PHA-797804
Rat tail cuff BP was measured. Results are displayed as mean values + SD. $p>0.05$

5.5 p38MAPKα inhibition by PH-797804 reduces serum IL-6 levels

It has previously been shown that serum levels of IL-6 correlate prognostically with outcome in pulmonary arterial hypertension. We have shown potential mechanistic data supporting IL-6 in pulmonary vascular remodelling. Therefore in this study with the selective alpha isoform we decided to look at the effect on the serum levels of IL-6 in the animals.

The serum levels of Il-6 were found to be elevated in animals with chronic hypoxic exposure (Figure 5-12). These levels were increased at both the 14- and 28-day mark. When the animals were given the PH-797804 the levels were found to be lower in these animals (Figure 5-12). This suggests that the p38MAPK alpha inhibition was resulting in less serum IL-6 being produced.

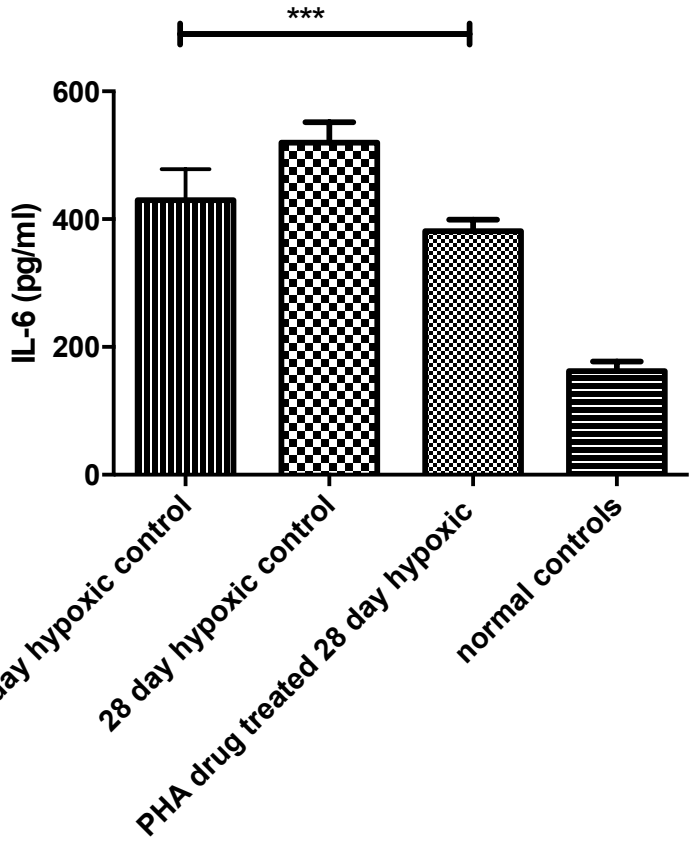

Figure 5-12 PH-797804 reduces serum IL-6 levels in reversal of chronic hypoxic pulmonary hypertension

Serum was collected from animals at the time of cardiac puncture and stored at -80^{o}C until analysis could be performed. ELISA for IL-6 was performed on serum samples. Values shown are mean values + SD. samples were analysed in duplicate and total animal number n=11. ***p<0.005

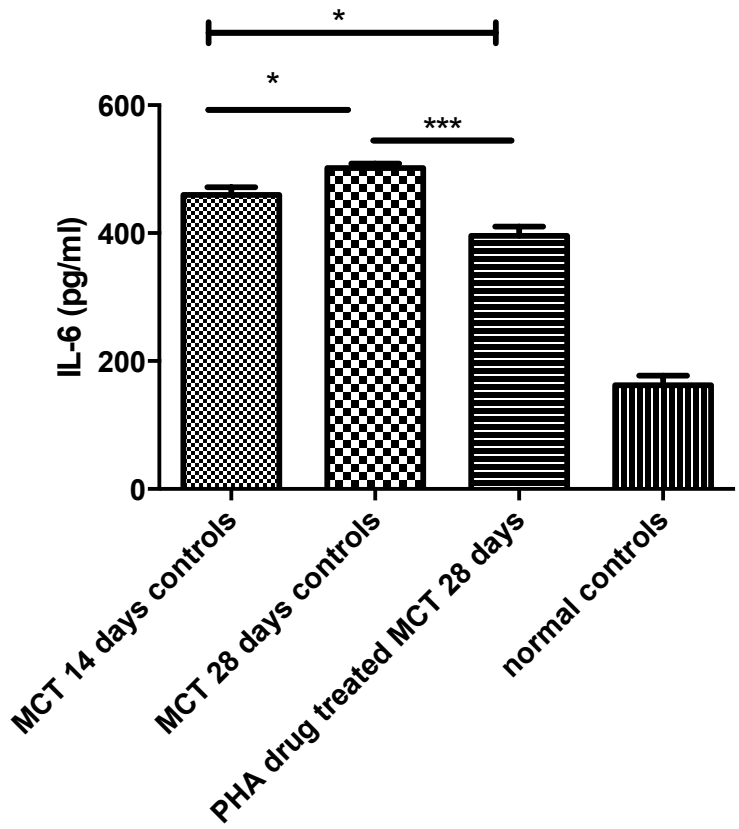

Figure 5-13 PH-797804 reduces serum IL-6 in reversal of MCT-induced pulmonary hypertension

Serum was collected from animals at the time of cardiac puncture and stored at -80^{o}C until analysis could be performed. ELISA for IL-6 was performed on serum samples. Values shown are mean values + SD. samples were analysed in duplicate and total animal number n=12. ***p<0.005

Again similar findings were obtained in the MCT reversal experiments. The PH-797804 drug led to a reduction in the level of circulating IL-6 (Figure 5-13).

Taken together these findings would suggest that an important effect of the p38MAPK alpha inhibition is to lead to a reduction in the serum IL-6 in these two animal models of pulmonary hypertension. As already shown we postulate that this reduction in IL-6 may be a mechanism leading to the reduction in pulmonary vascular remodelling.

5.6 p38MAPKα inhibition reduces serum pro-collagen 1 and pulmonary vascular fibrosis

In order to investigate the effects of p38MAPK on collagen in the animals, we looked at the serum levels of procollagen type 1. This was found to be reduced in the drug treated animals compared to hypoxic controls and normal animals (Figure 5-14A).

Next we looked at the degree of fibrosis surrounding the vessels to see whether the inhibition of p38MAPK had any effect on this (Figure 5-14B,C). There was a reduction in the degree of fibrosis seen around the smaller vessels in the drug treated animals. This suggests that the inhibition of p38MAPKα led to a reduction in the collagen deposition in the pulmonary circulation and this was mirrored with a reduction in the serum level of pro-collagen 1.

It is also noticeable that there is an increase in the amount of collagen present in the alveolar walls in the chronic hypoxic animal model versus normoxic which is also reduced after p38MAPK inhibition.

