The Effects of Statins on Hypoxia-Induced Proliferation and Cell Signalling Pathways in Pulmonary Artery Fibroblasts

A thesis presented by

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List of Publications

Some of the results within this thesis have been published, details of which are given below.

Full paper

Carlin CM, Peacock AJ, Welsh DJ. Fluvastatin inhibits hypoxic proliferation and p38 MAPK activity in pulmonary artery fibroblasts. *Am J Respir Cell Mol Biol.* Oct 2007;37(4):447-456.

Abstracts

Carlin CM, Peacock AJ, Welsh DJ. Cell signaling and phenotypic changes in chronic hypoxic pulmonary adventitial fibroblasts: positive effects of fluvastatin are not seen with established pulmonary hypertension agents. *Thorax* 2007; 62 (S III): A1

Carlin CM, Peacock AJ, Welsh DJ. The antiproliferative effects of statins on pulmonary vascular cells are stimulus and dose dependant: implications for clinical trials in pulmonary hypertension. *Am J Respir Crit Care Med* 2007; 175: A412

Carlin CM, Peacock AJ, Welsh DJ. Fluvastatin selectively inhibits hypoxic proliferation and activation of p38 MAP kinase in pulmonary artery fibroblasts: implications for pulmonary hypertension treatment. *Thorax* 2006; 61 (S II): ii19

Welsh DJ, **Carlin CM,** Peacock AJ. Hypoxia-induced p38 MAP kinase activation in pulmonary artery fibroblasts is inhibited by simvastatin. *Am J Respir Crit Care Med* 2006; 173: A316

Carlin CM, Peacock AJ, Welsh DJ. Fluvastatin inhibits hypoxia-induced proliferation in pulmonary artery fibroblasts: role of prenylated signaling proteins. *Am J Respir Crit Care Med* 2006; 173: A315

Carlin CM, Peacock AJ, Welsh DJ. Statins inhibit hypoxic proliferation of pulmonary artery fibroblasts: potential for the treatment of pulmonary hypertension. *Thorax* 2005; 60 (S II): ii6

List of Abbreviations

ADP	Adenine diphosphate
ATP	Adenine triphosphate
BPASMC	Bovine pulmonary artery smooth muscle cell
BPAF	Bovine pulmonary artery fibroblast
cAMP	Cyclic adenine monophosphate
cGMP	Cyclic guanine monophosphate
CH-RPAF	Chronic hypoxic rat pulmonary artery fibroblast
CH	Chronic hypoxia
CH-RSAF	Chronic hypoxic rat systemic artery fibroblast
COPD	Chronic obstructive pulmonary disease
CO_2	Carbon dioxide
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulphoxide
EC	Endothelial cell
ECL	Enhanced chemiluminescence
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
FPP	Farnesyl pyrophosphate
GDP	Guanine diphosphate
GEF	Guanine exchange factor
GGPP	Geranylgeranyl pyrophosphate
GTP	Guanine triphosphate
HCL	Hydrochloric acid
HMG-CoA reductase	3-hydroxy-3methylglutaryl coenzyme A reductase
HPV	Hypoxic pulmonary vasoconstriction
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
NaOH	Sodium hydroxide
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
O_2	Oxygen
PAF	Pulmonary artery fibroblast
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline / tween
PDE	Phosphodiesterase
PH	Pulmonary hypertension
RIPA	Radioimmunoprecipitation assay (buffer)
ROCK	Rho kinase
ROS	Reactive oxygen species
RPAF	Rat pulmonary artery fibroblast
SAF	Systemic artery fibroblast

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMC	Smooth muscle cell
SPVU	Scottish Pulmonary Vascular Unit
TCA	Trichloroacetic acid
TGF-β	Transformin growth factor β
WHO	World Health Organisation

Declaration

This thesis is entirely my own composition and the experimental work detailed within was undertaken wholly by myself.

Signed ……..

Date ……...

Summary

Chronic hypoxia, in animals and man, results in remodelling of the pulmonary vasculature with consequent pulmonary hypertension. The pulmonary artery fibroblast (PAF) has been shown to play an early and important role in hypoxia-induced pulmonary vascular remodelling. In acute and chronic hypoxia there is excess proliferation of PAFs. Morevoer, it is likely that cell-cell interactions between hypoxia-stimulated PAFs and other vascular cells – particularly smooth muscle cells - initiates and progresses the changes that occur in pulmonary vascular remodelling in the other vessel compartments. Although hypoxic proliferation of PAFs has been shown to be circulation specific and dependant on phosphorylation of p38 mitogen-activated protein (MAP) kinase, the cell signalling pathway(s) underlying this are incompletely characterised. Hypoxic activation of PAFs is a potential therapeutic target but, as p38 MAP kinase inhibitors are not established for clinical use, work was proposed to better characterise this pathway and identify agent(s) which may inhibit p38 MAPK indirectly.

The HMG-CoA reductase inhibitor simvastatin was recently shown to inhibit hypoxic pulmonary vascular remodelling in rats, but the applicability of this finding to clinical practice is incompletely established and the mechanism of action of the statin is unclear. Statins have been shown to influence MAP kinase pathways in other cell types and, as their modes of action are well established, they can be used to interrogate uncharacterised upstream cell signalling pathways. On this basis, the aims of this study were firstly to determine whether statins had a therapeutically useful inhibitory effect on hypoxia-induced, p38 MAP kinase-mediated PAF proliferation. A second aim was to exploit the known effects of statins to better characterise hypoxic cell signalling upstream of p38 MAP kinase in PAFs. Lastly, comparison of the effects of statins with established pulmonary hypertension therapeutics and a preliminary assessment – also using statins as an experimental tool - of cell-cell interactions between PAFs and pulmonary artery smooth muscle cells (PASMCs) was proposed.

1μM of fluvastatin was found to selectively inhibit acute and chronic hypoxia-induced p38 MAP kinase phosphorylation and proliferation in rat PAFs. At this dose, fluvastatin had no effect on serum-induced proliferation in PAFs, no effect on systemic adventitial fibroblast proliferation, and no effect on the phosphorylation status of other MAP kinases. Selective use of mediators and inhibitors related to the HMG-CoA pathway indicated that a geranylgeranylated protein, probably Rac1, had an obligatory role upstream of p38 MAPK, in this signalling pathway. Co-culture and conditioned media experiments with bovine PAFs and PASMCs demonstrated the release of PASMC mitogens from hypoxic PAFs. 1μM fluvastatin and the p38 MAP kinase inhibitor SB203580 selectively blocked the hypoxic PAF-PASMC interaction. Results with hypoxic PAF proliferation with the prostacyclin analogue treprostinil, the phosphodiesterase-5 inhibitor sildenafil and the endothelin-1 antagonist bosentan were negative. Bosentan, however, inhibited the hypoxic PAF-PASMC interaction, suggesting endothelin-1 release by hypoxic PAFs, with pro-proliferative effects on PASMCs.

The results reported in this thesis provide new information on hypoxic signalling, PAF proliferation and PAF cell-cell interactions in hypoxic states. A circulation and stimulus specific anti-proliferative effect of fluvastatin on PAFs was identified and this may be of clinical relevance in hypoxia-associated pulmonary hypertension.

Chapter 1

Introduction

1.1 General Introduction

Pulmonary hypertension is now recognised to be a common and important condition in humans. The last 10 years has seen significant progress in our understanding of the pathophysiology and epidemiology of the different forms of pulmonary hypertension. These advances have allowed an appropriate classification of the spectrum of disorders causing pulmonary hypertension, recognition and validation of specific treatments for selected forms of pulmonary hypertension, and a resultant improvement in the prognosis for patients, in parts of the world where these new drugs are available. In addition, it is now appreciated that pulmonary hypertension is an important complication of common hypoxic lung diseases[1]. If we can improve our understanding of the pathophysiology of pulmonary hypertension, in particular hypoxia-related pulmonary hypertension, and identify agents which can tackle this selectively, inexpensively and without major side effects, then we would have therapies which could potentially improve the quality of life and survival prospects of the millions of people worldwide suffering from this disorder[2].

Despite progress, our understanding of the pathophysiology of pulmonary hypertension is incomplete. What we do know now is that, regardless of the aetiology, the pathology of the disease at the time symptoms develop is similar. Structural remodelling of the pulmonary vascular bed leads to an increase in pulmonary vascular resistance which causes respiratory and right heart failure. Data from animal models indicates that an acceleration of pulmonary adventitial fibroblast (PAF) proliferation is a primary event in the development of pulmonary vascular remodelling and pulmonary hypertension, when hypoxia is the initial stimulus[3]. Although hypoxia is only one of the causes of pulmonary hypertension, it is a useful model, particularly when considering the behaviours of pulmonary adventitial fibroblasts. Assessment of the cell signalling processes, proliferation and other behaviours of PAFs, in hypoxic conditions may provide important insights into the early events in the pathogenesis of hypoxic pulmonary hypertension, and potentially other forms of the disease. Also, use of this established model system allows for the assessment of the potential new pulmonary vascular therapies.

Recently, statin drugs have been shown to significantly attenuate the development of pulmonary hypertension (PH) in rats exposed to chronic hypoxia[4] and these findings have subsequently been replicated in other experimental pulmonary hypertension models[5-10]. Using statin drugs to treat human pulmonary hypertension is an attractive prospect: there is considerable clinical experience in the use of these drugs for hyperlipidaemia and cardiovascular disease, they are relatively inexpensive and major side effects are rare[11-17]. There are, however, large gaps in our knowledge in relation to the potential utility of statins for pulmonary hypertension, which need addressed before large clinical trials. Notably, we do not know the mechanism(s) of the statin effect, we have no information to help us judge how to combine or compare statins with other established or proposed pulmonary hypertension therapies, and we do not know which statin, at which dose, to select for clinical studies.

Though the specific mechanism(s) of action of statins in the animal models of pulmonary hypertension are incompletely explored, the effects of statins on cell signalling pathways, in general, are well known[18]. It was considered possible that the effects of the statin may be mediated via an effect on pulmonary artery fibroblasts. Also, it is notable that whilst hypoxic signalling in PAFs is incompletely understood, lipid raft structures and membrane GTPase systems – both of which are sensitive to statin inhibition – are signalling systems which might potentially be involved. Based on these possibilities, it was concluded that if positive effects of statins on hypoxic PAFs were identified, it might present an opportunity to better characterise hypoxic signalling in this important cell type and add to the body of preclinical knowledge of statin therapy for pulmonary hypertension. Accordingly a body of work on statins and hypoxic PAFs, to address three specific issues was proposed. Firstly we sought to determine whether early signalling events in hypoxic PAFs were statin sensitive, and to exploit any positive findings, in relation to the known mechanism of action of statins, in order to better characterise the nature of these signalling events. Secondly we wished to evaluate whether PAF inhibition was a potential mechanism(s) of action of statins in hypoxic pulmonary hypertension and use this as an approach to provide relevant insights for future potential clinical use of statins in human pulmonary hypertension. Lastly, there is an increasing awareness that fibroblast-smooth muscle cell interactions are important in the pathogenesis of

hypoxic PH and we sought to extend our established PAF cell model and develop a PAF-pulmonary artery smooth muscle cell (PASMC) experimental model, and study the effects of statins on this.

1.2 Pulmonary Hypertension: Definitions and Classification

Pulmonary hypertension is, by definition, a condition of abnormally high pressure within the pulmonary circulation. It can develop in many species, including humans, either as a *de novo* condition, as a complication of common heart, lung and systemic diseases, or as a complication of prolonged residence at high altitude.

Haemodynamic definitions of pulmonary hypertension have some deficiencies, as information on normal and abnormal pulmonary haemodynamics at all situations of rest and exercise is limited: invasive measurement of pulmonary haemodynamics carries small but notable risks. A definition consisting of a persistent elevation in mean pulmonary artery pressure >25mmHg at rest, or >30mmHg on exercise is, however, widely accepted, and the recent 4th World Symposium on Pulmonary Hypertension (see below) will hopefully provide further consensus on this. Pulmonary arterial hypertension (where the pathology originates in the pulmonary circulation, rather than being a consequence of high flow state or passive pressure elevation due to left atrial hypertension and venous backpressure) additionally requires the exclusion of elevated left heart pressures by identification of a normal left atrial pressure (<15mmHg; estimated either from pulmonary capillary wedge pressure or left ventricular end-diastolic pressure). Also, demonstration of an elevated pulmonary vascular resistance is required (resistance = pressure/flow, where flow is the cardiac output), to exclude passive rise in pulmonary artery pressure simply due to a high flow state[19].

Dramatic rises in the incidence of pulmonary arterial hypertension (PAH) were noted in relation to the introduction of the appetite suppressant drugs aminorex[20] and dexfenfluramine/fenfluramine[21, 22]. In response to these, the World Health Organisation (WHO) convened international symposia first in 1973 (following the aminorex outbreak) and 1998 (following the dexfenfluramine outbreak). These summarised the state of knowledge in relation to pulmonary hypertension,

standardised disease classification and considered future research priorities[23]. In the face of considerable scientific and clinical developments further WHO supported world symposia were convened in 2003 (Venice) and 2008 (Dana Point, California; symposia summary not yet published). The 2003 meeting in Venice provided a state of the art overview of the current knowledge of disease pathology[24], pathogenesis[25], genetics[26], treatment[27] and consensus statements on clinical assessment of patients with suspected PH[28] and clinical trial design[29]. Also, the conference revised the clinical classification of pulmonary hypertension which had originally been made at the Evian symposium in 1998[30]. The current comprehensive clinical classification, shown in Table 1.1, is useful both as a clinical standard and as a summary of the principle aetiological factors from which pulmonary hypertension may arise.

1.3 Pulmonary Hypertension: Epidemiology

Pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH) are the forms of pulmonary hypertension for which disease specific medical or surgical treatment (rather than general supportive measures) are now validated[31], and it is for these that the most robust epidemiological data, in the developed world, is available. PAH and CTEPH are rare disorders, though probably not as rare as previously thought. Epidemiological studies in this area are challenging as these are difficult diseases to recognise and classify and non-expert care can lead to both under and overdiagnosis. With these reservations, recent reports suggest that pulmonary arterial hypertension has a prevalence between 26 and 52 / million[32]. This may still be an underestimate as it is likely that PAH is under-recognised in patients with HIV infection, portal hypertension and other associated conditions. Considering CTEPH, it has recently been reported that significant pulmonary hypertension may develop in as many as 4% of patients following an acute, symptomatic pulmonary thromboembolism[33]. Symptomatic pulmonary thromboembolism is common and, additionally, a substantial number of patients develop CTEPH in the absence of antecedent acute thromboembolism[34]. Accordingly, CTEPH may be a relatively common condition. Even taking our

1. Pulmonary arterial hypertension (PAH)

- 1.1 Idiopathic (IPAH)
- 1.2 Familial (FPAH)
- 1.3 Associated with (APAH)
  - 1.3.1 Collagen vascular disease
  - 1.3.2 Congenital systemic-to-pulmonary shunts
  - 1.3.3 Portal hypertension
  - 1.3.4 HIV infection
  - 1.3.5 Drugs and toxins
  - 1.3.6 Other (thyroid disorders, glycogen storage disease, Gaucher disease, hereditary haemorrhagic telangiectasia, haemoglobinopathies, myeloproliferative disorders, splenectomy)
- 1.4 Associated with significant venous or capillary involvement
  - 1.4.1 Pulmonary veno-occlusive disease (PVOD)
  - 1.4.2 Pulmonary capillary haemangiomatosis (PCH)

2. Pulmonary hypertension with left heart disease

- 2.1 Left-sided atrial or ventricular heart disease
- 2.2 Left-sided valvular heart disease

3. Pulmonary hypertension associated with lung diseases and/or hypoxaemia

- 3.1 Chronic obstructive pulmonary disease
- 3.2 Interstitial lung disease
- 3.3 Sleep-disordered breathing
- 3.4 Alveolar hypoventilation disorders
- 3.5 Chronic exposure to high altitude
- 3.6 Developmental abnormalities

4. Pulmonary hypertension due to chronic thrombotic and/or embolic disease

- 4.1 Thromboembolic obstruction of proximal pulmonary arteries
- 4.2 Thromboembolic obstruction of distal pulmonary arteries
- 4.3 Non-thrombotic pulmonary embolism (tumor, parasites, foreign material)

5. Miscellaneous

Sarcoidosis, histiocytosis X, lymphangioleiomyomatosis, compression of pulmonary vessels (adenopathy, tumour, fibrosing mediastinitis)

Table 1.1 Revised Clinical Classification of Pulmonary Hypertension[30]

current understanding of prevalence, the number of patients suffering from PAH and CTEPH justifies continued research and development in this field.

The true public health burden of pulmonary hypertension is likely to be much greater[1]. In particular, the number of patients with pulmonary hypertension related to respiratory disease and/or hypoxaemia is likely to be substantial. Three points seem relevant when considering this. Firstly, the number of individuals worldwide who live chronically at high altitude is substantial, and severe pulmonary hypertension develops in a significant proportion of these[35]. Secondly, the prevalence of the commonest respiratory disorder associated with pulmonary hypertension - chronic obstructive pulmonary disease (COPD) - has increased significantly in recent years, and is projected to continue to do so[36]. It is likely that the number of patients with pulmonary hypertension related to this will increase similarly. The number of patients with COPD who develop complicating severe PH is uncertain, but one recent study found that, in patients with severe COPD, PH develops in 50% of patients, with severe resting PH in 7% of patients[37]. Even if only the patients with severe COPD and severe resting PH experience morbidity or mortality related to their pulmonary hypertension, given how common COPD is, this still represents a substantial burden of PH in the worldwide community. Thirdly, although most patients with PH related to underlying lung disease have only mild-moderate resting PH[38], this may be a significant underestimate of the impact of PH on their health and on their symptoms. Pulmonary hypertension in these patients - measured as mild-moderate at rest - may worsen significantly with exercise, sleep and during exacerbations and therefore if we consider only their resting haemodynamics (whether they are invasive or non-invasive) we may be significantly underestimating the contribution of the pulmonary vascular component of these patient's conditions to the overall morbidity and mortality[39].

Taking all of this together it seems clear that severe pulmonary hypertension - including pulmonary hypertension related to lung disease and/or hypoxaemia - is an important human disease, worthy of further research.

1.4 The Normal and Diseased Pulmonary Artery

1.4.1 Structure of Normal Pulmonary Arteries[40, 41]

The normal pulmonary circulation is a high-flow, low pressure system and, by design or by consequence, the pulmonary arteries are thin-walled and compliant. There is also, in health, a substantial reservoir vascular bed which is relatively unperfused, at rest. These properties give the pulmonary circulation the capacity to passively cope with large changes in volume (when cardiac output rises), without the requirement for high filling pressures (which the normal right ventricle cannot generate).

A normal artery consists of circumferential layers. From the lumen outwards these are the intima (comprising endothelial cells and their basement membrane), the internal elastic lamina (a thin layer of elastin fibres), the media (consisting of smooth muscle cells and their associated extracellular matrix, an external elastic lamina and the adventitia (containing fibroblasts, extracellular matrix and connective tissue). In the proximal elastic arteries the medial smooth muscle layer is thinner, may partly be arranged longitudinally and the adventitia contains small blood vessels (vaso vasorum) from the systemic (bronchial) circulation. Progressing distally, the muscular (resistance) arteries have a relatively thicker media, with the smooth muscle cells arranged spirally. Further out, the smaller arteries have an incomplete media, and lose their elastic laminae. At the most distal arteries and arterioles there is no laminae or smooth muscle cell layer: media consists solely of undifferentiated pericyte cells.

1.4.2 Pathology of Pulmonary Hypertension

The primary pathological processes which can cause pulmonary arterial hypertension are vasoconstriction, constrictive pulmonary vascular remodelling (consisting of thickening / hypertrophy of the vessel wall layers), distortion or loss of the pulmonary vascular bed and pulmonary vascular thrombosis[24]. Additionally, in idiopathic pulmonary arterial hypertension (and rarely in other forms of PH) complex pathological lesions, in particular so-called plexiform lesions, develop in the pre-acinar and intra-acinar pulmonary arteries. Plexiform lesions consist of endothelial channels lined by myofibroblasts, extending out from destroyed arterial walls, often associated with intravascular thrombus. It is hypothesised that these lesions may

relate to a monoclonal proliferation of an abnormal endothelial cell clone, and that this may be the primary / initiating pathological defect in idiopathic PAH [42, 43].

The relative contribution and the exact pathological features of each of these processes vary in the different forms of pulmonary hypertension. For example, certain individuals with IPAH have long term symptomatic responses to calcium channel blocker therapy[44], and it is assumed that vasoconstriction plays a dominant role in these patients. In contrast, extensive arterial thrombosis, in proximal and distal vessels is typical in CTEPH, whilst it is an inconsistent feature, typically present only in the small arterioles, in PAH[45]. In most instances, however, it is now considered that the pathological process responsible for the clinical manifestations in severe PAH is pulmonary vascular remodelling. In severe pulmonary hypertension, remodelling of the pulmonary arteries causes luminal restriction and loss of vessel compliance, leading to a rise in pulmonary vascular resistance, an increase in right ventricular afterload and, consequently, respiratory and right heart failure.

1.4.3 Pulmonary Vascular Remodelling

Pulmonary vascular remodelling is a complex and incompletely understood process. There is some heterogeneity in the features, when the different forms of severe pulmonary hypertension in humans are compared, and also in the different forms of experimental pulmonary hypertension. However, it appears to be the common endpoint to the diverse genetic errors or injurious stimuli which the pulmonary circulation is susceptible. Medial hypertrophy / increased muscularisation of muscular arteries and distal extension of smooth muscle into normally non-muscular arterioles are often considered to be the morphological changes responsible for most of the haemodynamic upset in severe PH. All vessel wall compartments are however involved and intimal thickening, neointima formation, thickening and distal extension of elastic laminae and adventitial thickening (+/- complex lesions) are also hallmarks[46]. A schematic representation of some of the pathological features of pulmonary hypertension is shown in figure 1.1.

As discussed in Chapter 1.2, chronic hypoxia is one of the main causes of pulmonary vascular remodelling and severe pulmonary hypertension in humans. The nature of the pulmonary vascular remodelling in hypoxic pulmonary hypertension has been

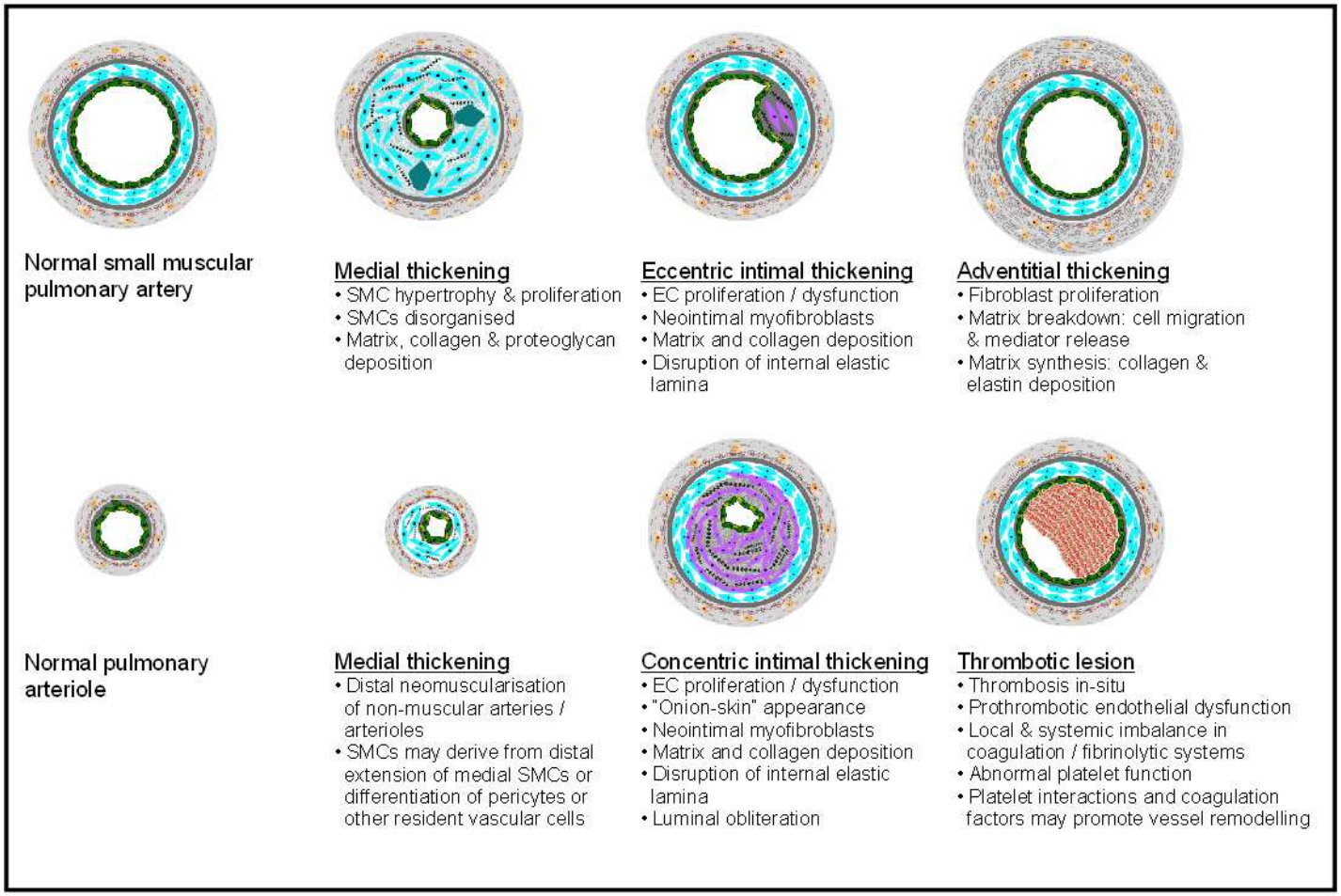


Figure 1.1: Schematic Representation of Key Aspects of Pulmonary Vascular Remodelling

described in humans, with some differences in the remodelling - notably longitudinal intimal neomusclarisation of small pulmonary arteries – compared with other forms of severe PH[47]. Remodelling in hypoxic animal models has been extensively studied and summarised[48]. In animal models it is notable that the earliest changes are seen in the adventitia, where the resident adventitial fibroblast is seen to proliferate before other cell types[49].

The view that luminal narrowing and vessel loss consequent on pulmonary vascular remodelling are the key pathological features of pulmonary hypertension in chronic hypoxia has recently been challenged. Emerging experimental data indicates that rather than a reduction in the vascular bed, there may actually be angiogenesis and an expansion of the pulmonary circulation[50]. The subsequent identification by this group of acute reversal of chronic hypoxic pulmonary hypertension with a Rho kinase inhibitor, led to the conclusion that sustained and relatively intractable pulmonary vasoconstriction, arising on a background of structurally altered pulmonary vessels, may be much more important than remodelling in causing the haemodynamic upset in chronic hypoxic pulmonary hypertension[51]. With the multiple failures of vasodilator therapies in clinical practice (other than calcium channel blockers for a small subset of patients with idiopathic PAH) it had been concluded that reversible vasoconstriction was an unimportant aspect of established severe PH[52]. It is intriguing to speculate that vasoconstriction, potentially reversible by novel vasodilators acting via new mechanisms, may be relevant in hypoxia-associated PH in humans, or other forms of severe PH[53].

Regardless of whether the nature of the vessel remodelling is luminal narrowing consequent on inward remodelling, reduction of the pulmonary vascular bed consequent on proximal remodelling or sustained vasoconstriction arising in structurally abnormal vessels, pulmonary vascular remodelling seems to be the primary pathological process which leads to severe pulmonary hypertension. This makes it an important area for ongoing research and an important target for new treatments, for all forms of pulmonary hypertension.

1.5 Pulmonary Vascular Remodelling: Components & Mechanisms

Despite advances, our understanding of the pathophysiology of pulmonary vascular remodelling remains limited: in particular the sequence of events in human disease is far from clear. There have, however, been numerous experimental advances in recent years, with considerable insights into the cellular and molecular factors which contribute to the process. The specific components of pulmonary vascular remodelling and the cellular and molecular mechanisms thought to be involved are considered individually.

1.5.1 Cellular and Extracellular Matrix Changes in Pulmonary Hypertension

1.5.1.1 Endothelial Cells

The normal pulmonary endothelium acts a semipermeable barrier (between the vascular and extracellular spaces) and importantly functions to maintain normal vessel homeostasis, vascular tone, proliferation and the coagulation system. It is the vessel component most readily exposed to injury (eg shear stress, drugs or toxins) and the origins of many of the hallmark features of pulmonary vascular remodelling have been related to alterations in normal endothelial cell (EC) function.

Increased endothelial cell proliferation is seen as an early feature in experimental models[49, 54], and endothelial cells from pulmonary hypertensive lungs have a hyperproliferative phenotype[55]. This endothelial proliferation is thought to contribute to intimal thickening, and is the primary constituent driving the formation of plexiform lesions. Though plexiform lesions are not thought to contribute significantly to vascular obstruction, and are only rarely seen in forms of PAH other than IPAH, the recognition that the endothelial cells in IPAH plexiform lesions are monoclonal[42] – suggesting a semi-malignant process – provides interesting insights into possible disease mechanisms. In addition, specific mutations in a TGF-β receptor protein in ECs from plexiform lesions[43], and infection of ECs with the angioproliferative agent human herpes virus 8[56] have been reported (though this secondt finding is contentious and unreplicated)[57, 58]. Taken together, however, these findings suggest the possibility that various events (somatic mutations, viral infection) may trigger clonal proliferation of endothelial cells as an early event in

idiopathic PAH pathogenesis, with subsequent cell-cell and cell-matrix interactions driven by these phenotypically abnormal, proliferative ECs causing the development of the other features of pulmonary vascular remodelling[25].

Endothelial cell proliferation and dysfunction may be the initiating factor in some forms of PAH, and a secondary – but eminent - factor in others. Regardless, it is likely that the EC contributes significantly to the propogation and maintenance of pulmonary vascular remodelling in most forms of the condtion. Endothelial dysfunction, leading to reduced production of vasodilators / antiproliferative mediators (eg nitric oxide and prostacyclin) and increased production of vasoconstrictors / pro-proliferative mediators (eg endothelin-1, angiotension II), seems to be a ubiquitous feature in severe PH[59]. Lastly, loss of the endothelial barrier (either as a consequence of endothelial cell dysfunction or disruption of the elastic lamina) may contribute to vessel oedema, disruption of the extracellular matrix and allow influx of disruptive mediators from the circulation to the media and adventitia[60].

1.5.1.2 Smooth muscle cells

Under normal laboratory or *in vivo* conditions the smooth muscle cell (SMC) population in general demonstrates minimal proliferation and synthetic activity. There are, however, different subpopulations of SMCs identifiable within the medial layer and these have been shown to have different proliferative and synthetic properties and responses to injury or growth factors[61]. During the development of severe PH, SMCs hypertrophy, proliferate, migrate and synthesise matrix proteins, contributing significantly to pulmonary vascular remodelling[59]. In some situations these changes in SMC behaviour may be intrinsic to the SMC (eg due to changes in potassium channel activity[62-64]) but there is significant evidence that the SMC is a relatively passive player, responding to paracrine signals from endothelial cells[65] or fibroblasts[66]. Also, the apparent SMC population in the neointima and media of remodelled arteries may originate from differentiation of other vascular cells, rather than expansion of the native SMC population[59].

1.5.1.3 Fibroblasts

The adventitia was previously considered to be a relatively inert structure and many observers fail to comment on it, when describing pulmonary hypertension. It is now considered likely that the adventitial fibroblast acts as a 'concert leader', particularly in hypoxia-associated pulmonary hypertension, where it is the first cell to respond to the injury[67]. Also, there is an evolving understanding that generalised excessive fibroblast proliferation is a primary pathogenic step in the development of complications in connective tissue disease[68]. Based on this it is speculated that the pulmonary adventitial fibroblast (PAF) is affected along with dermal and systemic vascular fibroblasts in connective tissue diseases and that this may be an important and early pathogenic feature of pulmonary vascular remodelling in connective tissue disease-associated PAH. It seems increasing likely that the fibroblast is a major contributor in all forms of pulmonary vascular remodelling. Adventitial thickening is generally difficult to assess histologically and is not consistently commented on[24] but, where it is assessed it is found to be a prominent feature in forms of severe PH other than hypoxia-associated PH[69]. Migrated and differentiated adventitial fibroblasts likely also contribute significantly to the population of myofibroblasts which are found in the neointima and plexiform lesions in these other forms of PAH. PAFs may also be the origin of mitogenic factors and facilitated changes in the extracellular matrix, which may contribute to the intiation / progression / persistence of the other cellular changes in pulmonary vascular remodelling[59].

The evidence for the role of the PAF in pulmonary vascular remodelling is most established in hypoxic rat and bovine models. In these models, in addition to increased proliferation, hypoxic PAFs synthesise matrix proteins, demonstrate increased migration and secrete paracrine factors which cause SMC proliferation[48]. Pro-angiogenic factors (eg endothelin-1) secreted by hypoxic PAFs may also contribute to the vasa vasorum neovascularisation which is seen in remodelled vessels[70] and which may serve as a conduit via which circulating leukocytes and progenitor cells (potentially attracted by the inflammatory mediators also released by adventitial fibroblasts) enter the vessel wall[71].

1.5.1.4 Circulating progenitor cells

Various circulating cells seem to have an important role in the normal maintenance and repair of the pulmonary vasculature, and dysregulation of this function (leading either to deficiency or excess activity) may be an initiating or contributing factor in PAH pathogenesis[72]. There is strong evidence for an important role of circulating progenitor cells, particularly fibrocytes, in hypoxic pulmonary vascular remodelling[71]. In hypoxic animal models, there is an increase in bone-marrow derived circulating cells, and these cells, particularly the circulating fibrocytes, are recruited into, and become resident in, the pulmonary arteries. These may contribute directly (eg increase resident cell population, proliferate, transform to myofibroblasts, modify extracellular matrix) or indirectly (eg via paracrine effects on resident vascular cells) to pulmonary vascular remodelling.

1.5.1.5 Inflammatory cells

Inflammatory cells may be more important contributors to pulmonary vascular remodelling than previously thought. PAH does not appear to be a typical inflammatory disorder however a small, selected group of patients with SLE-related PAH respond well to immunosuppressant therapies[73]. Systemic activation of inflammatory pathways in PAH patients and local production of inflammatory mediators by pulmonary vascular cells has been identified. Also, inflammatory infiltrates can be seen in plexiform lesions. These changes may be secondary phenomena and there is certainly not evidence of a widespread arteritis in pulmonary vascular remodelling. It is notable, however, that significant numbers of macrophages and neutrophils are seen transiently in the pulmonary circulation in the early stages of hypoxic PH in animal models (a model not conventionally seen as inflammation-related) and pursuing the role of inflammatory cells and inflammatory signalling pathways in pulmonary vascular remodelling seems likely to be an important future research direction[74].

1.5.1.6 Extracellular matrix

Though the extracellular matrix in the normal vessel appears relatively static, there is, in fact, important dynamic turnover of matrix. Matrix protein synthesis (by ECs, SMCs and fibroblasts) and breakdown (by metalloproteinases, regulated by tissue inhibitors of metalloproteinases) is usually balanced. Interactions with the normal

extracellular matrix – eg sequestration of growth factors by matrix proteins and facilitation of cell migration – are likely important for normal vascular cell function[75].

Changes in the extracellular matrix (increased collagen deposition, disruption of elastic lamina) in remodeled pulmonary arteries are notable and the importance of these changes is underscored by observations that agents which enhance or disrupt matrix protein degradation significantly modify experimental pulmonary vascular remodeling[76].

1.5.2 Cell Signaling and Molecular Events in Pulmonary Vascular Remodelling

Regardless of the cause of PAH, the vascular pathology seen is stereotyped. This suggests that the different forms of the disease may have similar cellular and molecular mechanisms. Diverse initiating cellular or molecular events may result in stereotyped upsets in normal vasodilator / vasoconstrictor, pro/anti-proliferative and anticoagulant / procoagulant balances in the pulmonary vasculature.

It is difficult to see that we will ever be fully confident about the early pathogenesis of all forms of PH, given our inability to recognise the disease clinically until it is most advanced, and given the deficiencies of our animal models. The discovery, however, that mutations in the bone morphogenetic protein (BMP) type II receptor (BMPR-II) gene were responsible for most forms of familial PAH was a major breakthrough[77, 78]. Familial PAH is a rare form of PAH, sporadic mutations in BMPR-II do not appear to be common in other forms of PAH and also it is observed that only a minority of individuals carrying BMPR-II mutations develop PAH and accordingly the importance of this, at first glance, seems limited[26]. Recognition of the gene for familial PAH has, however, led to considerable insights. Broadly speaking, normal BMP-BMPR2 signalling has an antiproliferative and pro-differentiation effect on pulmonary vascular cells[79]. Deficient and/or dysfunctional BMP-BMPR2, or other TGF-β family signaling systems, is apparent in other forms of PAH, and it seems likely that disruption of these systems is an early and important contributor to the pathogenesis of forms of PH other than familial PAH[80].

Study of the molecular nature of the underlying defect in the underlying primary disorder has also led to observations about potential molecular mechanisms implicated in the early pathogenesis in specific forms of associated-PAH eg HIV infection[81], HHT[82], portal hypertension[83], anorexigen-induced PAH[84], myeloproliferative disease[85].

The majority of molecular signaling pathways implicated in PH pathogenesis have been initially characterized in animal models and/or patients with established IPAH, anorexigen associated PAH or CTEPH. It is not completely clear which of these are disease initiating mechanisms and which are secondary mechanisms contributing to disease propogation. A summary of some of molecular mechanisms currently considered important are illustrated in figure 1.2.

When considering the aims of this thesis, the most relevant pathways for further elaboration are those which currently seem to be fundamental to the disease process and for which modifying therapies have had clinical success (ie the prostacyclin, endothelin-1 and nitric oxide-cGMP pathway). These can be considered as 'benchmarks' when considering any potential new therapy (such as statins) and are considered in specific detail below. Also relevant to this thesis are those pathways potentially involved in hypoxia / MAPK signaling. These are considered in detail in Chapters 1.6 and 1.7.

1.5.2.1 Prostacyclin

Prostacyclin is an arachidonic acid metabolite, produced by the vascular endothelium. It is a potent pulmonary and systemic vasodilator and inhibitor of vascular cell proliferation. It also has antiplatelet and positive inotropic effects. Prostacyclin acts via a G-protein / adenyl cyclase coupled cell membrane receptor, causing an increase in intracellular cyclic AMP which, in pulmonary vascular smooth muscle cells, leads to relaxation and decreased proliferation. Studies in the late 1980's and early 1990's led to a recognition of a relative imbalance of arachidonic acid metabolites (reduced prostacyclin and excess of thromboxane A2, which has opposing effects) in humans and PH animal models[86]. Pulmonary endothelial cells from PAH patients have subsequently been shown to have reduced levels of prostacyclin synthase[87]. It was considered likely that there was relative

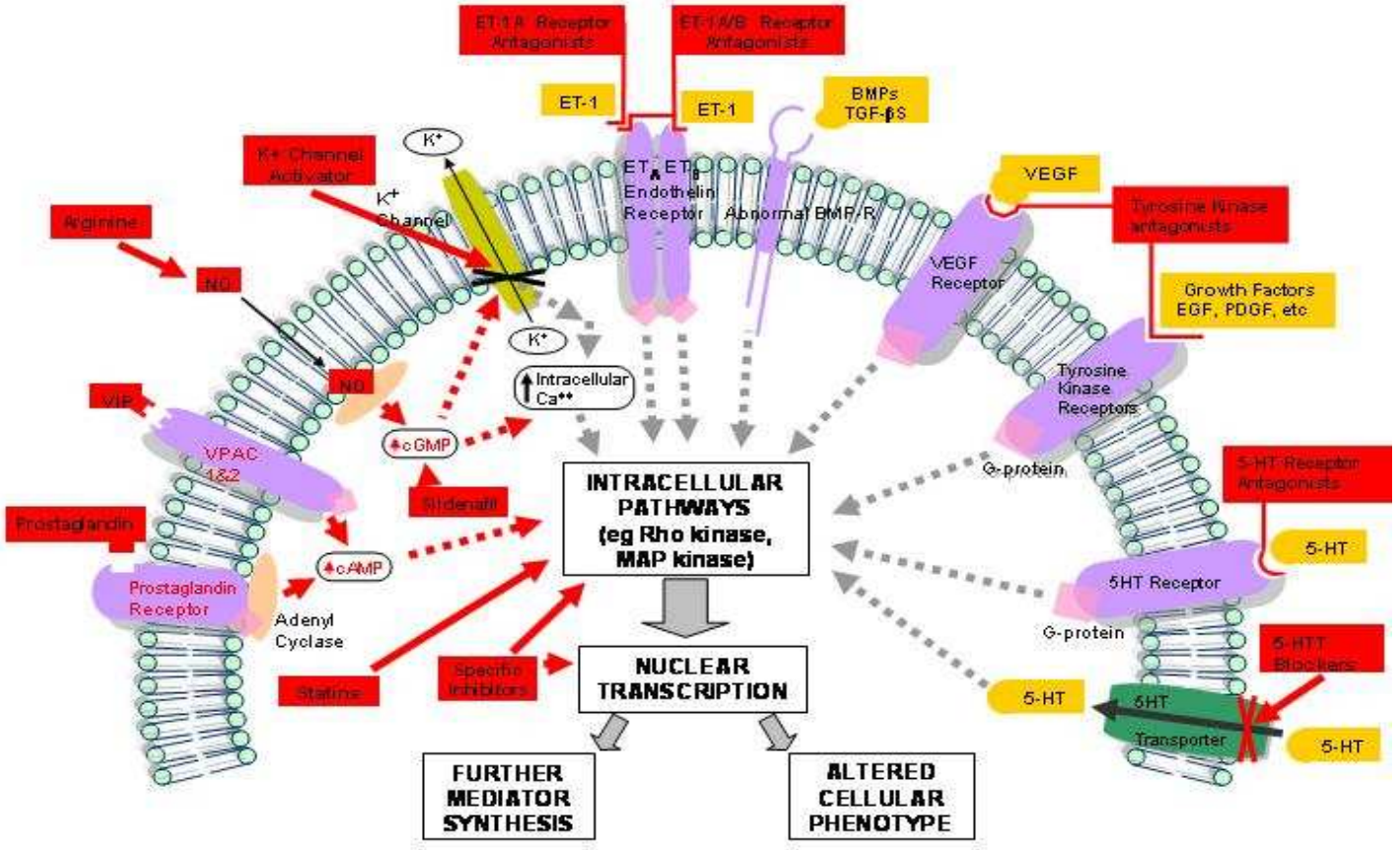


Figure 1.2 Molecular Signaling Pathways & Potential Therapies in Pulmonary Arterial Hypertension

Selected extracellular factors (yellow), cell membrane receptors (purple), membrane channels (green) and intracellular pathways (white boxes) implicated in PAH pathogenesis are shown on a generic cell diagram. Specific factors and receptors have been implicated in different vascular cells (eg ET1 principally originates from endothelial cells but may act primarily on smooth muscle cells). Potential disease modifying / therapeutic interventions (red boxes) are also shown.

prostacyclin deficiency in PAH, and that prostacyclin supplementation may have therapeutic benefit. Initial observations of benefit from continous intravenous infusion of prostacyclin (epoprostenol) in individual patients were striking, and this led to randomised control trials of these agents for PAH, and the subsequent development and validation in further clinical trials of novel prostanoids / delivery mechanisms[88]. Currently, intravenous prostacyclin remains the 'gold standard' drug treatment for PAH with considerable evidence demonstrating survival benefit. Inhaled and subcutaneous prostacyclin is also effective, but oral analogues have been less successful. Though prostacyclin therapies significantly benefit large numbers of patients, treatment is very expensive, has substantial side effects and risks (making it inapplicable to a large number of patients, even in resource rich countries) and it only retards disease progress: it doesn't cure the disease.

There is also evidence for a role for the prostacyclin pathway in the pathogenesis of hypoxia-associated pulmonary hypertension. Hypoxia inhibits the production of prostacyclin by pulmonary endothelial cells[89]. Also, the development of hypoxic PH is accentuated in mice lacking prostacyclin receptors[90] and diminished in transgenic mice overexpressing prostacyclin synthase[91]. Lack of benefit of prostacyclin monotherapy in the chronic hypoxic rat model might suggest that prostacyclin is not a major contributor in hypoxic PH[92] but, in contrast, other observers have seen reduction in experimental hypoxia-induced pulmonary vascular remodeling with prostacyclin therapy[93] and some success with prostacyclin treatment of hypoxic lung disease related PH in humans has been seen[94].

1.5.2.2 Endothelin-1

Endothelin-1 (ET-1) is a peptide hormone / paracrine molecule. In the lung, it is principally considered to be synthesised and released by endothelial cells and act, via G-protein coupled receptors, on adjacent smooth muscle cells with vasoconstrictive and pro-proliferative effects. As shown in figure 1.3, ET-1 is also known to have effects on fibroblasts, platelets and inflammatory cells and interact with nitric oxide and prostacyclin pathways. From this it can be seen that excess of ET-1 would potentially cause the development and / or progression of pulmonary hypertension.

Increase in production and reduced clearance of ET-1 is evident in PH models[95] and patients[96], and endothelin blockade has substantial positive effects on experimental PH[97, 98]. The importance of ET-1 in PH pathogenesis is underscored by the success of endothelin antagonist therapy in PAH[99] and chronic thromboembolic pulmonary hypertension[100]: oral endothelin antagnosists have variously but consistently been shown to improve symptoms and survival in PAH[101]. Many patients, however, benefit from these drugs for only a short period and effects on pulmonary haemodynamics are minimal: as with prostacylin, these drugs do not cure PAH and additional therapies are required.

In the normal pulmonary artery, vasoconstrictor and proliferative actions of ET-1 seem to be mediated via the endothelin A and B receptor on smooth muscle cells, whilst on endothelial cells the ET-B receptor facilitates a negative feedback loop on ET-1 synthesis, triggers release of vasodilator molecules and mediates clearance of endothelin-1 from the vascular interstitium and the circulation. The situation appears more complex in the diseased state, as there is more extensive expression of the B receptor in smooth muscle cells from the distal arteries and activation of both the A & B receptor may contribute to adverse effects of ET-1 in the diseased pulmonary artery[102].

Both selective endothelin A receptor antagonists and dual A/B receptor antagonists have shown benefit in PH treatment, but whether selective or dual inhibition is better in clinical practice remains uncertain[102, 106].