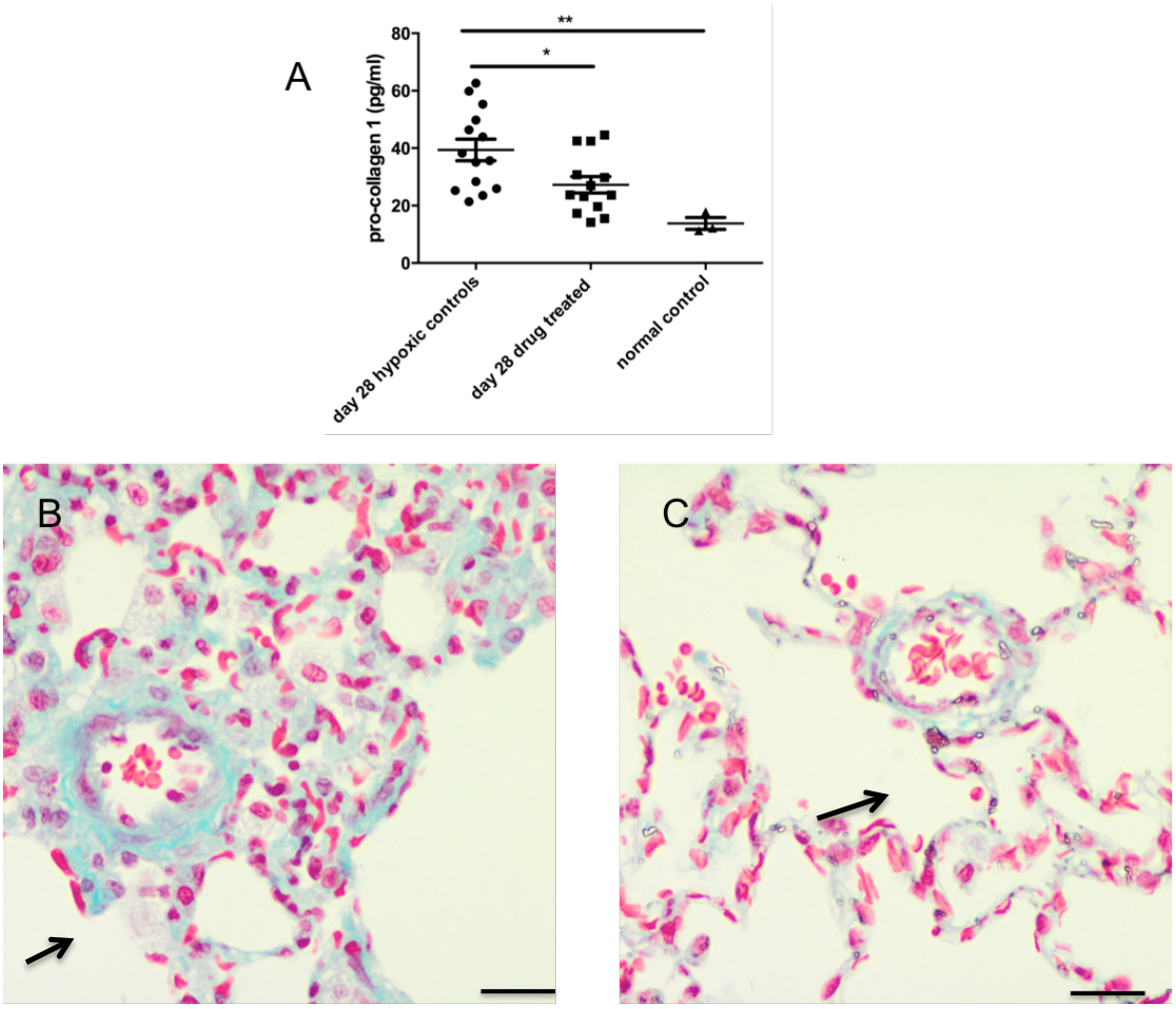

Figure 5-14 Inhibition of p38MAPKα results in lower serum pro-collagen type 1 and reduced peri-vascular fibrosis
Pro-collagen serum levels as measured by ELISA in (A). Trichrome staining performed on representative slides in (B) chronic hypoxic untreated and (C) drug treated animals (arrow). Bar represents 75μm. X40 objective lens.

5.7 Summary

The hypothesis of this chapter was that the alpha isoform of p38MAPK was important in the development of pulmonary hypertension *in vivo*. Using a selective alpha specific drug, which has been well characterised in other animal models of inflammation, the pulmonary hypertensive phenotype was targeted in both chronic hypoxic and monocrotaline animal models of pulmonary hypertension. The compound PH-797804 has been shown in kinase assays to have a IC_{50} of 26nM for p38-α compared to 102nM for p38-β. Other studies have shown it to be between 10-20 times more potent for p38-α. In addition it has been shown to have an anti-inflammatory action in a wide variety of animal models including inflammatory bowel disease and rheumatoid arthritis.

The use of this compound *in vivo* led to a reversal in the haemodynamics and right ventricular hypertrophy of established pulmonary hypertension in two animal models. In addition and perhaps most importantly there was an observed attenuation in the pulmonary vascular remodelling seen in the animals that had been treated. This certainly suggests that the inhibition of the p38MAPK alpha isoform leads to a reduction in the propagation of pulmonary vascular remodelling and there is a trend in both models at least that there might actually be a reversal of pulmonary vascular remodelling. The reason that there was a greater observed reduction in the remodelling in the SB203580 treated animals may reflect that the dose used was not optimal for this compound or that the length of administration was not long enough. The dose and length of administration for SB203580 in other animal models has been clearly identified.

The mechanism that may be important in this result is the reduction in observed IL-6 levels in the serum. This corresponds to similar findings in the previous chapter which showed that there was reduction in both lung tissue IL-6 and signalling via STAT-3 pathway.

Of course, although *in vivo* findings are very important in understanding the pathology and biology of a disease process, determining true relevance in human disease is key. This quest was undertaken and led to investigation of the

hypothesis that the p38MAPKα pathway is dysregulated in human disease, as outlined in the next chapter.

6 p38MAPK activity and expression is increased in human pulmonary arterial hypertension

6.1 Introduction

The hypothesis of this thesis is that p38MAPK is important in the development of pulmonary vascular remodelling and hence pulmonary hypertension. Evidence has been shown of the role of p38MAPK in cellular proliferation, migration and the release of cell mitogens. In two animal models of pulmonary hypertension the expression and activity of p38MAPK seems to be increased. More importantly inhibition of this pathway can lead to the prevention and reversal of pulmonary hypertension in those animal models.

However it is not clear whether p38MAPK is dysregulated in human pulmonary arterial hypertension. This chapter sought to explore the answer to this question by using immunohistochemistry techniques to observe the expression levels of p38MAPK in the lungs of patients with pulmonary hypertension.

6.2 Relevant methods employed

A collaboration was formed with the Lung Transplantation centre from Newcastle,UK. All patients with pulmonary arterial hypertension in Scotland who require lung transplantation for PAH are referred to Newcastle. They have established a biobank of tissue, which has been taken from the native lungs of patients at the time of transplantation after appropriate consent. This is then available for further histological research.

Sections from two patients with Idiopathic pulmonary arterial hypertension who had undergone lung transplantation were kindly donated to us. Control slides were used from patients who were undergoing thoracic operations at a local cardiothoracic centre (Golden Jubilee Hospital, Glasgow) for solitary nodules. The control patients used were having lung resection for what eventually showed to be benign conditions, such as pulmonary hamartoma. The control patients had no clinical, radiological or echocardiographical evidence of pulmonary hypertension, although had not undergone a right heart catheterisation. Preparation procedures at both centres were identical. Lungs were removed

from the individuals at the time of operation and immediately placed in 10% buffered formalin.

The details of the patients are as follows:

	Age at transplant (years)	Condition	Type of transplant
Patient 1	54	IPAH	Double lung transplant
Patient 2	60	IPAH	Heart-lung transplant

Table 6-1 Clinical characteristics of samples used for immunohistochemistry

The sections were stained for phospho-p38MAPK and p38MAPKα. Initially the slides were stained using a number of ranges of dilutions (1:100-1:700) in order to establish the optimal dilution for staining.

In order to determine the difference in staining intensity between cells an intensity scoring system was employed. This mechanism is based on the Allred score for estrogen receptor positive cells in breast cancer(342). This is a quantitative scoring system which takes into consideration of the proportion of positive staining cells and the staining intensity. The total number of vessels present in the field was determined and then the staining intensity of each vessel determined by a blinded observer (score 0-2). The staining intensity is determined using an arbitrary system. The product of the score and the number of vessels determines the overall value.

6.3 Histology from normal and IPAH lungs

The following slides are from patients who have normal lungs and who have IPAH. The key differences are in the vasculature (Figure 6-1 and 6-2). The IPAH patients have the thickened vessels demonstrating all the hallmarks of pulmonary vascular remodelling:

1. thickened intima, media and adventitia

2. increased cellular numbers in vascular layers

3. plexiform lesion

This is in contrast to the normal histological appearance of the lungs from patients without pulmonary hypertension.