ET-1 release, endothelin receptor blockade and endothelin receptor deficient mice have been studied in hypoxic conditions and, ET-1 release, and its effects, likely contribute to vasoconstriction and vascular remodeling[107, 108], although there is conflicting data[109, 110]. Hypoxia triggers release of endothelin-1 from bovine PAFs and this may act as an autocrine fibroblast growth factor and chemoattractant[111]. Hypoxic bovine PAF derived ET-1 has also been shown to promote growth of vasa vasorum endothelial cells[105], and this ET-1 release may contribute to adventitial neovascularisation and consequent influx of circulating mesenchymal cells in hypoxic pulmonary vascular remodeling in these animals[71]. Lastly, differences in

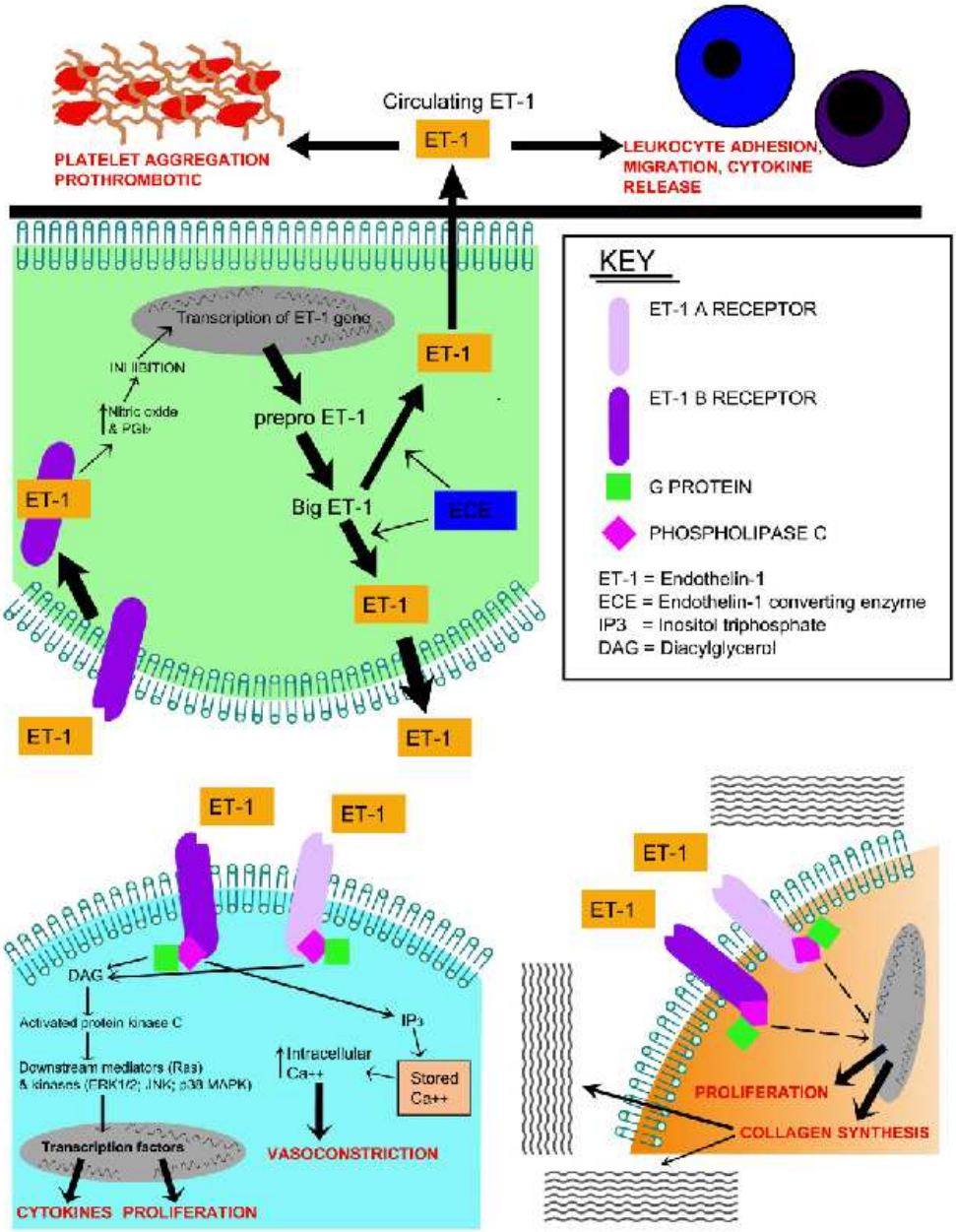


Figure 1.3 Endothelin-1 Signalling in Pulmonary Vascular Remodelling

Endothelin-1 (ET-1) synthesised and released by endothelial cells (green) acts on smooth muscle cells (blue), fibroblasts (brown) and enters the circulation[102]. ET-1 mediates diverse effects which promote pulmonary vasoconstriction and pulmonary vascular remodeling, via the endothelin A & B receptors[103], as shown. Smooth muscle cells[104] and fibroblasts[105] may also produce ET-1 and ET-1 derived from these cells may also contribute to the initiation and propogation of pulmonary vascular remodelling.

upregulation of ET-1 release, in response to hypoxia have been suggested as a contributory factor explaining the differential susceptibility of different rat strains to chronic hypoxic pulmonary hypertension[112]. This last observation raises the possibility that species differences in ET-1 responses may explain some of the contradictory results seen with ET-1 studies in hypoxic pulmonary vascular cell interactions. Overall, it is concluded that ET-1 is likely to be a hypoxia-responsive paracrine mediator, contributing to pulmonary vascular remodeling, but that additional work is required to clarify its role in different cell-cell interactions, in different animals.

1.5.2.3 Nitric oxide

In pulmonary artery smooth muscle cells, via activation of guanylate cyclase and increase in intracellular cyclic GMP, endothelial cell-derived nitric oxide acts as a vasodilator and antiproliferative agent. Disruption of endothelial nitric oxide synthase (eNOS) is seen in PAH blood vessels[113] and deficiency of nitric oxide is seen in experimental chronic hypoxic PH[114]. Supplemental, inhaled nitric oxide (NO) is used clinically in the acute assessment of vasoreactivity as part of the diagnostic evaluation of patients with severe pulmonary hypertension and prolonged inhalation is also used in neonatal intensive care units for the treatment of respiratory failure of prematurity and pulmonary hypertension. Chronic ambulatory nitric oxide inhalation systems have been developed and trialed in PAH[115] and PH-associated with COPD[116] with some success but the high cost and very cumbersome nature of this makes it an unattractive prospect for further development.

More successful has been an indirect approach to amplify the residual nitric oxide signal in PAH, by inhibiting the degradation of the intracellular 2nd messenger, downstream of nitric oxide. In the pulmonary circulation, cGMP is principally degraded by phosphodiesterase - PDE-5. Selective inhibition of PDE-5 – an enzyme which has relatively restricted expression in other tissues of the body - would potentially allow relatively lung specific amplification / persistence of any residual NO-cGMP signal in the pulmonary arteries[117]. Therapy with sildenafil – a PDE-5 inhibitor - has proven successful in clinical trials in the treatment of PAH[118].

Disturbance of NO-cGMP signaling likely contributes to chronic hypoxic PH. Chronic hypoxia leads to a decrease in nitric oxide production[119] and treatment with sildenafil attenuates chronic hypoxic PH in rats[120] and mice[121]. In human trials, PDE-5 inhibition with sildenafil improves haemodynamics in acute hypoxic states[121, 122] and chronic therapy has been shown to improve chronic hypoxic PH[123, 124].

1.5.2.4 Other molecular signaling pathways in PAH

Disturbances of a variety of other signaling pathways – including serotonin, potassium channel, angiopoietin, vasoactive intestinal polypeptide, adrenomedullin and tyrosine kinase growth factor/receptors – have been implicated in PAH pathogenesis and/or, based on experimental observations, highlighted as pathways of interest for potential therapeutic manipulation in PAH. These are not expanded on here – they are outwith the focus of this thesis – but they are described in detail in recent reviews[125, 126].

1.5.2.5 Summary

Disturbances of a variety of molecular pathways are seen in pulmonary hypertensive blood vessels. Establishing whether these pathways are restricted to established disease, to subtypes of the disease, to restricted cells within the vessel and the interactions / relative contributions of these pathways is work in progress. The work in the BMPR-2 field seems most promising and it may be that it will lead to novel pathogenetic insights and therapeutic breakthroughs.

At the current time, however, the pathways best characterised are the three elaborated on in the preceding sections (prostayclin, endothelin-1 and nitric oxide). Though the established therapeutics for PAH are imperfect, any potential new therapy needs to be considered in relation to these and any effects additional or alternative to these should be sought. For example, an agent found initially to be beneficial in an experimental PH model but subsequently shown to simply duplicate the molecular/cellular effects of sildenafil would be of limited value. Work to explore how these signaling pathways may complement one another in experimental models, and in clinical scenarios, is ongoing[93, 127]. With regards to the work in this thesis, comparison of statins (the focus of this thesis) with a drug from each of the established therapeutic classes, acting on these three key pathways seems relevant.

1.6 Hypoxia

1.6.1 Definition

Hypoxia is a physiological term for the condition in which the oxygen supply to tissue, organs or whole animals is insufficient to maintain a normal function. Hypoxia is a relative condition: eg exercising skeletal muscle (where blood supply is reduced and oxygen consumption is increased) is 'used' to much lower oxygen tensions in comparison to the brain or kidneys. The response of a given organ or cell type to a specific oxygen tension, and whether this constitutes 'hypoxia' or not, depends on the usual oxygen tension (under resting or active conditions) that the tissue under study is exposed to. In response to relative hypoxia, mammals exhibit systemic responses that decrease cellular oxygen requirements and dependence and increase tissue oxygen supply. Alterations in oxygen tension also elicit important stereotyped responses at the organ, tissue and cellular level, which are site specific.

Hypoxia, either generalised to the animal (caused by reduction in inspired oxygen, cardiopulmonary disease or other disturbance of oxygen transport) or occurring in a specific organ (localised vascular insufficiency), results in what are now relatively well characterised changes in molecular, cellular and organ status. For the purpose of this thesis, the most relevant effect of hypoxia in the body is its effect on the lungs where hypoxia leads to pulmonary vasoconstriction and pulmonary vascular remodelling

1.6.2 Hypoxic Lung Disease and Pulmonary Hypertension

Exposure to chronic hypoxia causes pulmonary hypertension in most mammalian species. Hypoxic pulmonary hypertension is associated with changes in vascular tone and is nearly always associated with significant structural changes within the pulmonary vascular bed (pulmonary vascular remodelling)[128]. There are also marked changes in the phenotype of endothelial, smooth muscle and fibroblast cell populations. Over the last few years it has become clear that the vascular responses to hypoxia involve extremely complicated cell-cell interactions mediated by growth factors, cytokines and other biological messengers[48].

The first recognition of hypoxia-induced pulmonary hypertension in mammals was brisket disease. This entity arose in cattle taken to new high pastures in Colorado and was subsequently shown to reflect right heart failure, secondary to pulmonary hypertension, at this altitude (10,000 feet)[129]. Pulmonary hypertension secondary to chronic hypoxia, consequent on residence at high altitude is thought to be one of the commonest forms of the disease worldwide[2].

Diverse respiratory diseases can be associated with pulmonary hypertension. These include COPD, cystic fibrosis, restrictive lung diseases (eg kyphoscoliosis), parenchymal lung diseases (eg idiopathic pulmonary fibrosis, sarcoidosis) and sleep-disordered breathing[30]. The pulmonary vascular component of these conditions has proven difficult to characterise: it is difficult to determine how much is due to effects of hypoxia on the pulmonary vasculature (vasoconstriction and vascular remodelling) vs non-specifc destruction of the pulmonary vascular bed due to progressive lung disease vs direct effects of the primary disorder on the pulmonary vasculature[130]. Also, there seems to be individual susceptibility factors which modify the effects of hypoxia: some individuals develop severe pulmonary hypertension despite only modest lung disease and/or hypoxia whilst some individuals seem relatively 'immune' to pulmonary hypertension, despite severe lung disease or prolonged residence at very high altitude[35]. Different animal species exhibit different susceptibilities to chronic hypoxic PH[112] and also ethnic origin seems to modify human susceptibility to altitude-related pulmonary hypertension[131]. These observations suggest genetic factors which modify susceptibility to hypoxic pulmonary hypertension and recently, polymorphisms in the serotonin transporter gene have been suggested to modify the severity of PH in COPD patients[132]. Also, polymorphisms in the angiotensin converting enzyme gene seem to be associated with the varying susceptibility to high altitude pulmonary hypertension in Kyrgyz highlanders[35].

The importance of pulmonary hypertension in chronic hypoxic diseases has been disputed but there is now a substantial body of data indicating that PH is a common complication of chronic hypoxic disorders and that, when it occurs, it carries a very adverse prognosis. Altitude-related pulmonary hypertension is definitely and COPD-related pulmonary hypertension is probably a major public health problem.

Pulmonary hypertension does not necessarily regress when hypoxia is corrected (eg by providing supplemental oxygen in COPD or moving residence to lower altitude) and correction of hypoxia in these conditions is often impossible. A better understanding of the pathogenesis of hypoxic pulmonary hypertension is therefore required, and seeking therapeutic strategies to manipulate the pulmonary vascular response to hypoxia seems very relevant for human disease.

1.6.3 Hypoxic Pulmonary Vasoconstriction and Pulmonary Vascular Remodelling

The ability of the pulmonary resistance arteries to constrict in response to alveolar hypoxia – hypoxic pulmonary vasoconstriction (HPV) - is of fundamental importance to normal pulmonary vascular physiology: a generalised but reversible constriction of the mammalian pulmonary circulation facilitates the change from fetal to infant circulation when the lungs are first ventilated, at birth, *in utero*[133]. Also, the maintained ability of the pulmonary arteries to constrict in response to lung pathology (eg consolidation or atelectasis) allows for the maintenance of ventilation/perfusion matching and systemic circulation oxygen tensions in the face of acute lung illnesses. This response - constriction of the pulmonary arteries to alveolar hypoxia – is circulation specific (systemic arteries dilate to hypoxia) and is conserved across species.

Whilst essential for normal health, HPV is also important in human disease. Generalised alveolar hypoxia (eg shift from sea level to high altitude) results in generalised constriction of the pulmonary arteries, with consequent rise in pulmonary vascular resistance and development of pulmonary hypertension[134]. Dysfunctions or maladaptions of the molecular and cellular mechanisms of HPV have also long been considered potential pathogenetic factors in PAH.

Although the hypoxia sensing mechanism at a cellular level remains controversial, the effector mechanism of HPV, at both molecular and cellular level has been well characterised. PASMCs (either in endothelium-denuded vascular rings or isolated in cell culture) contract in hypoxia. This is dependant on influx of extracellular calcium through L-type calcium channels and, to a lesser extent, release of intracellular calcium from the sarcoplasmic reticulum. Hypoxia also activates smooth muscle cell

RhoA & Rho-kinase (ROCK) and this acts to sensitise myosin to the effects of intracellular calcium, facilitating and enhancing acute hypoxic vasoconstriction. Persistence of Rho-ROCK activation may be an important contributor to the sustained vasoconstriction which occurs in chronic hypoxia. The secondary, sustained phase of HPV is also dependant on extracellular calcium but also requires an intact endothelium, with both reduction in eNOS activity (leading to decreased nitric oxide signal to SMCs with consequent decreased intracellular cGMP causing cellular contraction) and endothelial cell release of ET-1 (causing SMC contraction via G protein coupled receptor), in response to acute hypoxia, implicated[134].

As previously outlined, chronic hypoxia leads to changes in the behaviour of pulmonary vascular cells and structural changes in all vessel compartments: pulmonary vascular remodelling (Chapter 1.4.3). Also previously discussed is the ongoing debate as to which aspect – remodelling *per se*, sustained vasoconstriction consequent on remodelling etc – is responsible for persistent pulmonary hypertension in animal and human chronic hypoxia.

The link between acute hypoxic vascoconstriction and pulmonary vascular remodelling (and the newly emerging concept of chronic hypoxic vascoconstriction) in chronic hypoxic PH, and also a connection between HPV mechanisms and other forms of PAH has been speculated on for some time. It is recognised that constriction and remodelling share common mechanisms – so called cell growth-vasomotor coupling – and these continue to be added to. Paracrine agents such as prostacyclin, nitric oxide and ET-1 are now readily recognised for their effects both on SMC contraction, and proliferation. Inhibition of Kv1.5 (a cell membrane potassium channel) activity is an important early event in acute hypoxia in SMCs, mediating cellular depolarisation with consequent opening of Ca++ channels[133]. Chronic hypoxia has been shown to downregulate the Kv1.5 and Kv2.1 potassium channels in rat pulmonary arteries[135]. Also, modification of potassium channel activity has been implicated in the pathogenesis of pulmonary vascular remodelling secondary to anorexigen drugs, suggesting a more generalisable link between mechanisms of acute HPV and non-hypoxia related forms of PH[136]. More recent studies using inhibitors of Rho kinase have shown inhibition of both HPV and chronic hypoxia-induced vascular remodelling, implicating the RhoA-Rho kinase

signalling pathway in both processes. Taken together, these findings significantly support the concept of cell growth-vasomotor coupling and suggest that the field of acute hypoxia and HPV remains important, offering the potential for improvements in our understanding of human PH, in general.

The specific mechanisms which may be involved in oxygen sensing in HPV are considered in more detail below (chapter 1.6.5).

1.6.4 Effects of Hypoxia on Pulmonary Vascular Cells

The contributions of the different resident vascular cells to pulmonary vascular remodelling have previously been considered (Chapter 1.5.1). The responses of each individual cell to acute or chronic hypoxia, in cell culture, have been extensively studied. The focus of this thesis is the adventitial fibroblast and, to a lesser extent, the pulmonary artery smooth muscle cell and the effect of hypoxia on these are considered in more detail.

There is some controversy in this field and contrasting / contradictory results have been published. Important aspects to consider are the different origins (species, size of vessel derived from, cell harvest technique used) of the cells studied by different investigators and what constitutes hypoxia to different investigators, and to different cell types. For example, smooth muscle cells from the central media of a proximal vessel, being distant from both the alveoli and the vessel lumen, are likely routinely exposed to low oxygen tensions *in vivo* and therefore may be relatively hypoxia 'resistant'. Smooth muscle cells from the distal vessel (where all vessel layers are thinner) may be more hypoxia sensitive – either because of a fundamental difference in their nature or because they are 'used' to higher oxygen tensions *in vivo*. As adventitial fibroblasts reside closer to the alveoli than medial smooth muscle cells, they may be 'used' to higher oxygen tensions and therefore appear more sensitive to a specific level of cell culture hypoxia vs proximal vessel medial SMCs[137].

Smooth muscle cells proliferate *in vivo* in the chronic hypoxic animal models but this is inconsistently replicated with explanted cells *in vitro.* Hypoxic proliferation of PASMCs may be a function of an isolated, specific subset of PASMCs, it may be that it is dependant on cell-cell or cell-matrix interactions, it may be that PASMC

proliferation is not consequent on hypoxia directly, but a function of release of mitogens by other hypoxic cells or it may simply be that we are inadequately modelling the media of pulmonary arteries with PASMCs in hypoxic cell culture. There is, however, significant evidence of crosstalk between hypoxic PAFs and PASMCs, and it seems likely that PAF activity is a major contributor to PASMC proliferation[3, 66].

The effects of hypoxia on pulmonary artery fibroblasts are considered in more detail in a following section (chapter 1.8).

1.6.5 Hypoxia as an Experimental Model of Pulmonary Hypertension

There are numerous animal models of pulmonary hypertension but the two most commonly used and best characterised are chronic hypoxia and monocrotaline (a direct pulmonary endothelial cell toxin, used alone or in conjuction with pneumonectomy)[59]. Whilst the monocrotaline-pneumonectomy model and other, newer animal models recapitulate some of the histological features of PAH not seen with other models, the initiating factors are not seen in human disease.[138] In contrast, the chronic hypoxic model seems to have advantages:-

- The haemodynamic, compensatory and pathological changes seen in this model have been extensively described and are reliably achieved with standard experimental conditions (maintenance of adult animals in hypobaric hypoxia for 2 weeks)[139].

- The model directly reflects one of the commonest initiating factors in pulmonary hypertension worldwide (pulmonary hypertension associated with chronic exposure to high altitude)[140]. Although imperfect (the pathological changes seen in the hypoxic rat model incompletely reflect those seen in human pulmonary arterial hypertension and the pathogenesis of the pulmonary vascular remodelling may be different) this model is the one where the initiating insult most closely reflects a human condition.

- The success of new pulmonary hypertension treatments has significantly reduced the number of patients with pulmonary hypertension requiring lung transplant. This limits the ability to perform work on pulmonary vascular

cells derived from remodelled human vessels. Given this restriction, work on animal models is essential. The relevance of work on animal cells to human disease remains uncertain and speculative but, for the experiments in this thesis, the recognition that pulmonary artery fibroblast responses to hypoxia are conserved between species (see Chapter 1.8) offers some reassurance about the relevance of the model. In addition, although this is not considered in the experimental work of this thesis, animal models allows study of the early stages of pulmonary vascular remodelling whereas work on explanted or post-mortem diseased human lung only allows assessment of end-stage remodelling.

Experimentally, the effects of acute and chronic hypoxia can be studied separately, or in tandem. When we consider the cell proliferation models under consideration in this thesis, work with acute and chronic hypoxia is related, but complementary. An acute hypoxic cell model has the advantage of allowing 'screening' studies of a battery of different drugs / drug concentrations on readily available tissue from normal animals. Studying cells from chronic hypoxic animals has the advantage of allowing study of disease tissue in comparison to normal 'control' tissue and allowing conclusions to be reached about mechanisms common to acute and chronic hypoxic signalling (helping to unify our understanding of the pathogenic links between acute hypoxic signalling and cellular changes in chronic hypoxic pulmonary vascular remodelling).

Experimentally, hypoxia can be induced by two mechanisms:-

- Normobaric hypoxia. Hypoxia is induced by reducing the fraction of oxygen (from 21%) in normal pressure air. This is the mechanism utilised in the acute hypoxic cell model in this thesis.
- Hypobaric hypoxia. The fraction of oxygen in air is maintained but the pressure is reduced. This directly reflects the aetiology of high altitude pulmonary hypertension in humans and is the mechanism utilised to generate the chronic hypoxic rat model in this thesis.

1.6.6 Hypoxia-Sensing Mechanisms

Our understanding of the fundamental mechanism(s) by which cells recognise and respond to low oxygen have progressed significantly in the last 15 years. In relation to pulmonary hypertension and chronic hypoxic pulmonary vascular remodeling the most relevant and most studied pathways are the HIF (hypoxia inducible factor) pathway and the sensing mechanism(s) of acute hypoxic vasoconstriction.

1.6.6.1 HIF-1 signalling

First recognised in relation to the hypoxia responsive element in the erythropoietin gene[141], the HIF-1 pathway is now known to function as a ubiquitous mediator of cellular responses to hypoxia, being present in all nucleated cell types. HIF-1 is a heterodimeric protein consisting of 2 constitutively transcribed proteins HIF-1α and HIF-1β[142]. In the presence of oxygen, proline residues on HIF-1 α are hydroxylated. Hyrdoxylated HIF-1α binds to the Von-Hippel Lindau protein which leads to recognition by cellular ubiquitination mechanisms with consequent proteasomal protein degradation. In hypoxia, prolyl hydroxylase activity is reduced leading to reduced degradation of HIF-1α with consequent stabilisation of the HIF-1 protein complex[143]. HIF-2α and 3α subtypes of the protein, expressed in a more restricted panel of cell types, are now being characterised[144].

Stabilised HIF-1 protein complexes (whose existence is promoted by hypoxia) translocate to the cell nucleus where they are associated with coactivator proteins (eg CREB or p300) and this association is also facilitated by hypoxia[145]. The HIF protein-coactivator complex promotes transcription of genes containing a hypoxia responsive element in the gene promoter. The ultimate effect of this modified gene transcription is cell specific but can involve increased cellular proliferation, increased production of mitogens etc.

The HIF protein system is likely important in the pathogenesis of pulmonary vascular remodeling. Proteins whose transcription is mediated by HIF-1 include VEGF, ET-1 and voltage dependant calcium channels[146] and these are mediators implicated in pulmonary vascular remodeling. Studies with knockout / transgenic mice suggests activity of HIF-1α in PASMCs and HIF-2α in PAECs contributes to hypoxic pulmonary vascular remodeling[147].

1.6.6.2 Oxygen Sensing in Acute Hypoxic Pulmonary Vasoconstriction[133]

The connection between acute HPV and vascular remodeling is underscored by the observation that the cellular processes mediating PASMC constriction (Kv channel closure, increase in intracellular Ca++, activation of Rho-ROCK) can also influence PASMC proliferation.

Consideration of the ongoing debate about oxygen sensing mechanisms in acute HPV allows for an elaboration of the experimental difficulties which exist in this field. There currently exist two contradictory models of oxygen sensing in HPV and there is good experimental evidence for both. In both these models, reduction in cellular oxygen is 'sensed' both at the inner plasma membrane and in the mitochondria. This leads to alterations in activity of NAD(P)H oxidases (either in the cell membrane or the mitochondria) and/or in the mitochondrial electron transport chain and/or in heme oxygenase-1 activity. What is contentious is whether alterations in these leads to an increase or decrease in cellular reactive oxygen species (ROS) and where the connection between these processes (eg is the NADPH oxidase activation a cause or a consequence of changes in ROS) and the downstream processes lies[148].

1.6.6.3 Summary

Difficulties with the experimental definition and application of hypoxia to different cell types and the deficiencies of the current knowledge base and contentions which exist in relation to oxygen sensing mechanisms have been noted. When results of the experimental work – using 'hypoxia' as a very generic term - in this thesis are considered, all of these problems are acknowledged.

1.7 Mitogen Activated and Other Protein Kinases in Cell Proliferation

1.7.1 Mitogen activated protein kinases

Cell activities (growth, differentiation, apoptosis, gene activation, cytoskeletal conformation, mediator synthesis and secretion, etc) are regulated and influenced by diverse extracellular signals. Extracellular signals are integrated and amplified with

signal-specific responses generated (eg gene activation) by complexes of signalling cascades. The mitogen-activated protein kinases (MAPK) process various extracellular signals and constitute a key intracellular signalling complex.

Extracellular stimuli (mitogens[149], hypoxia[150], UV light[151]) activate diverse cell receptor complexes with consequent activation (eg by phosphorylation or GTP-loading) of upstream kinase proteins in the MAPK cascade. The signal is amplified at two preceding kinase steps, leading to phosphorylation (at tyrosine and threonine residues) of the MAPK protein (figure 1.4). MAPK exists in the cytoplasm and phosphorylated MAPK can act directly, phosphorylating and activating substrate cytosolic proteins[152]. Also, once phosphorylated, MAPKs can translocate to the cell nucleus and act on cell type specific transcription factors[153]. The MAPK pathway therefore acts as a physical link and also a signal amplification step from extracellular signals to cell nuclear responses.

Three distinct MAPK subfamilies are recognised in mammalian cells[154]. The classical MAPK pathway, consisting of ERK1 & ERK2 (extracellular regulated kinases) and their associated upstream kinases and downstream substrates, is typically implicated in mediation of conventional, constitutive signalling by growth factors. The stress activated protein kinase pathway (SAPK) is recognised as a key mediator of cellular responses to stress signals (eg hypoxia, UV light). p38α MAPK and c-Jun N-terminal kinase 1 (JNK-1) are the prototype SAPKs. Along with ERK these constitute the three MAPK subfamilies. Within each subfamily there exist various isoforms which seem to have tissue-specific functions[155]. Aspects of the p38 MAPK signalling cascade are represented in figure 1.5.

1.7.2 Upstream Signalling Events in MAP kinase activation

As illustrated in figure 1.5, phosphorylation of MAPKs is dependant on initial processing of the extracellular signal by receptor or other cell membrane-associated proteins with consequent activation of MEKK proteins. The small GTP binding proteins comprising the Ras superfamily have attracted considerable experimental attention in recent years, firstly based on their apparent involvement in the pathophysiology of diverse conditions and secondly based on the recognition of these as potential therapeutic targets[156]. A schematic of potential interactions between cell

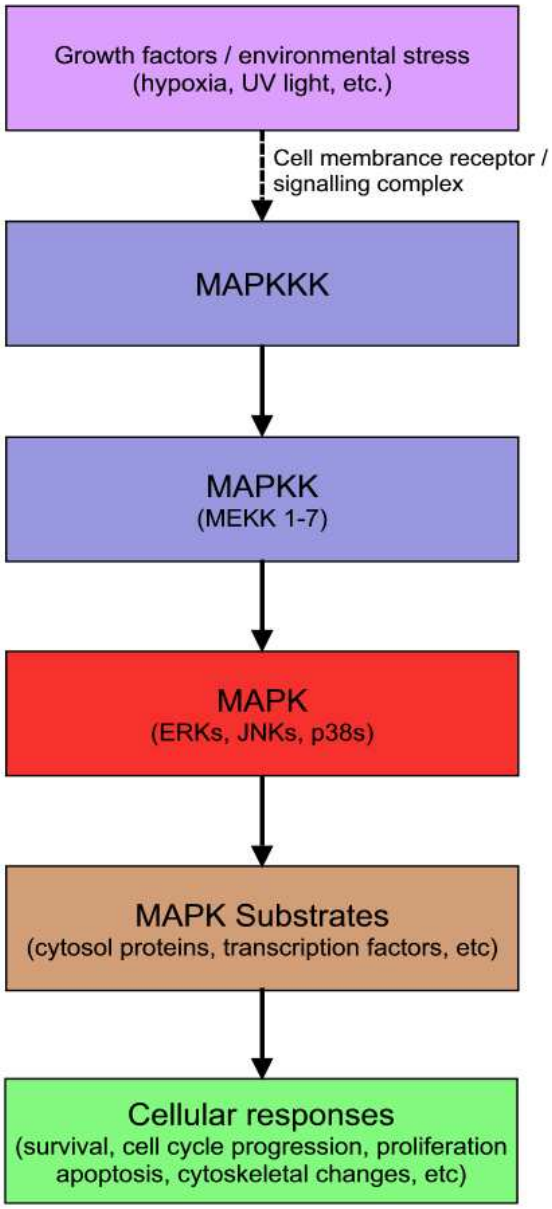


Figure 1.4 Mitogen-Activated Protein Kinase Signalling Pathways

Mitogen-activated protein kinases (MAPKs) integrate & process various extracellular signals. The MAPK cascade consists of 3 protein kinases: a MAPK and 2 upstream kinases a MAPK kinase (MAPK or MEKK) and MAPKK kinase (MAPKKK eg raf or rho kinase)

ERK: extracellular signal-related kinase. JNK: c-Jun-N-terminal kinase.

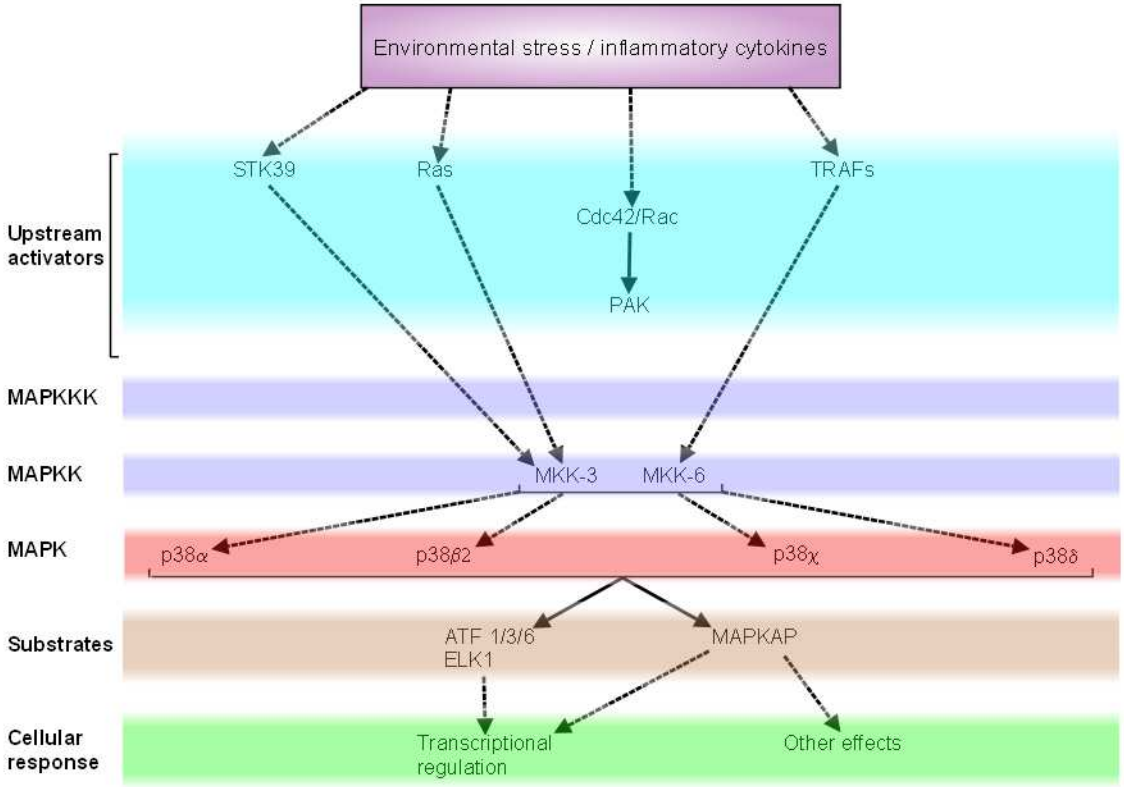


Figure 1.5 Overview of the p38 MAP Kinase Signalling Cascade

Extracellular signals activate MAPKKKs via variety of signalling mechanisms. MAPKKKs activate MAPKKs, which in turn phosphorylate and activate MAPKs. Activated MAPKs then phosphorylate downstream substrates, leading to altered cellular activity.

STK39: serine threonine kinase. PAK: p21-activated kinase. MAPKAP: MAPK-activated protein kinase.

TRAF: Tumour-necrosis factor receptor-associated factor ATF: activating transciption factor.

membrane receptors, extracellular signals, Ras family proteins and MAPKs is shown in Figure 1.6.

Signalling from extracellular growth factors via Ras-Raf1-ERK is well characterised[157]. More recently a connection between Rho family proteins and the SAPKs has been shown in various cell types[158, 159]. Experimental evidence linking the individual involvement of these proteins (Ras, ERK, RhoA, Rac1, p38 and JNK) to the cellular processes of interest in this thesis (ie hypoxia sensing, adventitial fibroblast proliferation, pulmonary vascular remodeling) is notable. Also notable is the emerging evidence that other processes extracellular / cell membrane systems potentially disrupted in PAH pathogenesis may mediate their effects via SAPK pathways: eg certain mutations in the BMPR-2 gene have been shown to disrupt normal antiproliferative downstream signaling (via phosphorylation of SMAD proteins) and result in activation of p38 MAPK (presumably with resultant dysregulated cellular proliferation)[160]. It is considered likely that Ras superfamily – MAPK signaling pathways are 'final common pathways' in PH pathogenesis and further investigation of these should provide further insights into fundamental disease mechanisms and potential therapeutic strategies[161].

Individual signaling systems that we are speculating may be involved in hypoxia-induced p38 MAP kinase activation in PAFs have been implicated in hypoxic pulmonary vascular remodeling. There is significant interest in the RhoA-Rho kinase signaling pathway. Rho kinase inhibition (eg with fasudil) inhibits hypoxic pulmonary vasoconstriction, reverses experimental pulmonary hypertension, inhibits pulmonary vascular remodeling and is likely to be safe for human use[51, 162-164]. Clinical trials of Rho kinase inhibitors for PAH are proposed. There is, however, some evidence that activation of RhoA-ROCK may be a secondary phenomeon, rather than an initiating factor in PH pathogenesis[165]. Knockout of a specific subunit protein of the NADPH oxidase complex (NOX2 / $gp91^{phox}$) inhibits the production of reactive oxygen species and prevents pulmonary vascular remodeling in chronic hypoxic mice[166]. The Rho GTPase family proteins Rac1 and Rac2 are involved in signal transduction from extracellular signals, leading to the activation of NOX (and subsequent reactive oxygen species generation) in most cell types, including fibroblasts[167]. The recent observation that specific inhibition of the NOX4 protein

reduced hypoxic proliferation and p38 activation in PAFs is particularly intriguing and NOX4 has been specifically implicated in the pathogenesis of both experimental hypoxic PH and IPAH[168]. This last point also supports the relevance of continuing to study experimental hypoxic PH, and relating findings to PAH in humans.

This converging evidence suggests a fundamental role for the RhoA-ROCK and Rac-NOX signaling pathways in hypoxia sensing and the pathogenesis of hypoxic pulmonary vascular remodeling. When we were speculating about pathways potentially upstream of p38 MAPK in hypoxic PAFs, we considered that these two pathways would likely be of specific interest for further study (if the initial experiments conducted supported the involvement of a GTPase protein).

Other signaling proteins are likely involved upstream of MAPKs in hypoxia, in other cell types and situations. In acute hypoxia in PAFs from neonatal calves, phosphatidylinositol-3-kinase, Akt and mTOR have been shown to be upstream activators of ERK MAP kinase, and this signaling pathway is necessary for acute hypoxic proliferation of this cell type[169].

In addition to the downstream substrates of MAPKs illustrated in Figure 1.4, activation of MAPKs has been linked to increased HIF-1α stabilisation and HIF-1α phosphorylation (phosphorylation promotes nuclear translocation of the HIF complex, see Figure 1.6)[170]. In particular, inhibition of p38 MAP kinase in acutely hypoxic PAFs has been shown to reduce HIF-1α stabilization and consequent cellular proliferation, indicating a link between these fundamental hypoxia-responsive signaling systems, in a cellular process important in pulmonary vascular remodeling[155, 171, 172].

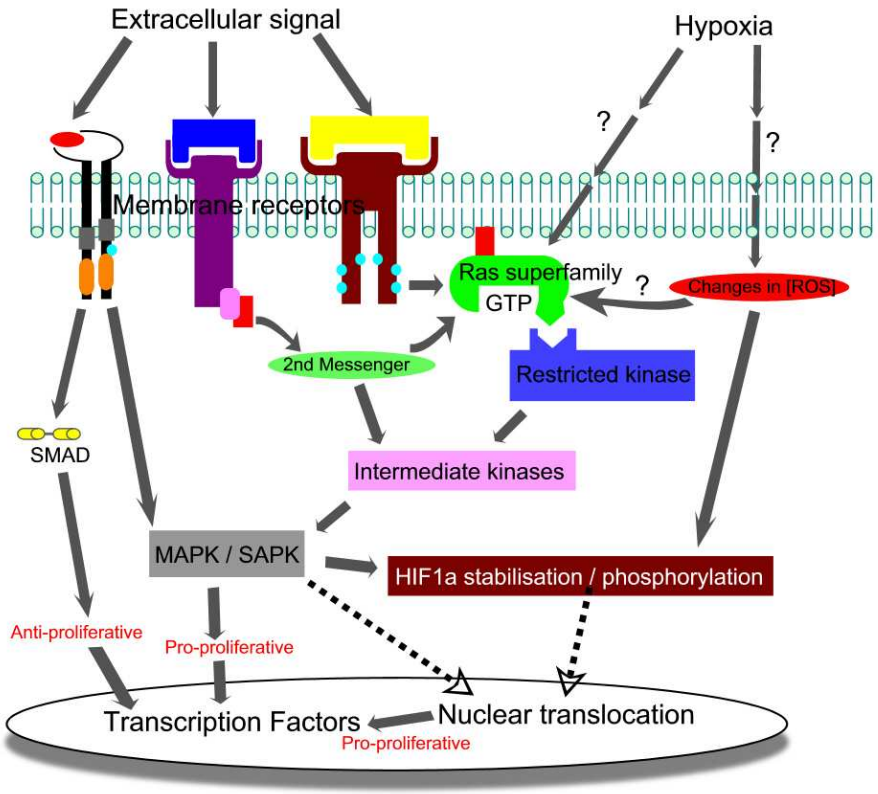


Figure 1.6 Pivotal Role of Ras Superfamily Proteins in Cell Signal Transduction

Via G-protein coupled receptors, tyrosine or serine-threonine kinase receptors or other mechanisms, Ras family guanine exchange factors (not shown) are activated, resulting in conversion of a Ras protein-GDP complex to Ras-GTP[170]. This active complex then activates specific kinases (eg in the case of Ras, this can be Raf-1 which is activated by translocation from the cytoplasm to the inner plasma membrane) which in turn phosphorylate downstream kinases (eg MKK3) with consequent MAPK phosphorylation. Hypoxia and/or changes in reactive oxygen species have been implicated in the activation of RhoA and Rac1 (members of the Ras superfamily) and this may be important in the molecular pathogenesis of hypoxic pulmonary vascular remodelling.

1.8 Fibroblasts in Pulmonary Vascular Remodelling and Effects of Hypoxia on Fibroblast Proliferation and MAP Kinase Pathways

In comparison to other resident vascular cells, adventitial fibroblasts seem to have a very notable capacity to proliferate, migrate and transdifferentiate in response to hypoxia. They play an important role in the initiation and evolution of pulmonary vascular remodeling in chronic hypoxic pulmonary hypertension. Some of the experimental evidence to support this has been discussed previously (chapter 1.5.1) but is summarised here:-

- Adventitial thickening (with increased cell numbers and matrix deposition) is the earliest and most prominent change in hypoxic pulmonary vascular remodelling. Increased proliferation and matrix deposition by pulmonary artery fibroblasts contribute to this adventitial thickening[173].
- Pulmonary artery fibroblasts proliferate faster in hypoxia than other vascular cells[174]
- In response to hypoxia, growth factors or injury PAFs synthesise collagen precursors. Collagen subsequently accumulates in the adventitia, contributing to loss of vessel distensibility. PAFs also migrate to the medial and intimal layers of the vessel and lay down collagen here, further contributing to reduction in vessel lumen and loss of vessel distensibility[48]
- In response to pulmonary artery injury and hypoxia PAFs transform and contribute to the population of subintimal, medial and adventitial "myofibroblasts."[175] These myofibroblasts seem to have a key role in the generation of the typical histological lesions seen in pulmonary hypertension.
- Signals generated by PAFs influence endothelial and smooth muscle cell activities eg pulmonary artery smooth muscle cells have been found to proliferate in hypoxic conditions only when cultured in association with PAFs (or in PAF conditioned media)[66].
- The adventitial fibroblasts in the remodelled vessel may be heterogenous. Different subsets may have different proliferative, synthetic and migratory phenotypes[176]. Some subsets of PAFs in the diseased vessel may be derived from a hypoxia-induced influx of vascular mesenchymal stem cells, rather than from resident adventitial fibroblasts[71].

Previous work in the Scottish Pulmonary Vascular Unit (SPVU) laboratory[150, 155, 171, 177-181] has characterised the effects of acute and chronic hypoxia on MAPK phosphorylation and proliferation of PAFs. Notable findings are:-

- Bovine, rat and human PAFs (from normal subjects) proliferate excessively when exposed to acute hypoxia.
- PAFs from chronic hypoxic rats are constitutively hyperproliferative: they demonstrate increased proliferation in comparison to PAFs from normal animals, even when cultured and passaged in normoxia.
- Acute hypoxic proliferation in PAFs is associated with a biphasic increase (at 4 and 16 hours) in phosphorylation of the α and γ isoforms of p38 mitogen-activated protein kinase. p38 MAPK is constitutively phosphorylated (abnormally) in PAFs from chronic hypoxic rats.
- ERK is always, and JNK may be, phosphorylated (dependant on species studied) in hypoxic PAFs, but as hypoxic proliferation is not affected by ERK or JNK inhibition these do not seem important in this hypoxia-induced proliferative signalling pathway.
- p38 MAPK inhibition completely and selectively blocks acute hypoxic proliferation and reverses the hyperproliferative phenotype of PAFs from chronic hypoxic rats.
- No changes are seen in proliferation or p38 MAPK phosphorylation in systemic artery fibroblasts in acute hypoxia or when derived from chronically hypoxic animals: the p38 MAPK pathway would seem to be a mechanism responsible for the circulation specific response to hypoxia.
- p38 MAPK phosphorylation was found to be partially responsible for HIF-1α stabilisation in acutely hypoxic rat and human PAFs.

There is some data in the literature which contradicts these findings. In particular, Stenmark's group typically report that PAF proliferation is dependant on ERK phosphorylation, rather than p38 MAPK[169]. This may be a function of different cells studied by this group (eg derived from neonatal calves) or study of the cells at different time points (eg p38 MAPK phosphorylation is only present at 2 time peaks and is absent at other times during a 24 hour acute hypoxic period). The

reproducibility of the SPVU lab findings throughout publications is notable and similar findings (p38 MAPK dependant hypoxic proliferation of fibroblasts) have been reported by others[172]. The experiments subsequently conducted in this thesis, however, sought to examine this controversy again.

Based on the SPVU's previous experimental results, p38 MAPK inhibition would seem an attractive therapeutic strategy, potentially achieving a circulation specific inhibitory effect on one of the fundamental cellular processes in chronic hypoxic pulmonary hypertension. This possibility is supported by the positive effects (attenuation of pulmonary hypertension) seen with p38 MAPK inhibitors in experimental pulmonary hypertension[182], and also the recognitions that p38 MAPK mediates sustained hypoxic pulmonary vasoconstriction[183] and pulmonary artery endothelial dysfunction in chronic hypoxic rats[184] (adding yet further support to the concept of cell growth-vasomotor coupling- see chapter 1.6.3) . A significant concern, however, is potential toxicity of p38 MAP kinase inhibitors in humans. Although there have been clinical trials (for other diseases) with p38 MAPK inhibiting agents, therapeutic development of p38 MAPK inhibitors is at an early stage and safety has not been absolutely demonstrated[185-188].

The signaling pathway upstream of p38 MAPK in PAFs is incompletely explored but an intriguing observation - that mitochondrial ROS activation was necessary for p38 MAPK phosphorylation - has been made. This raises the possibility that signaling protein(s) under scrutiny in hypoxic pulmonary vasoconstriction eg Rho kinase (ROCK) or NADPH oxidase (NOX) may be involved in hypoxic signal transduction and p38 MAPK phosphorylation in PAFs. This seems plausible in the context of MAPK in general: MAPKs have been linked to Ras superfamily proteins in pulmonary vascular cell proliferation[189]. Also specific proteins from this family (RhoA, Rac1 & CDC-42) have been linked to ROCK[190] , NOX[167] and p38 MAP kinase[158, 159].

Based on this, we considered the literature further, in order to determine an experimental strategy which would allow us to clarify hypoxic cellular signaling upstream of p38 MAPK in PAFs. Also we sought a potential indirect inhibitor of this p38 MAPK pathway which might be more immediately therapeutically

applicable to our patients than the direct p38 inhibitors currently only in early drug development. Given their known effect on small protein GTPase (ie Ras superfamily) dependant signaling[191] we considered the possibility that statin drugs may fit these requirements, and that they would be a potential tool to interrogate hypoxic signaling upstream of p38 MAPK, in PAFs.