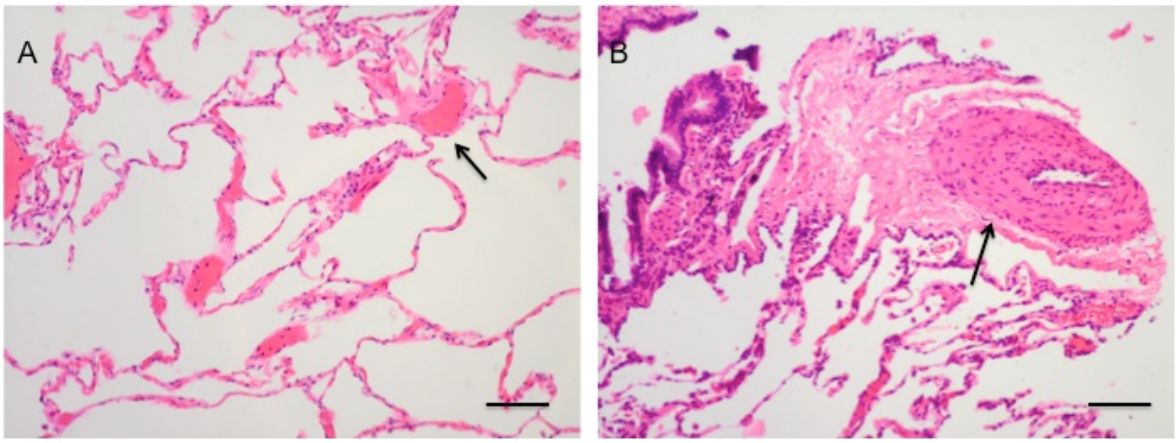

Figure 6-1 Contrast in the histological appearance between normal lung and pulmonary vascular remodelling in the IPAH lung
Normal lung (A) shows thin walled vessels (arrow) compared to thickened vessel (arrow) as seen in the remodelled lung (B). Sections were cut and stained with H&E. Microscopy taken with objective X20. Bar = 150μm

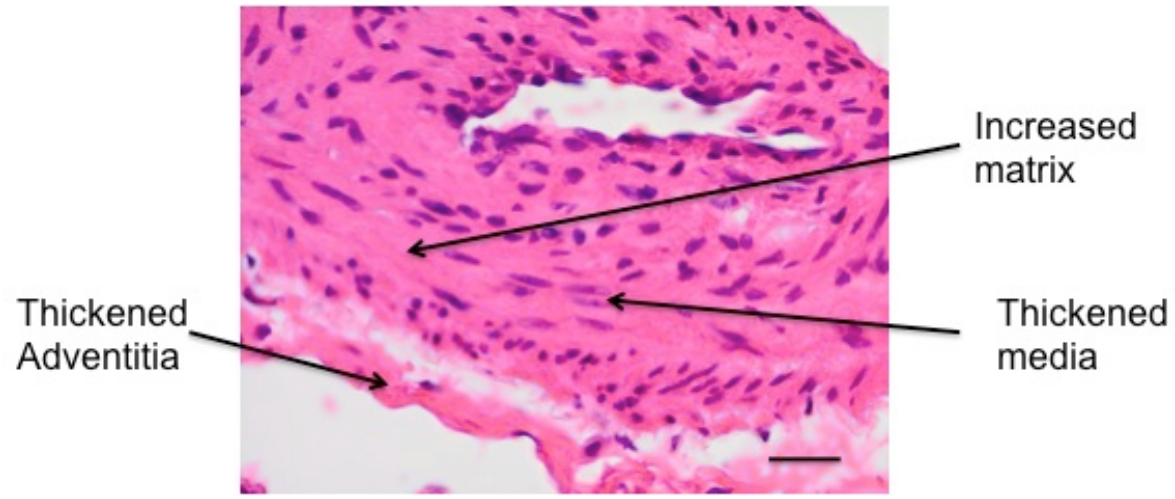

Figure 6-2 Classical features of pulmonary vascular remodelling
High power view of remodelled pulmonary vessel with intimal, medial and adventitial thickening. Increased cellularity and matrix deposition is seen. objective X40. Bar=50μm

6.4 Phospho-p38MAPK is increased in IPAH lungs

Using specific antibodies for phospho-p38MAPK, the relative differences in staining between IPAH and control lungs was assessed. Immunohistochemical staining of sections from the lungs of patients with IPAH who had undergone transplantation showed increased phospho-p38MAPK expression compared to control lungs (Figure 6-3). At higher power there is clear staining for phospho-p38MAPK in the intima, and adventitia and in the nuclei of the smooth muscle cells (Figure 6-4).

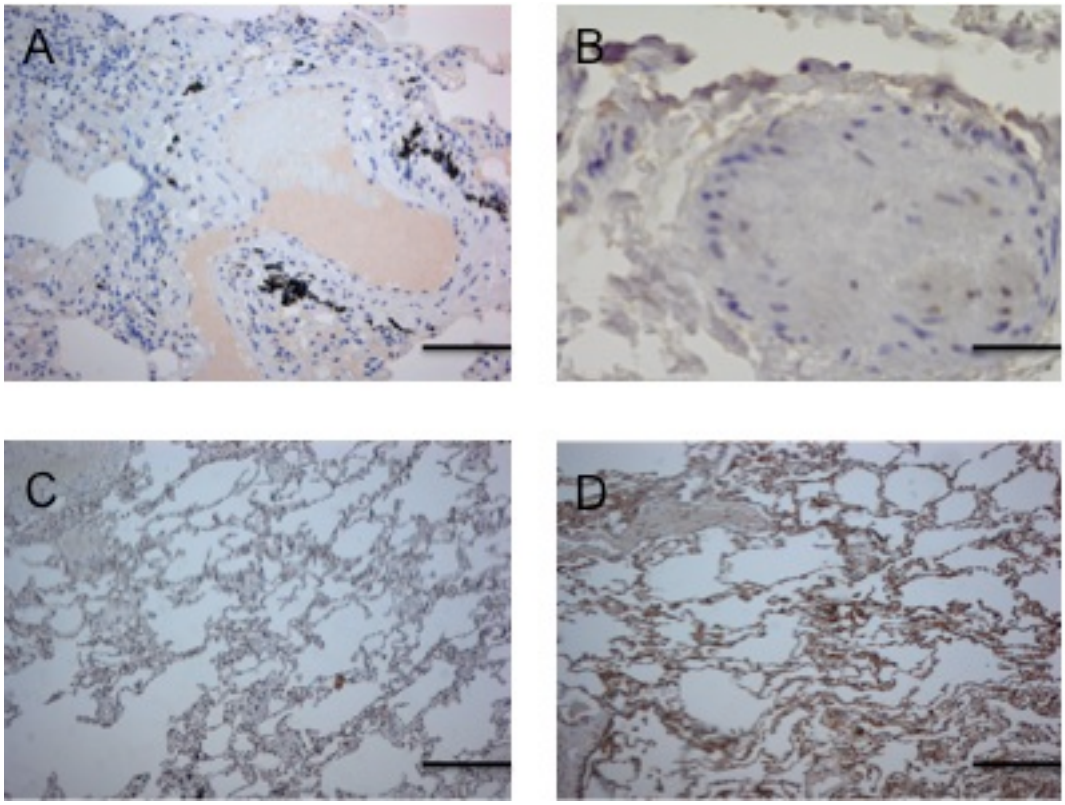

Figure 6-3 Increased phosphorylated-p38MAPK demonstrated in PAH lung
Sections (5mm) are taken and normal control lung stained for isotype (A) low power and (B) high power. Then normal control lung (C) and IPAH lung (D) are stained for pp38MAPK at dilution of 1:300. This dilution was optimally assessed for. Objective X20 except (B) X40. Bar = 150μm.