1.9 Statins

1.9.1 Clinical Effects of Statins: Lipid Lowering and Cardiovascular Disease

Statin drugs are one of the great successes of modern pharmacology. Based on the (now considered relatively simplistic) observations that elevated blood LDL-cholesterol levels were associated with atherosclerosis and cardiovascular disease and that statin drugs could inhibit hepatic cholesterol synthesis and thus reduce blood cholesterol, clinical trials to determine the effect of statins on the primary and secondary prevention of cardiovascular disease were initiated and reported in the mid-1990's[11, 12, 15]. With their beneficial effects and good tolerability (side effects are usually minor, the major side effect of rhabdomyolosis is very rare)[192] statins have revolutionised the management of atherosclerotic disease and are now prescribed almost ubiquitously for patients with established cardiovascular disease or who are at high risk of developing cardiovascular disease.

Statins are competitive inhibitors of the rate limiting step in cholesterol biosynthesis: they inhibit the enzyme 3-hydroxy-3methylglutaryl (HMG) CoA reductase. Inhibition of this in hepatocytes leads to a decrease in LDL-cholesterol (ie 'bad cholesterol) synthesis by the liver and also an upregulation in hepatocyte LDL receptor expression, with resulting clearance of LDL complexes from the bloodstream. This results in a reduction in total blood cholesterol, LDL-cholesterol and an improvement in the blood lipid profile. Clinical trials have latterly compared the differential clinical efficacy of the different statins, clarifying that the different pharmacology of these drugs is clinically relevant and that larger doses of statins have additional benefits[193]. Also, these large clinical trials and the widespread experience with statins in routine clinical practice has provided substantial reassurance about the safety and tolerability of these drugs, even at higher doses[194, 195].

1.9.2 Clinical Effects of Statins: Pleiotropic Effects and Cardiovascular Disease

Following on from the initial positive clinical reports there have been innumerable experimental studies of the effects of statins in cell culture and animal models, in order to elucidate their mechanism(s) of action in cardiovascular disease. From these experiments it has been realised that statins, in addition to their lipid lowering effects, have diverse other effects on molecular and cellular processes implicated in atherosclerosis pathophysiology, which may contribute to their clinical utility[196]. In the systemic circulation, statins have been shown to increase production of endothelial nitric oxide synthase, reduce production of endothelin-1 and reactive oxygen species and inhibit smooth muscle cell and macrophage proliferation[18]. It is observed that, if these effects were duplicated in the pulmonary circulation, statins would potentially inhibit pathogenic processes in pulmonary vascular remodeling.

Whether these so called 'pleiotropic effects' of statins contributes partly or wholly to their clinical benefits, is unclear and much debated[197]. It is argued that, as their benefit is on a par with their effects on lowering serum LDL-cholesterol, additional mechanisms of effect are not present. The evidence supporting the existence of pleiotropic effects is, however, currently considered to be stronger. In particular, hydrophilic statins (which have effects limited to hepatocytes, rather than other cells in the body) are generally less clinically potent for a given effect on serum cholesterol. Beneficial effects on systemic vascular reactivity, thrombotic and inflammatory profiles are seen early after statin initiation, before effects on serum cholesterol are apparent[198].

1.9.3 Clinical Effects of Statins: Pleiotropic Effects and Other Diseases

Experimental observations about the 'broad spectrum' positive effect of statins on disease-implicated cellular signaling systems and their putative pleiotropic benefits in cardiovascular disease led to the exploration of the benefits of statin therapy for non-cardiovascular disease[199]. Retrospective analysis of database/registry data and substantial laboratory research suggests that statin use may be beneficial in other diseases (notably cancers, sepsis, neurodegenerative diseases, osteoporosis and respiratory diseases)[200-205]. Based on more specific positive experimental

observations, small clinical trials of statin therapy in various 'non-cardiovascular' diseases have been reported. Some of these have been negative but in each it is acknowledged by the investigators that it is unclear if this is a true negative result or a function of the trial design[206]. It seems, however, that our knowledge about disease mechanisms, statin pharmacology and choice of specific statin and dose needs to be refined for any future trials of this nature[207]. That this is likely to be worthwhile is supported by the two notable positive prospective trials of statin therapy for rheumatoid arthritis[208] and multiple sclerosis[209].

1.9.4 Clinical Pharmacology of Statins

Mevastatin and lovastatin were first isolated from aspergillus species and their effects (potent inhibition of the HMG-CoA reductase enzyme) were characterised in the 1970's. Concerns about animal toxicity with mevastatin delayed clinical application until safety data for lovastatin was established. Initial trials, however, with this drug showed substantial reductions in LDL-cholesterol levels, much greater than that seen with agents acting on serum cholesterol via other mechanisms[210]. This led to the recognition of naturally occurring similar compounds (pravastatin and simvastatin) and synthesis of related compounds (including atorvastatin and rosuvastatin) and their subsequent clinical validation. The different statins differ in their pharmacology and cholesterol lowering potency. The lipophilic compound atorvastatin is currently considered by most observers to be the benchmark for clinical applications. The chemical structures and pharmacological characteristics of the statins in current clinical use are shown in figure 1.7 and table 1.2.

The different pharmacological properties of the different statins provides theoretical reasons why they may differ in their clinical utility for non-cardiovascular diseases[211]. When considering lipid lowering, the target organ for statin action is the liver and it would seem to matter little if they act only on hepatocytes (as pravastatin and rosuvastatin are thought to do) or whether they are extensively first pass metabolised, achieving only very small drug levels in the pulmonary or systemic circulations (as simvastatin and atorvastatin do). This last point is difficult as metabolites of simvastastin and atorvastatin (which reach the pulmonary and

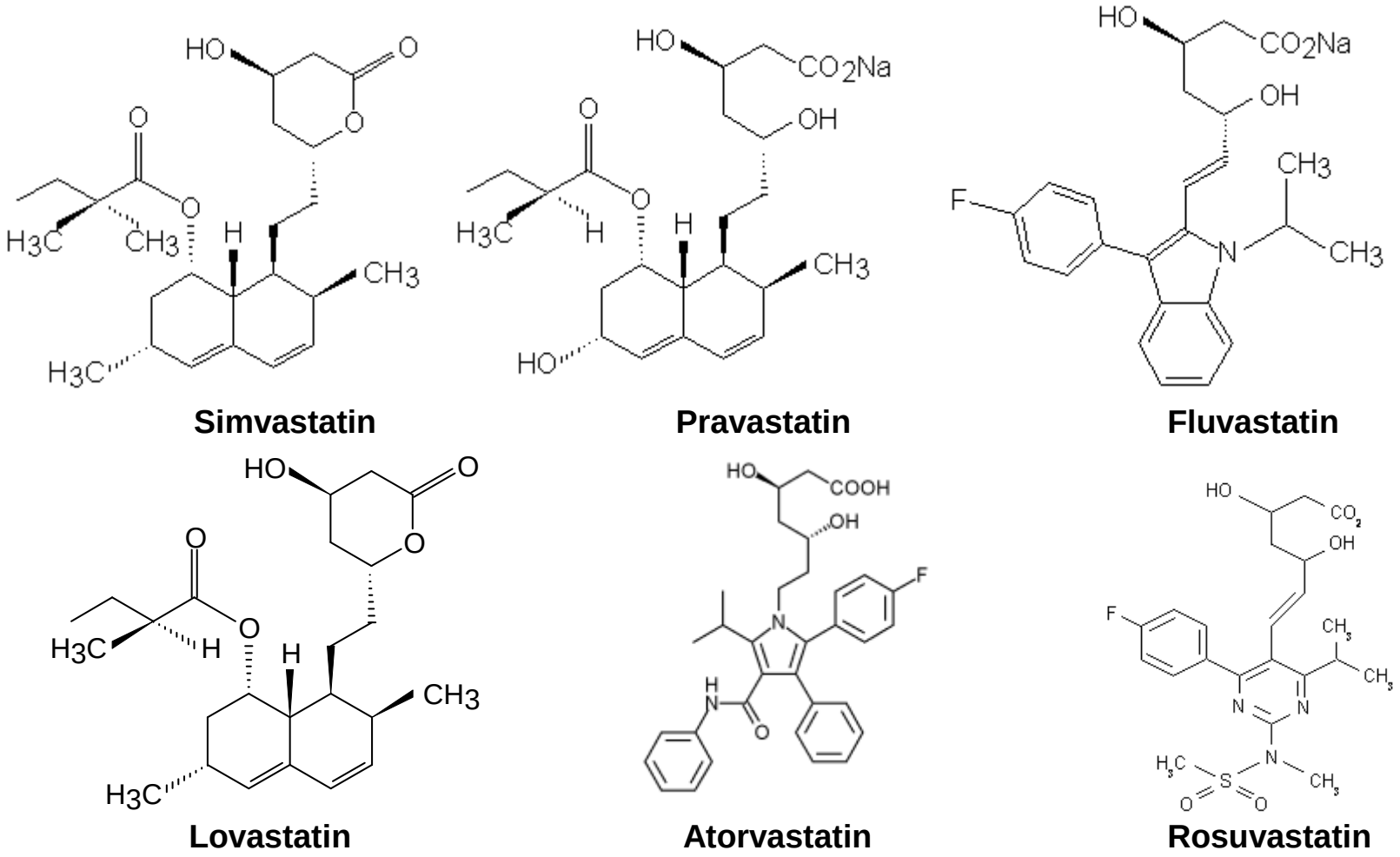


Figure 1.7: Chemical Structure of HMG-CoA Reductase Inhibitors

Parameter	Atorvastatin	Fluvastatin	Simvastatin	Lovastatin	Pravastatin	Rosuvastatin
C_{max} (μM/L)	0.02 – 0.05	1.035	0.024 – 0.082	0.024–0.048	0.1 – 0.123	0.045
Solubility	Lipophilic	Lipophilic	Lipophilic	Lipophilic	Hydrophilic	Hydrophilic
Metabolism	CYP3A4	CYP2C9	CYP3A4	CYP3A4	Sulfation	CYP2C9 / C19

Table 1.2 Clinical Pharmacokinetics of HMG-CoA Reductase Inhibitors

Relevant properties of statins in clinical use are summarized. Maximum serum concentrations are following a 40mg oral dose of statin. Lipophilic statins have effects on non-hepatic cells whereas hydrophilic statins (ie rosuvastatin and pravastatin) typically do not. Statins metabolised via the cytochrome P (CYP) 3A4 enzyme have an increased potential for drug interactions. Adapted from Bellosta *et al*[192]

systemic circulation) may be active, but this is unconfirmed. Nevertheless, it seems relevant to consider that a drug which doesn't exhibit these problems, and also has reduced drug-drug interactions (eg fluvastatin) may be most relevant to consider for non-cardiovascular disease studies.

1.9.5 HMG-CoA Reductase: The Cholesterol Biosynthesis Pathway and Mechanisms of Action of Statins

At a cellular level, statins mediate their effects via competitive inhibition of HMG-CoA reductase, inhibiting the intracellular synthesis of cholesterol and intermediates of the cholesterol pathway. Importantly, the production of the intermediate compounds farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) are also inhibited (figure 1.8). FPP is the substrate for many important intracellular molecules including ubiquinone, dolichol and transfer RNA but cells tend to have a stable pool of these molecules present and statin therapy, at clinical doses, has not been shown to impact on cellular functions requiring these molecules. In contrast, the membrane GTPases of the Ras superfamily require a ready supply of either FPP (Ras family[212]) or GGPP (RhoA family[213]) for normal function. The GTPase proteins undergo post-translational modification (prenylation) with addition of a FPP or GGPP molecule. This prenylation step (which may be dynamic) is necessary for normal association of these GTPases with the cell membrane (Figure 1.9). Inhibition of the prenylation step - either indirectly via HMG-CoA reductase inhibition, or via inhibition of a specific prenyltransferase enzyme (which catalyses the addition of the prenyl molecule to the protein) - prevents their function. The majority of the pleiotropic effects of statins have been associated with effects on protein prenylation[18].

The plasma cell membrane is not a homogenous structure: specific channels and receptor proteins are maintained in relative association by substructures in the plasma membrane, such as lipid rafts (Figure 1.10). Inhibition of intracellular cholesterol synthesis results in a failure of normal plasma membrane lipid raft maintenance. Statins have been shown to disrupt plasma membrane lipid rafts in vitro and in vivo and it is speculated that some of their pleiotropic effects may be mediated via this mechanism[214].

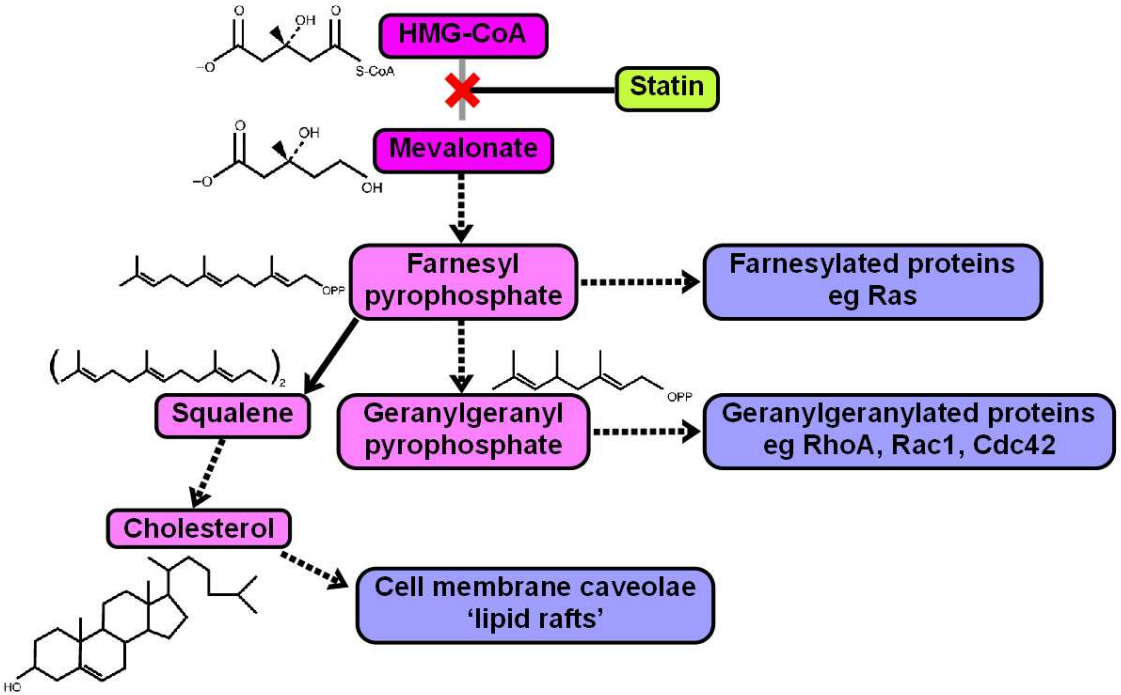


Figure 1.8 Cholesterol Biosynthesis Pathway

The conversion of HMG-CoA (derived from Kreb's cycle intermediates) to cholesterol involves a number of intermediate enzymes, but HMG-CoA reductase (which catalyses the conversion of HMG-CoA to mevalonate) is the rate limiting step. Cholesterol can subsequently be converted on to other steroid molecules but a ready intracellular supply is required for the maintenance of cellular lipid raft structures (see Figure 1.10). Specific cholesterol pathway intermediates - the prenyl compounds (aka isoprenoids) farnesyl pyrophosphate and geranylgeranyl pyrophosphate (purple boxes) - are required for diverse cellular functions, including transfer RNA and ubiquinone synthesis. These prenyl compounds are also required for the normal post-translational modification of proteins involved in centromere assembly and also GTPase proteins of the Ras and Rho superfamilies, which couple extracellular-intracellular signaling (see Figure 1.9). Adapted from Liao[18].

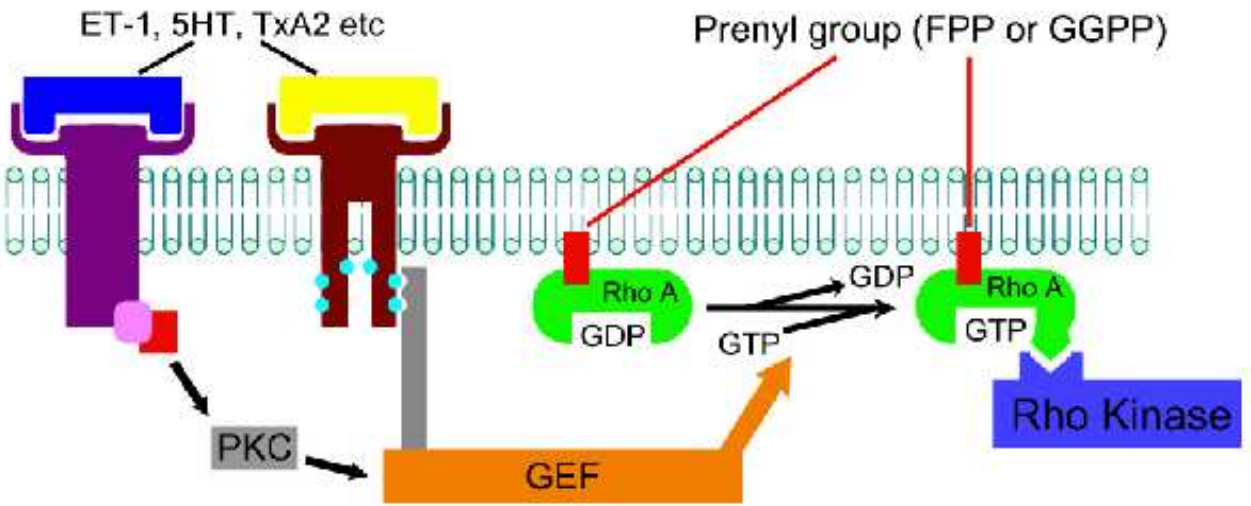


Figure 1.9 Prenylated GTPase Signalling Systems

Normal post-translational modification of Ras and Rho superfamily GTPase proteins (RhoA shown here as an example) involves the addition of a prenyl moiety, either farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP), depending on the specific GTPase involved. Specific transferase enzymes catalyse the addition of FPP or GGPP to the protein.

The molecular structure of the prenyl compounds (see figure 1.8) involves a long lipophilic portion and it is thought that the attachment of this, to the protein, facilitates association with the inner aspect of the plasma membrane, where GTPases couple signalings from extracellular receptors via guanine exchange factors (GEF). Removal of GDP and attachment of GTP by the GEF activates the GTPase protein causing downstream phosphorylation and activation of target proteins (eg Rho Kinase). Constitutive activation is prevented by the rapid intrinsic phosphodiesterase activity of the GTPase, whereby GTP is rapidly broken back down to GDP. Prenylation of GTPase proteins appears to be a high-turnover protein system and reduction in supply of prenyl compounds, or inhibition of prenyltransferase enzymes rapidly disrupts the normal function of GTPase dependant signaling systems, by blocking their normal association with the plasma membrane.

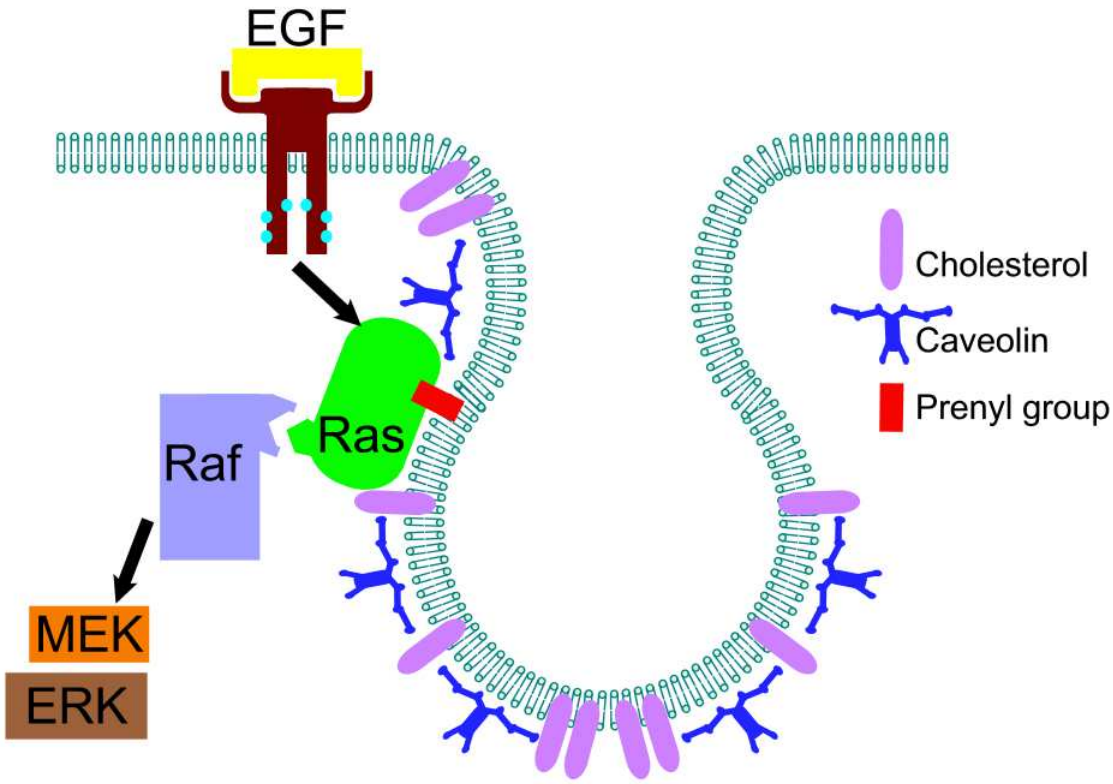


Figure 1.10 Cell Membrane Lipid Rafts

Plasma membrane lipid raft structures and associated signaling pathways (the EGF-Ras-Raf pathway is shown as an example). Cell membrane cholesterol, in conjunction with structural proteins such as Caveolin-1, localises in islands or invaginations (caveolae) in the plasma membrane. A ready supply of intracellular cholesterol seems required for the normal maintenance of these structures. These islands facilitate the normal association of cell membrane receptors / intracellular signaling proteins and, if disrupted, the associated signaling systems may be disconnected.

Lastly, simvastatin and lovastatin have been shown to bind directly and inhibit the integrin LFA-1 (a lymphocyte adhesion/activation molecule), independent of their effects on HMG-CoA reductase[215]. This property is not shared by all statins and this emphasises that a need exists to ensure that any positive experimental observations realised with an individual statin need to be fully explored with other statins and the mechanism of action interrogated, before clinical application.

Given the potential effects of statins on cell signaling systems (ie inhibition of protein prenylation and disruption of cell membrane lipid rafts), if statins are shown to have an effect on a cellular process (eg growth factor-induced proliferation) the mechanism of action of the statin can be deduced. If a subsequent, complementary experiment is conducted and a specific compound from the cholesterol pathway is added along with the inhibitory dose of the statin the experimental results achieved will allow conclusion about which 'arm' of the cholesterol pathway is implicated in the cell signaling system under consideration. Previous investigators have used this approach to determine the mechanism of action of statin on a specific cellular process[216]: we expanded on the experiments in this report to design the experimental protocol used in Chapter 4 of this thesis.

1.9.6 Statins in Experimental Models of Pulmonary Hypertension

Statins demonstrate notable antiproliferative effects on smooth muscle cells[217], including pulmonary artery smooth muscle cells[218], in vitro. It was also observed that all of the noted pleiotropic effects of statins in the systemic circulation (eg increase in eNOS activity, reduction in ET-1 synthesis[219]) would be potentially beneficial in PH. Based on this, investigators studied the effects of simvastatin on the chronic hypoxic rat model. Simvastatin given intraperitonealy or orally at a dose of 80mg/kg/day before or subsequent to 2 weeks of chronic hypoxia resulted in normalization of pulmonary artery pressures and reductions in medial hypertrophy, right ventricular hypertrophy and secondary polycythaemia. These effects were negated by co-administration of mevalonate (implicating an HMG-CoA reductase dependant mode of action of the statin) and were associated with reductions in ROCK activity (which may have been via a direct or indirect effect of the statin)[4, 220]. Similar results were reported - prevention and reversal of pulmonary vascular remodeling, pulmonary hypertension and compensatory right ventricular hypertrophy

– along with improved animal survival, in the monocrotaline rat model[5, 6] Subsequent to this, these effects have been confirmed in other PH models[8], and extended to other statins[221]. There has, however, been some conflicting data. Some of this may relate to problems with preparation of the statin drug[9] (verbal communication from authors). The negative results of McMurtry et al with simvastatin and atorvastatin in the monocrotaline-PAH model are however, not explained, in comparison to the many other positive reports[222]. The conclusion of the authors and specific correspondence is that further pre-clinical study of statins for pulmonary hypertension is required, a statement which supports the experimental work of this thesis.

When considering statin benefits and potential mechanisms of action in pulmonary vascular remodeling, other notable experimental findings include the recognition that simvastatin can inhibit growth factor release and proliferation of lung fibroblasts[223], and that lovastatin can stimulate release of BMP-2 (which is an agonist for the BMPR-2 receptor, which has a primary role in the pathogenesis of familial PAH) from vascular smooth muscle cells[224]. Mevastatin has been shown to inhibit serum-induced rat PASMC proliferation[218] and atorvastatin inhibits serotonin-induced MAP kinase nuclear translocation and proliferation of bovine PASMCs[225]. Statins have also recently been shown to indirectly increase the activity of the nuclear peroxisome proliferators activated receptor γ (PPARγ)[226]. There is an evolving body of research relating insulin resistance (and consequent PPAR dysfunction) to PAH pathogenesis[227, 228]. PPARy is downregulated in human and experimental PAH and PPARy agonists are beneficial in animal models[229, 230]. It can be speculated that interactions with PPAR may partly explain statin's mechanism of action in PH animal models and, more importantly, that statin's positive effects on PPARγ may be clinically useful in PAH. One notable problem, however, with the experimental approaches in the above cited papers is the dose of statin used in the cell culture. This is typically in the range of 10-80μM of statin, which is many orders above the circulating dose of statin achieved after standard oral dosage (Cmax ranging from 0.082-1μM, see table 1.2). Accordingly, the relevance of any of these experimental studies to clinical application has to be questioned, and it is again considered by us that further experimental work, as we propose for this thesis, is required.

Whilst positive results have been reported in a small, observational case series of simvastatin treatment of PAH patients[231], it is our group's view that the currently running clinical trials of statin therapy for PAH (1 using simvastatin and 1 atorvastatin) are problematic. Firstly, there are important considerations in relation to statin choice for clinical use and dosage. For example, the CH rat experiments used 80mg/kg/day of simvastatin, whereas 80mg total is the current maximum dose of simvastatin used in humans. The problems with statin dosing in the reported cell culture experiments have been discussed in the previous paragraph. Drug interactions with currently used PAH therapies (warfarin, bosetan, sildenafil) are notable for both atorvastatin and simvastatin and it is not clear how these – eg simvastatin concentrations will typically be halved in patients taking bosentan - will be controlled for in these clinical trial protocols.

In conclusion, whilst there exists promising experimental data for statins in relation to pulmonary hypertension, a number of questions need resolved if we are to judge how best to exploit them in clinical practice. Based on the available knowledge about the effects of statins, and the cell signaling processes (ie p38 MAPK phosphorylation) involved in hypoxia-induced PAF proliferation it seemed plausible that statins would have an inhibitory effect on this. If an inhibitory effect were identitifed, it would allow further interrogation of the hypoxic-proliferative cell signaling pathway in these cells (in particular to determine if prenylated protein or membrane lipid rafts were involved). Also, our established cellular models would potentially allow experimental comparison of different statins, assessment of dose responses and comparison with other established pulmonary hypertension therapeutics. Given these conclusions, the experiments which form this body of work were conceived and undertaken.

1.10 Hypothesis

Review of the literature provided the following key points:-

- Hypoxia induces proliferation of PAFs and this is, either directly, or via interaction with other cells (eg PASMCs) an important aspect of hypoxic pulmonary vascular remodelling. Further study of the cellular and molecular mechanisms involved in this should provide important insights into the pathogenesis of chronic hypoxic pulmonary hypertension.

- Hypoxia-induced p38 MAPK phosphorylation in PAFs is a circulation and stimulus selective proliferative signaling mechanism. Consideration of signaling mechanisms upstream of MAPKs in other cell types and of hypoxic signaling in other contexts strongly suggests a role for Rho family GTPases. As direct inhibitors of p38 MAPK are not yet clinically available, inhibitors of upstream pathways may have important therapeutic potential.
- Statin drugs have promising effects on cellular and animal models of pulmonary hypertension but the pre-clinical knowledge is incomplete. As statins are now known to inhibit chronic hypoxic pulmonary vascular remodeling and have well established inhibitory effects on cell signaling processes including prenylated Rho family GTPases, it is plausible that they may have inhibitory effects on hypoxia-induced p38 MAPK phosphorylation and proliferation in PAFs.
- If an effect of statins on hypoxic PAFs were identified, further elaboration on this should provide answers to key questions about hypoxic signaling in these cells, and about the potential clinical utility of statins in hypoxic pulmonary hypertension.
- Whether the cellular effects of statins complement, duplicate or contradict the effects of established pulmonary hypertension therapeutics is unknown, and this cannot be adequately addressed in the available animal models. Experimental cell models of pathogenic processes in pulmonary hypertension provide an opportunity to compare different potential PH therapies.

The hypothesis was that statins would inhibit hypoxia-induced PAF proliferation and that exploration of this would allow interrogation of hypoxic cell signaling in PAFs and provide useful insights into the therapeutic potential of statin therapy for human pulmonary hypertension.

1.11 Aims

The aims of this thesis are:-

- To establish competency with a cell model of fibroblast proliferation in acute and chronic hypoxia.
- To establish the effects of statins on mitogen, acute hypoxia and chronic hypoxia induced proliferation and MAP kinase phosphorylation in pulmonary and systemic artery fibroblasts.
- To exploit the known effects of statins to interrogate hypoxic cell signaling upstream of p38 MAP kinase in pulmonary artery fibroblasts.
- To further explore the potential of statins as a new pulmonary hypertension treatment by studying dose responses, comparing different statins and differentiating any effects of statins from those of established pulmonary hypertension therapeutics.

Cell-cell interactions and release of PASMC mitogens by hypoxic PAFs is likely an important factor in chronic hypoxic pulmonary hypertension. A preliminary assessment of this was also proposed by:-

- Establishing a PAF-PASMC co-culture and conditioned media cell model.
- Assessing the effects of acute hypoxia on PAF-PASMC interactions / PASMC proliferation and establishing whether statins, p38 MAPK inhibitors or established PH therapeutics have any effect on these.

Chapter 2

Materials and Methods

2.1 Materials

All general chemicals were of analar grade and supplied by Sigma (Poole, Dorset UK). All tissue culture plastics were from Greiner Labortechnick Ltd (Gloucestershire.UK). All components of tissue culture medium were purchased from Gibco Life Technologies (Paisley, Scotland). Galaxy-R incubators were supplied by Wolf Laboratories (York, UK). [*methyl*-^{3}H]thymidine was from Amersham (Little Chalfont, Bucks). Mevalonate, squalene, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) were from Sigma (Poole, Dorset UK). Simvastatin, pravastatin, cholesterol pathway intermediates and inhibitors, Rho kinase inhibitor (hydroxyfasudil) and Rac1 GEF exchange inhibitor (NSC23766) were from Calbiochem (Nottingham, UK).

I am grateful to Professor A Jardine (Glasgow University) for the gift of fluvastatin. I am also grateful to Drs G Boutros (Pfizer, UK) for atorvastatin and sildenafil, J Jepf (Actelion, Switzerland) for bosentan and C Sterrit (United Therapeutics, USA) for treprostinil diethanolamine. All of these were donated by the manufacturing pharmaceutical company without restriction.

Antibodies used for Western Blot analysis were from New England Biolabs (Hertfordshire, UK). Gel electrophoresis equipment was purchased from Bio-Rad Laboratories (Herts, UK). Immobilon-P transfer membrane was from Millipore (Massachusets, USA). ECL Western blotting detection reagent was from Amersham (Buckinghamshire, UK).

2.2 Animal models

2.2.1 Rat model

Cells derived from the pulmonary artery and aorta of adult Sprague-Dawley rats from the "In House" breeding stock of the University of Glasgow's Central Animal Facility were used. Thirty to thirty-three day old rats were maintained in normoxic conditions on a 12 hour (h) light/dark cycle and allowed free access to standard diet and water.

2.2.2 Chronic hypoxic rat model

The chronic hypoxic rat model was established and maintained in the laboratory of Professor R Wadsworth (University of Strathclyde).

2.2.2.1 Chamber design

The chamber is designed to hold two standard rat cages, with up to four rats in each cage. The structure of the chamber is made from transparent high resistance Plexiglas. Air is continually removed from the chamber by the pump. Air constantly flows through the chamber at 45L/minute, ensuring that moisture and CO_2 do not build up. Temperatures are similar both inside and outside of the chamber.

2.2.2.2 Maintenance of animals

The chamber was housed in a specially designed environmental room which maintains temperature at approximately 21^{o}C, humidity at 55%, gives 20 changes of filtered air per hour and maintains a 12h on 12h off light cycle. Animals used in the experiments were obtained from credited commercial suppliers (Harlan UK Ltd, Nottingham). The Wistar rats supplied were male specific, pathogen-free and ordered in at age 28-30 days (approximately 60g weight). Animals were allowed to acclimatise within the environmental chamber for five days before being established in chronic hypoxia.

2.2.2.3 Maintenance of rats in hypobaric hypoxia

The chamber door was removed and the rat cage placed in the chamber with the food and water dispensers at the rear, thus allowing observation of the rats. The chamber door was replaced and the pump switched on. The chamber was taken down to the

desired pressure (55mbar – 418mmHg) in small steps by slowly closing the inlet valve and observing the pressure reading on the gauge. Once the stable experimental pressure was reached, rats were checked every 15min for the following hour to ensure that the pressure remained stable and the animals were not in distress. The chamber could then be left to run for the experimental time required. Checks were made 5 times a day to ensure that pressure readings were not fluctuating beyond desired levels, temperature inside and outside the chamber remained between 20 and 23^{o}C, animals were not showing signs of distress and that the pump was in good operating condition. When the animals required fresh food and water, usually every three days, the chamber was taken down to atmospheric pressure over 2h by opening the inlet valve. Once atmospheric pressure was reached, the pump was switched off and allowed to cool for half an hour while the rat cages were cleaned and animals given fresh food and water. Following this, the animals were placed back in the chamber, the pump switched on and the chamber taken back to 55mbar over a period of two hours. After 14 days in the chamber, the rats were removed and sacrificed.

2.2.3 Bovine model

Cells derived from bovine pulmonary and mesenteric arteries were used. Tissue was obtained fresh on the day of experimentation from a local abattoir (Sandyford, Paisley). Lungs and small intestine/mesentery were removed from healthy, freshly slaughtered cattle and transported to the laboratory in a container filled with chilled Krebs-Henseleit Solution (NaCl 118mM, $NaHCO_3$ 25mM, KCl 4.7mM, KH_2PO_4 1.2mM, $MgSO_4$ 1.2mM, $CaCl_2$ 2.5mM, and Glucose 11mM).

2.3 Primary cell culture

Fibroblasts and smooth muscle cells used throughout these studies were obtained from primary culture by explant using tissues derived either from bovine or rat. The explant techniques and cell lines have been established in the Scottish Pulmonary Vascular Unit laboratory for many years. That the techniques achieve pure fibroblast and smooth muscle cell lines has been previously clarified by staining for α-actin[150].

To prevent fungal and bacterial contamination of cell lines, all steps such as making up solutions, changing media or preparing experimental reagents were conducted

under sterile conditions, within a clean Microflow laminar flow hood (model number M25121/1) (Figure 2.1). The flow hood was dismantled and cleaned regularly and before use each day was sprayed liberally with 70% (v/v) ethanol. Anything taken inside the flow hood (i.e. pipettes and reagent bottles) was also sprayed with ethanol and sterile gloves were worn throughout. Non-sterile consumables and reagents were sterilised using a Prestige Medical "Classic 210" autoclave or sterile-filtered (0.2μM pore filter).

2.3.1 Primary adventitial fibroblast culture

Lobar pulmonary artery was dissected free from the lung of either a freshly dissected cow or rat (Figure 2.2). A section of artery located towards the apex (Figure 2.3) of the lobe was cut longitudinally and opened into a flat sheet (Figure 2.4). Pulmonary artery fibroblasts were prepared using the technique of Freshney[232], with some modifications[178]. Muscular tissue and endothelial cell layers were removed by gentle abrasion of the vessel using a sterile razor blade (Figure 2.5). The remaining tissue (adventitia) was then dissected into $5mm^2$ portions and approximately 25 portions of this size were evenly distributed over the base of a $25cm^2$ culture flask containing 2ml of Dulbeccos modification of Eagles medium (DMEM) with 20% fetal calf serum (FCS), supplemented with penicillin/streptomycin (400iu/ml and 400μg/ml) and amphotericin B (5μg/ml) (Figure 2.6). The explants were incubated in a humidified atmosphere of 5% CO_2 in air at 37ºC. Within a few days cells were observed growing out from the tissue fragments. Once a monolayer of cells had partially covered the flask (Figure 2.7), cells were lifted from the flask by trypsinisation as described in Chapter 2.3.3. Tissue fragments were removed by aspiration.

In the bovine and rat model the main branch of the pulmonary artery was used to acquire pulmonary artery fibroblasts. To study fibroblasts from the systemic circulation, the mesenteric artery was dissected from the cow and the aorta dissected from the rat with primary cell culture as for the pulmonary artery fibroblasts.

Figure 2.1 Microflow Laminar Flow Hood

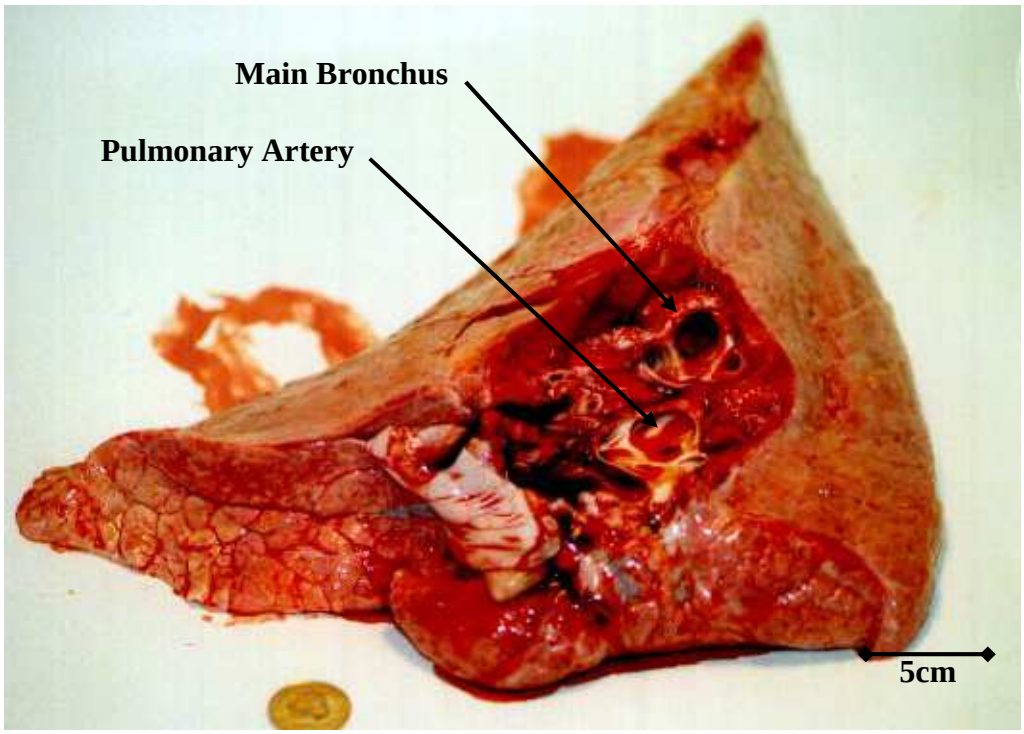


Figure 2.2 Location of Pulmonary Artery in Bovine Lung

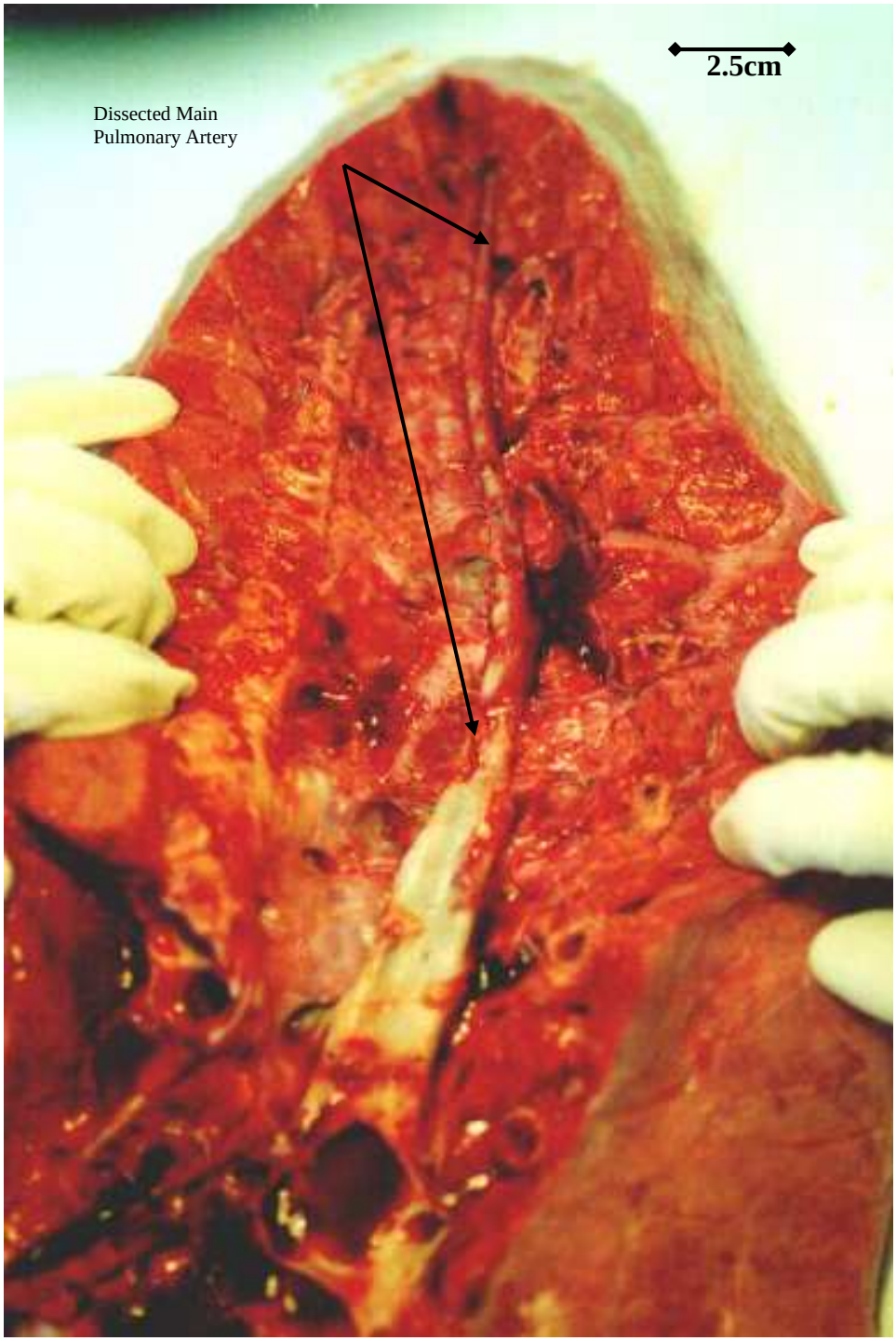


Figure 2.3 Longitudinally Dissected Pulmonary Artery in Bovine Lung

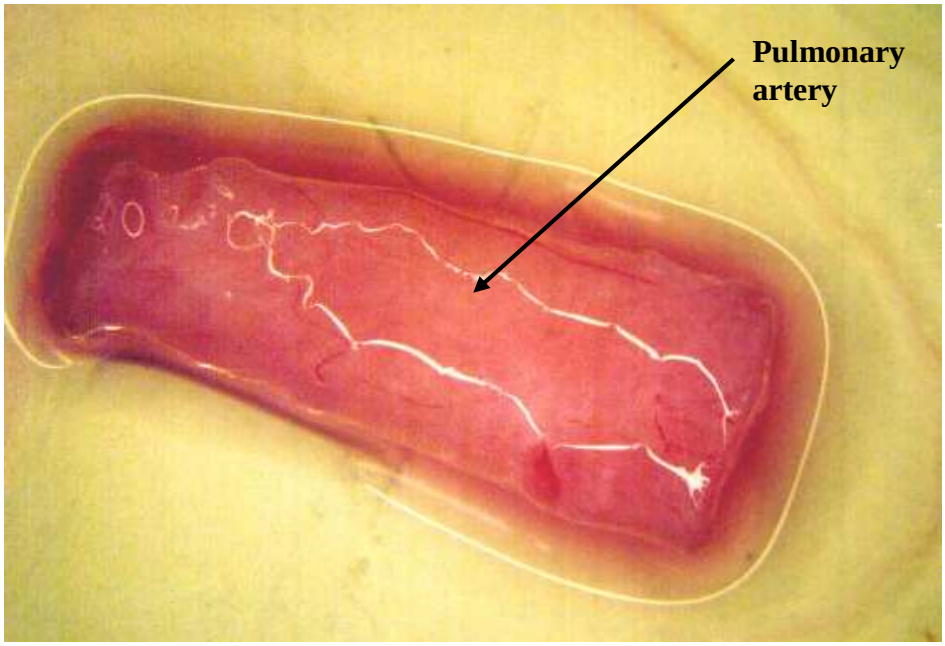


Figure 2.4 Cleaned and Dissected Pulmonary Artery

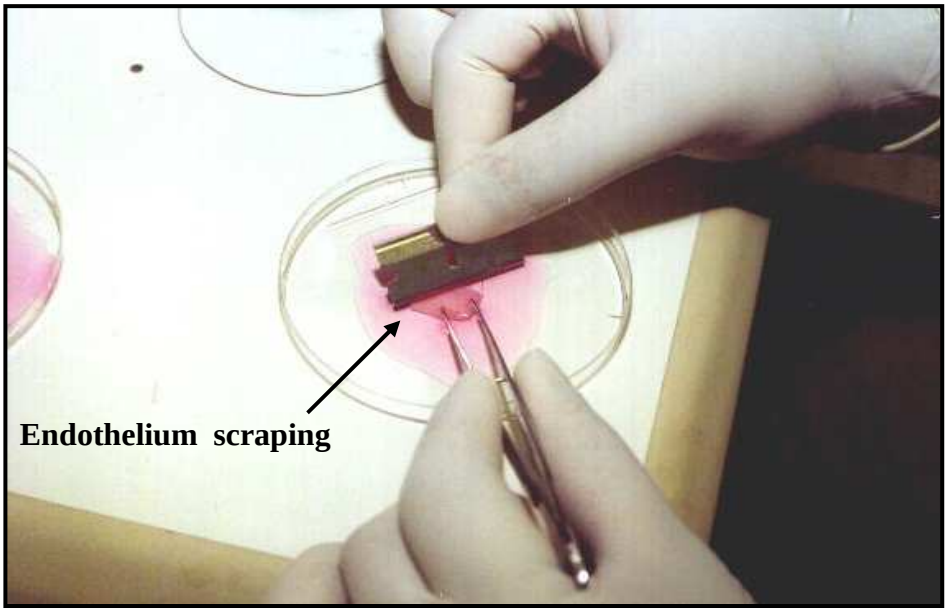


Figure 2.5 Removal of the Intimal and Medial Layers

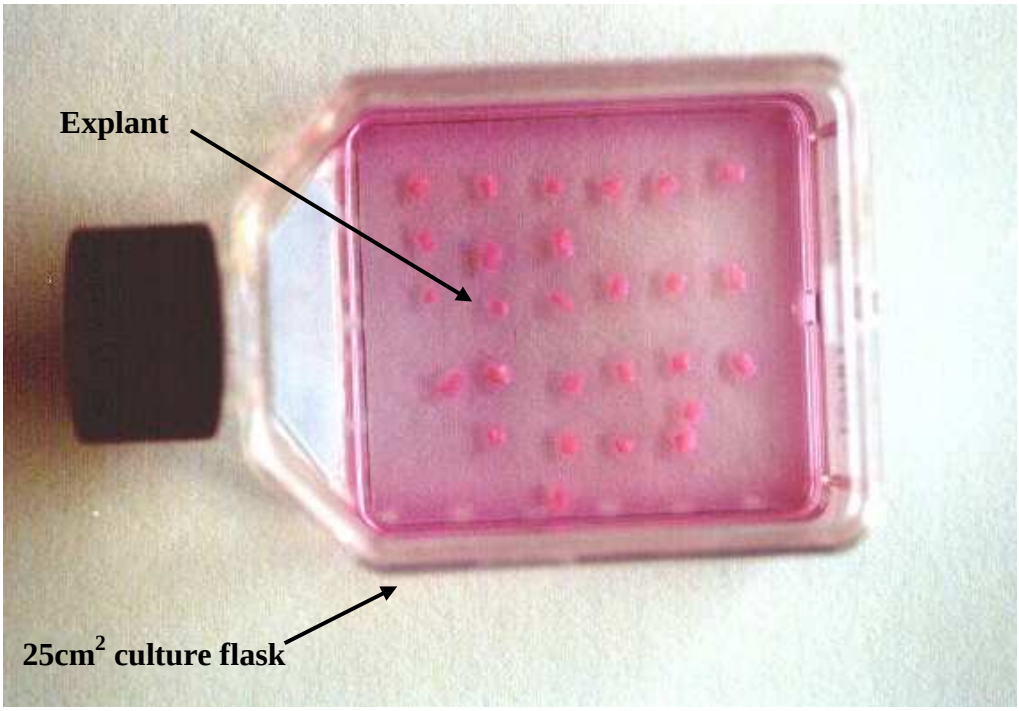


Figure 2.6 Explants in 25cm^3 Culture Flasks

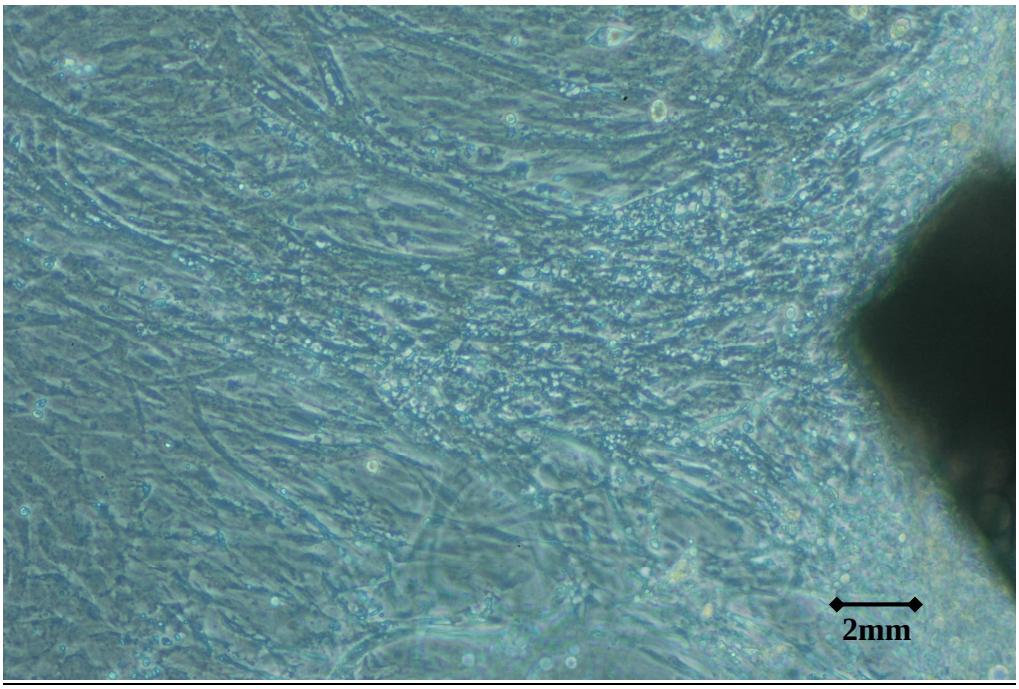


Figure 2.7 Pulmonary Artery Fibroblasts Growing From Explants

2.3.2 Primary vascular smooth muscle cell culture

Smooth muscle cells were prepared from bovine pulmonary arteries, using a modified explant technique. Other than the vessel preparation step (when intimal and adventitial layers were removed by gentle abrasion, leaving the medial layer) the technique was identical to that used to obtain adventitial fibroblasts (Chapter 2.3.1).