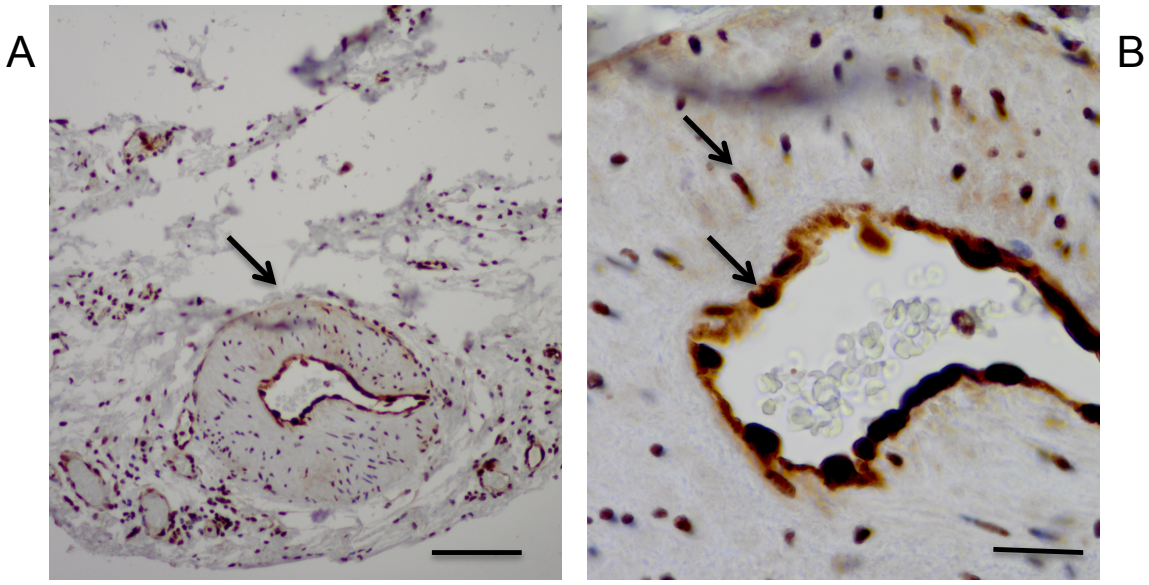

Figure 6-4 Increased phosphorylated-p38MAPK staining throughout vessel wall in IPAH
High power microscopy shows that there is strong staining for pp38MAPK in the intima, media and the adventitia (arrows). objective lens X20 and x40.

6.5 p38MAPKα is increased in IPAH lungs

The sections were stained for p38MAPKα expression. There was increased staining for p38MAPKα in the IPAH lung. There was staining in the control lung as expected and this was mainly in a nuclear distribution. However, in the IPAH lung the staining involved both nuclear and cytoplasmic staining in the IPAH patients (Figure 6-5 and 6-6). In the larger vessels increased cytosolic staining in particular was observed in the IPAH group.

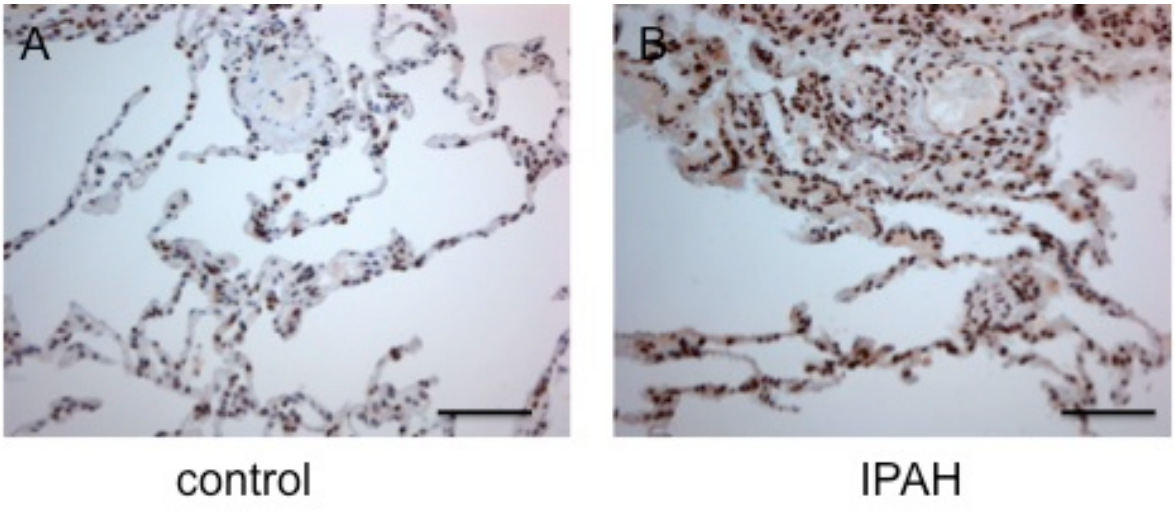

Figure 6-5 Increased staining of p38MAPKα is observed in IPAH lung
Sections of control lung (A) and IPAH lung (B) were stained for p38MAPKα at dilution of 1:300. Objective lens x20. Bar=150μm.

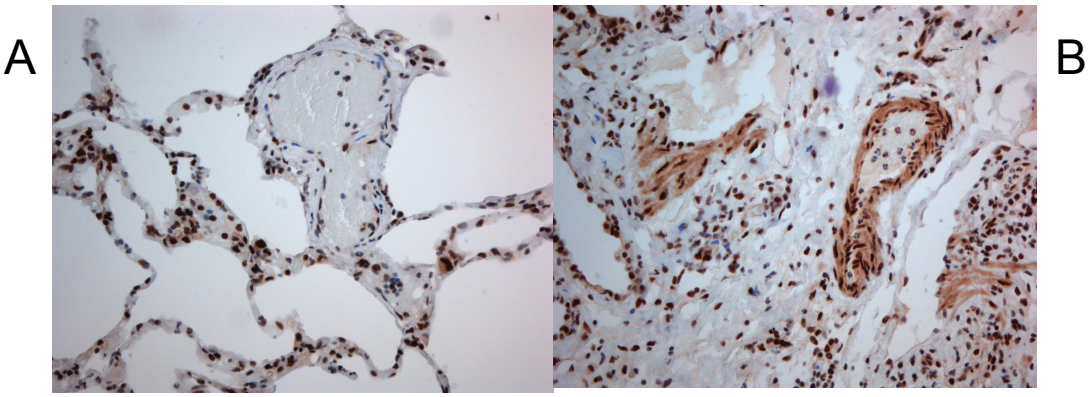

Figure 6-6 Increased cytosolic staining for p38MAPKα observed in IPAH lung
Staining for p38MAPKα showed increased cytosolic staining in the IPAH lung (B) compared to control lung (A).

Using the intensity scoring technique already discussed there was higher expression demonstrated for p38MAPKα in the vessels of patients with IPAH compared to controls (Figure 6-7). There was also an observation in some of the larger vessels that there was strong staining in the adventitial layer and the cells surrounding the adventitia, which are assumed to be fibroblasts based on location and morphology (Figure 6-8).

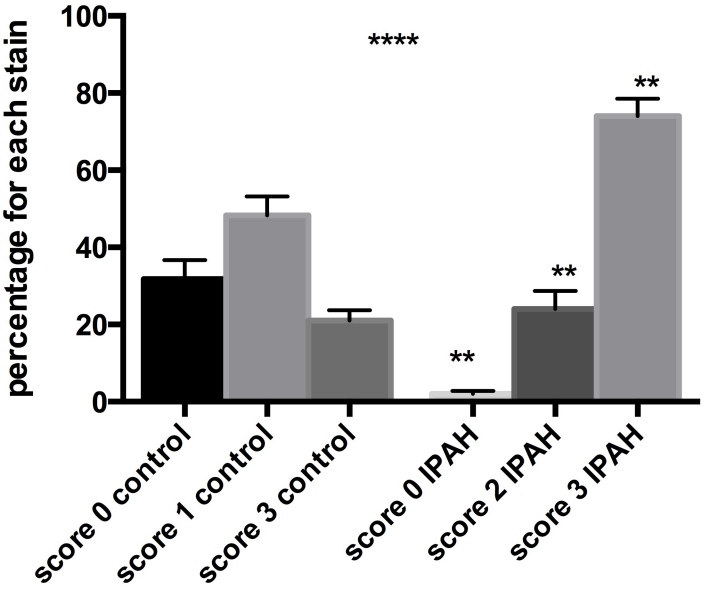

Figure 6-7 Histological scoring shows increased p38MAPK alpha staining throughout the vascular wall

Using a well validated histological scoring system (342), the vascular wall cells were scored for intensity of staining. The intensity multiplied by the number of vessels with that intensity determines the values. Values shown are from 5 random high power fields from 2 slides.**** $P<0.0001$ by ANOVA. ** represents $P<0.001$ for individual IPAH vs control columns

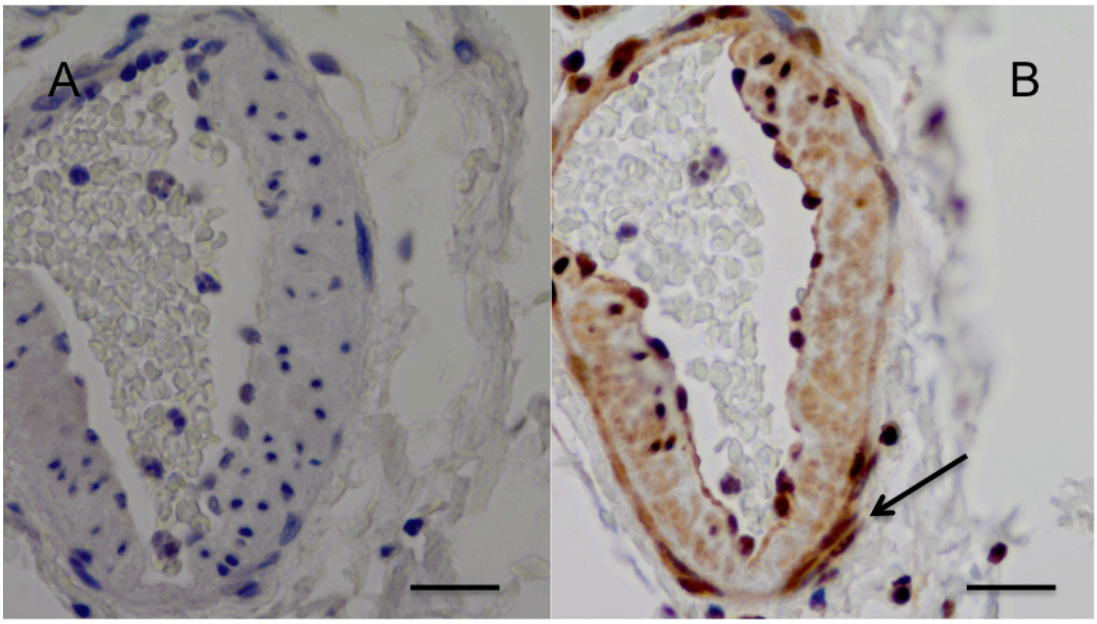

Figure 6-8 Increased p38MAPKα staining in adventitia in IPAH
High power view (x40) of staining with isotype (A) and (B) p38MAPKα in a vessel in IPAH lung. This shows staining throughout the vessel layers but especially in adventitia and fibroblast cells (arrow). Bar=50μm

6.5.1 Plexiform lesion

In the identified plexiform lesion there was strong cellular staining for p38MAPKα around the vascular channels in both cytosol and nucleus(Figure 6-9).

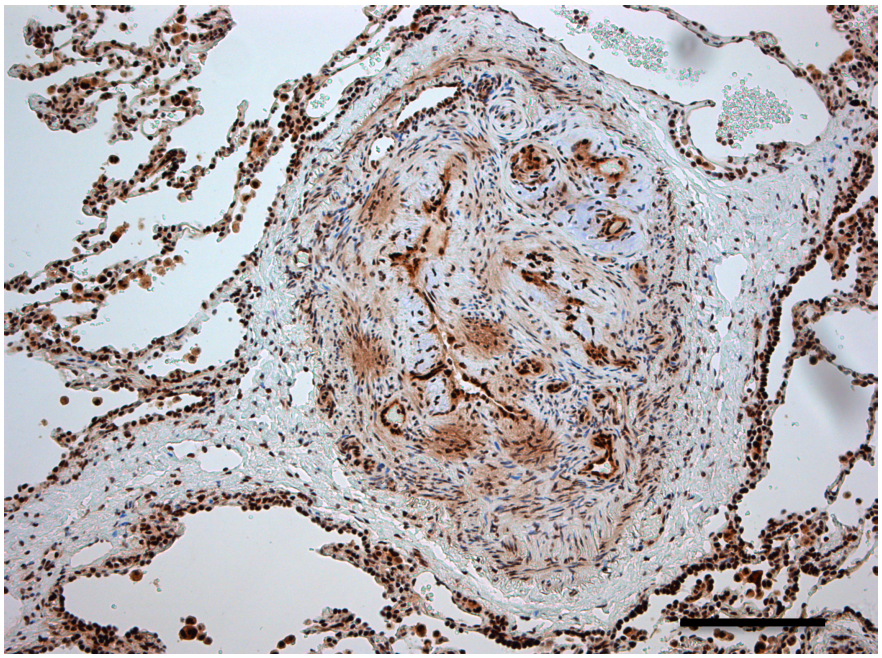

Figure 6-9 A plexiform lesion shows prominent staining in vascular channels for p38MAPKα
Low power view of a plexiform lesion. Staining for p38MAPKα using 1:300 dilution. Bar=150mm. Objective lens x20

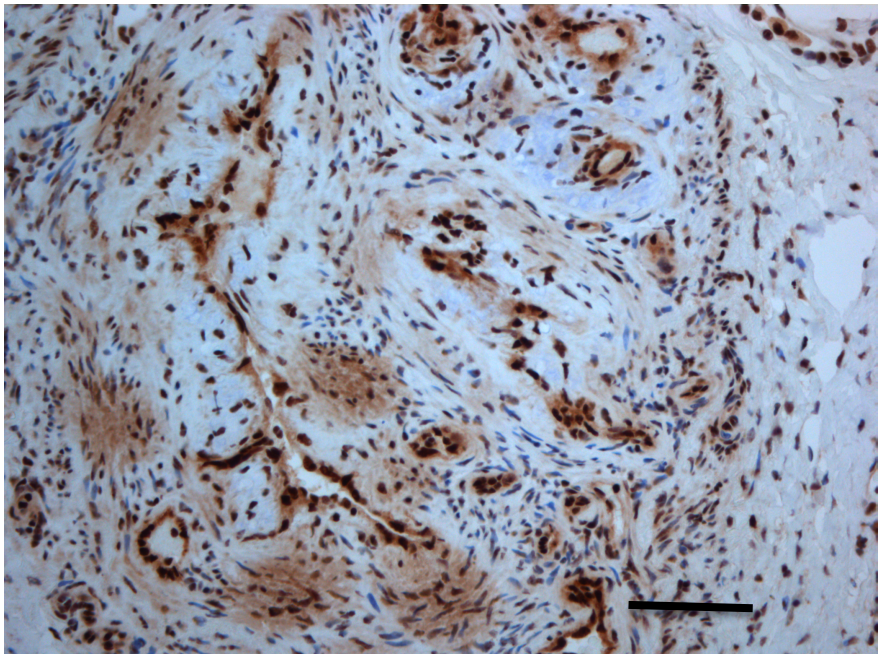

Figure 6-10 High power view of plexiform lesion
Objective lens X40 bar = 50μm

6.6 Summary

From this work it is clear that p38MAPK activity is increased in the lungs of the patients with Idiopathic pulmonary arterial hypertension. Although I have not analysed lungs from all different subcategories of WHO Group 1 PAH, it would seem reasonable that the increased activity would be similar in all types. Certainly it has been shown that the histological appearances are similar within these groups.

We have not undertaken double staining to closely scrutinise exactly which cells are staining the most intensely. However, as previously addressed the fibroblast lacks a unique histological marker so it is justifiable to assess staining on cell appearance and location in the vessel.