2.3.3 Routine cell maintenance

Cells were grown routinely in 75cm^3 culture flasks in Dulbecco's modification of Eagle's medium (DMEM), supplemented with penicillin (200 units/ml), streptomycin (200 ug/ml), L-glutamine (27 mg/ml) and 10% foetal calf serum (FCS). The cultures were kept in a humidified atmosphere of 5% CO_2 in air at 37^{o}C. Culture medium was changed every 2 days and cells were passaged just prior to confluency.

For cell passage, culture medium was removed and 2ml of trypsin solution (0.05% trypsin / EDTA 0.02%) was added to the layer of cells and then aspirated. A further 2ml of trypsin solution was then added and left on the surface of the cells for approximately 10s before aspiration. The cells were then incubated at 37^{o}C and observed at 2 minute intervals using a light microscope (Olympus CK2) until they were seen to have partially dislodged from the flask. Gentle tapping of the dish was used to completely dislodge the cells and 10ml of DMEM containing 10% FCS was then added to the flask to re-suspend them. A portion of this cell suspension (1ml) was then aliquoted into new flasks containing another 9mls of fresh medium. At this stage cells could be also be aliquoted into 6, 12 or 24 well plates, as required for experimentation. Cells were used for experimentation between passage 3-10.

2.3.4 Cell freezing / thawing

Confluent cells at passage 2 or 3 were trypsinised as above, resuspended in 10ml of 10% FCS culture medium and centrifuged at 1000g for 10 minutes. Medium was removed and the cell pellet was resuspended in 1ml of cryopreservation medium (DMEM containing 10% FCS and 10% dimethyl sulphoxide). This was transferred to a 2ml cryotubes and cooled for 1 hour each at +4°C and -20°C, then at -80°C overnight. Cryotubes were then maintained in the liquid phase of liquid nitrogen.

When required, frozen cells were removed from liquid nitrogen and thawed rapidly in a water bath at 37C. Cryotubes were swabbed with 70% v/v ethanol and the cell suspension was transferred to a 75cm^2 culture flask containing 9ml of culture medium with 10% FCS. After 4-6 hours the cells were seen to have attached to the flask and the medium was replaced with 10ml of fresh 10% FCS culture medium.

2.4 Acute hypoxia

Maintenance of cells in a nitrogen-supplemented, humidified, temperature-controlled incubator (Galaxy R, Wolf Laboratories, York, UK) allowed control of internal oxygen levels at 0-21%, whilst CO_2 level was maintained at 5% (Figure 2.8). Due to the large volume of nitrogen required to sustain a suitable degree of hypoxia, nitrogen cylinders were linked using a Pneuchange automatic gas cylinder change over unit (NTC, Woulton, Liverpool, UK) which activated a fresh supply of nitrogen as required.

Cells were routinely maintained for the acute hypoxic phase of experiments in an atmosphere of 5% O_2 and 5%CO_2. Previous assessment of the tissue culture supernatant with oxygen and pH probes has confirmed that these conditions PO_2 of 35mmHg for the hypoxic conditions, with pH stable over the 24 hour experimental period[233]. Experimental controls were achieved with the use of a second incubator which could simultaneously maintain control cells in normoxic experimental conditions.

2.5 Preparation of statin drugs and other reagents

Statins, cholesterol pathway intermediates and inhibitors were prepared in stock solution (0.04M and/or 200μM, depending on experimental concentrations required) with appropriate diluent, as summarised in table 2.1. All reagents had manufacturer or supplier data indicating stability, in appropriate storage, for >2 months.

Figure 2.8 Galaxy-R Hypoxic Chamber: Humidity, O_2 and CO_2 Regulated

Reagent	Supplied as	Molecular Weight	Diluent	Storage
Atorvastatin	Solid powder	1209.42	DMSO	-20℃
Bosentan	Solid powder	573.6	dH_2O @ 50℃	N/A*
Fluvastatin	Solid powder	433.46	DMSO	-20℃
FPP	200µg in 200µL methanol	433.4	Ethanol	-20℃
FTAse inhibitor I	Solid powder	470.7	DMSO	-20℃
GGPP	200µg in 200µL methanol	450.44	Ethanol	-20℃
GGTI-2133	Solid powder	456.5	DMSO	-20℃
Hydroxyfasudil	10mM solution in water	343.8	SFM	-20℃**
Methyl-β-cyclodextrin	Solid powder	1320	SFM	+4℃
Mevalonate	Solid powder	130.15	SFM	-20℃
NSC-23766	Dry powder	531	SFM	-20℃
PDGF-BB	Lyophilised Powder	28-31kDa	HCL solution***	-20℃
Pravastatin	Solid powder	446.5	SFM	-20℃
SB 203580	Solid powder	377.4	DMSO	-20℃**
Sildenafil	Solid powder	666.7	Ethanol	+4℃
Simvastatin	Solid powder	458.6	DMSO	-20℃
Squalene	Liquid (98%)	410.73	DMSO	-20℃
Treprostinil diethanolamine	Solid powder	495.66	SFM	+4℃
Zaragozic acid	Solid powder	756.7	SFM	-20℃

Table 2.1 Preparation and Storage of Reagent Stock Solutions

SFM: Serum free medium DMSO: Dimethyl sulphoxide

PDGF: Platelet derived growth factor

*Bosentan is unstable (precipitates) in solution for >24 hours

**Light sensitive

***HCL solution for preparing PDGF-BB comprises 0.4M hydrochloric acid with 1mg/ml bovine serum albumin

2.6 Determination of cell proliferation and viability

2.6.1 [^{3}H] thymidine assay

Cell proliferation was measured by determining the uptake of [^{3}H]thymidine into DNA.

Cells were seeded at a density of $5x10^3$ cells / well into 24-well plates in 500µl of culture medium and grown to 60% confluency. Cells were then quiesced for 24 hours by replacing the medium with 500µl serum-free DMEM. Cells were then stimulated with appropriate agonists and incubated for 24 hours, either in a normal CO_2 incubator (5% CO_2) or in the hypoxic incubator to obtain an acute hypoxic exposure (Chapter 2.4). In the latter case, the O_2 content of the atmosphere was reduced from 21% to 5% by flooding with N_2.

Dilute [*methyl*-^{3}H]thymidine solution was prepared by adding 10uL of [methyl^{3}H]thymidine (1.0mCi/ml) to 2.5ml serum-free DMEM. For the final 4h of agonist stimulation, 25uL of dilute [*methyl*-^{3}H]thymidine solution was added to each well of the 24 well plate, achieving a radiation concentration of 0.1µCi/well.

At 24 hours, the reaction was stopped by washing the cells twice in ice-cold phosphate buffered saline (PBS, 500µl/well). Proteins were precipitated by washing three times with 500µl/well 5% trichloroacetic acid (TCA) and lipid fractions were solubilised by washing twice with 100% ethanol (500µl/well) for 30mins. The acid / alcohol insoluble material was dissolved in 0.3M sodium hydroxide (500µl/well) for 30mins. The contents of each well were transferred to 1ml eppendorf tubes, to each of which was added 0.9mls of Ecoscint A (Ecoscint, Atlanta, Georgia, USA) scintillation fluid. Vials were vortexed for 1min and allowed to settle in darkness overnight. Radioactivity was measured by scintillation counting using a Wallac 1409 scintillation counter. Results are expressed as disintegrations per minute (DPM).

2.6.2 Cell counting and trypan blue staining

Cells were seeded into 12 well plates, grown to 60% confluency, quiesced and then stimulated in normoxic or hypoxic conditions with appropriate agonists, as above. At the end of the experiment cells were trypsinised with 2 brief exposures to 400μL of trypsin solution (0.05% trypsin / EDTA 0.02%) and, once detached, resuspended in 1ml PBS. 100μL of cell suspension was mixed with 100μL 0.4% w/v trypan blue solution. 20μL of the resulting solution was transferred to a Neubauer haemocytometer. Total cells and non-viable cells (blue-stained) lying in 4 corner squares of 1 chamber in the haemocytometer were counted under low power with the light microscope. Total number of cells in the original solution was then calculated as standard ie:-

$$\text{Number of cells in original solution} = 2 \times \frac{\text{Total number of cells counted in 4 squares}}{4} \times 10000$$

2.7 Co-culture and conditioned media

2.7.1 Preparation of pulmonary artery fibroblast conditioned media

PAFs were grown to approximately 60% confluency in 24-well plates and quiesced in serum free medium for 24 hours. PAF conditioned media was then prepared by removing the current medium and adding 0.5ml of fresh serum-free media to each well. Drugs or other investigational compounds were added, as required for the specific experiment.

Plates were then maintained for 24 hours in either normoxic (21%) or hypoxic (5%) conditions, as required. This process provided conditioned media containing any growth promoting or inhibiting mediators secreted from PAFs. The differential use of untreated / drug-treated and normoxic / hypoxic PAFs allows a comparison of the effects of these condtions on PAF mediator release.

2.7.2 Application of conditioned media to pulmonary artery smooth muscle cells

PASMCs were separately grown to approximately 60% confluency in 24 well plates and serum starved for 24 hours. This 24 hour quiescence period coincided with the 24 hours when PAF media was being conditioned.

At the end of the 24 hour media conditioning / PASMC quiescing period, serum-free media was removed from the PASMCs and replaced by the 0.5ml of experimental PAF conditioned media. Experimental mediators were added, as required, and plates were maintained for 24 hours in normoxic (21%) or hypoxic (5%) conditions. [^{3}H] thymidine assay was then performed, as before (Chapter 2.6.1) to determine PASMC proliferation.

2.7.3 Co-culture of PAFs and PASMCs

The use of 24 well plate co-culture cell inserts allows 2 different cell types to be maintained in direct contact – sharing the same media – for an experimental period. The base of the cell inserts comprises a semi-permeable membrane (Figure 2.9). We selected inserts with a membrane pore size of 1μM, which allows free diffusion of macromolecules within the shared cell culture media, but not cell migration. For these experiments we were interested in the effects of PAFs on PASMC proliferation. Accordingly, we grew the PASMCs on the 24 well plate, and the PAFs on the cell inserts (Figure 2.10).

PAFs were grown to 90% confluency on individual 24 well plate cell inserts; for this initial phase the cell inserts were maintained in the wells of a 'blank' 24 well plate, containing standard 10% supplemented DMEM. PASMCs were grown simultaneously to 60% confluency, on separate 24 well plates. Both cells types were then quiesced for 24-48 hours (variable period to 'synchronise' the two cell types at appropriate confluency) in serum free media.

Cell inserts containing PAFs were then placed within the wells of the 24 well plate containing the PASMCs. 0.5ml of serum free media +/- required experimental mediators were applied to the wells of the 24 well plates, containing the PASMC and the PAFs (on the cell inserts). These co-culture experimental plates were then maintained for 24 hours in normoxic (21%) or hypoxic (5%) conditions. The PAFs on cell inserts were discarded and PASMC proliferation was determined (chapter 2.6).

2.8 Detection and analysis of proteins

2.8.1 Preparation of samples for protein analysis

Cells were seeded at a density of $15x10^3$ cells/well into 6-well plates, grown to 90% confluency and then growth-arrested in serum-free DMEM for a period of 24 hours. Cells were then stimulated with agonist and maintained in normoxic or hypoxic conditions for an appropriate time period (determined by protein in question). Culture plates were thereafter maintained on ice. Culture medium was removed and the cell monolayer was washed x2 with 2ml of ice-cold PBS. The cells were then lysed with 50µl radioimmunoprecipitation assay buffer (RIPA buffer; comprises Tris-HCl 50mM pH 7.4, NP-40 1%, $C_{24}H_{39}O_4Na$ 0.25%, NaCl 150mM, EGTA 1mM, PMSF 1mM, Na_3VO_4 1mM, NaF 1mM, CLAP 1/1000, pH 7.4). Cells were mechanically disrupted using a cell scraper. The contents of each well were transferred to 1ml eppendorf tubes on ice and stored at -80°C.

2.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analysed using SDS-PAGE using precast 10% SDS-PAGE gels.

Protein samples to be analysed by SDS-PAGE were first diluted to 50µg in SDS Sample Buffer, (93mM Tris-HCl pH 6.8 and 20mM dithiothreitol (DTT), 1mM sodium-EDTA, 10% glycerol (v/v), 2% SDS (w/v) and 0.002% bromophenol blue (w/v)). Samples were boiled for 5 min to denature proteins and disrupt disulphide bonds. The required volume was loaded into the individual wells of the stacking gel using loading tips. Pre-stained SDS protein molecular weight markers (Biorad) of known size were also placed in one lane of the gel. After loading samples, polyacrylamide gels were assembled into a Bio-Rad electrophoresis unit. The two chambers of the unit were filled with Electrophoresis Buffer and the polyacrylamide gel subjected to electrophoresis for approximately 1 hour at a constant current of 240mA.

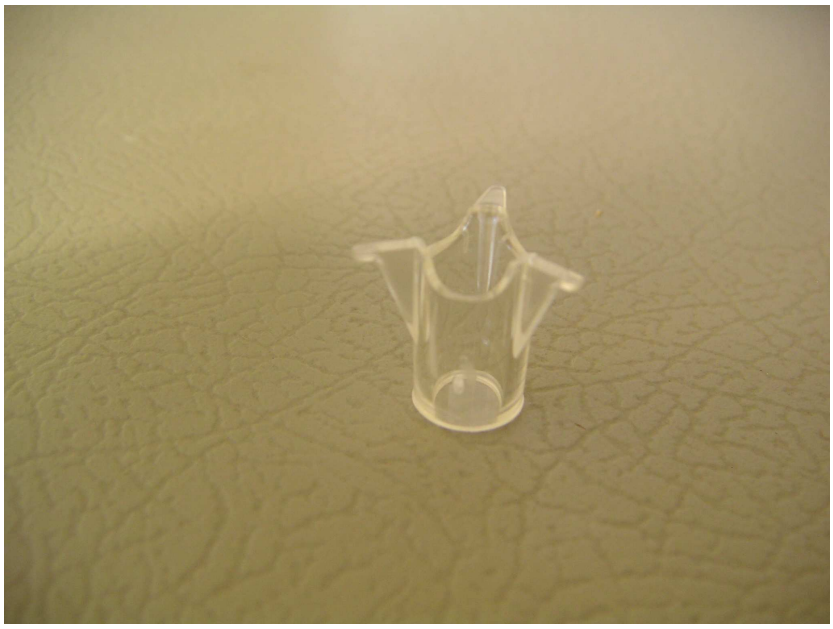


Figure 2.9 24 Well Plate Co-culture Cell Insert.

Figure 2.10 Co-culture Cell Insert in a 24 Well Plate.

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

2.8.3 Western blot analysis

2.8.3.1 Transfer to nitrocellulose membrane

Following completion of electrophoresis, polyacrylamide gels were removed from the apparatus and washed once in Blotting Buffer (20% methanol (v/v), 25mM Tris-base, 192mM glycine). Proteins were transferred from the polyacrylamide gel to the nitrocellulose by assembling a transfer cassette with the nitrocellulose juxtapositioned between the polyacrylamide gel and the cathode. By this method negatively charged proteins were transferred to nitrocellulose for 1.5-2h at a constant 1 amp current.

2.8.3.2 Immunoblotting

The nitrocellulose blots were washed in PBS/T (PBS containing 0.02% Tween-20 (v/v)) and blocked for non-specific binding for 3h on a rocking platform at room temperature in PBS/T supplemented with 10% non-fat milk (Marvel) (w/v). Blots were incubated for 1h in PBS/T supplemented with 5% Marvel (w/v) containing a 1:1000 dilution of primary antibody: either rabbit anti-dual phosphorylated MAPK antibody (to assess MAPK activation) or rabbit anti-MAPK antibody (to assess total MAPK and ensure equal protein loading). The blots were rinsed and washed 4 times over an hour with PBS/T and then incubated for a further 60 mins in PBS/T + 5% Marvel (w/v) + secondary donkey anti-rabbit IgG antibody conjugated to HRP (1:15,000). The blots were then washed as before.

2.8.3.3 Secondary antibody detection

Following completion of washing procedures proteins were detected using a method of enhanced chemilumenescence (ECL). Blots were incubated in ECL solution (Amersham) for 30s and sandwiched between acetate film. Care was taken to ensure all air bubbles were removed. Blots were then placed in an X-ray cassette and light emission from the horseradish peroxidase enzymatic action on its substrate contained

within the ECL solution was detected following exposure (1-10mins) of nitrocellulose blots to a piece of X-ray film using a KODAK M35-M X-OMAT processor.

2.8.3.4 Re-probing nitrocellulose membranes

In instances where the same membrane was used to probe for different proteins, primary and secondary antibodies were stripped from the nitrocellulose by incubating in Stripping Buffer (100mM B-mercaptoethanol, 2% SDS and 62.5mM Tris-HCl, pH 6.7) for 30 minutes at 50^{o}C with agitation. Blots were then rinsed with PBS/T 4x over 40 mins before the immunodetection protocol was repeated.

2.9 Data analysis

For cell counting and DNA synthesis experiments, data are expressed as mean ± S.D. for 4 replicate plates from the same experiment. Experiments were repeated in cells from a minimum of 4 different animals. The statistical significance of differences between mean values from control and treated groups were determined by Student's t-tests and all graph preparation and data analysis was conducted using GraphPad (Prism) software. Two-tailed probability values of less than 0.05 ($P<0.05$) were considered to be significant.

Chapter 3

Effects of Statins on Rat Pulmonary and Systemic Artery Fibroblast Proliferation

3.1 Introduction

As discussed in the general introduction (Chapter 1), regardless of the aetiology, pulmonary hypertension is characterised by vascular remodelling affecting the intimal, medial and adventitial layers of the pulmonary artery[25]. In hypoxic pulmonary hypertension, in animals and humans, the earliest changes are seen in the adventitial compartment of the pulmonary arteries, with fibroblast proliferation and adventitial thickening[48]. There is now a substantial body of work characterising cellular and molecular changes in pulmonary vascular remodelling. In particular, the available data supports a concept of the pulmonary artery fibroblast (PAF) as a "concert leader" of the remodelling in hypoxic pulmonary hypertension[3], indicating that this may be a valuable target for further research into therapeutic agents which may attenuate hypoxic pulmonary hypertension.

Previous work in the SPVU laboratory has characterised aspects of hypoxic PAF behaviour. In particular, PAFs have been shown to proliferate in acute hypoxia[178]. This response is selective for the pulmonary circulation (systemic adventitial fibroblasts shown no change in proliferation in hypoxia), is preserved across animal species[180] and is dependant on the phosphorylation of the stress responsive protein p38 MAP kinase[150, 155, 171]. Also, proliferative and signalling mechanisms in acute hypoxic PAFs have been shown to mirror those in PAFs from chronic hypoxic animals[177]. Firstly, this body of work provides a basis for further investigation of cellular and molecular pathways in hypoxic pulmonary vascular remodelling. Secondly, it demonstrates the utility of an acute hypoxic rat PAF model. In particular, the identical proliferative and cell signalling phenotype of acute hypoxic PAFs, when compared with either human PAFs and pulmonary hypertensive animal PAFs demonstrates the relevance of this cell model, for preliminary experiments exploring potential hypoxia-selective antiproliferative agents. As this cell model is robust and readily available it was selected for the initial 'battery' of experiments with statin drugs, proposed for this thesis.

Observations regarding the demonstrated pleiotropic effects of the HMG-CoA reductase inhibitors (statins) led to the suggestion that these drus may be effective in pulmonary hypertension. Various groups have now shown positive effects of statins

on experimental pulmonary hypertension, in different animal models, though there has been one recent contradictory report (see Chapter 1.10.6 for more details). Based on this, clinical trials of statins for pulmonary arterial hypertension have commenced but, as also previously discussed, there is still a substantial gap in the pre-clinical knowledge base in relation to statins and pulmonary hypertension.

We considered the possibility of further statin treatment of animals models, in particular the chronic hypoxic rat model. We discounted this as an approach to address the aims of this thesis at an early stage. Firstly, whole animal studies do not allow the assessment of the primary molecular mechanism of action of the statin. Secondly, dose-ranging and individual statin-comparison experiments would require a prohibitive number of animals. Also, it is not clear how well dosing studies in animals translate to human use.

As statins have been shown to have antiproliferative effects on diverse cell types - including fibroblasts[234] - we suspected that they may have useful anti-proliferative effects on PAFs. When the known effects of statins on cell signalling pathways (see chapter 1.10.5) are considered alongside hypoxic signalling pathways (eg RhoA-Rho kinase or Rac1-NADPH oxidase) it was considered possible that statins may specifically have effects on hypoxic proliferation in PAFs.

If statins were shown to have an effect on acute hypoxia-induced proliferation in rat pulmonary artery fibroblasts (RPAFs), this would provide a basis to further explore the therapeutic potential of statins, using our established cell models. Also, the known effects of statins on cell signalling could then be exploited to further interrogate hypoxic signalling in these cells. We hypothesised that statins would have an inhibitory effect on acute hypoxia-induced proliferation in RPAFs and the experiments in this chapter were conceived to clarify this.

3.2 Methods

As outlined in the general methods (Chapter 2) rat pulmonary artery fibroblast (RPAF) and rat aortic fibroblast (RAF) cell lines were established from normal adult male Sprague-Dawley rats. Cell lines were characterised (by negative smooth muscle α-actin staining) and morphology reviewed at each experimental stage to exclude cell transformation or infection. Proliferation and viability of RPAF and RAF cells was determined in experimental conditions - normoxia vs acute hypoxia (5%) +/- serum, statin or other mediator - by cell counting, thymidine assay and trypan blue staining. Results are expressed in the figures as mean +/- 1 standard deviation.

3.3 Results

3.3.1 Confirmation of the Fibroblast Cell Type

Initial cultures of individual cell lines were stained at the time of initial passage, after explant, for α-smooth muscle actin. Positively stained cells would indicate smooth muscle contamination and this cell line would not be used for experimentation.

Visual assessment of cell morphology undertaken at each passage and daily in cells undergoing experimentation confirmed the stability of the cell lines. Figure 3.1 shows fibroblasts derived from the pulmonary artery, confluent in a cell culture plate. In particular, the shape of the cell provided identification of the cell type. Fibroblasts appeared multipolar or bipolar and well spaced when subconfluent but, when confluent, typically appeared bipolar and less well spread[233].

3.3.2 Effect of Fluvastatin on Serum-induced DNA Synthesis And Proliferation of Rat Adventitial Fibroblasts

To determine the effects of fluvastatin on serum-induced proliferation of pulmonary and systemic adventitial fibroblasts, incremental doses of serum (1, 3 and 5%) and fluvastatin (0.1, 0.5, 1, 2.5, 5, 10, 20 and 50μM) were variously added to serum-starved semi-confluent cells during 24 hour proliferation assay. [^{3}H] thymidine uptake and total cell counts were determined, and cell viability was assessed using trypan blue staining.

Figure 3.2 illustrates the effect of fluvastatin on serum-induced DNA synthesis, measured by [^{3}H] thymidine uptake, in RPAFs (panel A) and RAFs (panel B). Figure 3.3 similarly outlines the effects of fluvastatin on serum-induced proliferation, as measured by total cell counts.

Increased DNA synthesis and proliferation of RPAFs and RAFs was seen with increasing concentrations of serum. Fluvastatin at doses ≤1μM had no effect on serum-induced proliferation. Doses of fluvastatin of 10μM or greater completely inhibited serum-induced proliferation ($p<0.001$) with [^{3}H] thymidine uptake and total cell numbers similar to serum-starved control cells. Fluvastatin 5μM had an intermediate effect on RPAF and RAF proliferation.

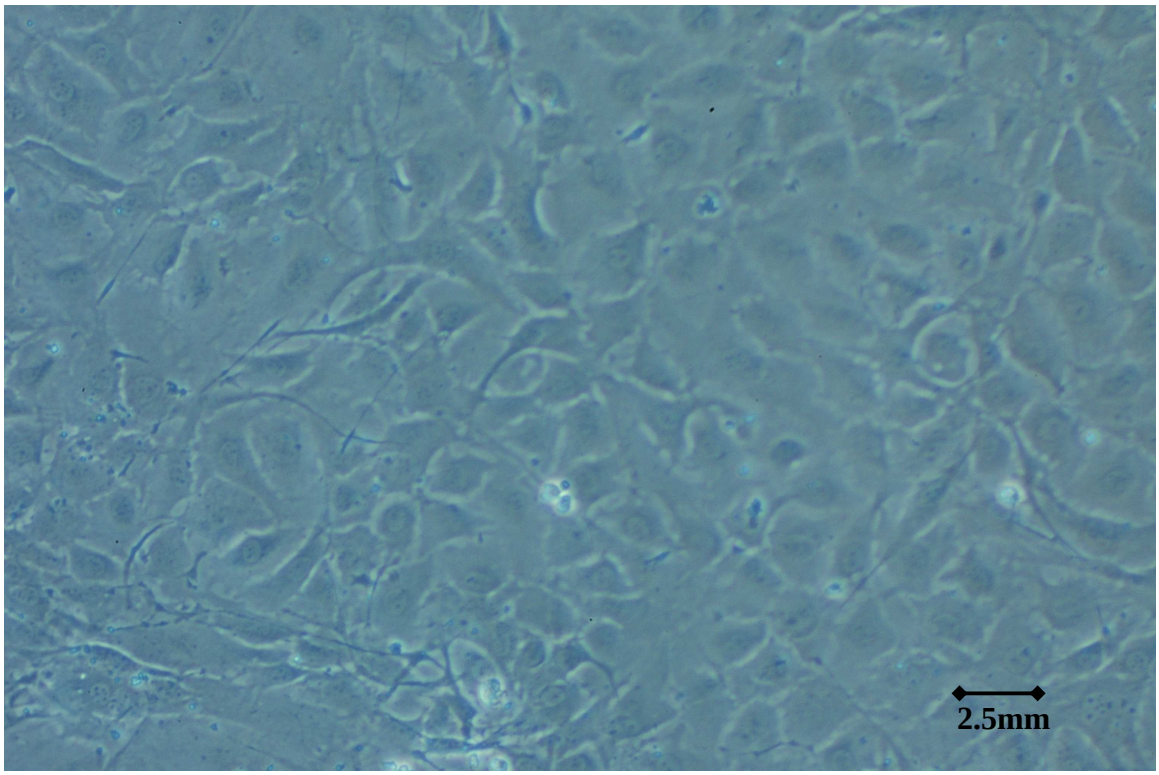


Figure 3.1 Confluent Pulmonary Adventitial Fibroblasts

Fibroblasts from the pulmonary artery were visualised by light microscopy.

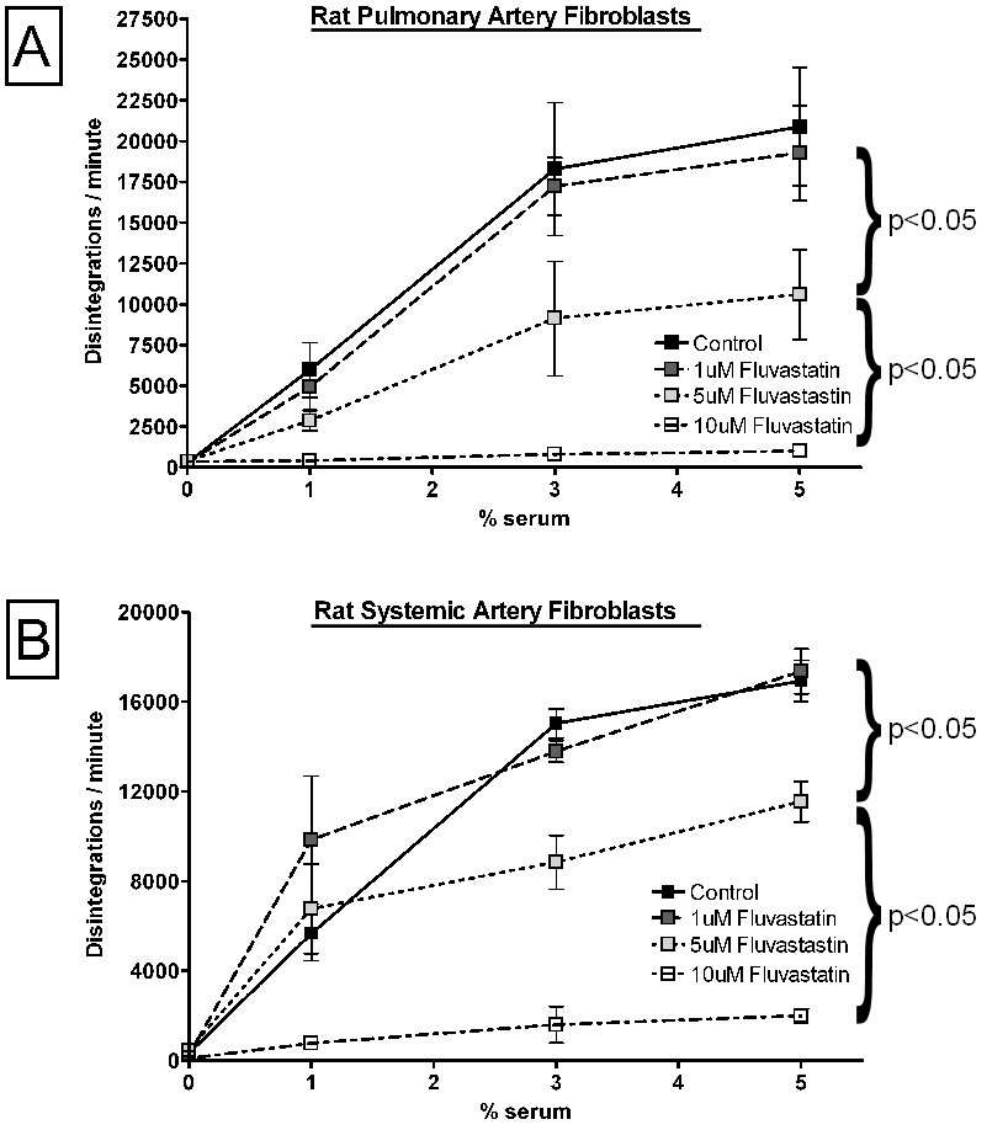


Figure 3.2 Effects of Fluvastatin on Serum-induced [^{3}H] Thymidine Uptake by Adventitial Fibroblast Cells

Growth arrested rat pulmonary artery fibroblasts (Panel A) and rat aortic fibroblasts (Panel B) were stimulated with graded doses of serum and fluvastatin, for 24 hours. DNA synthesis, as an index of cell proliferation, was measured by [^{3}H] thymidine uptake. Experiments with other doses of fluvastatin (0.1, 2.5, 20 and 50μM) were also undertaken but these are omitted from the graphs for clarity. Values shown are mean +/-SD from 4 replicate experiments on cells from the same animal; experiments were repeated on cells from >4 animals and representative graphs are shown here.

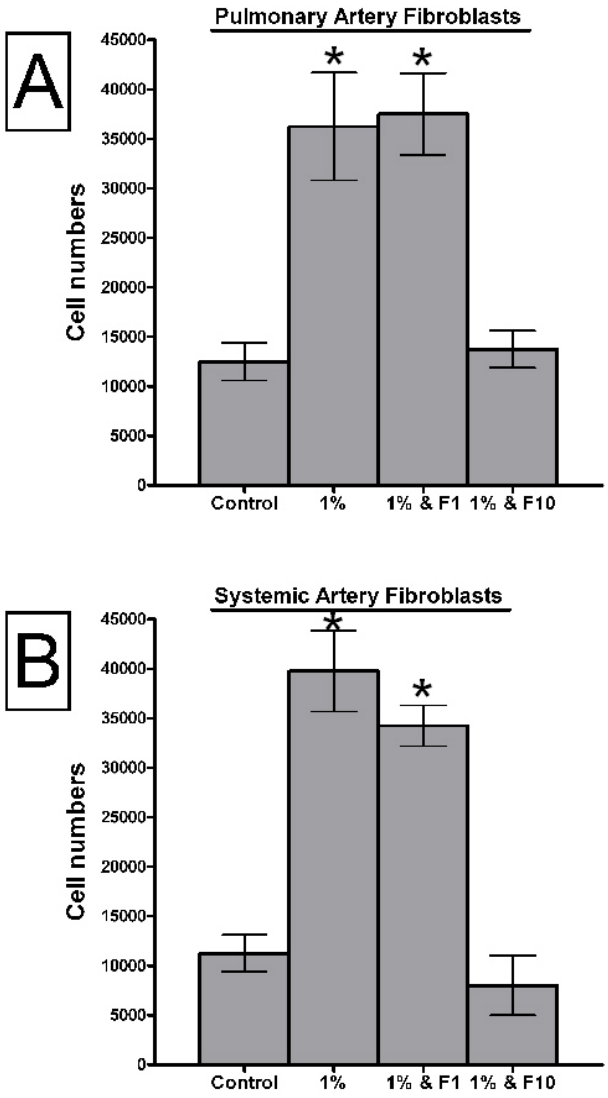


Figure 3.3 Effects of Fluvastatin on Serum-induced Proliferation of Adventitial Fibroblast Cells.

Growth arrested rat pulmonary artery fibroblasts (Panel A) and rat aortic fibroblasts (Panel B) were stimulated with graded doses of serum +/- fluvastatin 1μM (F1) or fluvastatin 10μM (F10), for 24 hours. Cells were stained with trypan blue and counted. Experiments with other doses of fluvastatin (0.1, 2.5, 20 and 50μM) were also undertaken but these are omitted from the graphs for clarity. Values shown are mean +/- 1SD from 4 replicate experiments on cells from the same animal; experiments were repeated on cells from >4 animals and representative graphs are shown here. (*value significantly greater than control $p<0.01$).

Proliferation responses of pulmonary and systemic adventitial fibroblasts to serum were similar. Also, no significant differences were seen when comparing the effects of fluvastastin on serum-induced proliferation of pulmonary vs systemic adventitial fibroblasts.

3.3.3 Effects of Fluvastatin on Rat Adventitial Fibroblast Viability

Cell viability (as assessed by morphology and failure to stain with trypan blue) was unaffected by fluvastatin at doses up to and including 10μM: <5% of cells stained with trypan blue for all experimental conditions.

Fluvastatin 20 and 50μM were seen to induce cell death by the end of the 24 hour experiment with minimal residual cells seen at light microscopy (figure 3.4). These residual cells stained with trypan blue, indicating loss of plasma membrane integrity / non-viable cells.

3.3.4 Effect of Fluvastatin on Platelet-Derived Growth Factor-induced Proliferation of Rat Pulmonary Adventitial Fibroblasts

To determine if the effects of fluvastatin on adventitial fibroblast proliferation were mitogen-specific, selected experiments were repeated, using PDGF-BB in place of serum. Given similar results obtained in the preceding experiments with serum in both [^{3}H] thymidine uptake vs cell culture and with RPAFs vs RAFs only [^{3}H] thymidine uptake in RPAFs was undertaken for this comparison.

PDGF-BB stimulated DNA synthesis in RPAFs, with a dose response seen, as previously shown. As with serum-stimulation, dose of fluvastatin <10μM had no effect in DNA synthesis. 10μM fluvastatin reduced DNA synthesis, but less so than in the serum stimulation experiments. As with the serum-stimulated cells, 20μM fluvastatin induced cell death, regardless of PDGF-BB concentration.

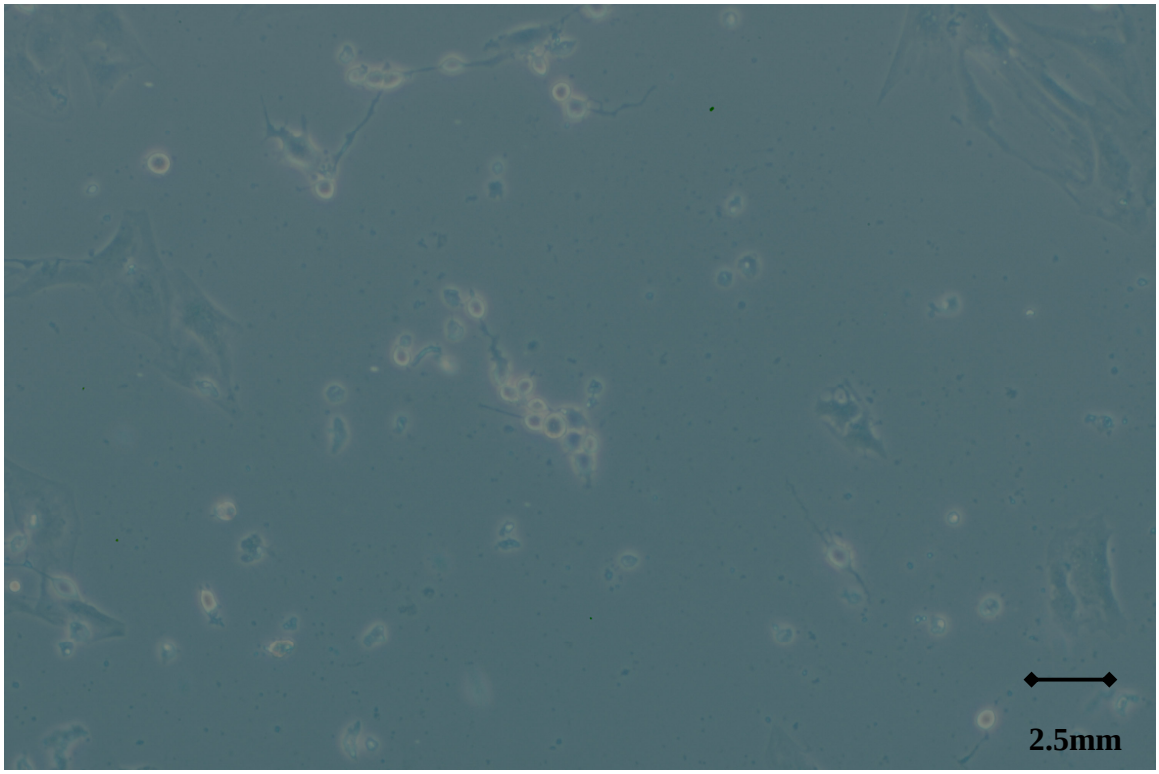


Figure 3.4 Effect of 20μM Fluvastatin on Rat Pulmonary Adventitial Fibroblast Cell Viability

Rat pulmonary adventitial fibroblasts were visualised by light microscopy, following a 24 hour proliferation experiment. Quiescent cells had been maintained in cell culture media supplemented with 5% serum and fluvastatin 20μM, in normoxic conditions for 24 hours.

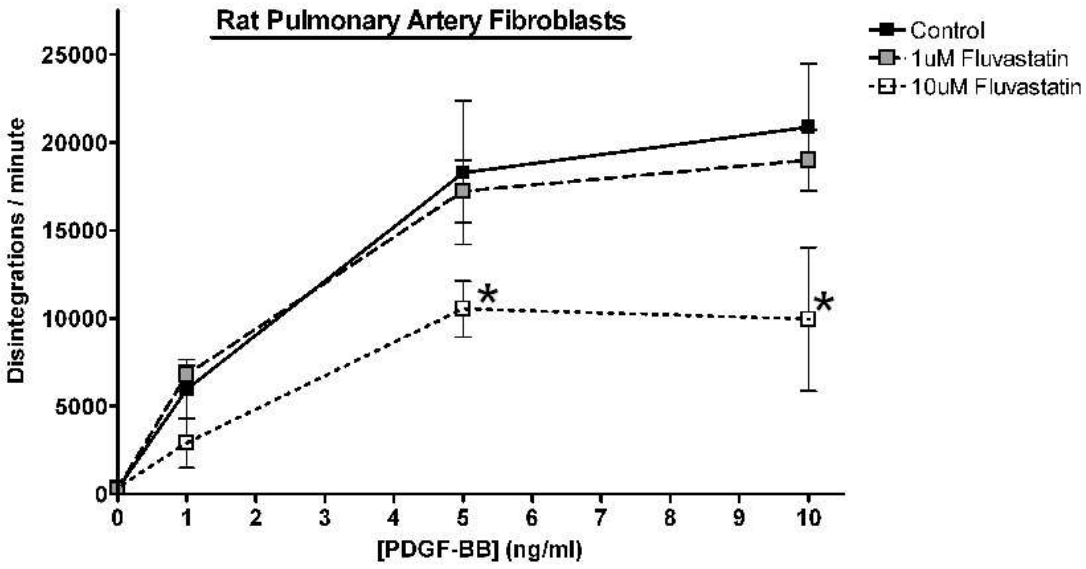


Figure 3.5 Effect of Fluvastatin on Platelet-Derived Growth Factor-Induced [^{3}H] Thymidine Uptake by Pulmonary Adventitial Fibroblast Cells.

Growth arrested rat pulmonary artery fibroblasts were stimulated with graded doses of serum for 24 hours +/- fluvastatin 1μM or 10μM. DNA synthesis, as an index of cell proliferation, was measured by [^{3}H] thymidine uptake. Values shown are mean +/- 1SD from 4 replicate experiments on cells from the same animal; experiments were repeated on cells from >4 animals and representative graphs are shown here. (*value significantly reduced vs control, $p<0.01$).

3.3.5 Effects of Acute Hypoxia and Fluvastatin on Adventitial Fibroblast Proliferation

The effects of acute hypoxia on RPAF proliferation was confirmed by thymidine experiments, with incremental serum concentrations, with [^{3}H] thymidine uptake compared in cells which had been maintained in normoxic (21%) and hypoxic (5%) conditions for the 24 hour experimental period (Figure 3.6). As had been previously shown, acute hypoxia had no effect on quiescent (control) cells but caused a significant increase in cellular DNA synthesis, in the presence of serum.