This is an exciting finding and confirms the hypothesis of this study that p38MAPK activity is dysregulated in pulmonary arterial hypertension. In addition inhibition of this in the clinical arena may prove to be an effective therapy for PAH and is worthy of further exploration.

7 Final Discussion

The results shown in this thesis demonstrate an important role for p38MAPK, and the alpha isoform in particular, in the pathobiology of pulmonary hypertension.

7.1 p38MAPKα activity controls pulmonary vascular remodelling at a cellular level

The p38MAPK pathway has been shown in this body of work to be essential in a number of key functions in the pulmonary artery fibroblast. The novel findings shown in this thesis include:

- hypoxia releases a number of pro-inflammatory signals and mitogens from the PAF, mediated by p38MAPK
- release of IL-6 from the PAF is regulated by p38MAPKα
- motility of the PAF is increased in hypoxia and regulated by p38MAPKα
- IL-6 and hypoxia can release collagen from PAF and this is regulated by p38MAPKα

The *in vitro* experiments performed in this thesis have shown the importance of p38MAPK in the biology of the pulmonary artery fibroblast. This cell resides in the adventitial layer of the pulmonary artery and is perhaps now becoming recognised as an important regulatory cell in the function of pulmonary vascular biology.

Previous work from the Peacock/Welsh laboratory have shown in the PAF a number of important p38MAPK dependent processes. Firstly that hypoxia can result in increased proliferation of the PAF and that p38MAPKα was implicated as a key control point in this activity. Secondly, work previously published has suggested the PAF derived from chronic hypoxic animals have undergone a 'phenotypic switch' in that the p38MAPK pathway is more constitutively activated, manifested by a pro-proliferative phenotype. Thirdly, PAF exposed to

a hypoxic environment can release a factor (or factors) which in turn can act on smooth muscle cells to stimulate proliferation.

Publications from other laboratories have confirmed these findings and shown that the pulmonary artery fibroblast can act as an important cell in the generation of pro-inflammatory mediators(48). Indeed we have shown here that PAFs derived from another animal model of PH, the MCT model, share the similar pro-proliferative phenotype observed in PAFs from the chronic hypoxic animals. This leads to the intriguing suggestion that one of the central events in development of pulmonary vascular remodelling is the change in the fibroblast biology. These cells also share upregulation of the p38MAPK pathway.

Work presented here using specific siRNA knockdown to the p38MAPKα isoform has confirmed the critical importance of this isoform in the hypoxia-induced proliferation of the PAF. Furthermore examination of PAF derived from both chronic hypoxic animals and monocrotaline animals have demonstrated that conditioned media isolated from stimulation of these cells with low dose serum can also stimulate the proliferation of smooth muscle cells.

A core part of this thesis work was to try and identify the mitogen(s) that was being released from the hypoxic fibroblast. Cytokine array technology was employed and identified a number of potential mediators that were released. Biological plausibility and evidence from the literature allowed us to focus on Interleukin-6. This has previously been shown to be a mitogen for smooth muscle cells and transgenic experiments with overexpression of IL-6 in mice have shown evidence of a pulmonary hypertensive phenotype(264). Therefore it seemed logical to explore this further.

Quantitative ELISA analysis of the conditioned media isolated from hypoxic PAF confirmed high levels of IL-6 were released from these cells in a time dependent manner. Moreover this process could be blocked by inhibition of the p38MAPKα using both pharmacological techniques with SB203580 and molecular knockdown with siRNA to the alpha isoform. Our experiments have confirmed that IL-6 is a mitogen for both smooth muscle cells and PAF and that STAT-3 signalling is activated during this.

Proliferation however is only one of the biological processes underpinning pulmonary vascular remodelling. Increase in the stiffening of pulmonary vessels with deposition of increased extracellular matrix is an additional factor. To explore this, the effect of both hypoxia and IL-6 on the release of soluble collagen from PAF was investigated. We have shown that both hypoxia and IL-6 can result in the release of increased soluble collagen and furthermore this can be reduced by p38MAPKα inhibition.

Taken together, the work from the *in vitro* experiments in this thesis suggests a strong role for p38MAPKα in the biology of pulmonary vascular remodelling. Through both pro-proliferative and pro-inflammatory routes this intracellular pathway can lead to a number of events which are consistent with our understanding of pulmonary vascular remodelling. A hypothetical outline of the cellular interactions which may be occurring are shown in Figure 7-1. However these are cellular experiments and in order to understand the relevance of this to the overall pathobiology we explored the role in the animal models of pulmonary hypertension.

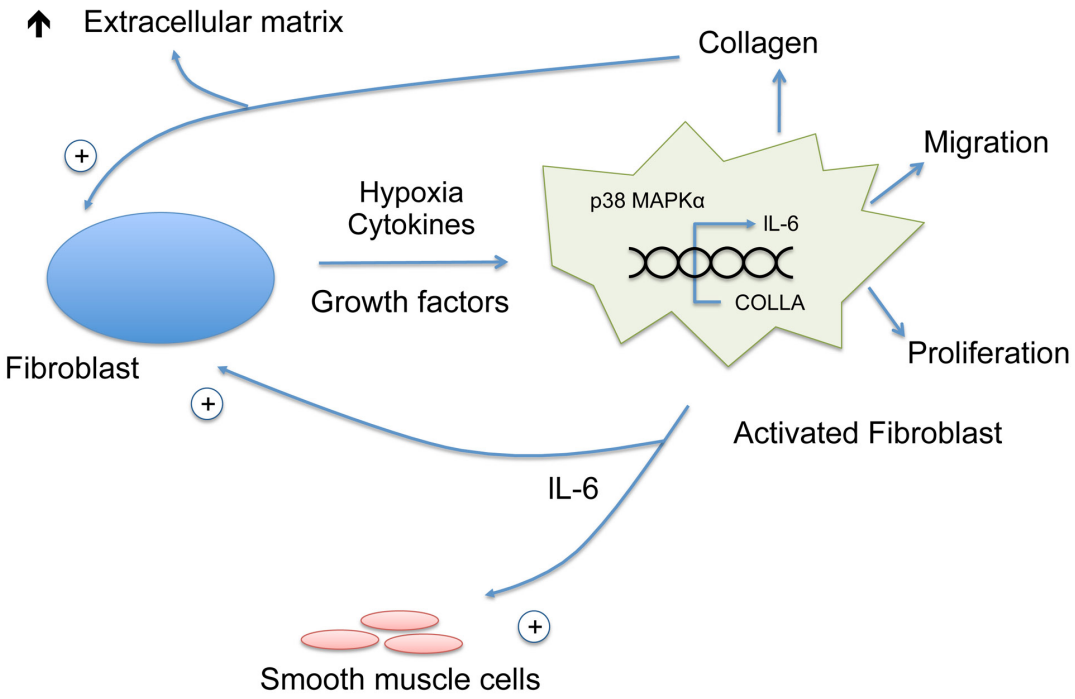

Figure 7-1 Outline of potential fibroblast role in pulmonary vascular remodelling

7.2 p38MAPKα is activated in animal models of pulmonary hypertension

There are a number of *in vivo* animal models available for the study of pulmonary hypertension. However as previously discussed all have drawbacks. Nonetheless these are accepted as the best experimental avenues to allow further exploration of various pathways which may be implicated in the disease process. The novel findings outlined here are:

- increased phospho-p38 MAPK activity in the chronic hypoxic and monocrotaline animal models of pulmonary hypertension

- increased levels of p38MAPKα in the chronic hypoxic and monocrotaline animal models of pulmonary hypertension

We examined two well-established *in vivo* models of pulmonary hypertension: the chronic hypoxic and the monocrotaline rat models. Using a kinase based p38MAPK assay we confirmed that p38 MAPK activity was increased in the lungs from these animals. Immunoblotting techniques confirmed increase in the phospho-p38MAPK activity but also in levels of p38MAPKα.

Histological examination of lungs from these animals identified increased staining for p38MAPKα in the remodelled vessels in the pulmonary vasculature.

Hence increased activity of p38MAPK and levels of p38MAPKα were observed in two animal models of pulmonary hypertension. Clearly novel animal models such as the SUGEN-hypoxia model would be interesting to examine and indeed this would contribute to future work. However confirmation of the findings in two robust animal models of pulmonary hypertension would suggest that this is an important and validated finding.

Although we have shown that p38MAPK is upregulated in animal models the next step was to see whether inhibition of the pathway could have favourable effects on the animal phenotype.

7.3 The inhibition of p38MAPKα prevents and reverses pulmonary hypertension in experimental animal models

This thesis has shown evidence that p38MAPKα is important in controlling cellular functions which are implicated in pulmonary vascular remodelling and is upregulated in two animal models of pulmonary hypertension. To demonstrate the key importance of this pathway in the biology of pulmonary hypertension we undertook inhibition of this pathway and observed the effects on the pulmonary hypertensive phenotype *in vivo*. The novel findings presented here are:

- p38MAPKα inhibition can prevent the development of chronic hypoxic induced pulmonary hypertension

- p38MAPKα inhibition reverses the pulmonary hypertensive phenotype in chronic hypoxic and monocrotaline animal models of pulmonary hypertension

- possible mechanisms underlying this are the reduction in both IL-6 and collagen observed in the drug treated animals

Since the p38MAPK inhibitor SB203580 has strong selectivity against the p38 MAPK-alpha isoform (as well as the beta isoform but to a lesser extent) and is the best characterised of the alpha isoform inhibitory agents we decided to use this initially. This has clearly shown that the development of hypoxia induced pulmonary hypertension and pulmonary vascular remodelling can be prevented with the administration of a p38 MAPK inhibitor. This finding correlates nicely with a previously published small animal study looking at the prevention of monocrotaline induced using a p38MAPK inhibitor(343). This study showed that p38MAPKα inhibition could prevent the development of pulmonary hypertension in animals that received monocrotaline and the inhibitor at the same time. This thesis demonstrates that inhibition of the p38MAPKα can prevent the development of pulmonary hypertension in the chronic hypoxic animal model.

Although prevention studies give insight into the biological pathways involved in a disease process, they do not really reflect the true clinical picture of human

disease. Patients present with established disease rather than before the disease starts. Hence studies which look at the reversal of the pulmonary hypertensive phenotype are perhaps more clinically relevant. Therefore this body of work aimed to see if the process of pulmonary vascular remodelling could be reversed. After allowing a period for development of established pulmonary hypertension in both chronic hypoxic and monocrotaline animal models, daily administration of SB203580 not only reversed the RVSP and RVH but also improved pulmonary vascular remodelling. These exciting findings suggest that inhibition of the p38MAPKα pathway can result in both prevention and reversal of pulmonary hypertension *in vivo*.

Although SB203580 is relatively specific for the p38 MAPK alpha isoform it does have some effects on the beta, gamma and delta isoforms and additional off-target effects, for example it can inhibit cyclo-oxygenases and thromboxane synthase. Indeed, one of the issues that have been apparent with the p38MAPK inhibitors is that of toxicity, principally hepatotoxicity. The explanation for this may lie in part with the cross talk between p38 and other intracellular pathways. For example it has been shown previously that in hepatocytes, p38MAPK integrates with IκB to protect them from TNF-induced cell death. This blockade of p38MAPK in certain cell types may lead to untoward effects. However toxicity may also reflect isotype specificity and that by targeting more specific isoforms in a cell specific or compartment specific way, these effects may be avoided.

For this reason we decided to use a novel new generation p38 MAPK alpha specific inhibitor, PH-797804. This has been previously shown to be extremely efficacious in animal models of rheumatoid arthritis and has been shown to be more specific for the alpha isoform of the p38MAPK family with a 10-20 fold increase in specificity over the p38 MAPK-beta isoform compared to existing inhibitors. Furthermore it is currently undergoing clinical trials in patients with chronic obstructive pulmonary disease which allowed us to use a drug with the potential to be translated from animal work to use in the clinical treatment of patients(312).

Our data with this drug, performed in reversal *in vivo* studies, produced similar findings to SB203580. There was a reduction in the RVSP and the RVH in the chronic hypoxic model with the PH-797804 drug.

Moreover there was a similar improvement in the RVSP in the drug treated animals in the monocrotaline group. Although the RVH was not found to revert to that of normal levels the progression of RVH was significantly attenuated. It may be that the dose employed in this treatment was not optimal for reversal of RVH compared to the maximal dose utilised in the SB203580 experiment or that treatment was not long enough for this particular agent. Further work will be needed to identify the optimal dose for this compound. Nevertheless a significant attenuation in the progression of RVH was seen.

To understand the molecular mechanisms which may be contributing to this we examined the levels of soluble collagen and IL-6 in the lungs and serum of animals. As previously outlined, IL-6 has been associated with both pathogenesis and outcomes in human PAH disease. Our *in vitro* work has shown that the fibroblast can be an important source of IL-6 and can be released in a localised environment. Therefore we examined the levels of Il-6 in the lungs of experimental animals and found that there were reduced levels in drug treated animals and also reduced STAT-3 signalling in the lungs. In addition in the animals treated with the more selective inhibitor PH-797804 there was a significant reduction in the serum IL-6 levels. These findings would suggest that alteration in IL-6 biology, mediated by the inhibition of p38MAPKα, leads to reversal of the pulmonary hypertensive phenotype in experimental animal models.

We have shown that p38MAPK inhibition can also control the release of collagen from fibroblasts after stimulation with hypoxia or IL-6. Collagen deposition leads to increased matrix deposition which can then cause reduced arterial compliance. This is being recognised as resulting in a detrimental effect on right ventricular function. In our experimental animal models we found reduced levels of soluble collagen in the lungs of animals after p38MAPKα inhibition. Hence inhibition of p38MAPKα can alter pulmonary vascular remodelling by decreasing the deposition of extracellular matrix.

These *in vivo* findings suggest that p38MAPKα inhibition can reverse the pathogenesis of pulmonary hypertension by reducing cellular proliferation, release of mitogenic mediators and deposition of extracellular matrix. However the question of whether p38MAPK is dysregulated in human PAH has not until now been addressed.

7.4 p38MAPK signalling is increased in human pulmonary arterial hypertension.

To complete a true translational aspect to this research we undertook immunohistochemistry to determine the activity of the p38MAPK pathway in explanted lungs of patients with pulmonary arterial disease. The novel findings obtained are:

- increased levels of phospho-p38MAPK in the vasculature of patients with PAH

- increased levels of p38MAPKalpha in the vasculature of patients with PAH

The finding of increased levels of phospho-p38MAPK in the sections of lungs from patients with PAH suggests that there is increased activity of the pathway. This is seen in all compartments of the vessel wall. In addition there is increased staining for the p38MAPK alpha isoform in the vasculature of the explanted lungs. These findings would suggest that there is increased activity of the p38MAPK pathway and in particular the alpha isoform, in human PAH. This obviously has great therapeutic implications as to whether the new p38MAPK inhibitors, which are becoming available in the clinical arena, could have an influence on the treatment of this devastating human disease.

7.5 Rationale for targeting p38MAPK in pulmonary hypertension

There have been various parallels drawn between the pathological processes of pulmonary vascular remodelling and that of neoplasia. Although pulmonary vascular remodelling does not breach tissue boundaries or metastasise, it does share disordered cell proliferation, migration and apoptosis(344,345). In addition there is a metabolic profile change towards favouring aerobic glycolysis as

previously discussed. Clinically this is detected by ^{18}Fluoro-deoxyglucose positron emission tomography (FDG-PET) scanning with increased uptake apparent in the lungs and right ventricle of PAH patients.

As in cancer, there is an extensive network of signalling pathways in vascular cells, which interact closely to determine the fate of the cell. It is clear that p38MAPK can also be involved in all these disordered processes in cancer(346,347). We have shown similar activation of p38MAPK in pulmonary vascular remodelling; so it would seem a logical target to focus on in pulmonary hypertension.