This effect – increased proliferation in acute hypoxia – was seen across all serum concentrations. The magnitude of the effect, however, appeared less with higher serum concentrations as the cells proliferated to the extent that they had become confluent in the wells before the end of the 24 hour period. Contact inhibition results and a relative reduction in DNA synthesis at the end of the experiment occurs. This artefact is seen to be most marked with 10% serum where early proliferation is so high even in normoxic conditions that the thymidine assay falsely suggests reduced proliferation.

Fluvastatin 1μM – a dose shown not to affect mitogen-induced proliferation of RPAFs in preceding experiments – completely blocked the acute hypoxia-induced increase in [^{3}H] thymidine uptake in RPAFs. Again, this was seen across the range of serum concentrations (Figure 3.6).

The effect of acute hypoxia on DNA synthesis and cell proliferation was found (as previously) to be pulmonary-selective: RAFs exhibited no increase in thymidine uptake (Figure 3.7) or cell numbers (Figure 3.8) in acute hypoxia. Also the anti-proliferative effect of fluvastatin 1μM was confirmed to be circulation and stimulus selective. Acute hypoxia-induced DNA synthesis and proliferation in RPAFs was completely inhibited by fluvastatin 1μM. Serum-induced proliferation in RPAFs and all proliferation in RAFs was again unaffected by fluvastatin at this dose. This is compared to the non-selective effect of fluvastatin at doses ≥10μM (significantly inhibited serum-induced proliferation in both cell types).

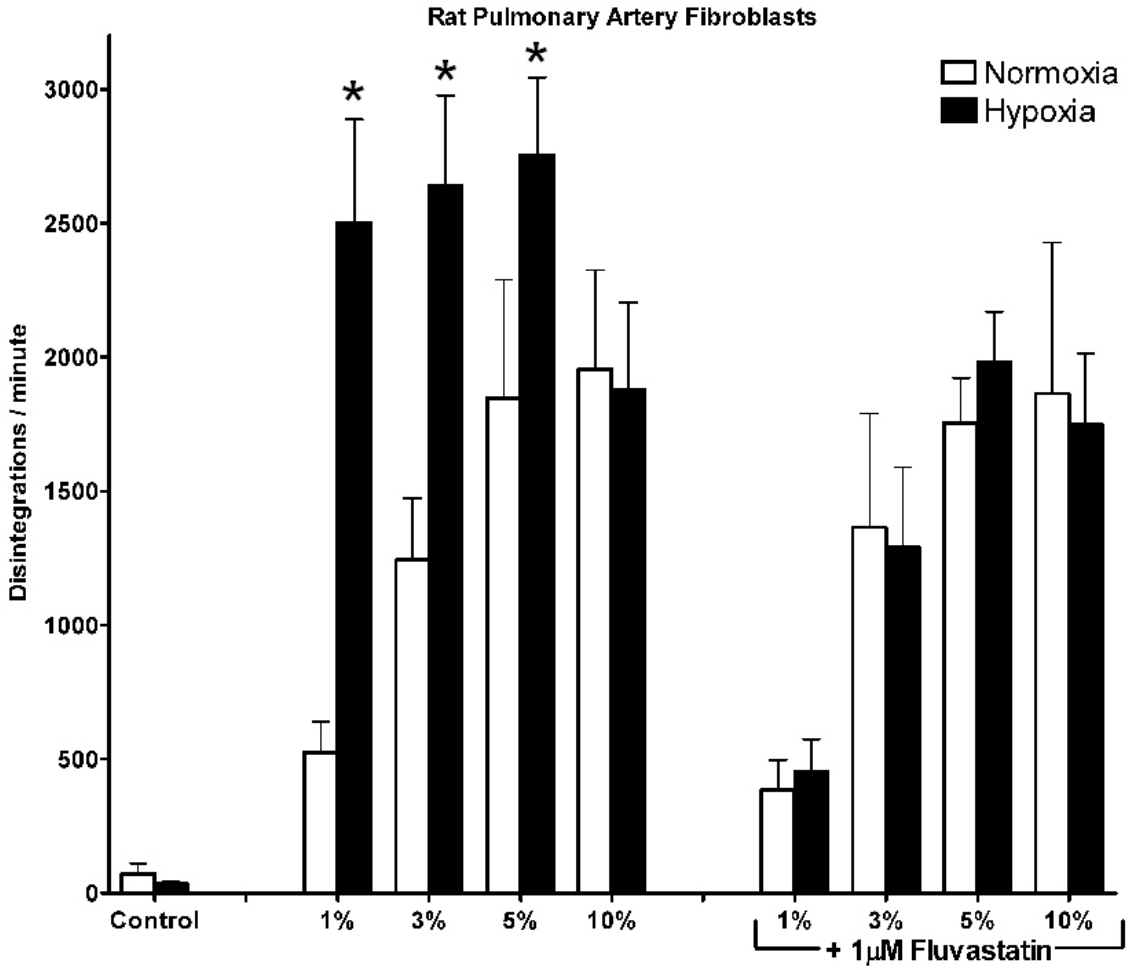


Figure 3.6 Effects of Incremental Serum Concentrations, Acute Hypoxia and 1μM Fluvastastin on [^{3}H] Thymidine Uptake by Rat Pulmonary Adventitial Fibroblast Cells.

Growth arrested rat pulmonary artery fibroblasts were stimulated in normoxia (21%) or hypoxia (5%, 35mmHg) for 24 hours +/- serum (1%, 3%, 5% or 10%) and fluvastatin 1μM. DNA synthesis, as an index of cell proliferation, was measured by [^{3}H] thymidine uptake. Values shown are mean +/- 1SD from 4 replicate experiments on cells from the same animal; experiments were repeated on cells from >4 animals and representative graphs are shown here. (*value significantly greater than serum-normoxia, $p<0.01$).

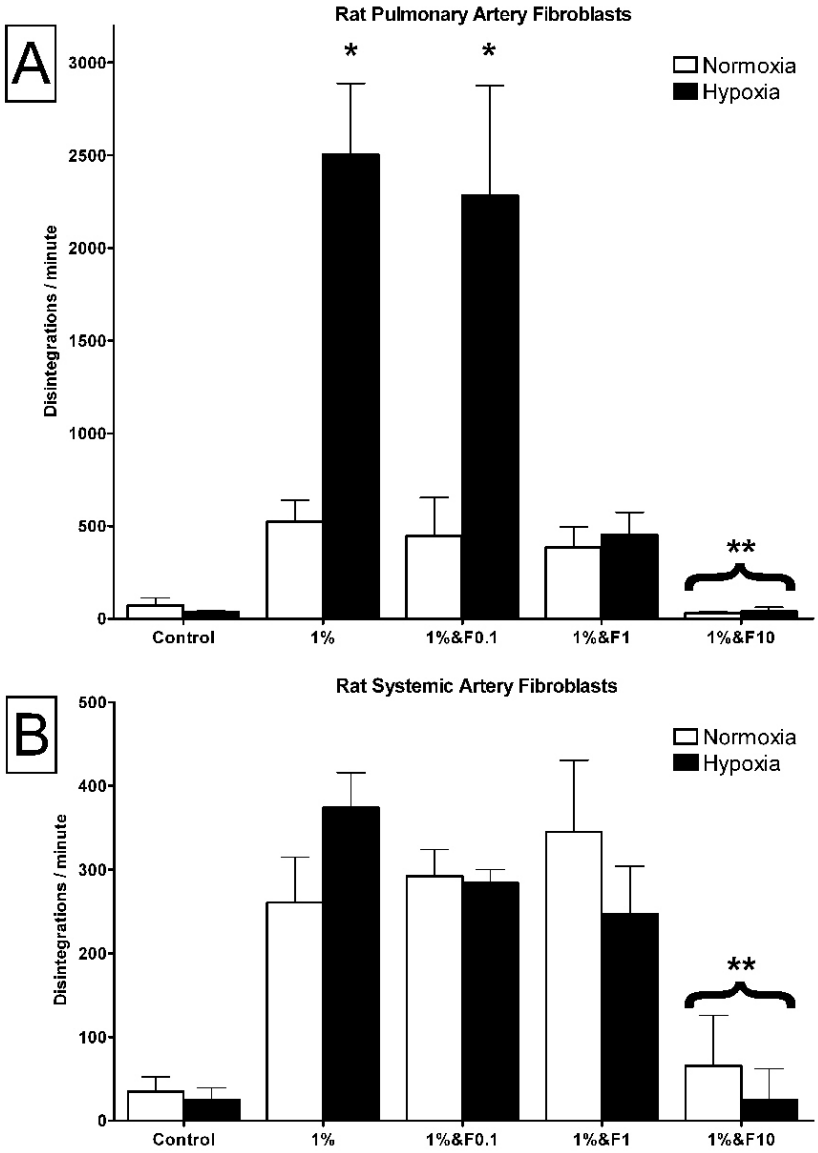


Figure 3.7 Effects of Acute Hypoxia and Fluvastatin on [^{3}H] Thymidine Uptake by Adventitial Fibroblast Cells.

Growth arrested rat pulmonary artery fibroblasts (Panel A) and rat aortic fibroblasts (Panel B) were stimulated in normoxia (21%) or hypoxia (5%, 35mmHg) for 24 hours +/- 1% serum and fluvastatin 0.1, 1 or 10μM (F0.1, F1 or F10). DNA synthesis, as an index of cell proliferation, was measured by [^{3}H] thymidine uptake. Values shown are mean +/- 1SD from 4 replicate experiments on cells from the same animal; experiments were repeated on cells from >4 animals and representative graphs are shown here. (* value significantly greater than normoxia-1%serum, $p<0.05$; ** value significantly less than normoxia-1% serum, $p<0.05$).

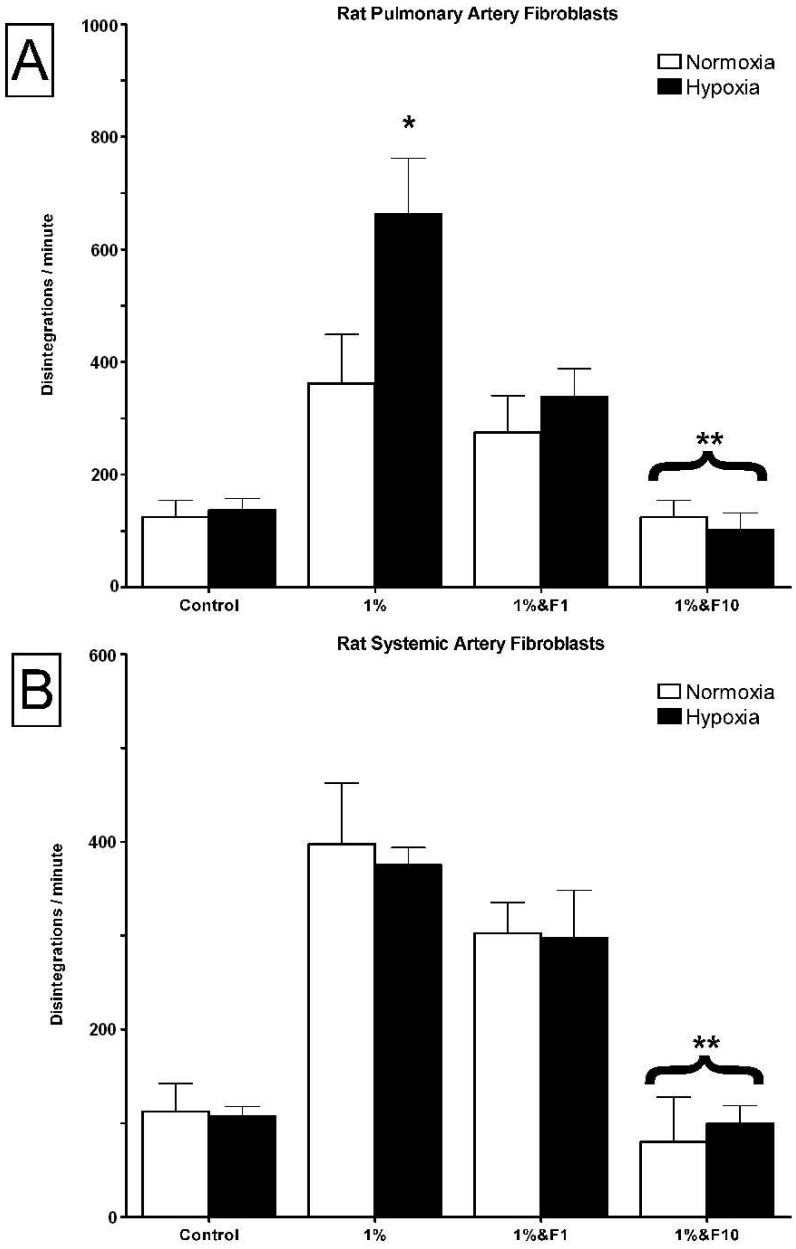


Figure 3.8 Effects of Acute Hypoxia and Fluvastatin on Proliferation of Adventitial Fibroblast Cells.

Growth arrested rat pulmonary artery fibroblasts (Panel A) and rat aortic fibroblasts (Panel B) were stimulated in normoxia (21%) or hypoxia (5%, 35mmHg) for 24 hours +/- 1% serum and fluvastatin 0.1, 1 or 10μM (F0.1, F1 or F10). Cells were stained with trypan blue and counted. Values shown are mean +/- 1SD from 4 replicate experiments on cells from the same animal; experiments were repeated on cells from >4 animals and representative graphs are shown here. (* value significantly greater than normoxia-1%serum, $p<0.05$; ** value significantly less than normoxia-1% serum, $p<0.05$).

3.3.6 Comparison of the Anti-Proliferative Potency of Different Statins

The effects of 3 lipophilic statins (atorvastatin, fluvastatin, simvastastin) and 1 hydrophilic statin (pravastatin) on serum and hypoxia-induced proliferation of RPAFs and RAFs were compared. As before, serum +/- incremental doses of statin was added to quiescent fibroblasts at the start of a 24 hour experiment. Cells were transferred to the normoxic or hypoxic incubator and [^{3}H] thymidine uptake was measured. Rigorous attention was taken to ensure accuracy of the concentration of the statin stock solutions and the doses added to the experimental wells. The number of wells for each experimental condition was also doubled for these experiments (to 8), so that any small differences in potency could be resolved.

No difference in the potency of the lipophilic statins was identified. In particular, hypoxia-induced proliferation of RPAFs was completely and selectively inhibited by 1μM fluvastatin, simvastatin and atorvastatin (Figure 3.9). These 3 statins exhibited similar anti-proliferative potency across a narrow dose range around this (0.5–2.5μM).

The hydrophilic statin pravastatin had no effect on cell viability, serum-induced proliferation of RPAFs or RAFs or hypoxia-induced proliferation of RPAFs, at doses up to 50μM.

3.3.7 Effects of Established Pulmonary Hypertension Therapeutic Agents on Adventitial Fibroblast Proliferation

The effects of a prostacyclin analogue (treprostinil diethanolamine, UT15c), a phosphodiesterase-5 inhibitor (sildenafil) and an endothelin antagonist (bosentan) on serum and acute hypoxia-induced proliferation of RPAFs was determined. As before, graded concentrations of serum and experimental mediators were added to quiescent cells and thymidine uptake was measured after 24 hours in either normoxic or hypoxic conditions.

50μM of UT15c, sildenafil and bosentan had a non-specific inhibitory effect on serum-induced proliferation (Figure 3.10) and also on cell viability as visualised by light microscopy. With sildenafil and bosentan 50μM, precipitated material (most likely drug) was visualised in the wells at 24 hours.

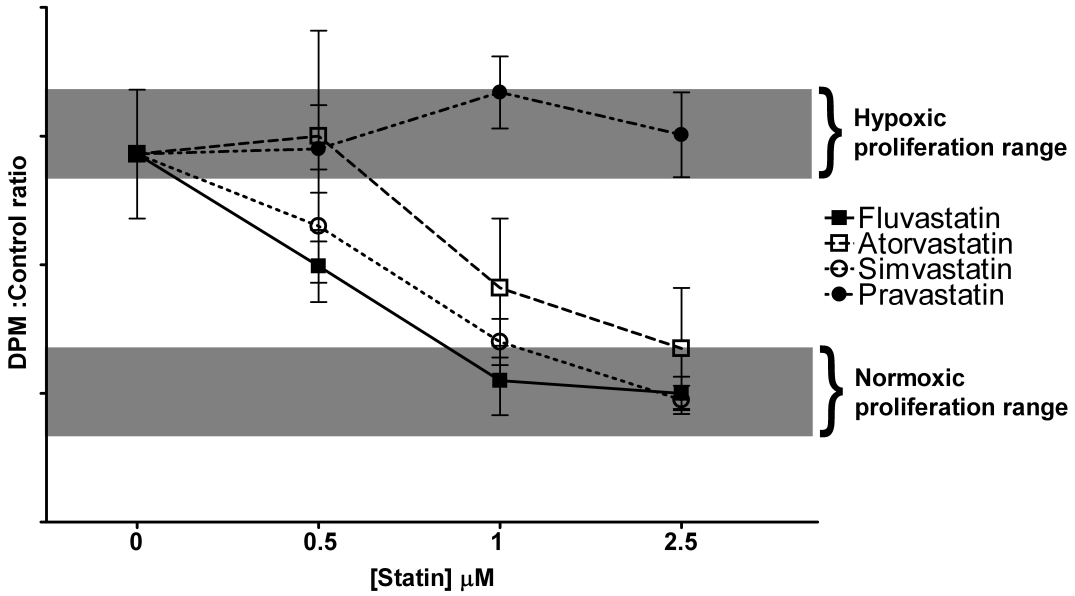


Figure 3.9 Comparison of the Effects of 4 Different Statins on Hypoxia-Induced [^{3}H] Thymidine Uptake by Pulmonary Adventitial Fibroblast Cells.

The effect of a range of doses of lipophilic statins (fluvastatin, atorvastatin, simvastatin) and a hydrophilic statin (pravastatin) on [^{3}H] thymidine uptake in quiescent RPAFs, stimulated with 1% serum and 24 hours of hypoxia (5%, 35mmHg), was determined. Results are plotted as ratios of measured thymidine uptake vs thymidine uptake in control cells (mean of 8 experiments on cells from the same animal; respresentative results from experiments on 4 different animals are shown). The mean +/- 1SD [^{3}H] thymidine uptakes of RPAFs stimulated with 1% serum in normoxia or hypoxia without statin are shown as shaded areas. ($p<0.05$ for fluvastatin, simvastatin, atorvastatin 1 & 2.5μM treated cells, vs serum-hypoxia treated cells)

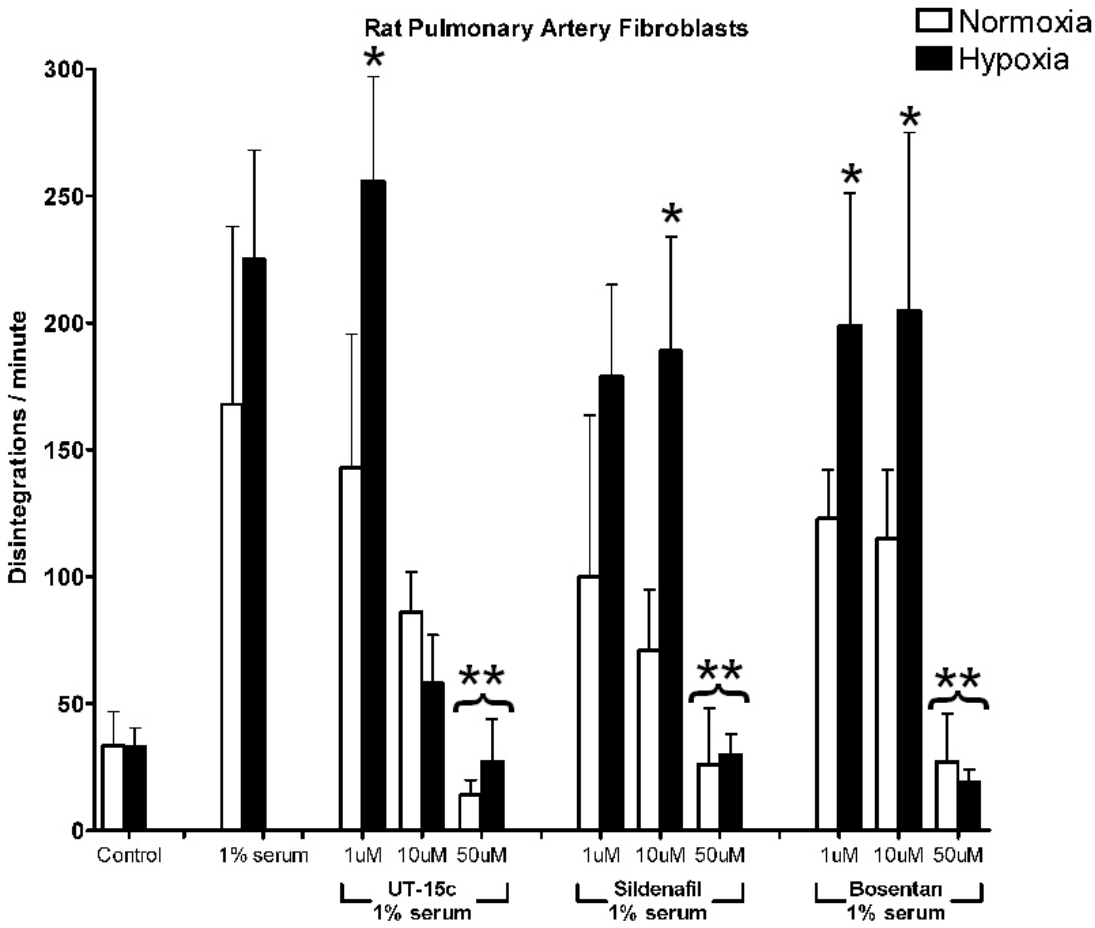


Figure 3.10 Effects of a Prostacyclin Analogue, PDE-5 Inhibitor and ET-1 Receptor Antagonist on Serum and Acute Hypoxia-Induced [^{3}H] Thymidine Uptake by Pulmonary Adventitial Fibroblasts.

The effect of a range of doses of treprostinil diethanolamine (UT-15c), sildenafil and bosentan on [^{3}H] thymidine uptake in quiescent RPAFs, stimulated with 1% serum and 24 hours of hypoxia (5%, 35mmHg), was determined. Values shown are mean +/- 1SD from 4 replicate experiments on cells from the same animal. Experiments were repeated once in cells from a different animal and respresentative results are shown. (* value significantly greater than normoxia-1%serum, $p<0.05$; ** value significantly less than normoxia-1% serum, $p<0.05$).

10μM of UT-15c partially inhibited serum-induced proliferation, with no effect on cell viability. 1μM of UT-15c and 1-10μM of sildenafil and bosentan had no effect on serum-induced RPAF proliferation. None of these drugs, across the range of cell culture concentrations, exhibited any definite hypoxia-selective effect. The error bars in the experiments with serum-normoxia and serum-normoxia+10μM UT-15c overlap – accordingly 10uM of UT-15c may be considered to possibly have partially, non-specifically inhibited proliferation or alternatively to have had no effect on serum-induced proliferation and a specific effect on hypoxia-induced proliferation.

3.4 Discussion

In this chapter, the ability to establish and maintain a previously characterised cell model (pulmonary and systemic adventitial fibroblasts from normal rats, in normoxia and acute hypoxia) was demonstrated. Techniques to assess cell proliferation were undertaken and the effects of statin drugs on adventitial fibroblast proliferation were determined.

For the initial experiments of this project, cell proliferation was estimated by both [^{3}H] thymidine uptake and cell counting. [^{3}H] thymidine uptake is widely used as a proxy measure of DNA synthesis and cell proliferation. Though there are potential sources of error with this (some of the [^{3}H] thymidine may be incorporated into macromolecules other than DNA; some of the DNA into which it is incorporated may be for cell repair rather than cell proliferation), using [^{3}H] thymidine uptake to determine cell proliferation has previously proven robust in our laboratory. In particular, the optimal timing of [^{3}H] thymidine addition (at 4 hours prior to experiment completion in a 24 hour experiment) had been previously verified[233].

For further clarification, [^{3}H] thymidine and cell counting experiments were initially used in tandem. As the tandem experiments in this chapter using these two techniques mirrored one another completely, [^{3}H] thymidine was used as a lone assessment tool for cell proliferation hereafter.

Fluvastatin was the statin initially available to us for experimental work. The effects of fluvastatin on serum and PDGF-BB induced proliferation and viability of PAF and SAFs are in line with numerous previous investigators findings with other (normal or tumor) cell types[200, 233-4]. A cell culture dose of 5μM caused partial inhibition of proliferation and doses of 10μM or greater caused complete inhibition of proliferation and cell death. This effect (inhibition of mitogen-induced adventitial fibroblast proliferation +/- cell death) was not specific to the mitogens studied, though effects mediated by PDGF-BB may be slightly more resistant to statin inhibition.

Based on previous investigators findings, it is speculated that this non-specific antiproliferative effect of fluvastatin, at doses >5μM, was a consequence of HMG-CoA reductase inhibition with consequent disruption of prenylated G-protein signalling and/or cell membrane lipid raft integrity. This contention was not pursued further in this project, firstly as hypoxic proliferation (rather than mitogen-induced proliferation) was the focus and secondly, as the cell culture dose required for these effects (5-10μM) is very much higher than that achieved in the circulation, *in vivo*, in humans, after standard oral dosing of this drug[192]. Accordingly, it was not considered likely that this effect is of major relevance to pulmonary vascular disease in humans, or potential treatment.

Acute hypoxia has been previously shown to stimulate significant increase in rat PAF cell proliferation, with no effect on systemic adventitial fibroblast proliferation[155]. This was confirmed again in this chapter's experiments. We also established that statins have a selective inhibitory effect on hypoxic proliferation, at a lower cell culture dose than is required for inhibition of mitogen-induced proliferation. Fluvastatin, at a dose of 1μM, demonstrated striking inhibition of acute hypoxia-induced proliferation. At this dose, the effect of fluvastatin was selective for hypoxia (fluvastatin 1μM had no effect on serum or PDGF-BB induced proliferation) and this cell type (fluvastatin 1μM had no effect on systemic adventitial fibroblasts proliferation). This result – selective inhibition of hypoxic PAF proliferation by fluvastatin 1μM - is the foundation for the other chapters in this thesis.

Inhibition of pulmonary vascular cell proliferation by statins has previously been reported, but effects were only recognised with high cell culture doses of statins (30–80 mM)[218], which are much higher than those achieved with standard dosage of statins, *in vivo*, in humans. Furthermore, in these previous studies, the pulmonary specificity of this antiproliferative effect was not studied: at these cell culture doses statins have nonspecific antiproliferative effects on virtually any cell studied, including systemic vascular cells[235], possibly via disruption of centromere assembly[234].

A dose dependant / stimulus specific / cell type specific *in vitro* effect of statin has been noted previously. A suggested explanation is that the cell signalling mechanisms unique to the stimulus or cell under study may be very reliant on ready supply of individual compounds produced by the HMG-CoA reductase pathway, accordingly rendering them very sensitive to statin inhibition. It seemed likely that acute hypoxia-induced proliferation of RPAFs was dependant on a HMG-CoA reductase product, but other mechanisms of action of the statin were considered possible. Experiments to fully address this were proposed and these are considered in detail in the following chapter.

There are 5 statins currently in mainstream clinical use in the UK (pravastatin, simvastastin, atorvastatin, fluvastatin and rosuvastatin) and the effects of some of these on cell proliferation have been compared[237]. Simvastatin, atorvastatin and fluvastastin are typically equipotent in *in vitro* proliferation studies, whilst pravastatin has much less, or no antiproliferative effect. To clarify this in our cell model, the acute hypoxia-selective antiproliferative effect in RPAFs was compared across a range of statin doses, with 3 different lipophilic statins (fluvastatin, atorvastatin and simvastatin). No difference in either the nature of the effect, or in statin dose-response was seen: we found the lipophilic statins to be equipotent. This result is compatible with the statin effect on acute hypoxic proliferation being mediated by HMG-CoA reducatase inhibition (for which these statins would be expected to be equipotent at a cellular level) rather than an HMG-CoA reductase independent effect (which depends on the specific statin's molecular structure)[215]. Our findings -equipotency of fluvastatin, atorvastatin and simvastatin in antiproliferative cell culture experiments - are in line with most of the literature. For our experiments, preparation and dilution of stock solutions of these statins was minimised and meticulous attention was taken, in order to minimise any cumulative error from repeated pipetting etc.

Lipophilic statins diffuse freely across cell membranes and have been shown to have effects on cellular processes in diverse cell types. In contrast, water soluble statins (eg pravastatin, rosuvastatin) do not diffuse across cell membranes and are expected only to have an effect on cells with specific membrane receptor mechanism to internalise them (eg hepatocytes). There have, however, been occasional reports of

positive effects of pravastatin on cell lines other than hepatocytes. To clarify whether there was an effect of hydrophilic statins on this cell model, pravastatin was studied. No effect on hypoxic or serum mediated PAF proliferation or cell viability was seen, at doses up to 50μM. This data suggests adventitial fibroblasts lack a cellular mechanism to internalise pravastatin. This result is typical for non-hepatocyte cell culture studies[238].

Combined with the previous results of other groups (statin-mediated inhibition / reversal of experimental pulmonary hypertension), it was considered that the observed cellular effects of statin - selective inhibition of acute hypoxia-induced PAF proliferation by low dose fluvastatin, atorvastatin and simvastatin - may be theoretically beneficial in clinical application in pulmonary hypertension. It was therefore felt relevant to determine if this cellular effect was novel, or simply duplicates an effect achieved by drugs already established in clinical use in pulmonary hypertension. To address this, an agent from each of the 3 established drug classes (prostacyclin analogue, phosphodiesterase-5 inhibitor, endothelin-1 antagonist) in PAH was obtained and evaluated. Prostacyclin acts via cell membrane receptors stimulating cAMP production and via PPAR-receptors, with direct effects on intracellular processes. Phosphodiesterase 5 (PDE-5) inhibitors inhibit the breakdown of cGMP (thereby increasing the intracellular concentration of cGMP). Endothelin-1 (ET-1) antagonists reversibly block cell membrane ET-1a +/- ET-1b receptors. All of these mediator pathways have been implicated in hypoxic signaling and pulmonary vascular cell proliferation (see Chapter 1.5.2). The manufacturing drug companies provided treprostinil diethanolamine (UT-15c, a prostacyclin analogue), sildenafil (a PDE-5 inhibitor) and bosentan (a dual ET-1 A/B receptor antagonist).

UT-15c, sildenafil and bosentan had non-specific inhibitory / cytotoxic effects, on serum-induced RPAF proliferation, at high cell culture doses. We found no definite specific effect of these drugs on hypoxia-induced proliferation which argues against a role for prostacyclin, cGMP or ET-1 signalling pathways in acute hypoxic proliferative signaling pathways in this cell type. The inconclusive results with 10μM UT-15c are, however, as acknowledged in section 3.3.7. The results with these agents should not be overinterpreted, particularly as time constraints limited the

number of experimental repeats undertaken for this section, in comparison with all of the other experiments presented in this chapter. Temporary issues with cell-culture infection interrupted experiments at this time and the decision was made to focus on the most relevant results (with the low dose statin) rather than the equivocal result with a cell-culture dose of UT-15c which is of doubtful clinical relevance. With caveats about less experimental repetition, however, the result with bosentan is notable. Stenmark's group have previously shown endothelin-1 release by PAFs in acute hypoxia and implicated autocrine/paracrine release of mediators (ET-1 and adenosine triphosphate) in hypoxia-induced PAF proliferation[105, 239]. The results we obtained with bosentan are not incompatible with those findings, but they do indicate that autocrine and/or fibroblast-fibroblast paracrine pro-proliferative effects of ET-1 do not significantly contribute to hypoxic proliferation of rat PAFs, over a 24 hour period.

Hypoxia-induced proliferation of pulmonary adventitial fibroblasts is likely to be a key process in pulmonary vascular remodelling in pulmonary hypertension. In previous work, and in the current series of experiments, the only agents which have been identified as selective inhibitors of hypoxia-induced proliferation of PAFs are p38 MAP kinase inhibitors[155] and low-dose statins[240]. Though these findings suggest therapeutic potential for statins in hypoxia-associated pulmonary hypertension, we also considered that there was a more immediate experimental application. The effects of statins on cellular processes have previously been shown to be mediated by a restricted panel of effects on intracellular proteins and signalling, and specific inhibitors and mediators for each of these individual mechanisms is available. Combining an awareness of the HMG-CoA reducatase signalling pathway with the key results from this chapter (selective inihibition of acute hypoxic PAF proliferation by 1μM fluvastatin) offered the possibility to further interrogate hypoxic proliferative signalling in this important pulmonary vascular cell. The experiments conceived in order to achieve this interrogation are the focus of the following chapter.

Chapter 4

Characterisation of Acute Hypoxic Signalling Pathways in Pulmonary Adventitial Fibroblasts: Mechanism of the Antiproliferative Effect of Fluvastastin

4.1 Introduction

In the previous chapter competencies with routine cell culture, an acute hypoxic cell model and standard proliferation assays were established. Results from that chapter showed the effects of statins on proliferation of adventitial fibroblasts. In particular, acute hypoxia-induced proliferation of rat pulmonary adventitial fibroblasts was selectively inhibited by 1μM of fluvastastin, atorvastastin or simvastastin. We sought to determine the mechanism of this inhibitory effect of statin, in order to characterise hypoxic signalling pathways in rat pulmonary adventitial fibroblasts.

The general mechanisms of action of statins have been previously outlined (see Chapter 1.10.5 and figure 4.1). Notably, intermediates of the cholesterol biosynthesis pathway and inhibitors of specific downstream steps related to this pathway are now available for experimental use. Based on this, a sequence of experiments to determine the effects of these inhibitors and intermediates (alone and in combination with fluvastatin), on acute hypoxia-induced RPAF proliferation were conceived, in order to clarify the mode of action of the statin.

Phosphorylation of p38 MAP kinase α and γ has been shown to be an obligatory step responsible for the differential response of pulmonary and systemic adventitial fibroblasts to acute hypoxia, mediating acute hypoxic proliferation[155]. p38 MAP kinase inhibitors selectively inhibit hypoxia-induced PAF proliferation (ie identical effect to 1μM fluvastatin) whereas inhibitors of other MAP kinase proteins have no effect[233]. This hypoxia-p38 MAPK-proliferation signalling pathway has been confirmed in acute and chronic hypoxia[177], and in rat, bovine and human cells[180]. There is, however, some contradictory data from a neonatal calf acute hypoxia PAF model. In these cells, there is also biphasic p38 MAP kinase phosphorylation. Acute hypoxic proliferation, however, of these PAFs is dependant on phosphorylation of ERK, rather than p38 MAPK[241]. Accordingly, given the similar effect of 1μM statin and p38 MAPK inhibitor on acute hypoxic RPAF proliferation and this controversy about MAP kinase signalling in hypoxic adventitial fibroblasts, we also elected to determine the effects of acute hypoxia, statins and the other inhibitors and mediators on MAP kinase phosphorylation.

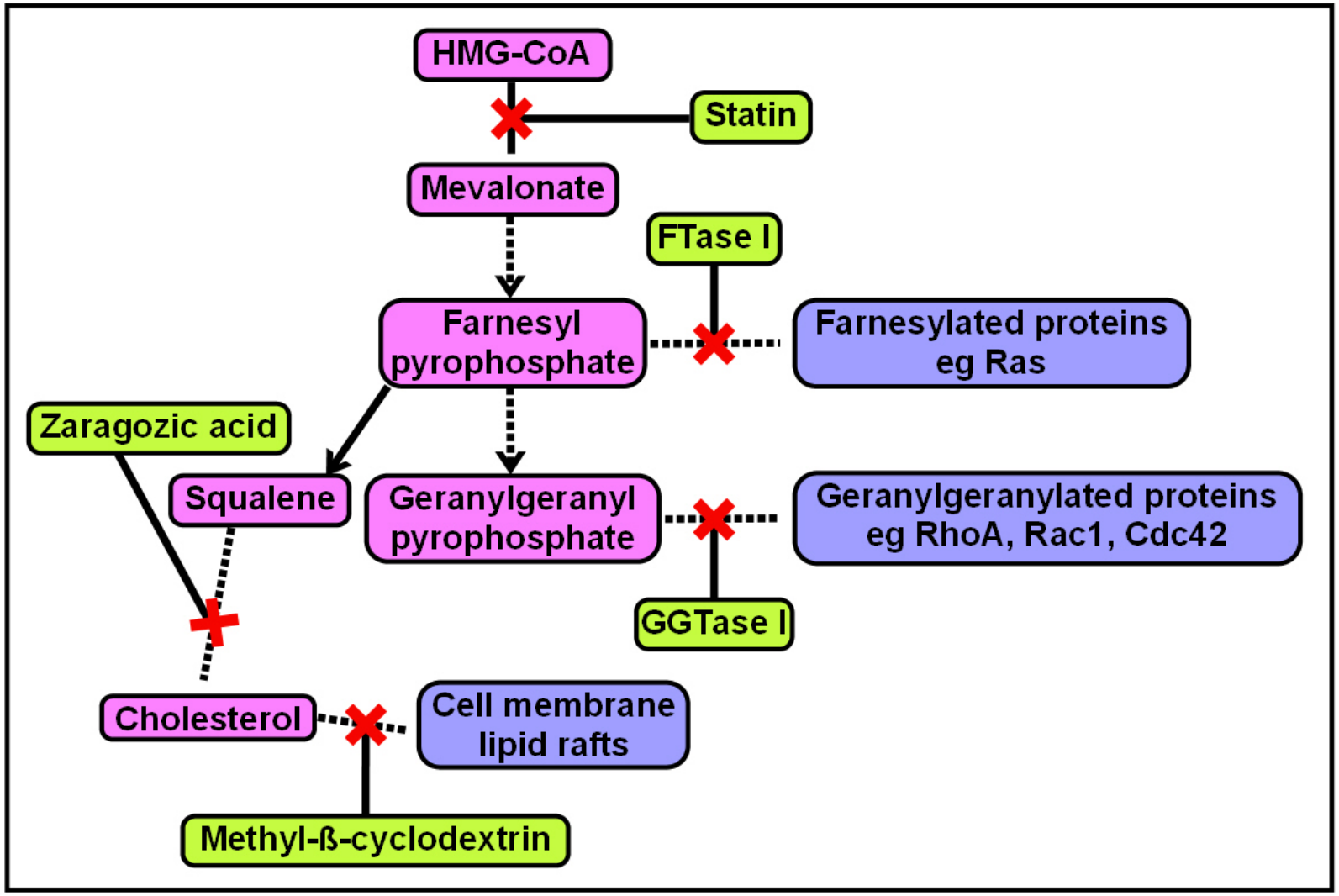
Considering the known effects of statins, we considered it likely that the positive effect (ie inhibition of acute hypoxic RPAF proliferation) identified in Chapter 3 might be mediated via effects on a prenylated GTPase protein. Based on observations about acute hypoxic signalling in general (see Chapters 1.6.5 and 1.7.2), we additionally speculated that this GTPases may be RhoA or Rac1, acting upstream of p38 MAP kinase. Based on this, we proposed to evaluate the effects of specific inhibitors of Rho kinase and Rac1 activation on hypoxia-induced MAP kinase phosphorylation and proliferation or RPAFs, if the results with the HMG-CoA reductase pathway compounds were suggestive of a role for a geranylgeranylated signalling protein.

Figure 4.1 Cholesterol Biosynthesis Pathway: Intermediates and Related Inhibitors

The cholesterol biosynthesis pathway with its related cell signalling systems (see chapter 1.10.5 for further description) is shown annotated with available inhibitors (green boxes). Statins inhibit the synthesis of mevalonate (the rate limiting step in this pathway) and accordingly have the potential to interfere with the indicated cell signalling pathways (blue boxes). Statins have also been shown to have effects on cell activity independent of this pathway, via direct interaction with cell receptors (eg the LFA-1 receptor on T lymphocytes).

Prenyltransferase enzymes (farnesyl transferase or geranylgeranyl transferase I and II) facilitate the post-translational modification of selected target GTPase proteins, attaching the specific isoprenoid molecule (FPP or GGPP) onto the protein. Specific inhibitors are available for farnseyl transferase (FTase I) and geranylgeranyl transferase I (GGTase I). Zaragozic acid A (squalestatin) is a squalene synthase inhibitor, blocking the conversion of sqalene to cholesterol. There is, however, some crossover inhibition of farnesyltransferase with this compound[242] and an additional agent was sought to comprehensively assess this 'lipid raft arm' of the cholesterol pathway. Methyl-β-cyclodextrin doesn't affect the cholesterol biosynthesis pathway directly but it is an avid binding molecule of cholesterol, retaining it within its molecular ring structure. When added to cell medium for a short period (30mins to 1 hour) methyl-β-cyclodextrin disrupts lipid raft structures by leaching cholesterol from the plasma membrane and sequestering it in the water soluble culture media[243].

Where statins are shown to have an inhibitory effect in cell culture (as with acute hypoxic proliferation of RPAFs, Chapter 3) the mechanism of this effect, and the cell signalling system implicated can be clarified with reference to this pathway. In particular, the cell signalling events can be interrogated by a sequence of experiments using statin inhibition and sequential repletion with each of the pathway intermediates, with concurrent experiments utilising each of the indicated downstream inhibitors.


HMG-CoA
Statin
Mevalonate
FTase I
Farnesyl pyrophosphate
Farnesylated proteins eg Ras
Zaragozic acid
Squalene
Geranylgeranyl pyrophosphate
Geranylgeranylated proteins eg RhoA, Rac1, Cdc42
GGTase I
Cholesterol
Cell membrane lipid rafts
Methyl-ß-cyclodextrin

4.2 Methods

As outlined in the general methods (Chapter 2), rat pulmonary artery fibroblast (RPAF) cells were studied in experimental conditions - normoxia vs acute hypoxia (5%) +/- serum, statin or other mediator. Under these experimental conditions, cell proliferation was assessed by thymidine assay and phosphorylation of mitogen-activated protein kinases was determined by the application of relevant antibodies to nitrocellulose membranes containing proteins separated from cell extracts using an SDS-PAGE / western blot technique. Results are expressed in the figures as mean +/- 1 standard deviation.

4.3 Results

4.3.1 Effects of Hypoxia and Fluvastatin on MAP Kinase Phosphorylation

Previous experimental work in the SPVU laboratory had demonstrated the effects of serum and acute hypoxia, at various timepoints over a 24 hour period, on MAP kinase phosphorylation in rat PAFs. There is constitutive phosphorylation of ERK1/2 in normoxic or hypoxic RPAFs. There are peaks of p38 MAP kinase phosphorylation at 4 and 16 hours and inhibitor experiments confirm that hypoxic proliferation is dependant on these. JNK is not phosphorylated in these cells in normoxia or hypoxia[180].

In order to establish competency in protein analysis techniques and as a precursor to experiments with statin, phosphorylated MAP kinase and total MAP kinase in cells maintained in normoxia or hypoxia for 16 hours were determined by Western Blot analysis (Figure 4.2). Subsequent experiments demonstrated complete inhibition of p38 MAP kinase phosphorylation after 16 hours of acute hypoxia, by 1μM fluvastatin. There was no effect of fluvastatin 1μM on phosphorylated ERK or JNK, or total MAP kinase, at this time point.

4.3.2 Inhibition of Hypoxic Proliferation and p38 MAP Kinase Phosphorylation by Low Dose Fluvastatin: Effects of Mevalonate Replacement

The first question regarding the statin's mechanism of action on hypoxic signalling in RPAFs was whether this was a consequence of HMG-CoA reductase inhibition, or whether there was an HMG-CoA reductase independent statin effect. To answer this, we added supplemented statin-inhibited cells simultaneously with mevalonate, 'bypassing' the effects of statin-mediated HMG-CoA reductase inhibition (figure 4.3A).

Mevalonate, at doses up to 1mM had no effect on serum or hypoxia-induced RPAF proliferation. Mevalonate repletion completely negated the inhibitory effect of 1μM fluvastatin on acute hypoxia-induced RPAF proliferation after 24 hours and p38 MAP kinase phosphorylation at 16 hours (Figures 4.3B & 4.3C).

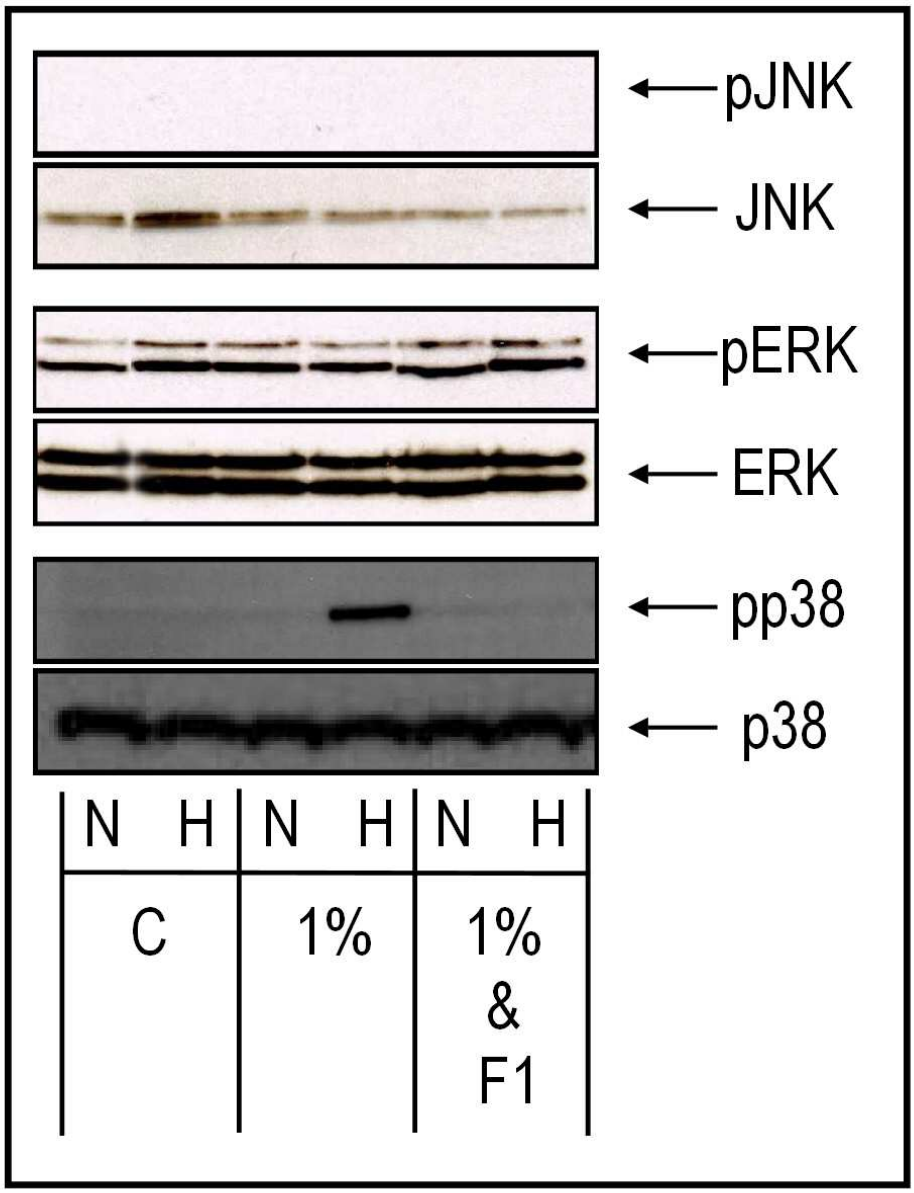


Figure 4.2 Effects of Acute Hypoxia and Fluvastastin 1μM on MAP Kinase Phosphorylation in RPAF cells

Quiescent RPAFs were maintained in normoxia (21%) or hypoxia (5%) for 16 hours. Cell extracts were subjected to SDS-PAGE and western blot analysis of phosphorylated (p) and total (t) JNK, ERK and p38 MAP kinase was conducted. Each blot is representative of at least 3 independent experiments on cells from different animals.