7.6 Potential Clinical Applications

Ultimately biomedical research is limited unless it can aid our understanding and treatment of human diseases. This translational study has drawn on *in vitro* findings which have then been confirmed with *in vivo* models and finally linked to human pathology by immunohistochemistry of lung tissue from PAH patients. Perhaps the most exciting aspect of this work is the potential use of p38 MAPK inhibitors in human disease. Recently work looking at the use of p38MAPK inhibition in both cardiovascular and respiratory diseases has been reported.

A recent study has shown that the administration of a p38 MAPK alpha inhibitor to patients with hypercholesterolaemia can improve nitric oxide mediated forearm vascular flow, which is impaired in these patients, and reduce inflammatory responses as measured by C-reactive protein(314). Although only proof of concept studies using surrogate markers of vascular dysfunction, they do suggest the tolerability, safety and efficacy of the new generation p38MAPK inhibitors.

One of the most important and novel findings in this study was that we were able to use the drug PH-787904 in our experiments, which is currently undergoing clinical evaluation in chronic obstructive pulmonary disease (COPD)(348). A recent publication has again shown this to be efficacious and safe in patients with moderate to severe COPD(312). Our data has shown that in pulmonary hypertension associated with chronic hypoxia there is a significant improvement in vascular remodelling and haemodynamics. The WHO

classification of pulmonary hypertension includes Group 3 which identifies those patients who have pulmonary hypertension as a result of respiratory diseases such as COPD and interstitial lung disease (ILD). The association of PH with these diseases heralds a poor prognosis for the patient and currently there are no beneficial pulmonary vascular targeted therapies to address this.

In addition ILD has a significant fibrotic component to its pathology and the pulmonary parenchymal fibroblast is responsible for this. Indeed increased p38MAPK activity has been linked to aberrant TGF-β stimulation in the pulmonary fibroblast(349). One possibility for further investigation would be whether the inhibition of p38MAPK could reduce the fibrotic component as well as alter the pulmonary vascular remodelling process. This study presents the possibility that p38MAPK inhibition in pulmonary hypertension associated ILD should be investigated further and could potentially offer a treatment.

However blocking the complex intracellular signalling that p38MAPK represents can lead to problems. It is clear that activation of p38MAPK can have different effects depending on the cell or indeed organ that it is activated in. For example, as confirmed in this thesis, it can generate pro-inflammatory cytokines. However it can also produce in certain circumstances anti-inflammatory mediators, perhaps as an attempt as ever in nature to obtain homeostasis. Thus the balance between the activity in certain cell types may perhaps hold the key to the main effect blocking p38MAPK would have.

However the focus of this work is on PAH. As previously discussed the treatment of PAH now requires a more concerted effort to try and identify treatments which can reverse the remodelling process rather than only alleviating pulmonary vasoconstriction. Our findings suggest that this is now possible with this p38MAPK inhibitor and furthermore because of the potential selectivity of the alpha isoform for the fibroblast in the pulmonary circulation we believe that this agent could offer a targeted approach.

7.7 Important outstanding questions in the biology of p38MAPK and pulmonary vascular disease

The work in this thesis has extended our understanding of the biology of p38MAPK in pulmonary hypertension. However this research has also led to further important questions that need answered in order to drive the understanding of this field further.

The work presented here suggests that p38MAPKα is involved in the process of pulmonary vascular remodelling. This is based on evidence in terms of biochemical analysis and inhibition of the pathway using specific pharmacological inhibitors. However to prove beyond doubt, genetic animal studies should be used. p38MAPK alpha knockout mice are lethal at early gestation(291). However *in vivo* siRNA specific for the alpha isoform could be adapted and given to animals. Ideally a mouse model of pulmonary vascular remodelling could be used, as the doses required for the siRNA would be less. Murine models such as the chronic hypoxic model or indeed the novel Sugen-hypoxia murine model could be used. This would allow very specific targeting to the alpha isoform.

Exploration of novel drug delivery methods could also be employed. For example nebulised or aerosolised formulations of the p38MAPK inhibitor may allow a more selective targeting to the pulmonary vasculature(350,351). Again this would reduce the off target effects that may arise from p38MAPKα inhibition.

In some of the *in vivo* animal experiments there was a dramatic improvement in the degree of right ventricular hypertrophy accompanied by more modest changes in the degree of reversal of pulmonary vascular remodelling. One could postulate that there is a direct effect of the p38MAPK inhibition on the right ventricle separate from that on the pulmonary vasculature. Indeed there is mounting *in vivo* evidence of a pathogenic role for p38 MAPK in systemic vascular dysfunction(352-354). In animal models of myocardial infarction the p38 MAPK pathway is activated in the infarct zone of the left ventricle and this can lead to increased fibrosis, increased cardiac inflammation and increasing cardiomyocyte mitoses resulting in cardiac hypertrophy(355). Future studies in

pulmonary hypertension might address whether p38 MAPK pathways are activated in the right ventricle in animal models.

For example, use of the novel animal Pulmonary artery banding model would allow to investigate whether the reversal of pulmonary vascular remodelling with the p38MAPK inhibition was sufficient alone for reversal of the RVH. However more likely is that there would be a direct effect on the 'stressed' myocardium which could be analysed in greater detail. In this model the pulmonary artery is restricted such that the RV faces an increased afterload. This allows a model to be developed to assess the effects of PH on the RV directly as they can develop pressure overload RV hypertrophy. Interestingly though is the observation that they do not necessarily develop RV failure in contrast with the PH models, suggesting the effect of pulmonary vascular remodelling is not just due to increased afterload. Investigating the role of p38MAPK in RV biology and pathophysiology would be a fascinating area and one that I hope to pursue.

Although we have identified IL-6 as a key mediator, It is unlikely that IL-6 is the only pro-inflammatory agent which is responsible for the pathogenesis in pulmonary hypertension. From our cytokine array we have identified a number of inflammatory cytokines and chemokines which were released from the fibroblast under hypoxic conditions. The role of chemokines in PAH is unclear and this may be an avenue for fruitful research. Indeed recent studies have shown CXCL12 and CXCL4 to be implicated in the development of pulmonary hypertension at least in chronic hypoxia(272).

The downstream molecular signalling which links p38MAPK and the synthesis of IL-6 has not been fully investigated in these cells. There is evidence that p38MAPK can be activated in cardiac myocytes by MKK6 and this then can lead to activation and translocation of NF-κB to the cell nucleus. This then activates the transcription of IL-6 at a promoter level. Experiments in the hypoxic PAF looking at the effect of inhibition of NF-κB on the transcription of IL-6 would be most interesting.

On a similar note the upstream signalling pathway which results in the p38MAPK activation needs clarification. There are a number of important upstream

kinases such as TGF-β Activating Kinase-1 (TAK-1) and Apoptosis Signalling Kinase-1 (ASK-1) which may be involved and may present more selective targeting to minimise the chance of theoretical off target effects presented by inhibition of p38MAPK.

There are still a number of exciting avenues to be explored and in our laboratory we are already tackling some of these questions.

7.8 Final comments and summary

This body of work has generated significant novel findings which could have potential clinical applications.

We have demonstrated that the p38MAPK pathway is implicated in the pathogenesis of pulmonary hypertension. We show evidence for the importance of the p38 MAPK alpha isoform in mediating the hypoxic induced proliferation of pulmonary artery fibroblasts and the release of IL-6 from these cells. p38 MAPK activity is increased in two *in vivo* animal models of pulmonary hypertension compared to normal animals, with increased expression in the walls of the vessels and increased expression of the alpha isoform in whole lungs. Administration of two p38 MAPKα inhibitors *in vivo* has demonstrated that the pulmonary hypertension phenotype can be prevented and reversed with improvements in pulmonary vascular remodelling. These effects were associated with a reduction in serum and lung IL-6 and soluble collagen. Furthermore increased phospho-p38 MAPK activity and levels of p38MAPKα were noted in vessels and plexiform lesions from the lungs of patients with IPAH.

Taken together the data presented herein provide a contribution to a greater understanding of the biology of p38MAPK and its role in pulmonary vascular remodelling.

Hopefully one day this research will lead to a cure for human pulmonary arterial hypertension.

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