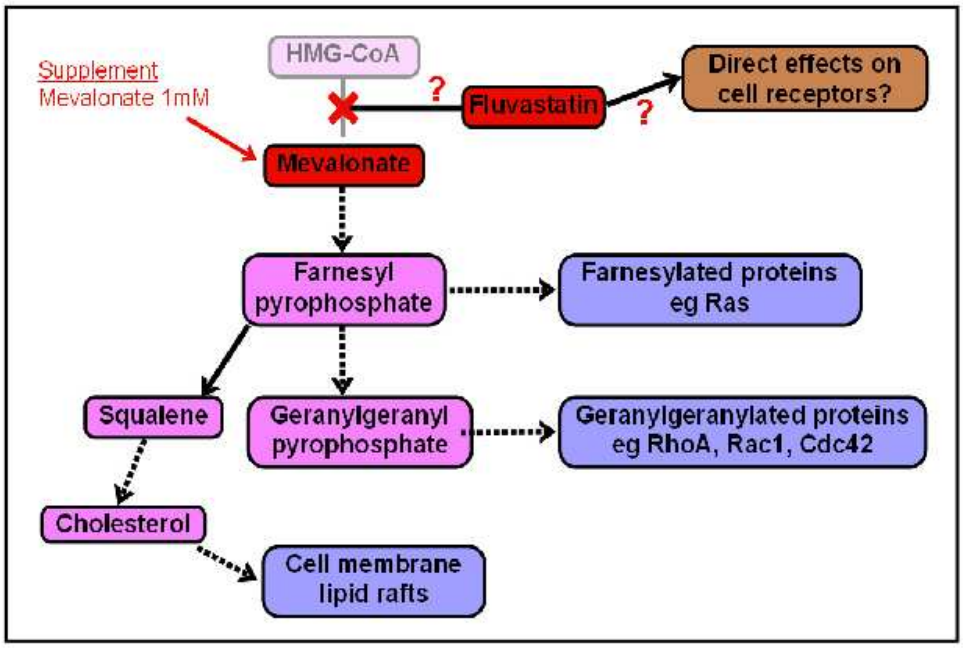
Figure 4.3 Assessment of the HMG-CoA Reductase Pathway in Acute Hypoxic Proliferative Signalling in RPAFs

Panel A shows the cholesterol biosynthesis pathway annotated to indicate the assessment of statin mechanism (via HMG-CoA-reductase inhibition or cholesterol pathway independent effect) achieved by mevalonate repletion.

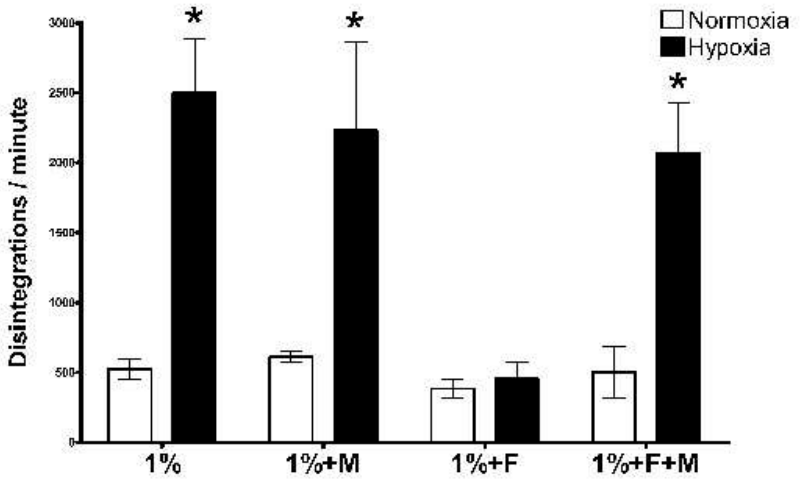
Growth arrested rat pulmonary artery fibroblasts were stimulated with serum +/- fluvastatin 1μM (F1) or mevalonate 1mM (M) for 24 hours in normoxic (21%) or hypoxic (5%) conditions. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours (Panel B). Values shown are mean +/-SD from 4 replicate experiments on cells from the same animal (*value significantly greater than 1% serum-normoxia, $p<0.01$).

p38 MAP kinase phosphorylation was assessed by western blot analysis after 16 hours (Panel C). Data in panel B and C is representative of >3 repeat experiments on cells from different animals.

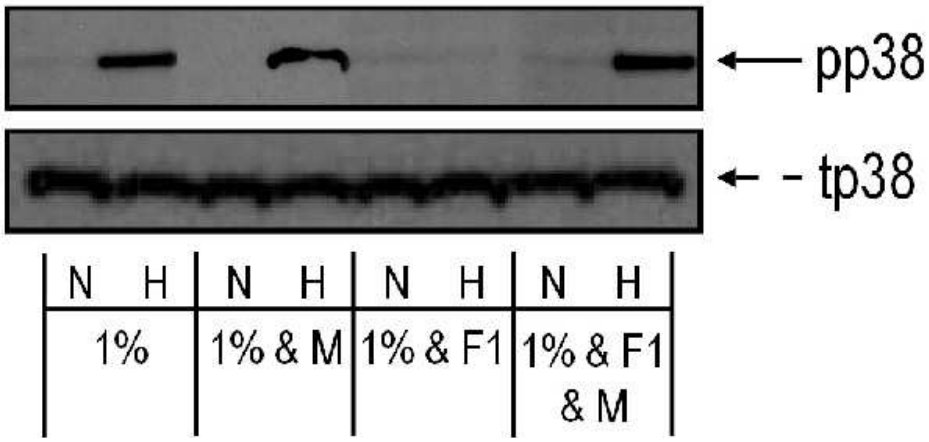
A


Supplement
Mevalonate 1mM
HMG-CoA
?
Fluvastatin
?
Direct effects on cell receptors?
Mevalonate
Farnesyl pyrophosphate
Farnesylated proteins eg Ras
Squalene
Geranylgeranyl pyrophosphate
Geranylgeranylated proteins eg RhoA, Rac1, Cdc42
Cholesterol
Cell membrane lipid rafts

B


Normoxia
Hypoxia
Disintegrations / minute
3000
2500
2000
1500
1000
500
0
*
*
*
1%
1%+M
1%+F
1%+F+M

C


pp38
tp38
N H N H N H N H
1% 1% & M 1% & F1 1% & F1 & M

4.3.3 Effects of Cholesterol Pathway Intermediates on Rat Pulmonary Adventitial Fibroblast Proliferation

Having confirmed that fluvastatin's effects on acute hypoxic RPAFs was mediated by HMG-CoA reductase, we sought to determine which intermediate compound from the HMG-CoA pathway was required for acute hypoxia-induced p38MAPK phopshorylation and RPAF proliferation.

We initially repeated the statin/acute hypoxia proliferation experiments with repletion of squalene, FPP and GGPP, using 0.1-1mM of these compounds (these cell culture doses were based on previous reports and experience with mevalonate in the preceding experiments). After 24 hours of exposure to these cholesterol pathway intermediates, at these concentrations, RPAF proliferation was completely inhibited and distortion of cell architecture / cell lysis was seen under light microscopy (photomicrographs not retained).

A direct toxic effect of these HMG-CoA reductase pathway compounds on the RPAF cells was suspected. In order to determine a safe concentration of these for future experiments, the individual effects of a graded series of concentrations of squalene, FPP and GGPP on serum and hypoxia-induced proliferation of RPAFs was determined.

Threshold concentrations of squalene (25μM), FPP (1μM) and GGPP (0.5μM) (which had no effects on cell architecture or on serum or acute hypoxia-induced proliferation of RPAFs) were identified (Figure 4.4). At higher concentrations of squalene, FPP and GGPP, non-specific antiproliferative and toxic effects on RPAFs were seen.

4.3.4 Lipid Raft Disruption as a Potential Mechanism for the Fluvastastin Effect on Hypoxia-induced RPAF proliferation and p38 MAP Kinase Phosphorylation

Lipid raft integrity has been implicated in abnormal proliferation of pulmonary vascular cells. Statins disrupt lipid raft integrity by interrupting the supply of intracellular cholesterol necessary for lipid raft maintenance. To determine if an effect of fluvastatin 1μM on lipid rafts was mediating the inhibitory effect on acute

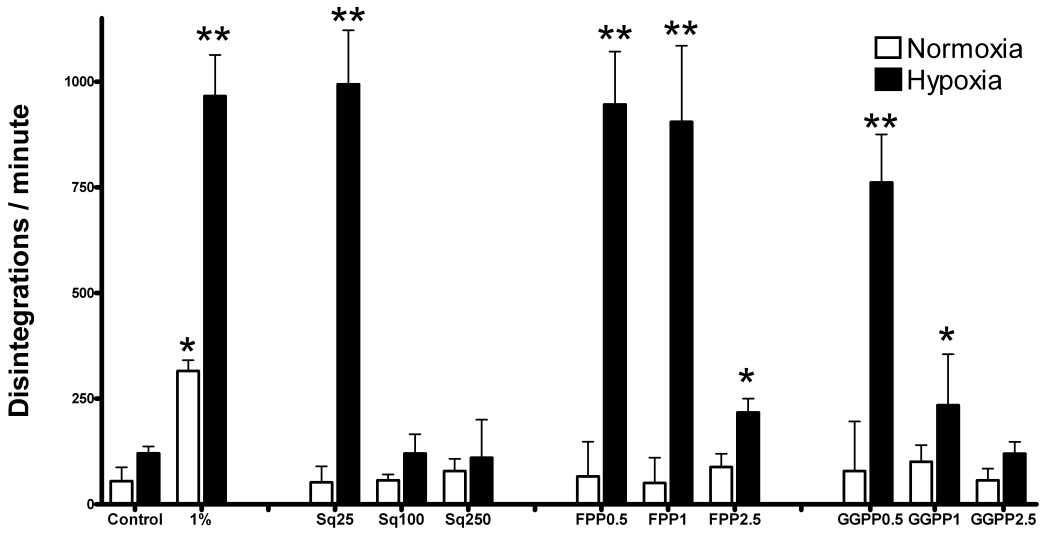


Figure 4.4 Effects of Cholesterol Pathway Intermediates on Serum and Hypoxia-Induced [^{3}H] Thymidine Uptake by RPAFs

RPAF cells were grown to 60% confluence and quiesced. The cholesterol biosynthesis intermediates - squalene (Sq) 25, 100 and 250μM, farnesyl pyrophosphate (FPP) 0.5, 1 and 2.5μM or geranylgeranyl pyrophosphate (GGPP) 0.5, 1 and 2.5μM - were added as indicated, with 1% serum. Cells were maintained in normoxic (21%) or hypoxic (5%) conditions for 24 hours with cell proliferation assessed by [^{3}H] thymidine uptake. Values shown are mean +/-SD from 4 replicate experiments on cells from the same animal and are representative of experiments from cells from >3 animals. (*values significantly greater than control, ** values significantly less the 1% serum-normoxia, $p<0.01$).

hypoxic proliferation and p38 MAPK phosphorylation the effect of squalene repletion on the statin effect (ie did it negate it) was established. Also, the effects of direct intracellular cholesterol synthesis disruption via squalene synthase inhibition (zaragozic acid) or membrane cholesterol depletion (methyl-β-cyclodextrin) – specifically to enquire if these agents mimicked the effect of fluvastatin 1μM – were studied (Figure 4.5A).

Co-administration of squalene 25μM with fluvastatin 1μM had no influence on the statin effect (ie inhibition of hypoxic proliferation and p38 MAPK phosphorylation in RPAFs; Figures 4.5B & 4.5C). Zaragozic acid (60μM) had no effect on serum or hypoxia-induced RPAF proliferation, or on hypoxia-induced p38 MAP kinase phosphorylation. Pre-incubation of cells with methyl-b-cyclodextrin 1mM (a concentration previously shown to disrupt lipid raft integrity) had visible effects on cell architecture on light microscopy (not shown) but no effect on proliferation or p38 MAPK status in RPAFs in either normoxia or hypoxia.

4.3.5 Disruption of Protein Prenylation as a Potential Mechanism for the Fluvastastin Effect on Hypoxia-induced RPAF Proliferation and p38 MAP Kinase Phosphorylation

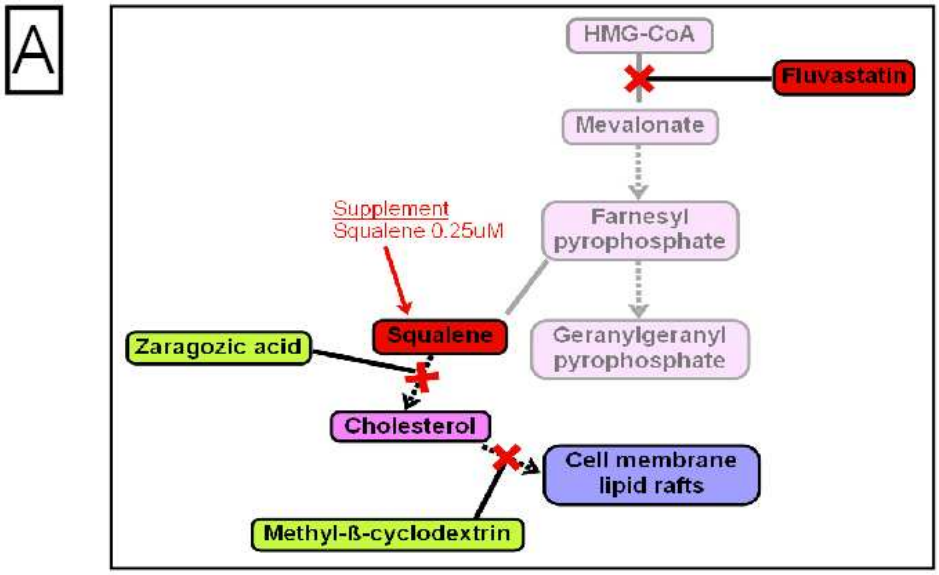
As discussed above (chapter 4.1), we suspected a prenylated protein, possibly one of the Rho GTPase family, as a cell signalling intermediate in acute hypoxic signalling, upstream of p38 MAPK. To clarify this, and in order to focus the search, we investigated the effect of FPP and GGPP repletion on the statin/acute hypoxia RPAF proliferation and p38 MAPK protein experiments. The prenyltransferase inhibitors FTAseI and GGTI-276 were selected based on their potency and selectivity, in comparison to other available prenyltransferase inhibitors. The effect of these prenyltrasferase inhibitiors on acute hypoxia-induced proliferation and p38 MAPK phosphorylation in RPAFs was studied in conjunction with acute hypoxia / statin / FPP & GGPP repletion experiments (figures 4.6A and 4.7A).

Co-administration of FPP (0.5μM) with fluvastatin (1μM) restored hypoxia-induced p38 MAPK phosphorylation and proliferation in RPAFs. FTAseI 5μM had, however, no effect on acute hypoxic proliferation (figure 4.6B). Considering the effect of FTAseI on p38 MAPK phosphorylation in these RPAFs (figure 4.6C) there

Figure 4.5 Effects of Cholesterol Deprivation on Acutely Hypoxic RPAFs

Panel A shows the cholesterol biosynthesis pathway annotated to indicate the assessment of the cholesterol biosynthesis pathway and lipid raft integrity achieved by fluvastatin / squalene co-administration, squalene synthase inhibition (by zaragozic acid, ZA) and membrane cholesterol depletion (by methyl-β-cycoldextrin, MBCD).

Fluvastastin 1μM, squalene 25μM, zaragozic acid 60μM and 1% serum were added as indicated to quiescent RPAFs, prior to 24 hour incubation in normoxic (21%) or hypoxic (5%) conditions. Selected cells were pre-treated with MBCD 1mM for 30 mins prior to the start of the 24 hour experiment. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours (Panel B). Values shown are mean +/-SD from 4 replicate experiments on cells from the same animal. p38 MAP kinase phosphorylation was assessed by western blot analysis after 16 hours (Panel C). Data shown is representative of >3 repeat experiments on cells from different animals. (*values significantly greater than 1% serum-normoxia, $p<0.01$).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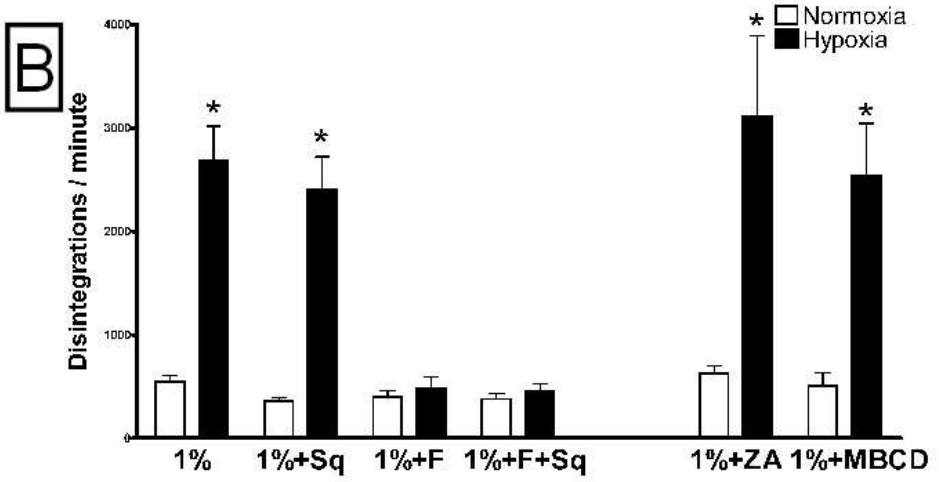
HMG-CoA
Fluvastatin
Mevalonate
Supplement
Squalene 0.25uM
Farnesyl pyrophosphate
Squalene
Geranylgeranyl pyrophosphate
Zaragozic acid
Cholesterol
Cell membrane lipid rafts
Methyl-ß-cyclodextrin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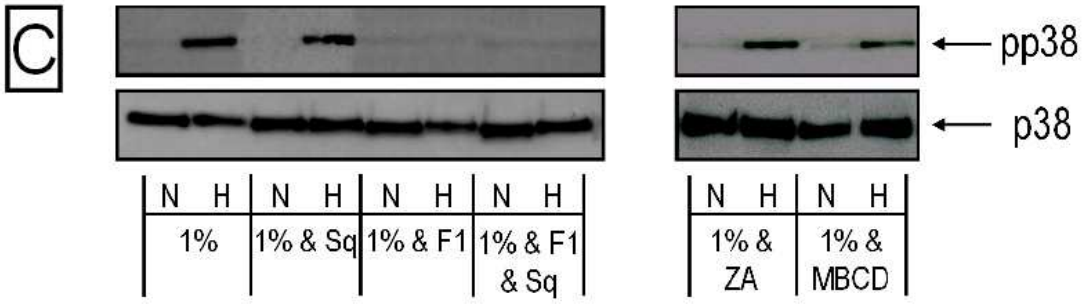
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N H N H
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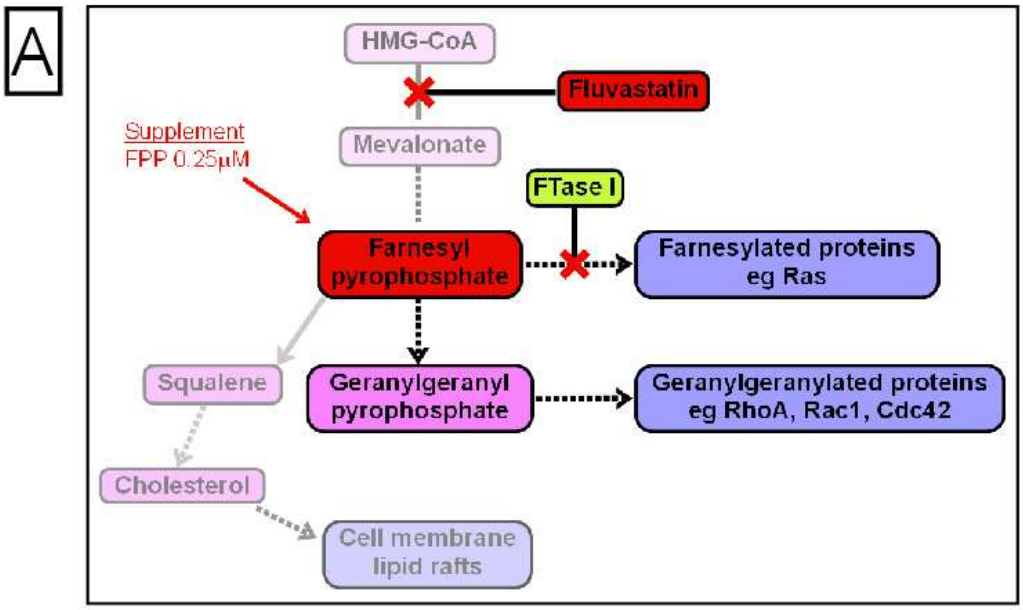
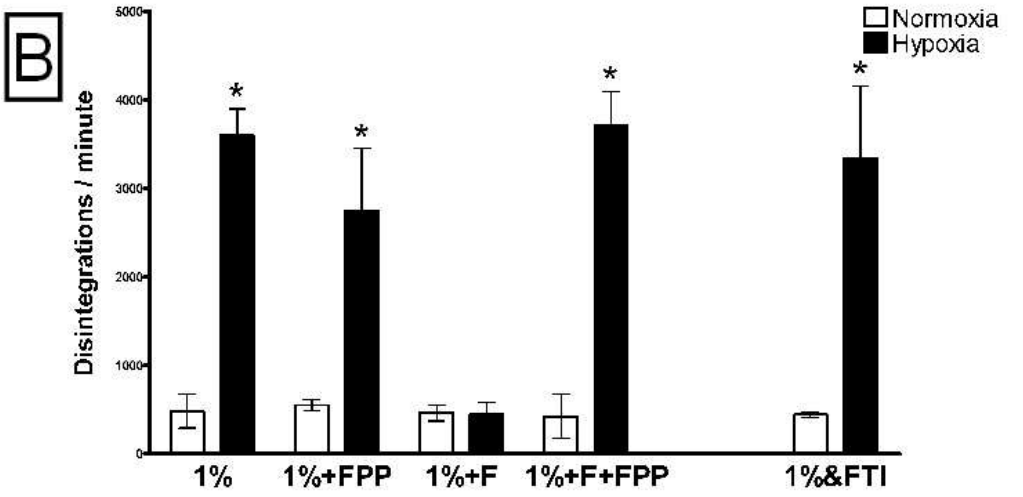
Figure 4.6 Farnesyl Pyrophosphate Dependant Pathways in Acutely Hypoxic RPAFs

Panel A shows the cholesterol biosynthesis pathway annotated to indicate the assessment of this achieved by fluvastatin / FPP repletion and a farnesyl transferase inhibitor.

Fluvastastin 0.5μM, the farnesyl transferase inhibitor FTaseI (FTI 5μM) and 1% serum were added as indicated to quiescent RPAFs, prior to 24 hour incubation in normoxic (21%) or hypoxic (5%) conditions. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours (Panel B). Values shown are mean +/-SD from 4 replicate experiments on cells from the same animal. p38 MAP kinase phosphorylation was assessed by western blot analysis after 16 hours (Panel C). Data shown is representative of >3 repeat experiments on cells from different animals. (*values significantly greater than 1% serum-normoxia, $p<0.01$).


A
HMG-CoA
Fluvastatin
Supplement
FPP 0.25μM
Mevalonate
FTase I
Farnesyl pyrophosphate
Farnesylated proteins eg Ras
Squalene
Geranylgeranyl pyrophosphate
Geranylgeranylated proteins eg RhoA, Rac1, Cdc42
Cholesterol
Cell membrane lipid rafts


B
Normoxia
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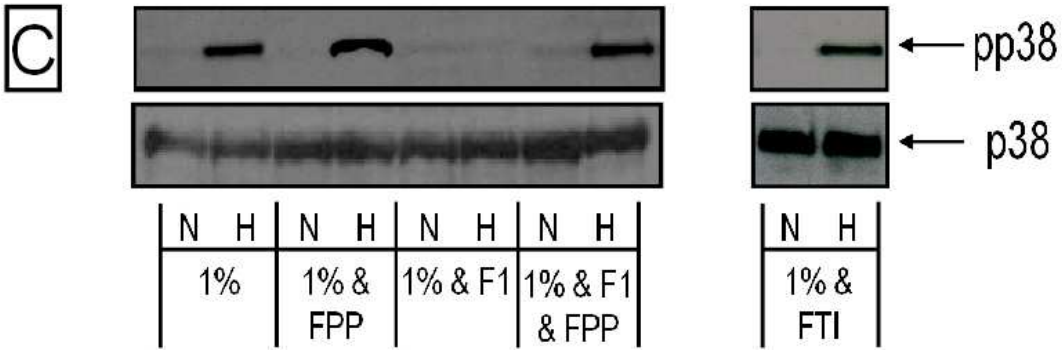
C
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p38
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1% & F1
1% & F1 & FPP
1% & FTI

is an apparent slight reduction in the pp38 blot in hypoxia, with this inhibitor. This is considered equivocal, particularly when considered in comparison to the abrogation of hypoxic p38 phosphorylation seen with fluvastatin and other the inhibitors investigated subsequently, and as it was not consistent with other results.

GGPP repletion (0.5μM) also completely negated the inhibitory effect of fluvastatin effect. GGTI-276 (5μM) selectively inhibited acute hypoxia-induced proliferation and p38 MAP kinase phosphorylation in RPAFs, mimicking the effects of fluvastatin 1μM (figure 4.7B & 4.7C).

4.3.6 Effects of a Rho Kinase and a Rac1-specific Guanine Exchange Factor Inhibitor on Hypoxia-induced RPAF Proliferation and p38 MAP Kinase Phosphorylation

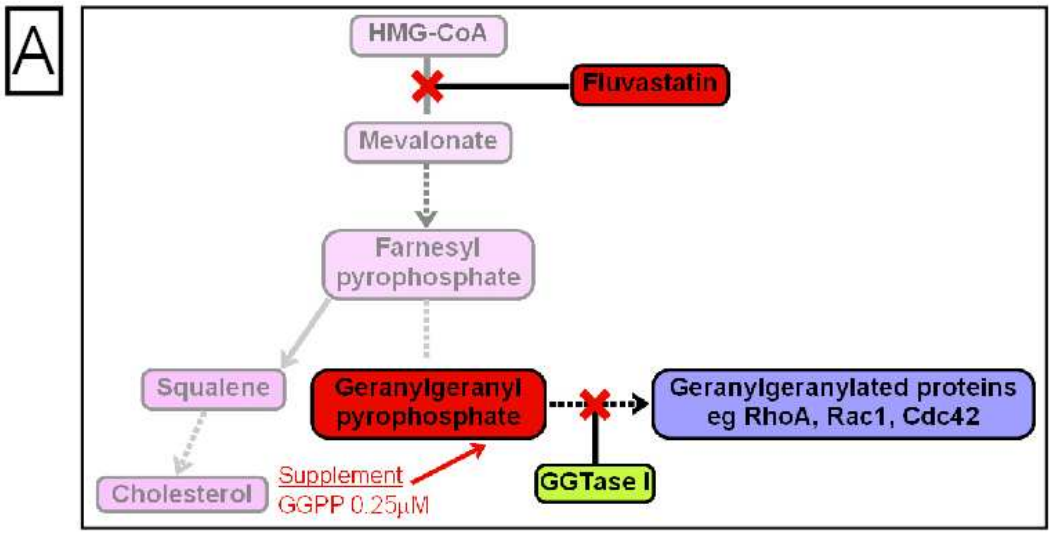
The preceding experiments implicated a geranylgeranylated protein (ie from the Rho superfamily) in acute hypoxic prolfierative signalling, upstream of p38 MAP kinase phosphorylation, in RPAFs. We had previously suspected a RhoA-Rho kinase or Rac1-NADPH oxidase pathway in hypoxic signalling in RPAFs (chapter 1.7.2). As a further (preliminary) assessment of these possibilities we obtained a selective Rho kinase inhibitor (hydroxyfasudil). Guanine exchange factor (GEF) proteins, when activated, convert GTPase proteins from the inactive GDP-bound state to an active GTP-bound state. NSC-23766 is a recently described compound which selectively inhibits the interaction between Rac1 and its specific GEFs (Trio and Tiam1)[244]. We also obtained this and repeated normoxia/hypoxia RPAF proliferation and p38 MAP kinase phosphorylation experiments with these 2 inhibitors.

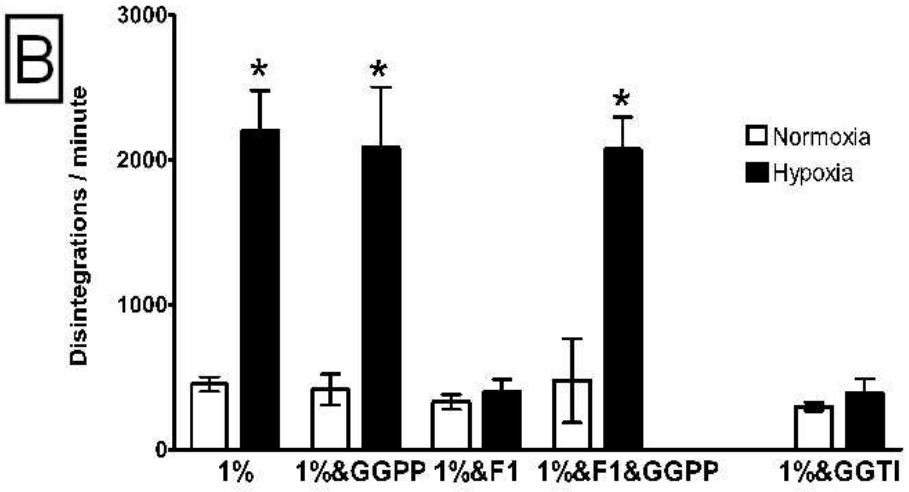
Hydroxyfasudil had no effect on serum or hypoxia-induced RPAF proliferation or hypoxia-induced p38 MAPK phosphorylation, even at high cell culture dose (20μM. In contrast, NSC-23766 (100μM) selectively inhibited hypoxia-induced p38 MAPK phosphorylation and proliferation in RPAFs (figure 4.8).

Figure 4.7 Geranylgeranylpyrophosphate Dependant Pathways in Acutely Hypoxic RPAFs

Panel A shows the cholesterol biosynthesis pathway annotated to indicate the assessment of this achieved by fluvastatin / GGPP repletion and a geranylgeranyl transferase inhibitor.

Fluvastastin 0.5μM, the geranylgeranyl transferase inhibitor GGTI-276 (GGTI 5μM) and 1% serum were added as indicated to quiescent RPAFs, prior to 24 hour incubation in normoxic (21%) or hypoxic (5%) conditions. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours (Panel B). Values shown are mean +/-SD from 4 replicate experiments on cells from the same animal. p38 MAP kinase phosphorylation was assessed by western blot analysis after 16 hours (Panel C). Data shown is representative of >3 repeat experiments on cells from different animals. (*values significantly greater than 1%serum-normoxia, $p<0.01$).


A
HMG-CoA
Fluvastatin
Mevalonate
Farnesyl pyrophosphate
Squalene
Geranylgeranyl pyrophosphate
Geranylgeranylated proteins eg RhoA, Rac1, Cdc42
Cholesterol
Supplement GGPP 0.25μM
GGTase I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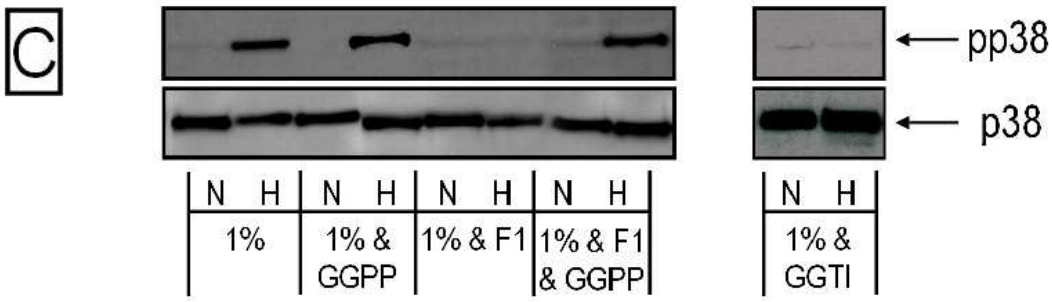
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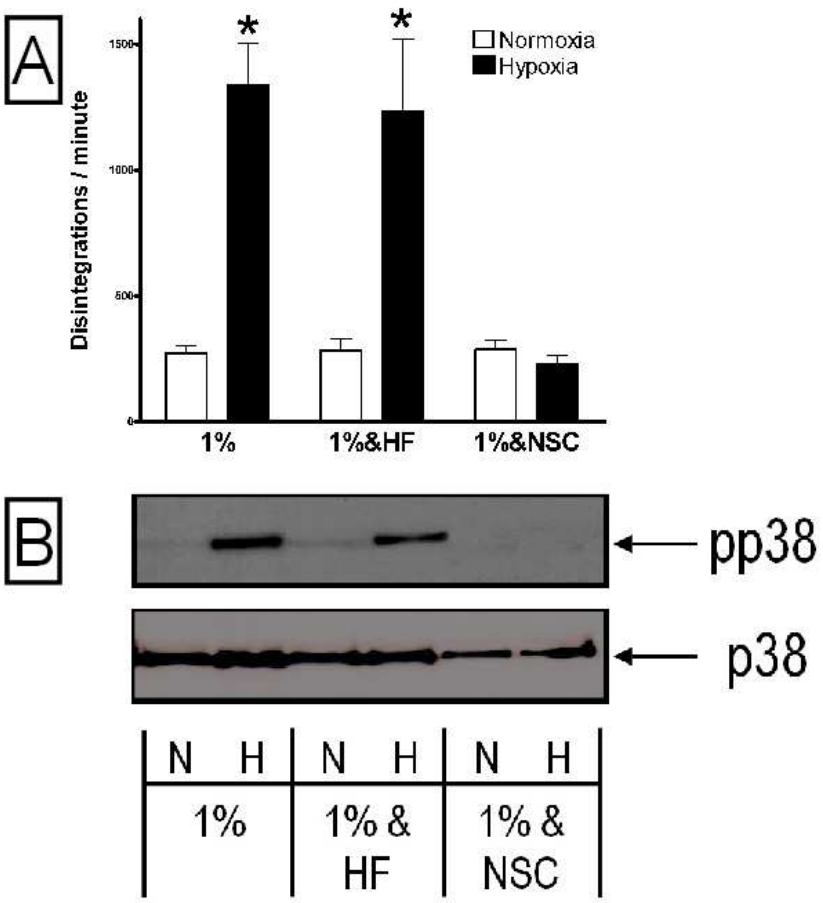

Figure 4.8 Effects of a Rho Kinase and a Rac1-GEF Exchange Inhibitor on Acutely Hypoxic RPAFs

The Rho kinase inhibitor hydroxyfasudil (HF, 20 μM), the selective Rac1 GEF-exchange factor inhibitor NSC-23766 (NSC 100μM) and 1% serum were added as indicated to quiescent RPAFs, prior to 24 hour incubation in normoxic (21%) or hypoxic (5%) conditions. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours (Panel A). Values shown are mean +/-SD from 4 replicate experiments on cells from the same animal. p38 MAP kinase phosphorylation was assessed by western blot analysis after 16 hours (Panel B). Data shown is representative of >3 repeat experiments on cells from different animals. (*values significantly greater the 1% serum-normoxia, $p<0.01$).

4.4 Discussion

In this chapter, the mechanism of the inhibitory effect of fluvastatin on acute-hypoxia induced proliferation of RPAFs (as identified in Chapter 3) was studied.

An obligatory role for p38 MAP kinase phosphorylation in acute-hypoxia induced PAF proliferation has been shown previously[150, 155, 171]. Statins have been shown to indirectly inhibit MAP kinase phosphorylation in other cell types and it was notable that the effect of fluvastatin 1μM on acute hypoxic RPAF proliferation (Chapter 3.3.5) duplicated those previously seen with the p38 MAP kinase inhibitor SB203580[233]. We considered it likely that the inhibitory effect of 1μM fluvastatin seen in the experiments in Chapter 3 would be via inhibition (directly or indirectly) of a signalling component upstream of p38 MAP kinase. It was also considered possible that fluvastatin may have a 'bystander' effect on the other MAP kinases and also that the effect of fluvastatin may be on a parallel signalling pathway, independent of p38 MAP kinase. These contentions were, however, refuted by the initial data in this chapter where we found that fluvastatin 1μM completely inhibited acute hypoxia-induced p38 MAP kinase phosphorylation, with no effect on total or phosphorylated ERK1/2 or JNK, either in normoxia or hypoxia.

Having confirmed that the inhibitory effect of fluvastatin was mediated upstream of p38 MAPK, it was considered that exploitation of this effect, in conjunction with available cholesterol biosynthesis pathway intermediates, offered the possibility of clarifying the nature of the upstream signalling pathway in the acute hypoxia-p38 MAP kinase–HIF-1a–proliferation pathway (previously identified in RPAF cells, in this laboratory).

When an antiproliferative effect of statin on a cell has been identified, co-administration of mevalonate, in order to clarify if the effect of statin is via HMG-CoA reductase inhibition, is a long established experimental modality[245]. Mevalonate, however, has been reported in other cell types to have independent effects on cell activity [246]. In our experiments, mevalonate had no effect on RPAF proliferation, even at high dose. Repletion with mevalonate negated the effect of

fluvastatin 1μM, implicating a cholesterol biosynthesis pathway component as an obligate compound for acute hypoxic proliferative signalling in RPAFs.

Other investigators have variously used FPP and GGPP to further study statin effects on proliferative cell signalling pathways, in other cell types and with other mediators. However, there is no known report where all 3 'arms' of this pathway have been assessed together, as completely as was undertaken here. Based on observations from other cell types, each of the individual cell signalling systems in question (lipid rafts, farnesylated and geranylgeranylated proteins) could conceivably be implicated in hypoxic signalling and/or MAP kinase pathways in RPAFs. As overlap or redundancy between these different systems was considered a possibility (eg a cell membrane based signalling protein may require both prenylation and lipid raft association for normal function), it was considered that, even if positive or negative results were found on early experiments with one or other 'arm', that a comprehensive experimental interrogation of this pathway was required.

The observation that standard experimental concentrations of squalene, FPP and GGPP could disrupt normal RPAF cell status (see section 4.3.3) provided some challenges. We were able to identify 'safe' concentrations of each of these compounds which did not disrupt RPAF cell status or serum/hypoxia-induced proliferation. This left us, however, with uncertainty as to how to interpret a partial or negative proliferation result. For example, if the effect of fluvastatin was unchanged when co-administered with the 'safe' concentration of a cholesterol pathway intermediate, would this be a true negative result or a function of inadequate repletion at the concentration of the intermediate used? Also, if a lack of effect was seen with a downstream inhibitor from the cholesterol pathway, it would be unclear if this were a true negative, or due to inadequate dosing or other experimental problem with the inhibitor under study.

To counter these concerns, we conceived and performed complementary experiments for each arm of the pathway: statin/cholesterol pathway intermediate repletion experiment + specific inhibitor experiments combined, for each arm. Moreover, when we considered the lipid raft arm, we noted the potential for crossover inhibition of farnesyltransferase with zaragozic acid. The negative result with this compound

implied that products of squlaene synthase and farnesyltransferase were not required for acute hypoxic RPAF proliferation. However, as we also obtained negative results the fluvastatin/squalene repletion experiment, we sought a third experiment to ensure that these results were 'true' negatives, rather than a function of a problem with these compounds or their cell culture dosing. Pre-incubation of RPAFs with methyl-β-cyclodextrin - to deplete membrane cholesterol and directly disrupt lipid rafts - was therefore also done. Though we haven't determined whether fluvastatin 1μM and/or MBCD had any effect or not on lipid raft integrity, the negative results with fluvastatin/squalene repletion, zaragozic acid and MBCD strongly suggest that the inhibitory effect of fluvastatin 1μM, on acute hypoxic proliferation and p38 MAPK phosphorylation, is not mediated via lipid raft disruption. These results also argue against a requirement for lipid raft integrity for normal acute hypoxic proliferative signalling in these cells.

Repletion of RPAFs with FPP negated the inhibitory effect of fluvastatin 1uM on acute hypoxic RPAF proliferation and p38 MAPK phosphorylation. With review of the cholesterol pathway, it can however be seen that this effect does not help to discriminate the statin's mechanism: FPP is a direct precursor of farnesylated proteins and other compounds involved in the cell cycle, and an indirect precursor (via GGPP) or geranylgeranylated proteins. The data on acute hypoxic RPAF proliferation and p38 MAPK phosphorylation which follow this - no effect with the farnesyl transferase inhibitor, negation of the effect of fluvastatin with GGPP repletion and duplication of the fluvastatin effect by the geranylgeranyl transferase inhibitor – led us to conclude, however, that the statin effect is mediated via inhibition of GPP synthesis. This conclusion does have a minor caveat in relation to the slight reduction in pp38 intensity in the acute hypoxia blot with the FTase inhibitor (chapter 4.3.5): this may have been a spillover effect of this inhibitor to GGTase at the experimental concentration explored but further study of this at different concentrations was not considered necessary, given the clear cut result with the GGPP compound and the geranylgeranyl transferase inhibitor.

These results also allow us to conclude that a geranylgeranylated protein is involved in acute hypoxic proliferation in RPAFs, upstream in the signalling pathway from p38 MAP kinase. Specifically, we considered the possibility that either RhoA or

Rac1 may be the geranylgeranylated protein in this pathway. The negative results with the Rho kinase inhibitor and positive results with the Rac1-GEF inhibitor (mimicking the effects of fluvastatin ie selective inihibition of acute hypoxic RPAF proliferation and p38 MAPK phosphorylation) provide circumstantial evidence for an acute hypoxia-Rac1-p38 MAPK pathway in these cells. Lack of time and resources meant that it was not possible to conduct experiments to determine Rac1-GDP vs Rac1-GTP (activated) status in normoxic, hypoxic and statin-treated RPAFs directly. The positive results with NSC-23766, however, do provide strong circumstantial evidence for the signalling pathway that is proposed in Figure 4.9.

Data from other researchers supports this proposed pathway. Rac1 has been identified as an upstream activator of p38 MAP kinase in other cell types[248]. Small inhibitory RNAs to NADPH oxidase 4 (NOX4) have been shown to inhibit acute hypoxia-induced increase in ROS production and proliferation in PAFs[247]. Function of NOXs and their transduction of external signals via intracellular ROS generation is known to be dependant on association of the complex with intracellular membranes[249]. Activated Rac1 is known to be a constituent of NADPH oxidase protein complexes, stabilising the interaction between inactive membrane-bound components of the complex, and intracellular activator components of the complex. Based on the findings in this chapter, it seems appropriate to speculate that, in RPAFs, acute hypoxia results in geranylgeranyl pyrophosphate dependant translocation of Rac1-NOX to the (plasma or endoplasmic reticular) membrane, leading to a localised increase ROS production which triggers a signalling cascade which results in phophorylation of p38 MAPK, stabilisation and phosphorylation of HIF-1α[155] and increased cellular proliferation. The previous findings that atorvastatin can inhibit intracellular ROS production via inhibition of Rac1 geranylgeranylation in vascular smooth muscle cells[250] and that enhancement of Rac1 function leads to increased membrane associated NADPH oxidase complex and ROS production, support this contention[251].

Even if the identity of the geranylgeranylated protein inhibited by fluvastatin in hypoxic RPAFs is considered to be incompletely clarified, the results in this chapter provide insights into the intracellular processes which may be involved in the activity

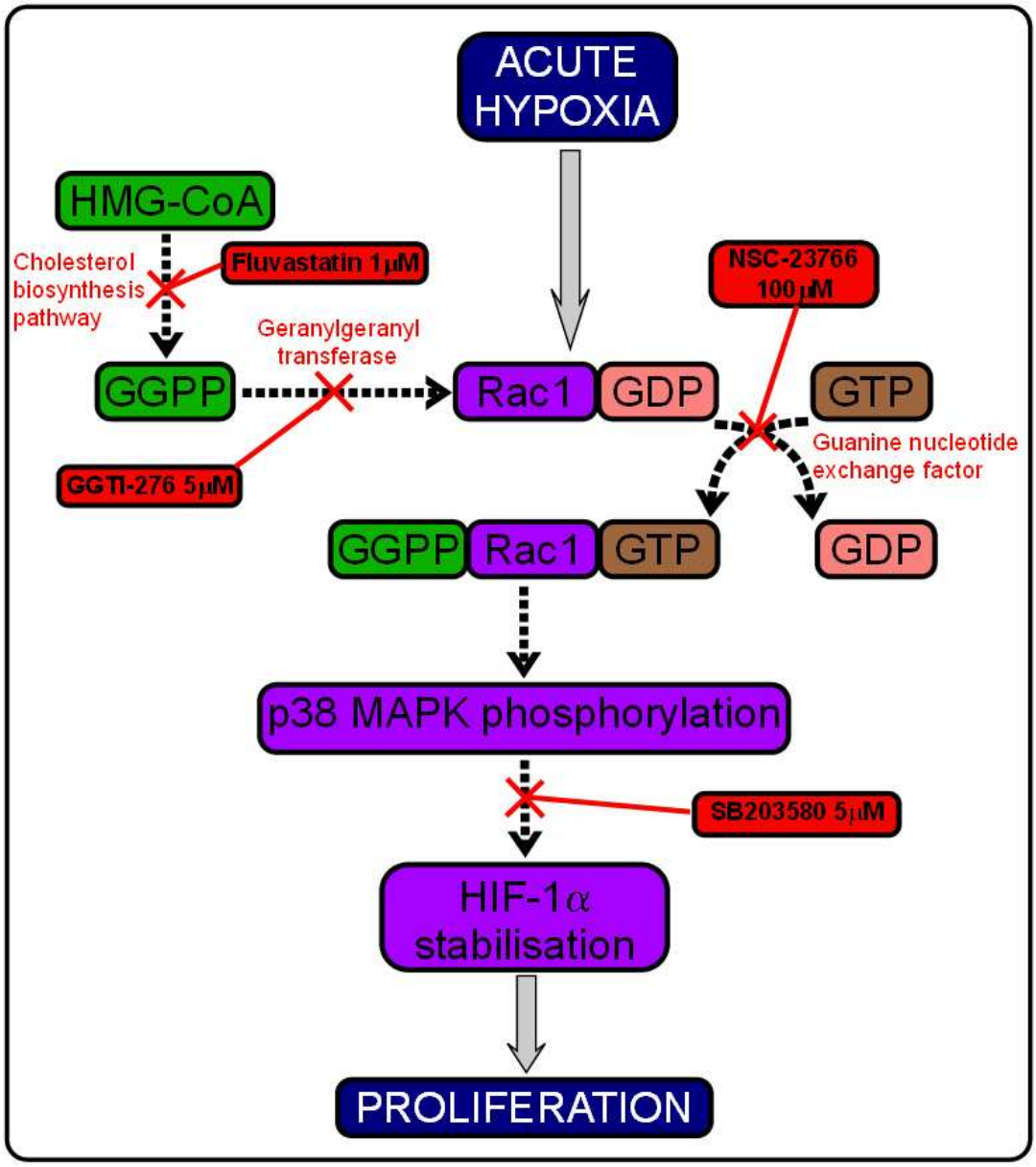

Figure 4.9 Proposed Model of Acute Hypoxic Proliferative Signalling in Rat Pulmonary Adventitial Fibroblast Cells

The results presented in this chapter, supplemented by previous work (on p38 MAPK inhibition and HIF-1α stabilisation) in the SPVU laboratory[155], suggest the above cell signalling pathway.

of this protein. Specifically, acute hypoxic signalling in RPAFs is blocked by a very small cell culture dose of statin (10-50 fold less than is typical for other inhibitory effects seen with statins). This finding - complemented by the results in this chapter - indicates a signalling process which is extremely dependant on ready 'supply' of GGPP. Differential sensitivity of individual cell signalling processes within a single cell type, to statin inhibition, has been documented in other cell types[252]. The observations in this chapter are also consistent with previous observations that – when activated - Rho-family proteins circulate from the cytosol to the plasma membrane[253]. The previous suggested explanation for this – which is supported by the results in this chapter – is that this intracellular circulation is dependant on dynamic prenylation of the protein.

In summary, a detailed interrogation of the HMG-CoA reductase pathway and the mechanism of fluvastatin's inhibitor effect on acute hypoxia-induced proliferation of RPAFs was conducted in this chapter. The results obtained provide significant new information on acute hypoxic signalling in this cell type and this is an important outcome of itself. That fluvastatin 1μM had a stimulus (hypoxia), circulatation (pulmonary) and MAP kinase (p38) specific inhibitory effect on this cell type, which is important in pulmonary vascular remodelling, seems highly notable. When we consider, however, potential application of these results to the pathogenesis or treatment of human hypoxia-induced pulmonary hypertension, acute hypoxia has problems as a model. In this human condition, by the time treatment is sought, hypoxia has been chronic. It therefore seemed relevant to consider whether statins have a positive effect on chronic hypoxic PAF behaviour, and whether geranylgeranylpyrophosphate, Rac1 and p38 MAP kinase have a role in chronic hypoxia-induced PAF proliferation. This is considered further in the following chapter.

Chapter 5

Effects of Fluvastatin on the Hyperproliferative Phenotype of Pulmonary Artery Fibroblasts from Chronic Hypoxic Rats

5.1 Introduction

In the preceding two chapters the ability of low dose fluvastatin to selectively inhibit acute hypoxia-induced RPAF proliferation, via inhibition of a geranylgeranylated protein-p38 MAPK pathway, was established. Though a drug which can selectively inhibit acute hypoxic proliferation may be considered to have therapeutic potential, more impressive would be the ability to reverse the hyperproliferative PAF phenotype seen after exposure to chronic hypoxia.

The important contributions of changes in pulmonary artery fibroblast behaviour in chronic hypoxia-induced pulmonary vascular remodelling have been discussed (see chapter 1.8). Following chronic hypoxia, pulmonary artery fibroblasts exhibit increased proliferative capacity and this effect persists on cell culture passage, regardless of whether the cells are maintained in normoxic or hypoxic conditions. This sustained, excessive proliferation of PAFs from chronic hypoxic rats (CH-RPAF) has been previously shown to be dependant on constitutive phosphorylation of p38 MAP kinase and reversed by p38 MAPK inhibition[177]. Also, as with acute hypoxia, this p38 MAPK response and increased proliferation is circulation specific: systemic adventitial fibroblasts (SAFs) from chronic hypoxic rats behave identically to SAFs from normal rats.

Based on all of this, we considered it possible that low dose fluvastastin (which had acted as an indirect p38 MAPK inhibitor on acute hypoxic RPAFs) might also influence CH-RPAF proliferation, if the geranylgeranylated protein (speculated to be Rac1), which acted upstream of p38 in acute hypoxic RPAFs, was also constitutively activated in CH-RPAFs. To explore these possibilities we proposed proliferation and Western Blot analysis experiments, comparing PAFs and SAFs from normal and chronic hypoxic rats, with assessment of the effects of fluvastatin, cholesterol intermediates and related inhibitors. We did not assess MAPKs other than p38, given the results obtained in Chapters 4.3.1.

5.2 Methods

For the chronic hypoxic model, rats were maintained in hypobaric hypoxia for 2 weeks (see general methods, chapter 2). Following this, pulmonary and aortic fibroblast cell lines were established and scrutinised for cell transformation and infection regularly. Quiescent cells were studied in experimental conditions – normoxia +/- serum, statin or other mediator- and cell proliferation was assessed by thymidine assay and phosphorylation of mitogen-activated protein kinases was determined by the application of relevant antibodies to nitrocellulose membranes containing proteins separated from cell extracts using an SDS-PAGE / western blot technique. Results are expressed in the figures as mean +/- 1 standard deviation.

5.3 Results

5.3.1 [^{3}H] Thymidine Uptake and p38 MAP Kinase Phosphorylation in Pulmonary and Aortic Adventitial Fibroblasts from Normal and Chronic Hypoxic Rats

Previous work in the SPVU laboratory on CH rats has confirmed the proliferation and p38 MAPK status in vascular fibroblasts of normal and chronic hypoxic rats. To clarify competency with the chronic hypoxic rat model and establish controls for the following work, these experiments were repeated. As 24 hours of acute hypoxia had previously been shown to have no additional effect on CH rat proliferation or p38 MAPK phosphorylation[233], all experiments in this chapter were conducted in normoxic (21%) conditions.

Increased proliferation of adventitial fibroblasts with increasing concentrations of serum was confirmed (figure 5.1A). Serum-treated PAFs from chronic hypoxic rats demonstrated increased [^{3}H] thymidine uptake at a given concentration of serum, in comparison with PAFs from normal animals. This hyperproliferative phenotype of CH-RPAFs was independent of cell passage (data not shown) and circulation specific: SAFs from normal and chronic hypoxic rats proliferated at similar rates with serum stimulation.

In CH-RPAFs, p38 MAP kinase was phosphorylated at all times points studied (16 hours is shown, to maintain consistency with previous experiments, figure 5.1B). No p38 MAP kinase phosphorylation was seen in PAFs or SAFs from normal rats or in SAFs from hypoxic rats.

The phenotype of CH rat systemic artery fibroblasts - normal proliferation to serum and absence of p38 MAPK phosphorylation - in comparison to pulmonary artery fibroblasts allowed the use of CH-RSAFs as control cells for the following experiments.

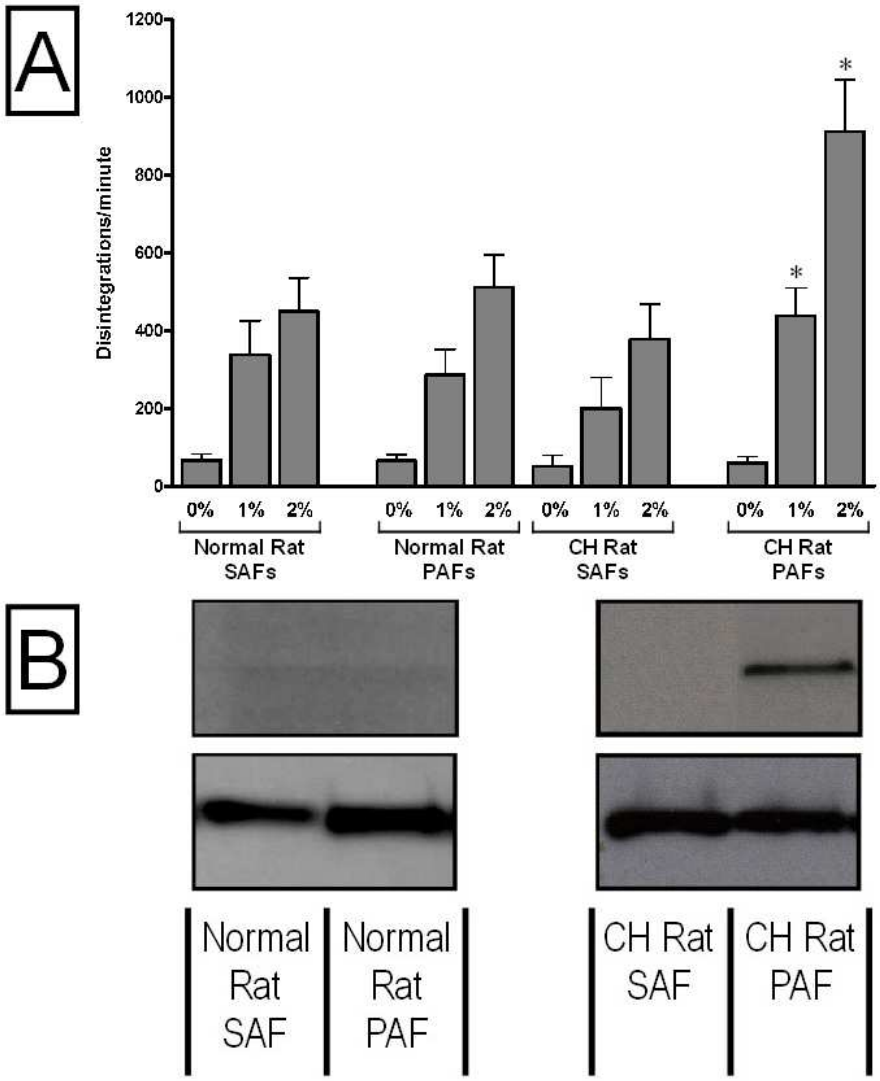

Figure 5.1 **[^{3}H] Thymidine Uptake and p38 MAP Kinase Phosphorylation in Pulmonary and Systemic Artery Fibroblasts from Normal and Chronic Hypoxic Rats**

Growth arrested pulmonary (PAF) and systemic artery fibroblasts (SAF) from normal and chronic hypoxic rats were stimulated with serum for 24 hours in normoxic (21%) conditions. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours (Panel A). Values shown are mean +/-SD from 4 replicate experiments on cells from different animals. p38 MAP kinase phosphorylation was assessed by western blot analysis after 16 hours (Panel B). Data shown is representative of >3 repeat experiments on cells from different animals. (*value significantly greater than that in normal rat PAF or CH-rat SAF, at equivalent serum dose, $p<0.05$).

5.3.2 Effects of Fluvastatin on the Chronic Hypoxic Rat Pulmonary Adventitial Fibroblast Phenotype

The effects of fluvastastin on serum-induced proliferation of CH rat PAFs and SAFs were determined, using doses (1μM and 10μM), based on experiments in Chapters 3.3.2 and 3.3.5.

Fluvastastin 1μM had no effect on CH-RSAF proliferation but it reduced serum-induced proliferation of CH-RPAF, such that 3[H] thymidine uptake was at a similar level to the control (SAF) cells (Figure 5.2A). Fluvastatin 1μM did not otherwise interefere with the proliferative response of CH-RPAFs or SAFs to incremental doses of serum. Fluvastatin 10μM had a complete / non-specific anti-proliferative effect on CH-RPAFs and SAFs, similar to that seen with PAFs and SAFs from normal animals.

Phosphorylated p38 MAP kinase was not detected (ie constitutive phophorylation was reversed) in CH-RPAF cells which had been incubated with fluvastatin 1μM for 16 hours (Figure 5.2B).

5.3.3 Effects of Cholesterol Pathway Intermediates and Related Inhibitors on the Chronic Hypoxic Rat Pulmonary Adventitial Fibroblast Phenotype

We next explored the mechanism of the selective pp38 MAPK inhibitory / anti-proliferative effect of fluvastatin 1μM on CH-RPAF cells. We duplicated a selected panel of experiments used on the acute hypoxic RPAF model (chapter 4.3). In particular, we used only the specific cholesterol intermediates and inhibitors (at the previously established appropriate dose) which had yielded positive results in the acute hypoxia model. This allowed an interrogation of the HMG-CoA reductase pathway in these chronic hypoxic rat cells, with a significantly reduced experimental workload.

As before, fluvastatin 1μM attenuated CH-RPAF [^{3}H] thymidine uptake to a level similar to control (SAF) cells, and inhibited/reversed constitutive p38 MAPK phosphorylation (Figure 5.3A and 5.3B).

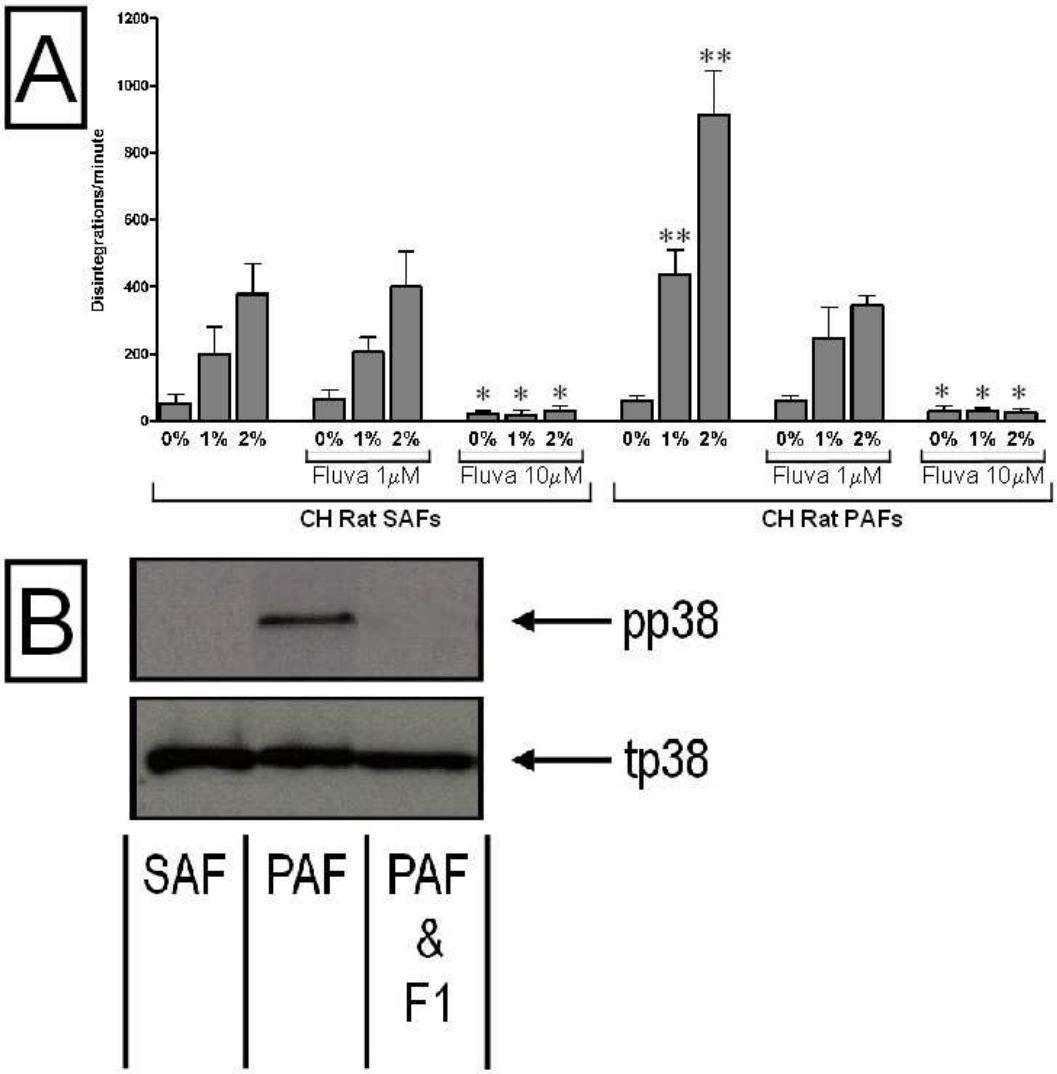

Figure 5.2 Effects of Fluvastastin on [^{3}H] Thymidine Uptake and p38 MAPK Phosphorylation in Chronic Hypoxic Rat PAFs

Growth arrested pulmonary (PAF) and systemic artery fibroblasts (SAF) from chronic hypoxic rats were stimulated with serum +/- fluvastatin (F) 1 or 10μM for 24 hours in normoxic (21%) conditions. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours (Panel A). Values shown are mean +/-SD from 4 replicate experiments on cells from different animals. p38 MAP kinase phosphorylation was assessed by western blot analysis after 16 hours (Panel B). Data shown is representative of >3 repeat experiments on cells from different animals. (*value significantly less than control cells, $p<0.05$; **value significantly greater than control cells, $p<0.05$)

Repletion of fluvastatin treated CH-RPAFs with mevalonate 1mM and GGPP 0.5μM negated the statin effect. Treatment of CH-RPAFs with the geranylgeranyl transferase inhibitor GGTI-276 (5μM), or the Rac1 GEF inhibitor NSC-23766 (100μM), attenuated [^{3}H] thymidine uptake to control cell levels. p38 MAPK phosphorylation was also inhbited/reversed by these inhibitors. These reagents had no effect on [^{3}H] thymidine uptake by chronic hypoxic rat systemic artery fibroblasts (data not shown).

5.3.4 Effects of Established Pulmonary Hypertension Therapeutics on [^{3}H] Thymidine Uptake in Chronic Hypoxic Rat Pulmonary Adventitial Fibroblasts

Having demonstrated a positive and selective effect of fluvastatin on CH-RPAFs, we sought to determine if this effect simply duplicated an effect seen with established pulmonary hypertension therapeutics.

Cell culture concentrations of treprostinil diethanolamine (UT-15c), sildenafil and bosentan which did not have an effect on normal rat PAF proliferation had been previously identified (chapter 3.3.6). These doses (1μM of UT-15c and 10μM of sildenafil and bosentan) were applied to CH-RPAFs and the effect on [^{3}H] thymidine uptake was determined.

The prostacyclin analogue, PDE-5 inhibitor and ET-1 receptor antagonist had no effect on CH-RPAF proliferation (figure 5.4), nor on systemic artery fibroblast proliferation (data not shown), at these doses. Given this negative result, the effect of these agents on p38 MAPK phosphorylation in CH-RPAFs was not studied.

Figure 5.3 Effects of Fluvastastin, Cholesterol Pathway Intermediates and Related Inhibitors on [^{3}H] Thymidine Uptake and p38 MAPK Phosphorylation in Chronic Hypoxic Rat PAFs

Growth arrested pulmonary (PAF) and systemic artery fibroblasts (SAF) from chronic hypoxic rats were stimulated with serum for 24 hours in normoxic (21%) conditions. Fluvastatin (F1, 1μM), mevalonate (M, 1mM), geranylgeranyl pyrophosphate (GGPP, 0.5μM), GGTI-276 (GGTI, 5μM) or NSC-23766 (NSC, 100μM) were added, at the start of the experiment, as indicated.

DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours (Panel A). Values shown are mean +/-SD from 4 replicate experiments on cells from different animals. p38 MAP kinase phosphorylation was assessed by western blot analysis after 16 hours (Panel B). Data shown is representative of >3 repeat experiments on cells from different animals. (*value significantly greater than CH-RSAF control cells, $p<0.01$).

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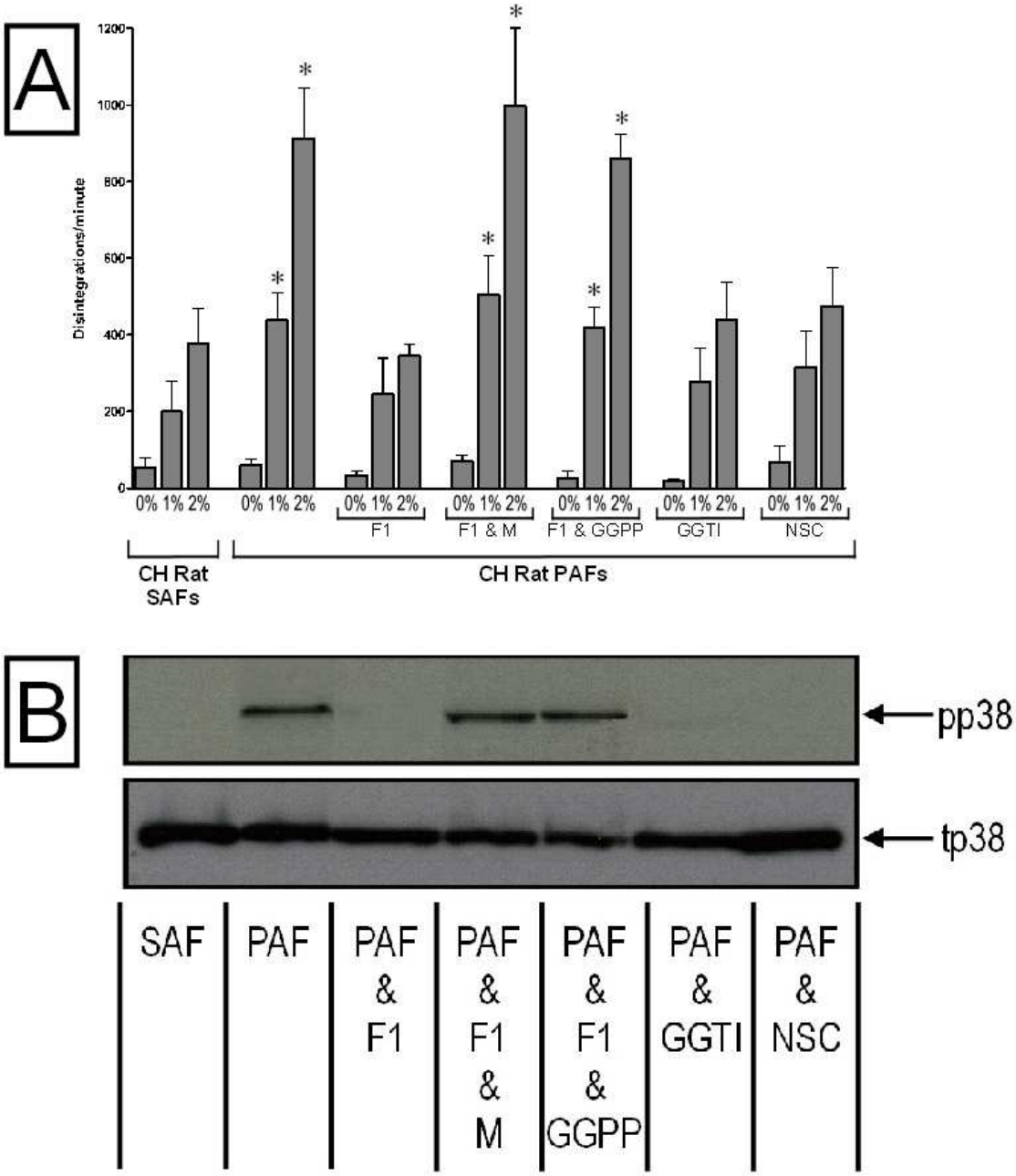
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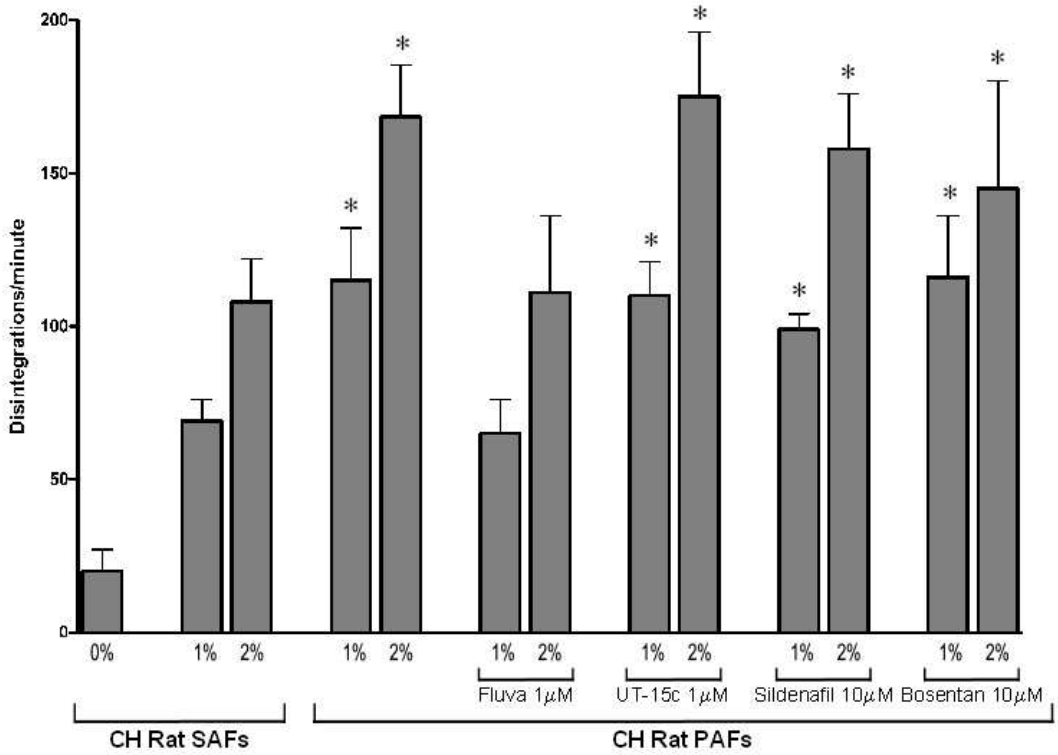

Figure 5.4 Effects of Fluvastastin and Established PH Therapeutic Agents on [^{3}H] Thymidine Uptake in Chronic Hypoxic Rat PAFs

Growth arrested pulmonary and systemic artery fibroblasts from chronic hypoxic rats were stimulated with serum for 24 hours in normoxic (21%) conditions. Fluvastatin, prostacyclin analogue (UT15c), phosphodiesterase-5 inhibitor (sildenafil) and endothelin A/B receptor antagonist (bosentan) were added along with serum, as indicated.

DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours. Values shown are mean +/-SD from 4 replicate experiments on cells from different animals. (*values significantly greater than CH-RSAF control cells, $p<0.01$).

5.4 Discussion

In this chapter, we sought to determine whether fluvastatin had a selective anti-proliferative effect on chronic hypoxic rat PAFs and, if this effect was present, if this was indicative of constitutive activation of a geranylgeranylated protein-p38 MAP kinase pathway in CH-RPAFs, similar to pathway proposed for acute hypoxic RPAF proliferation, in Chapter 4.4.

The results in this chapter with CH-RPAFs mirrored those seen with acute hypoxia applied to RPAFs from normal animals. The selective inhibitory effect of fluvastatin 1μM on CH-RPAF proliferation and p38 MAPK phosphorylation, negation of this effect by mevalonate and GGPP and duplication of this effect with geranylgeranyl transferase and Rac1 inhibitors strongly suggests that the hyperproliferative phenotype of CH-RPAFs is dependant on constitutive activation of the same signalling pathway as is activated by acute hypoxia in normal rat PAFs.

A phenotypic switch in PAF behaviour after exposure to chronic hypoxia, whereby explanted PAFs from chronic hypoxic rats exhibit enhanced proliferation, dependant on constitutive p38 MAPK phosphorylation, even once exposed to normoxic conditions / multiple cell passage, has been previously proposed and suggested as an important mechanism in chronic hypoxic pulmonary vascular remodelling[48]. In the experiments conducted in this chapter, this phenotypic switch has again been confirmed and linked to p38 MAPK status. The experiments with fluvastatin, cholesterol pathway intermediates, related inhibitors and PH therapeutics further characterise the molecular basis for this phenotypic switch and provide an indication of the potential of statin therapy to positively influence cellular mechanisms important in hypoxia-related pulmonary hypertension.

As with the acute hypoxic RPAF work in chapter 4, the data in this chapter implicates a Rac1-NADPH oxidase signalling complex in proliferative signalling, upstream of p38 MAPK, in chronic hypoxic PAFs. Moreover, the abrogation of excessive CH-RPAF proliferation by 1μM fluvastatin suggests that the molecular location of the phenotypic

proliferative switch in these cells lies at an upstream location, prior to Rac1. The sensitivity of this to statin inhibition is again notable, and combined with the repletion / complementary inhibitor experiments indicates again that the activated signalling pathway here is very dependant on a ready supply of GGPP. This suggests a dynamic process is continuing in these cells – eg Rac1 is continually cycling through a prenylated / unprenylated state – rather than a static, irreversible change in this signalling protein's status.

Lipophilic statins have been previously shown to attenuate and reverse chronic hypoxic pulmonary hypertension in rats[4, 220]. The results presented here complement and extend these, in several ways. The animal model experiments reported suggest that the positive effects of statins are mediated via HMG-CoA reductase (agreeing with our results) and exploratory work from the animal models suggested that modification of eNOS by statins is not involved in their mechanism of action, but that inhibition of Rho-kinase activity may be. Medial thickening in the pulmonary arteries of the CH-rats is also attenuated / regressed by statin therapy, suggesting a direct or indirect benficial effect of statins on smooth muscle cells. Our data suggests that the positive effects of statins on CH rat pulmonary hypertension *in vivo* may be mediated, at least partly, via positive effects on adventitial fibroblasts, and via a Rac1-p38 MAP kinase pathway. A particular difficulty with all experiments of potential new therapeutics on experimental PH models is that the effects of many drugs on these models (eg sildenafil, simvastatin, VIP, adrenomedullin) are, broadly speaking, similar, and typically out of proportion to the more modest effects seen when translating findings (so far) to human clinical disease. The similarity of the effects of these drugs on the animal models makes the identification of whether a new drug has a novel effect or simply duplicates the effect(s) of an established agent difficult. The results in this chapter and in chapter 3.3.6 – obtained in a cell-based model rather than an animal model - indicate that statins have a potentially beneficial effect on a disease-related cellular mechanism which does not simply duplicate an effect of a drug from each of the three established PH therapeutic drug classes.

An unanswered question in relation to statins (and many other drugs with demonstrated positive effects on chronic hypoxic rat PH) is how essential they are: do they simply duplicate the effects of re-oxygenation (ie the condition could as easily be treated by oxygen, as by other drugs) or do they enhance the effects of re-oxygenation? Our finding of a phenotypic switch in CH-RPAFs, persisting in normoxic cell culture could be interpreted as evidence that simple re-oxygenation would be inadequate to restore normal pulmonary vascular cell physiology. However, if the interpretation of the sensitivity of this phenotypic switch to low dose fluvastatin is correct (ie that the activation of the Rac1-p38 MAPK pathway is dynamic) this raises the possibility that the phenotypic switch may itself be dynamic, and potentially subject to reversal by re-oxygenation, *in vivo* where cell-cell and cell-matrix interactions may modify the effects of hypoxia then subsequent normoxia, in contrast to the explant cell situation.

When considering human hypoxia-related PH and its treatment, it is notable that the characteristics of the underlying lung problem often prohibit complete correction of hypoxia, even with supplemental oxygen. Also, patients may exhibit persistent or progressive pulmonary hypertension, even if hypoxia and the underlying condition is treated. Therefore, drugs which can duplicate or enhance the effects of re-oxygenation on the pulmonary vasculature seem relevant. The experimental data previously reported and presented in this chapter would suggest that statins may have the potential to complement re-oxygenation (whether achieved with oxygen supplementation or treatment of the lung disorder). The ability of fluvastatin to selectively normalise CH-RPAF proliferation at a cell culture dosage potentially relevant to human pharmacology is notable. Further work to extend this observation and assess re-oxygenation formally (*in vitro or in vivo)* was outwith the scope of this project. It would, however, seem relevant in future to assess the effects of re-oxygenation +/- statin therapy, in the chronic hypoxic rat model, in particular considering the differential effects of these on regression of pulmonary vascular remodelling and normalisation of the PAF phenotype.

In addition to general concerns about the applicability of cell culture experiments on animal tissue to human disease, there are specific drawbacks when considering the

clinical application of the results in this chapter. Experiments were only conducted on cells from Wistar rats. Different strains of rats are known to exhibit different pulmonary vascular cell responses to hypoxia[254], and it may be that the CH-RPAF proliferative phenotype, the Rac1-p38 MAPK signalling pathway, and the effects of fluvastatin on these, are unique to this rat strain. Future work should extend these observations to PAFs from other rat strains (eg Sprague-Dawley or high altitude susceptible rats) and to human cells, ideally from lung tissue from normal patients and patients with hypoxic lung disease related pulmonary hypertension. Access to human tissue from patients with hypoxic PH is very limited, and potentially confounded by the effects of the underlying primary lung disease. Some reassurance can, however, be taken from the previous observations indicating the identical proliferative and MAPK responses to acute hypoxia seen when comparing human PAFs with Wistar rat PAFs[180]. Overall, it is considered that the experiments conducted here provide important insights into molecular and cellular processes potentially involved in hypoxic pulmonary hypertension and reflect a reasonable compromise between expediency and applicability.

When we consider pulmonary vascular remodelling in animals or humans, although significant changes are seen in the adventitia, the most notable changes in established disease are in the media, where the smooth muscle cells reside. In the chronic hypoxic simvastatin experimental work, prevention / regression of medial hypertrophy was the notable histological finding (though the reports available provide no information on the status of the adventitia or the fibroblasts). Interactions between adventitial fibroblasts and medial smooth muscle cells are thought likely to be important in hypoxic pulmonary vascular remodelling and we considered it possible that the positive effects of simvastatin on medial hypertrophy in the chronic hypoxic rat model might be mediated via an initial positive effect on adventitial fibroblasts, with consequent favourable modification of fibroblast-smooth muscle cell interactions. Experimental work to better characterise PAF-PASMC interactions in hypoxia was ongoing in our laboratory at this time. Having identified and characterised a positive effect of fluvastatin on CH-RPAF proliferation in the experiments in this chapter, it seemed logical to progress to characterise the effects of fluvastatin on hypoxic PAF-PASMC interactions. This proposition led to the work which comprises the following chapter.

Chapter 6

Effects of Hypoxia and Fluvastatin on Bovine Pulmonary Adventitial Fibroblast – Pulmonary Artery Smooth Muscle Cell Interactions

6.1 Introduction

In the preceding chapters, the focus has been on hypoxia-induced adventitial fibroblast proliferation and the signalling systems involved. Although it is considered likely that this proliferation is an important contributor to pulmonary vascular remodelling in pulmonary hypertension (as discussed in Chapter 1.8) it is also acknowledged that the most profound changes (medial hypertrophy, sustained vasoconstriction, muscularisation of distal arteries, neointimal formation) involve the media of the vessel. It may be that migration and transdifferentiation of adventitial fibroblasts is important to the expansion in numbers of smooth muscle/myofibroblast cells in remodelled vessels but it seems likely that proliferation/phenotypic changes in medial SMCs contributes significantly.

Much of this work on pulmonary vascular remodelling has focused on the endothelial and smooth muscle cells, and their interactions. Notably, endothelial-derived vasodilators (eg prostacyclin, nitric oxide) and vasoconstrictors (eg endothelin-1, thromboxane) have been implicated in smooth muscle cell proliferation and consequent vascular remodelling: the processes of vasoconstriction and remodelling seem to be connected. In hypoxic pulmonary vasoconstriction, however, endothelial-independent constriction dominates and the signalling pathways involved (eg inhibition of membrane potassium channels, RhoA-Rho kinase activation) are intrinsic to the pulmonary artery smooth muscle cells (PASMC). These cell signalling pathways are also implicated in PASMC proliferation, supporting the concept of vasomotor coupling (see Chapter 1.6.3). As a counterpoint, however, many investigators (with caveats about definitions of hypoxia, Chapter 1.6.5) have been unable to demonstrate that the isolated PASMCs proliferate to hypoxia, *in vitro*[137]. Possible explanations are that the medial hypertrophy seen is due to an expansion of a subpopulation of hypoxia-responsive PASMCs (not represented in the PASMC population routinely cultured), or that the medial hypertrophy/distal muscularisation is caused by an influx of transdifferentiated cells originating from the adventitia, or the circulation. The explanation with the most experimental support, however, is that the presence of a paracrine co-factor (either intrinsic or hypoxia-dependant), released from other the other cells in the vessel wall, in the *in vivo* hypoxic pulmonary artery, is necessary

for PASMC proliferation. This mitogenic co-factor may facilitate PASMC proliferation in response to hypoxia, or may be a direct PASMC mitogen which is only released by adjacent cells or from the circulation, in response to hypoxia. Given that the earliest vascular changes in hypoxia are seen in the adventitia, it seems appropriate to speculate that the fibroblast may be the source of this PASMC mitogen or co-mitogen. Mitogens (including endothelin-1 and hypoxia-inducible mitogenic factor, HIMF) have been shown to be released by hypoxic pulmonary adventitial fibroblasts and these are known to stimulate PASMC proliferation, supporting this speculation[105, 255].

We considered the possibility that the cell signalling pathways responsible for proliferation of PAFs might also be responsible for secretion by hypoxic PAFs of a PASMC mitogen. Work on hypoxic PAF-PASMC interactions is ongoing in the SPVU laboratory and, based on the results in the preceding chapters, we proposed experiments with statins, cholesterol pathway mediators, related inhibitors and PH therapeutics in order to better characterise hypoxia-induced cell signalling events which lead to mitogen release by PAFs.

Cell-cell interactions *in vivo* can be modelled in the cell culture laboratory. Two complementary techniques were used here:-

- **Co-culture.** This involves the use of basket-like cell culture plate inserts with a permeable membrane. The 2 cells types of interest are grown separately, one in standard cell culture wells and the other on the cell culture inserts. During the experimental period the inserts (containing cell type 1) are placed in the cell culture wells (containing cell type 2). The permeable membrane allows any secreted paracrine factors(s) from the cells in the inserts to pass freely and interact with the other cell type. Inserts with different membrane pore sizes are available. Here we used pore sizes which freely allow transfer of large molecules (including any experimental reagents and secreted factors) but not cell migration.
- **Conditioned media.** Cell culture media which has been in contact with one cell type (in this situation, PAFs) for a period (under

experimental conditions) can be aspirated and applied to cells of another type (in this situation PASMCs). The effects of this conditioned media on proliferation can be compared with control media, allowing assessment of any mitogenic factors released.

Both of these have drawbacks. Co-culture cell culture inserts are expensive. Any experimental drug or inhibitor applied during co-culture experiments can diffuse freely and affect either cell type in the experiment. In conditioned media experiments, any drug or inhibitor applied to the initial cell type will remain in the conditioned media and can affect the second cell type also. These 2 experimental techniques are, however, complementary. In addition, any experiments which yield positive findings can be duplicated such that the experimental condition is applied to cell type 1 for the period where media is conditioned **and** separately after conditioning but before application to cell type 2. With these approaches, which cell type the experimental reagent is affecting can be deduced.

Rat PAFs are problematic for these experiments as they only respond to hypoxia in the presence of serum[180]. Any serum applied to the PAFs (in conditioned media or co-culture) would also affect PASMC proliferation and this would require an entire extra set of experimental control conditions. Also, the serum might confound / overwhelm the effect of any secreted mitogen. Human and bovine PAFs do not require a serum-cofactor to respond to hypoxia and, accordingly, bovine cells were selected for the work in this chapter.

Previous work has reassured that there are no differences in proliferative or MAP kinase responses to hypoxia with PAFs derived from different species. It was, however, considered relevant to exclude any difference (in comparison to rat PAFs) in terms of the effects of statins and other mediators on hypoxia-induced bovine PAF proliferation. Also, it was necessary to clarify the effects of acute hypoxia and statins on bovine PASMC proliferation, to characterise the isolated cell type. We therefore proposed proliferation experiments – with fluvastastin and other mediators - on bovine PAFs and PASMCs separately, as a prelude to studying the effects of acute hypoxia, fluvastatin and other reagents on BPAF-BPASMC interactions using conditioned media and co-culture experiments.

6.2 Methods

Bovine pulmonary artery fibroblast (BPAF) and bovine pulmonary artery smooth muscle cell (BPASMC) lines were established and characterised (Figure 6.1). Interactions between these cells were studied by conditioned media and co-culture techniques (see general methods, Chapter 2.7). The influence of experimental mediators on these cellular interactions, and the overall effect on BPASMC proliferation was determined by thymidine assay. Results are expressed in the figures as mean +/- 1 standard deviation.

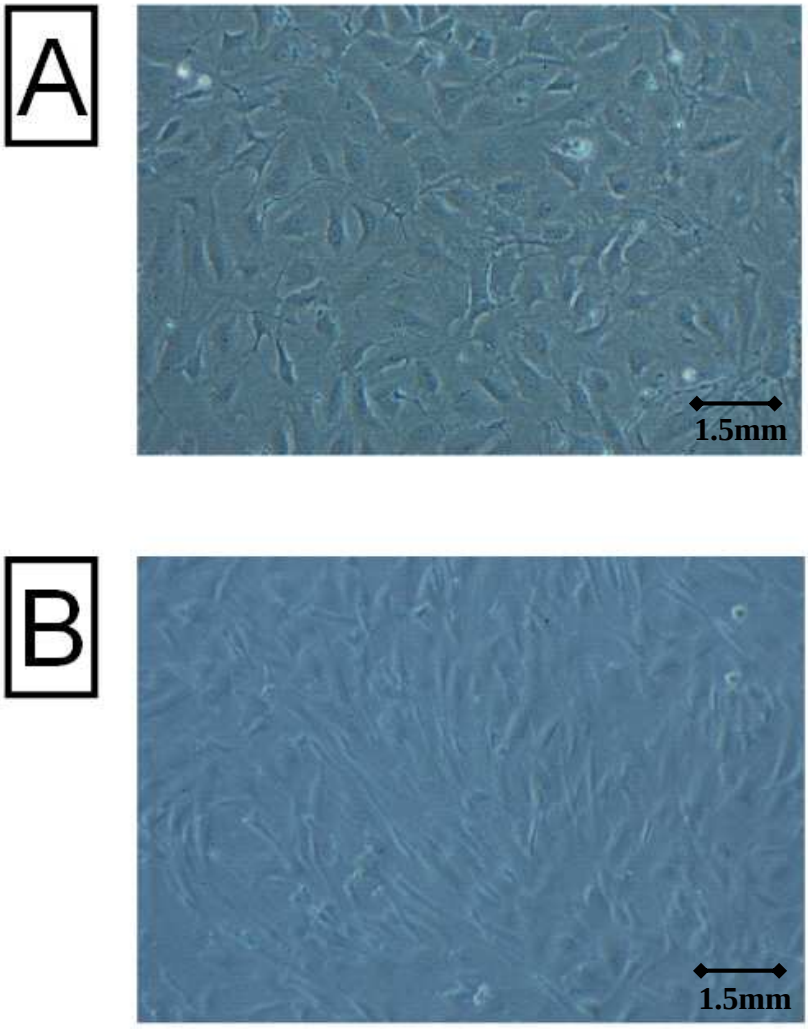

Figure 6.1 Bovine Pulmonary Artery Fibroblasts (A) and Pulmonary Artery Smooth Muscle Cells (B)

Cells were visualised by light microscopy. Characteristic morphology was noted and verified at each cell passage.

6.3 Results

6.3.1 Effects of Hypoxia, Serum and Fluvastatin on Bovine Pulmonary Adventitial Fibroblast Proliferation

Bovine PAFs have previously been shown to proliferate to acute hypoxia in the absence of serum[233]. In the presence of serum, the proliferative effects of acute hypoxia are augmented significantly.

The effects of fluvastatin on acute hypoxic and serum-augmented acute hypoxic proliferation of bovine PAFs were studied using [^{3}H] thymidine assay. 24 hours of acute hypoxia (5%) again resulted in increased proliferation of BPAFs, both in the absence or presence of serum (Figure 6.2). Fluvastatin 1μM selectively attenuated BPAF proliferation in acute hypoxia, restoring it to a level similar to normoxic cells, both with and without serum.

6.3.2 Effects of Choleterol Pathway Intermediates and Related Inhibitors on Bovine Pulmonary Adventitial Fibroblast Proliferation

We next sought to determine if the effects of cholesterol pathway intermediates and related inhibitors on acute hypoxia-induced bovine PAF proliferation were similar to those seen with rat PAFs (Chapter 4).

The effects of fluvastatin 1μM on acute hypoxia-induced BPAF proliferation were negated by mevalonate and GGPP (figure 6.3). The p38 MAP kinase inhibitor SB203580 (5μM), geranylgeranyl transferase inibitor (GGTI-276, 5μM) and the selective Rac1-GEF inhibitor (NSC-23766, 100μM) selectively inhibited serum-augmented, acute hypoxia-induced BPAF proliferation.

6.3.2 Effects of Hypoxia, Serum and Fluvastatin on Bovine Pulmonary Artery Smooth Muscle Cell Proliferation

Effects of acute hypoxia on bovine PASMCs, under our standard experimental conditions, had not previously been reported. The effects of serum, 24 hours of acute hypoxia (5%) and fluvastatin on DNA synthesis by bovine PASMCs were determined.

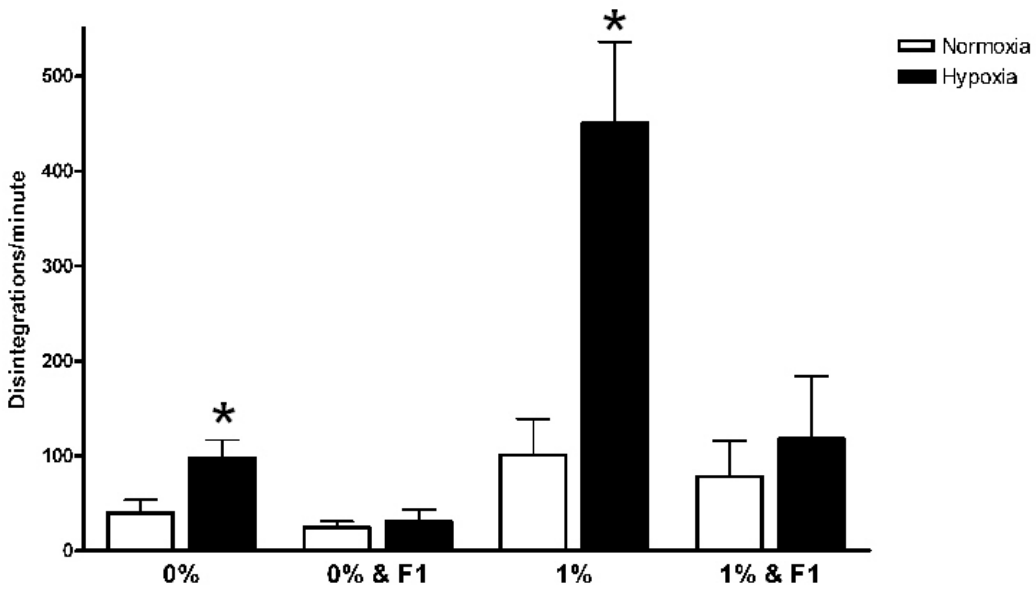

Figure 6.2 Effects of Acute Hypoxia and Fluvastatin on [^{3}H] Thymidine Uptake by Bovine Pulmonary Artery Fibroblasts.

Growth arrested bovine PAFs were stimulated in normoxia (21%) or hypoxia (5%) for 24 hours +/- 1% serum +/- fluvastatin (F) 1μM. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours. Values shown are mean +/-SD from 4 replicate experiments. (*value significantly greater than normoxic cells, $p<0.01$).

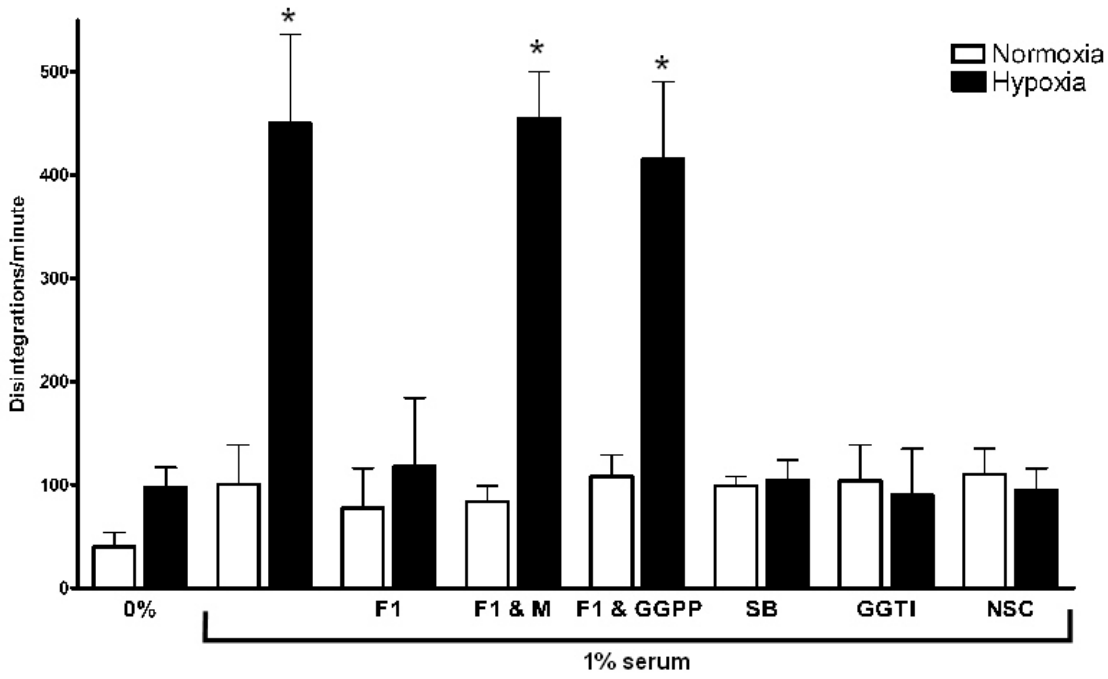

Figure 6.3 Effects of Acute Hypoxia, Fluvastatin, Cholesterol Pathway Intermediates and Related Inhibitors on [^{3}H] Thymidine Uptake by Bovine Pulmonary Artery Fibroblasts.

Growth arrested BPAFs were stimulated in normoxia (21%) or hypoxia (5%) for 24 hours. 1% serum, fluvastatin (F) 1μM, mevalonate (M, 1mM), GGPP (0.5μM), SB203580 (SB, 5μM), GGTI-276 (GGTI, 5μM) or NSC-23766 (NSC, 100μM) were added to selected wells on the cell culture plate. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours. Values shown are mean +/-SD from 4 replicate experiments. (*value significantly greater than normoxic cells, $p<0.01$).

Acute hypoxia had no effect on BPASMC [^{3}H] thymidine uptake, either in the presence or absence of serum (figure 6.4). Fluvastatin 1μM had no effect but fluvastatin 10μM significantly reduced serum-induced [^{3}H] thymidine uptake, similar to that previously seen with fluvastatin 10μM applied to serum-treated pulmonary and systemic artery fibroblasts (chapter 3.2).

6.3.3 Effects of Fibroblast Conditioned Media, Fibroblast Co-culture and Hypoxia on Pulmonary Artery Smooth Muscle Cell Proliferation

We considered two possibilities:- either that acute hypoxia caused PAFs to release a BPASMC mitogen or that PAFs secrete a mitogen (either constitutively or only in hypoxia) which facilitates a proliferative effect of acute hypoxia on PASMCs. To clarify this, we first studied the effects, on BPASMC proliferation, of different doses of conditioned media derived from normoxic and hypoxic BPAFs. Also, we determined the effects of BPAF-BPASMC co-culture, in normoxia and hypoxia, on BPASMC proliferation.

Conditioned media from normoxic BPAFs had no effect on BPASMC DNA synthesis over 24 hours, either in normoxia or hypoxia (figure 6.5). Conditioned media from hypoxic BPAFs led to increased BPASMC [^{3}H] thymidine uptake, and a dose response was seen. Maintaining the BPASMCs in normoxia or hypoxia had no effect on the proliferative response to conditioned media. Co-culture of BPAF-BPASMCs in normoxia had no effect on BPASMC DNA synthesis. Co-culture in hypoxia, however, led to an increase in [^{3}H] thymidine uptake by BPASMCs.

6.3.4 Effects of Fluvastatin, p38 MAP Kinase Inhibitor and Other Therapeutics on Fibroblast Conditioned Media-Induced Pulmonary Artery Smooth Muscle Cell Proliferation

Results from the preceding experiment were in keeping with previous reports, indicating that acute hypoxia induces PAFs to release (directly acting) PASMC mitogens[66]. The effects of fluvastatin, cholesterol pathway intermediates and related inhibitors on hypoxia-induced PAF proliferation had been established (Chapters 4.3, 6.3.1 and 6.3.2).

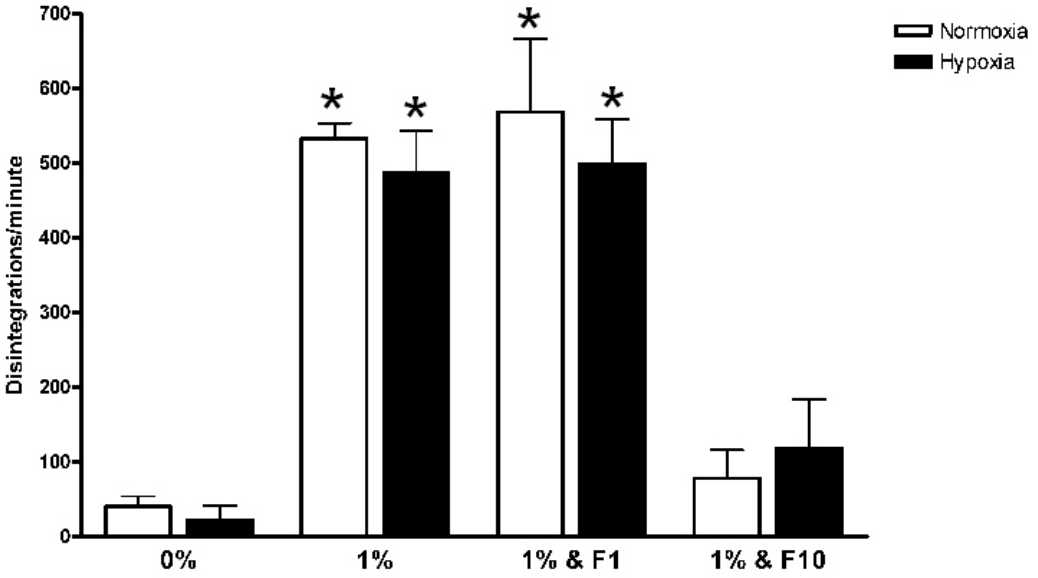

Figure 6.4 Effects of Acute Hypoxia and Fluvastatin on [^{3}H] Thymidine Uptake by Bovine Pulmonary Artery Smooth Muscle Cells.

Growth arrested bovine PASMCs were stimulated in normoxia (21%) or hypoxia (5%) for 24 hours. 1% serum and fluvastatin (F) 1 or 10μM were added to selected wells in the cell culture plate. DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake at 24 hours. Values shown are mean +/-SD from 4 replicate experiments. (*values significantly greater than 0%-normoxia, $p<0.01$).

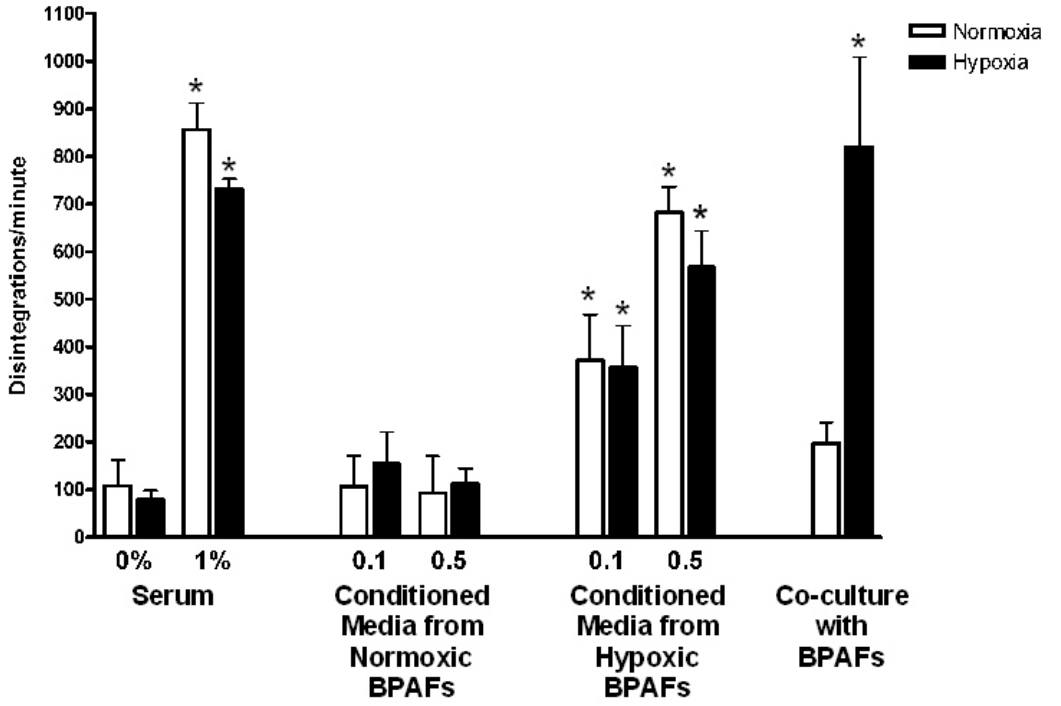

Figure 6.5 Effects of Application of Fibroblast Conditioned Media, Co-Culture with PAFs and Acute Hypoxia on [^{3}H] Thymidine Uptake by Bovine PASMCs

Bovine PASMCs were quiesced for 24 hours in serum free media. In selected cell wells, serum free media was then replaced by 0.1ml or 0.5ml of conditioned media taken from bovine PAFs which had been previously maintained for 24 hours in normoxia (21%) or hypoxia (5%). In other wells, co-culture cell inserts containing confluent bovine PAFs were placed. BPASMCs maintained in serum free media, and in media supplemented with 1% serum served as controls.

BPASMC plates were maintained for 24 hours in normoxia (21%) or hypoxia (5%) and DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake. Values shown are mean +/-SD from 4 replicate experiments. (*values significantly increased vs 0% serum-normoxia, $p<0.01$).

We therefore sought to determine if similar effects were seen on hypoxia-induced PAF mitogen release, using these drugs at previously established experimental concentrations.

In the previous experiment, the effects of conditioned media from hypoxic PAFs, on PASMC proliferation, were found to be identical, regardless of whether PASMCs were in normoxia or hypoxia. Given this finding, all of the following experiments were conducted with the PASMCs in normoxia.

Conditioned media from hypoxic BPAFs again stimulated BPASMC proliferation (figure 6.6). Conditioned media from BPAFs treated with fluvastatin 1μM had no mitogenic effect on BPASMCs but co-treatment of the BPAFs with fluvastation/mevalonate and fluvastatin/GGPP resulted in conditioned media which stimulated BPASMC proliferation.

Conditioned media from BPAFs treated with fluvastatin 10μM had an inhibitory effect on BPASMC prolilferation: this may have been a ‘spillover’ effect of the fluvastatin still present in the conditioned media, or it may reflect release of growth inhibitory substances from BPAFs damaged by this large dose of statin.

Treatment of the hypoxic BPAFs during the media-conditioning phase with p38 MAP kinase inhibitor (SB203580, 5 μM), geranylgeranyl transferase inhibitor (GGTI-276, 5 μM) or Rac1 inhibitor (NSC-23766, 100 μM) resulted in conditioned media which did not stimulate BPASMC proliferation. In contrast, treatment of the hypoxic BPAFs during media conditioning with a prostacyclin analogue (UT-15c, 1μM), phosphodiesterase-5 inhibitor (sildenafil, 10μM) or endothelin A/B receptor antagonist (bosentan, 10μM) had no effect on the conditioned media stimulated BPASMC proliferation.

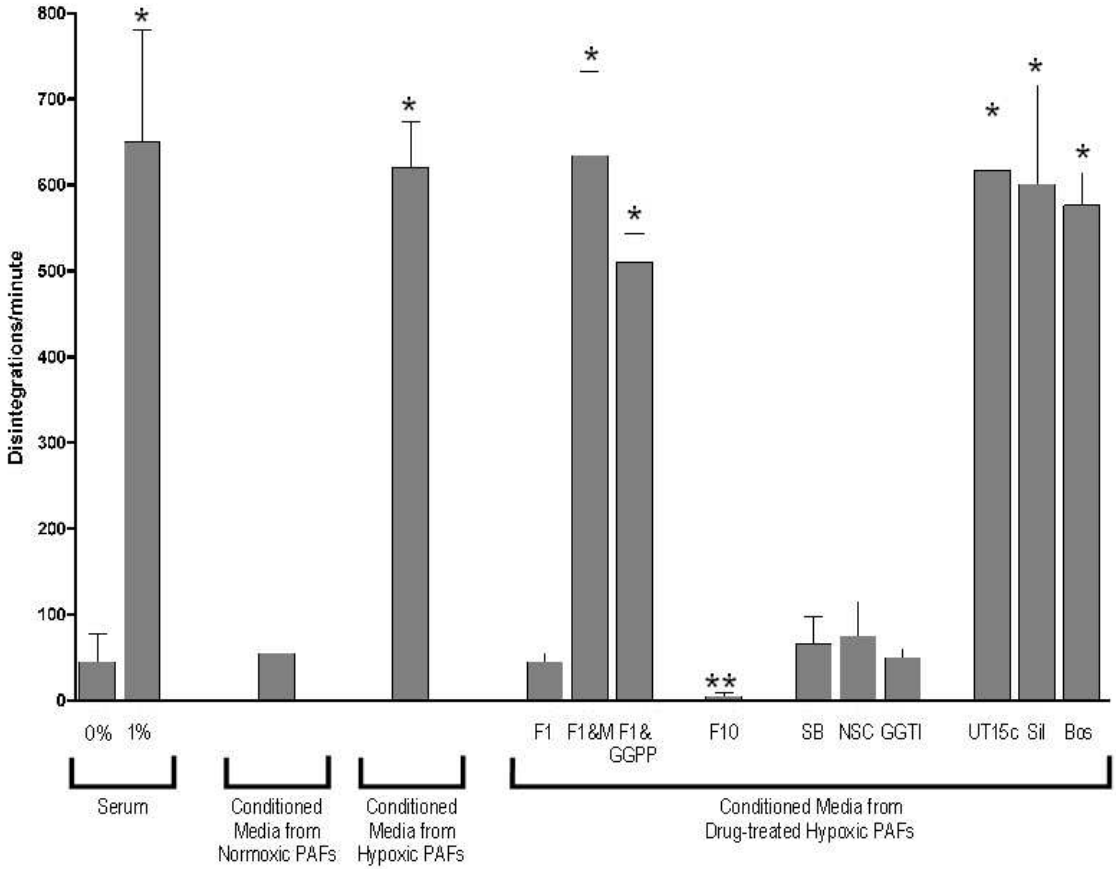

Figure 6.6 Effects of Application of Fibroblast Conditioned Media from Drug-Treated Hypoxic PAFs on Bovine PASMC Proliferation.

Bovine PASMCs were quiesced for 24 hours in serum free media. Serum free media was then replaced by 0.5ml of conditioned media taken from bovine PAFs which had been previously maintained for 24 hours in hypoxia (5%) +/- fluvastatin (F, 1 or 10μM), mevalonate (M, 1mM), GGPP (0.5μM), SB203580 (SB, 10μM), GGTI-276 (GGTI, 5μM), NSC-23766 (NSC, 100μM), treprostinil dethanolamine (UT-15c, 1μM), sildenafil (sil, 10μM) or bosentan (bos, 10μM).

BPASMC plates were then maintained for 24 hours in normoxia (21%) and DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake. Values shown are mean +/-SD from 4 replicate experiments. (*values significantly greater than 0% serum control cells, ** value significantly less than 0% serum control cells, $p<0.05$).

The lack of effect of fluvastatin 1μM on BPASMC proliferation (figure 6.4) offered some reassurance that the observed inhibitory effects of fluvastatin in these conditioned media experiments were a result of action of the drug on the BPAFs during the media conditioning, rather than a direct inhibitory effect of residual drug in the conditioned media, on the BPASMCs. To clarify this for fluvastatin and all of the other inhibitors, the conditioned media experiments were repeated, but on this occasion drugs were applied to the BPASMCs at the time of addition of conditioned media (from fibroblasts not treated with drugs).

Fluvastatin 1μM, SB203580 5μM, GGTI-276 5μM and NSC-23766 100μM had no effect on BPAF conditioned media stimulated proliferation of BPASMCs (figure 6.7). Fluvastatin 10μM inhibited BPASMC proliferation induced by serum or BPAF conditioned media, consistent with the non-specific effects of this dose of fluvastatin, identified throughout this thesis. UT-15c and sildenafil again had no effect but bosentan partially attenuated the stimulatory effect of hypoxic BPAF derived conditioned media on BPASMC proliferation.

6.3.5 Effects of Fluvastatin and Bosentan on Bovine Pulmonary Artery Fibroblast - Smooth Muscle Cell Coculture

Only a small number of co-culture inserts were available at this time and consequently only a very limited repertoire of inhibitor experiments could be undertaken. Effects of statins were the focus of these experiments but the results in the preceding experiment with bosentan were intriguing (endothelin-1 release by hypoxic PAFs has previously been shown). Accordingly, the effects of bosentan and low dose fluvastation on BPAF-BPASMC co-culture in normoxia and hypoxia were studied.

Co-culture with BPAFs in acute hypoxia led to increase in [^{3}H] thymidine uptake by BPASMCs. This increased DNA synthesis was prevented by fluvastatin 1μM and bosentan 10μM (figure 6.8).

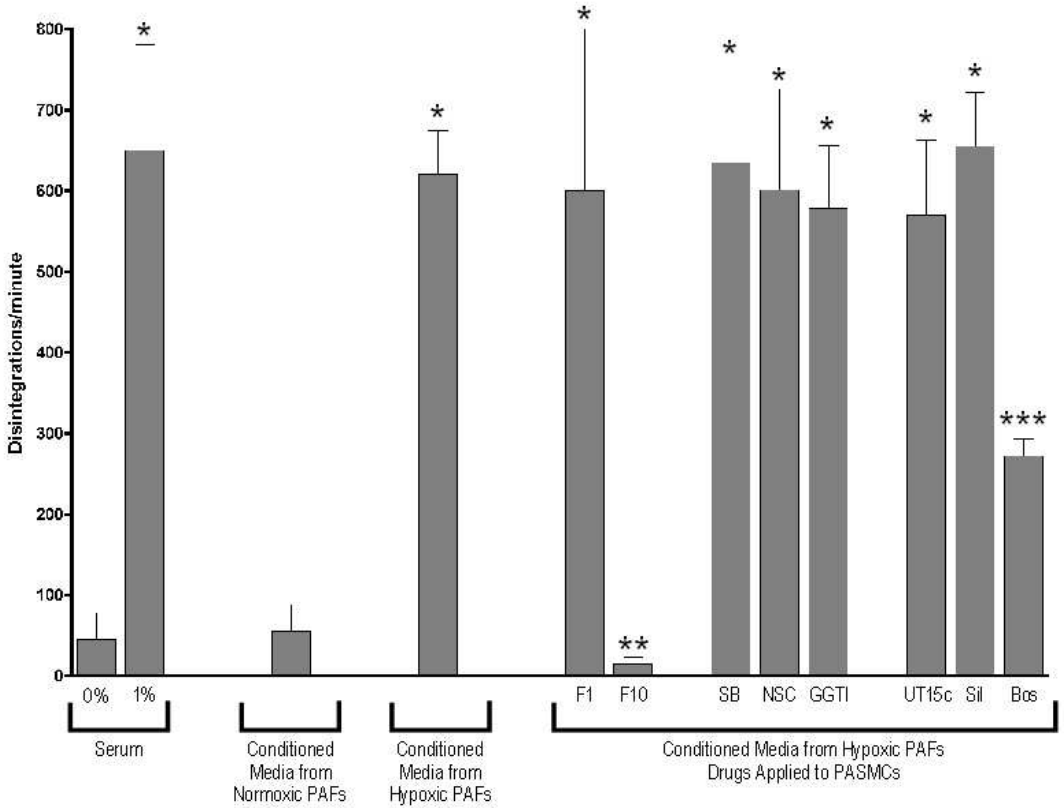

Figure 6.7 Effects of Simultaneous Application of Fibroblast Conditioned Media from Hypoxic PAFs and Investigational Drugs on Bovine PASMC Proliferation.

Bovine PASMCs were quiesced for 24 hours in serum free media. Serum free media was then replaced by 0.5ml of conditioned media taken from bovine PAFs which had been previously maintained for 24 hours in hypoxia (5%). Fluvastatin (F, 1 or 10μM), mevalonate (M, 1mM), GGPP (0.5μM), SB203580 (SB, 10μM), GGTI-276 (GGTI, 5μM), NSC-23766 (NSC, 100μM), treprostinil dethanolamine (UT-15c, 1μM), sildenafil (sil, 10μM) or bosentan (bos, 10μM) was added to the PASMC, along with the conditioned media.

BPASMC plates were then maintained for 24 hours in normoxia (21%) and DNA synthesis, as an index of cell proliferation, was assessed by [^{3}H] thymidine uptake. Values shown are mean +/-SD from 4 replicate experiments. (*values significantly greater than 0% serum, **value significantly less than 0% serum, ***value significantly greater than 0% serum and significanlty less than 1% serum, $p<0.05$)

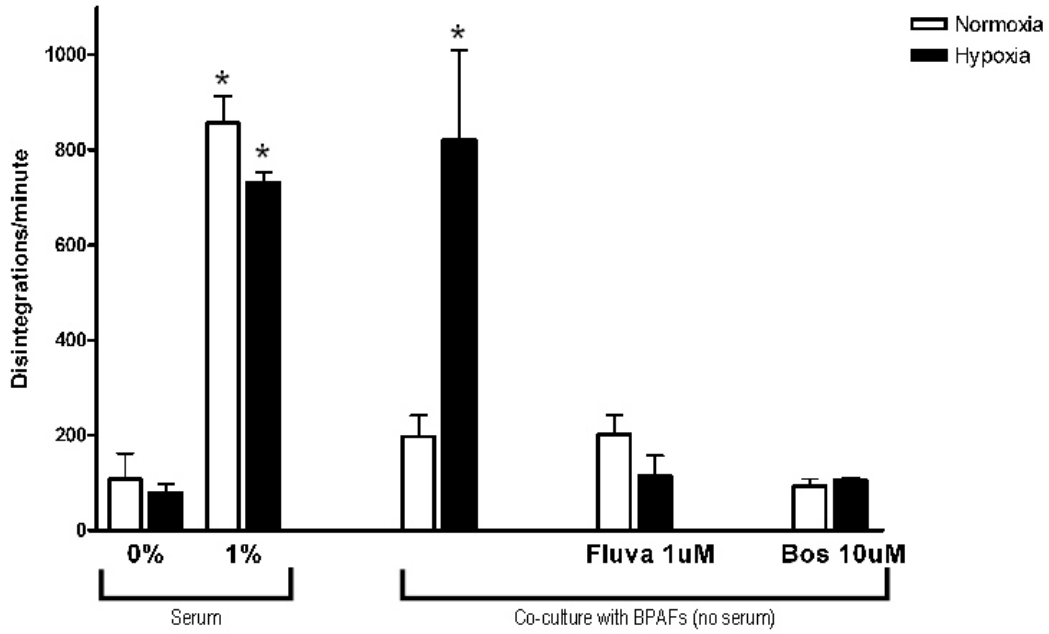

Figure 6.8 Effects of Fluvastatin and Bosentan on Bovine PASMC Proliferation in PAF-PASMC Co-culture.

Cell culture inserts containing confluent, quiescent bovine PAFs were applied to cell culture wells on plates containing growth arrested bovine PASMCs. Fluvastatin and bosentan were added to selected wells and the co-culture plates were maintained in normoxia (21%) or hypoxia (5%) for 24 hours, as indicated. [^{3}H] thymidine uptake by the PASMCs was then assessed at 24 hours and results are expressed as mean +/- SD from 4 replicate experiments. (*value significantly greater than 0% serum-normoxia, $p<0.01$).

6.4 Discussion

This chapter focused on the interaction between pulmonary artery fibroblasts and pulmonary artery smooth muscle cells in hypoxia: specifically studying whether acute hypoxia causes PAFs to release a PASMC mitogen, whether this is amenable to low dose statin inhibition and if the cell signalling pathway responsible for this is similar to that in hypoxia-induced proliferation.

As a prelude to addressing these questions about BPAF-BPASMC interactions, the effects of hypoxia and fluvastatin on BPAF and BPASMC proliferation were individually characterised. As previously seen with rat PAFs, the effects of acute hypoxia on BPAF proliferation were selectively and completely inhibited by fluvastatin. The results with mevalonate/GGPP repletion and p38 MAPK, geranylgeranyl transferase and Rac1 inhibitors indicate that the speculative Rac1-p38-proliferation signalling pathway identified in rat PAFs is also activated by acute hypoxia in bovine PAFs.

Bovine PASMCs showed no proliferative response to 24 hours exposure to 5% normobaric hypoxia. This result may be a function of the cell population obtained (explant from proximal pulmonary arteries and no specific single clone isolated) or the level of hypoxia used (5% may be relative 'hypoxia' to PAFs but relative 'normoxia' to medial PASMCs which typically reside distant from the PA lumen or the vasa vasorum). This result is, however, consistent with the majority of the literature and does support the contention that a substantial component of PASMC proliferation in hypoxic pulmonary vascular remodelling may be a consequence of indirect effects of hypoxia on other pulmonary vascular cells, rather than a direct effect of hypoxia on PASMCs. If we assume that the level of hypoxia (5%) studied here is appropriate, given that PASMCs are hypoxia sensitive in general (ie in acute hypoxic pulmonary vasoconstriction), why they should not exhibit a proliferative response to hypoxia is unknown. The Rac1-p38 MAPK pathway, present in PAFs, may be absent or insensitive to hypoxia, it may be activated but ineffective, or there may be additional hypoxia signalling pathways activated in PASMCs which counteract its effects. Further exploration of these possibilities was outwith the scope of this thesis.

Considering BPASMC proliferation further, fluvastatin 10μM again had a non-specific antiproliferative effect but fluvastatin 1μM had no effect on BPASMC proliferation. This result again supports the interpretations made previously (chapter 3.4): that 1μM dose of statin has a selective effect on hypoxia-activated proliferative signalling pathways.

Having characterised the effects of fluvastatin on BPAF and BPASMC proliferation individually, we proceeded to study the effects on BPAF-BPASMC interactions. The effects of hypoxic BPAFs on BPASMC proliferation have been previously reported by others[66] but results with our experimental conditions have not been described. We found an increase in BPASMC proliferation with BPAF in acute hypoxia, but not in normoxia. Conditioned media derived from hypoxic BPAFs caused a dose dependant increase in BPASMC proliferation but conditioned media from normoxic BPAFs had no effect. Also, proliferation of conditioned media treated BPASMCs were similar in normoxia and hypoxia. Taken together, these results suggest acute hypoxia causes secretion of soluble mitogen(s) by BPAFs, which have a direct pro-proliferative effect on BPASMCs, rather than facilitating a BPASMC proliferative response to hypoxia. These results are consistent with all previous data reported in this field.

We considered it possible that hypoxia-induced mitogen release by PAFs may be mediated by the same signalling pathway as is responsible for hypoxia-induced PAF proliferation. The results with fluvastatin 1μM in the conditioned media and co-culture experiments (where the fibroblasts were drug-treated) were indicative of complete inhibition of acute hypoxic BPAF mitogen release, supporting this contention. Negation of the effect of fluvastatin by mevalonate and GGPP again implicated a geranylgeranylated signalling protein. Moreover, inhibition of BPASMC proliferation, in the conditioned media experiments, by treatment of the BPAFs with the p38 MAP kinase inhibitor and Rac1-GEF interaction inhibitor strongly support a cell signalling model for acute hypoxia-induced PAF mitogen release (figure 6.9), similar to that

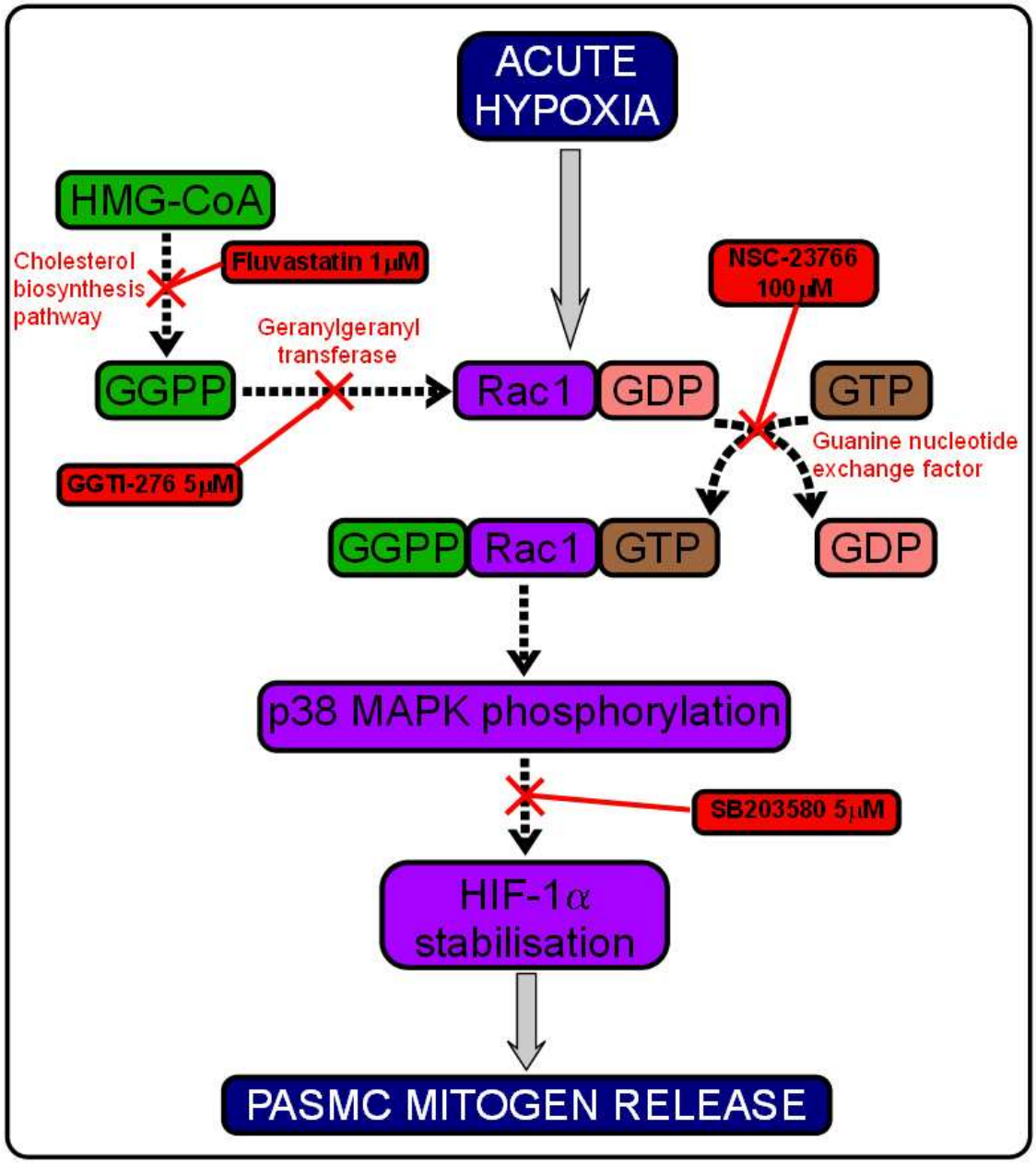

Figure 6.9 Proposed Model of Acute Hypoxic Signalling Leading to PASMC Mitogen-Release by Bovine PAFs

The results presented in this chapter, supplemented by results from Rose et al[66], suggest the above cell signalling pathway

proposed for PAF proliferation (figure 4.9). This model is also supported by the work of Rose et al[66]. They confirmed mitogen-release by hypoxic PAFs and confirmed that this was dependant on hypoxia-induced stabilisation of HIF-1α: previous SPVU research has linked acute hypoxic PAF proliferation to p38 MAPK dependant HIF-1α stabilisation. Considering these 2 reports alongside the results in this chapter adds further weight to the contention that proliferation and mitogen-release by acutely hypoxic PAFs occur as a consequence of activation of the same signalling pathway.

With these conditioned media experiments, the possibility that 'spillover' of drug still present in the conditioned media had a direct inhibitory effect on the BPASMCs, rather than on BPAF mitogen release, was considered. Fluvastatin 1μM and p38 MAPK, GGTI and Rac1-GEF inhibitors, when added to BPASMCs along with conditioned media derived from hypoxic BPAFs, had no effect on (conditioned media stimulated) BPASMC proliferation, excluding the possibility of a spillover effect.

We found the prostacyclin analogue UT-15c and the phosphodiesterase-5 inhibitor sildenafil to have no effect on BPAF-BPASMC interactions in these experiments. These findings argue against either a prostacyclin or a GTP / PDE-5 influenced signalling pathway mediating either hypoxia-induced BPAF mitogen release or the proliferative response of BPASMC to this mitogen(s). Prostacyclin is known to have inhibitory effects on BPASMC proliferation, in general. We did not explore this further but the lack of inhibitory effect of UT-15c on BPAF-induced BPASMC proliferation may have therapeutic implications: prostacyclin therapy may be ineffective or less effective as an anti-remodelling therapy in situations where an important pathophysiological mechanism is BPASMC proliferation driven by mitogen(s) derived from BPAFs (ie a mechanism thought to be important in chronic hypoxic pulmonary hypertension).

Hypoxic pulmonary artery fibroblasts have been shown to release a variety of mitogens, including ATP[239], HIMF[255] and ET-1[105]. These previous reports have identified an autocrine effect of these compounds on PAFs and a paracrine effect on vasa vasorum endothelial cells and smooth muscle cells. A comprehensive study of

compound(s) released by hypoxic PAFs with characterisation of the individual effects of each on PASMCs would seem very relevant but time constraints prevented inclusion of this work in this project. As a preliminary observation, however, the results with bosentan in the conditioned media and co-culture experiments suggest an important role for PAF-derived ET-1. In these experiments, co-treatment of BPASMCs with bosentan 10μM along with hypoxic PAF-derived conditioned media partly attenuated PASMC proliferation and application of bosentan to co-cultured BPAF-BPASMCs blocked the increase in BPASMC proliferation. Treatment of BPAFs with bosentan during the media conditioning period had no subsequent effect on BPASMC proliferation. This result could initially be considered contradictory: should residual endothelin antagonist in the conditioned media not block interaction of BPAF-derived ET-1 with ETA/B receptors on BPASMCs? It is known, however, that bosentan is only stable in solution for periods of less than 24 hours (personal communication, Dr J Jepf, Actelion). Consequently, by the time conditioned media was added to the BPASMCs, there would be no active bosentan present, and this result is therefore compatible. It is also considered notable that angiotensin-II induced ET-1 release by systemic vascular fibroblasts has been shown to be regulated by NADPH oxidases[256]: there are common themes in these cellular signalling pathways.

It was considered notable that the effects of hypoxic PAF-derived conditioned media were consistent, regardless of whether the PASMCs were maintained in normoxia and hypoxia. It seems appropriate to speculate that this – along with the previously discussed proliferative phenotypic switch in chronically hypoxic PAFs (Chapter 5.4) – is a cellular mechanism which could account for persistent/progressive pulmonary vascular remodelling and pulmonary hypertension in hypoxic lung disease, even if hypoxia is corrected therapeutically. If the proliferative phenotypic switch in PAFs also led to persistent release of PASMC mitogens - which seems likely, given the identical intracellular signalling pathways which have been identified here - whose effect is independent of oxygen tension, then this maladaptive proliferative cell-cell interaction would require drug treatment which could specifically block it (eg statin), if it were confirmed in humans, *in vivo*. Future work to characterise PASMC mitogen release by chronic hypoxic PAFs is proposed.

These results were obtained at the end of the experimental program comprising this thesis and time did not permit even experiments to directly confirm ET-1 release by these hypoxic PAFs (though ET-1 release by BPAFs has already been shown by others). As a result of these findings, however, experiments to fully characterise endothelin-1 (and other possible mitogen) release by hypoxic PAFs and further study receptor interactions and proliferative mechanisms on the PASMC are in progress in the SPVU laboratory.

In conclusion, the results in this chapter indicate that acute hypoxia causes release of BPASMC mitogens from BPAFs. This is likely to be mediated by a Rac1-p38 MAP kinase pathway in the PAFs and inhibition of this by a dual A/B endothelin antagonist suggests endothelin-1 is likely to be one of the mitogens responsible (figure 6.10). This BPAF-BPASMC hypoxic interaction is likely to be an important pathophysiological mechanism in the initiation and persistence of chronic hypoxic pulmonary vascular remodelling and the observation that this was completely and selectively inhibited by fluvastatin, at cell culture concentrations which are potentially clinically relevant, may have important therapeutic applications.

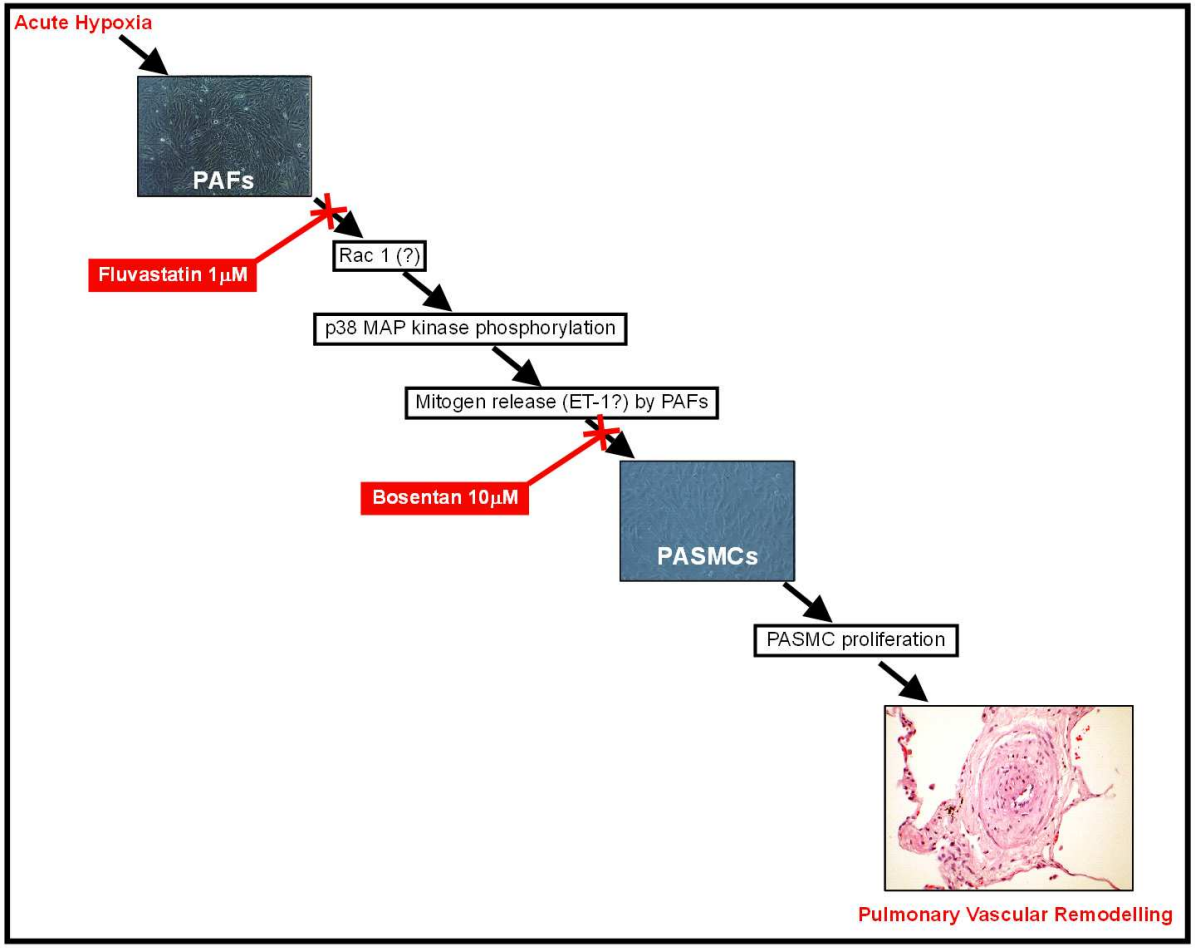

Figure 6.10 Schematic of Suspected Acute Hypoxic Signalling Pathways PAF-PASMC Cellular Interactions in Hypoxic Pulmonary Vascular Remodelling

Chapter 7

General Discussion

7.1 Summary of Results

The data presented in this thesis show that statins, at low cell culture dose (1μM), have a marked, but selective, inhibitory effect on hypoxia-induced pulmonary artery fibroblast cell proliferation. Using established acute and chronic hypoxic cell models, this effect of statin drugs has been characterised. We have shown that:-

- Large doses of statin (>5μM) have a non-specific antiproliferative effect on mitogen induced proliferation of all cell types studied (pulmonary and systemic artery fibroblasts from normal and chronic hypoxic rats and bovine pulmonary artery fibroblasts and smooth muscle cells).
- Low doses of statin (1μM) inhibit the augmented proliferation of pulmonary artery fibroblasts in acute hypoxia, or in cells derived from chronic hypoxic rats, via selective inhibition of hypoxia-induced p38 MAP kinase phosphorylation. This dose of statin has no effect on serum-induced proliferation of pulmonary artery fibroblasts or smooth muscle cells, or on proliferation of systemic artery fibroblasts. 1μM statin also had no effect on ERK or JNK MAP kinase status in normoxic or acutely hypoxic rat pulmonary artery fibroblasts.
- The lipophilic statins fluvastatin, simvastatin and atorvastatin were equipotent when the effects of these on acute hypoxic pulmonary artery fibroblast proliferation were compared.
- A geranylgeranylated protein, probably Rac1, is an obligatory component upstream of p38 mitogen-activated protein kinase, in the acute hypoxia-proliferative signalling pathway in pulmonary artery fibroblasts.
- This geranylgeranylated protein–p38 MAP kinase pathway appears to be constitutively activated in chronic hypoxic rat pulmonary artery fibroblasts and this is likely responsible for the ‘phenotypic switch’ in these cells which results in sustained increases in proliferative capacity, even when they are restored to normoxia. Despite this phenotypic switch low dose fluvastatin is able to attenuate the proliferation of these cells, such that their proliferation is at a rate similar to fibroblast cells from the systemic circualation..
- The hypoxia-selective antiproliferative effect of low dose statin on pulmonary artery fibroblasts is <u>not</u> duplicated by a drug from each of the established

pulmonary hypertension therapeutic classes (prostacyclin analogue, phosphodiesterase-5 inhibitor and endothelin-1 antagonist).

- Though human cells were not available for this project, we were able to show that the statin effect and hypoxic signalling pathways implicated were identical in pulmonary artery fibroblasts from 2 different species (rat and cow). This is consistent with previous reports on conservation of this p38 MAP kinase-proliferative hypoxic signalling pathways between species[180].

We also studied the potential for smooth muscle cell mitogen release by hypoxic pulmonary artery fibroblasts and were able to show that:-

- Acute hypoxia causes bovine pulmonary artery fibroblasts to release soluble mediator(s) which act directly to cause smooth muscle cell proliferation.
- This hypoxia-induced mediator release by PAFs again appeared related to activation of a Rac1-p38 MAP kinase signalling pathway, and was sensitive to inhibition by low dose fluvastation. This suggests that fluvastatin may have broad spectrum effects on pathophysiological mechanisms implicated in hypoxic pulmonary vascular remodelling.
- The effect of the hypoxic pulmonary artery fibroblast derived mediators on smooth muscle cells is not influenced by subsequent hypoxia.
- Bosentan attenuated the effects of hypoxic PAF-derived conditioned media on PASMC proliferation, implicating endothelin-1 as a principle PASMC mitogen released by the hypoxic pulmonary artery fibroblasts.

7.2 Cell Signalling in Hypoxic Pulmonary Artery Fibroblasts

The initial results with fluvastatin – selective inhibition of acute-hypoxic induced PAF proliferation – offered the potential to exploit the known effects of statins to interrogate PAF cell signalling events. A number of other investigators have reported on a limited panel of experiments using repletion of cholesterol intermediates along with statins. The experimental protocol that we devised and successfully employed involved a comprehensive sequence of experiments (more extensive than any previous reports that have been identified) in order to completely characterise the effects of the statin. The rationale for this protocol has been previously discussed (Chapter 4.4).

Four novel observations in relation to hypoxic signalling in pulmonary artery fibroblasts have been made:-

- A protein which requires a ready supply of geranylgeranyl pyrophosphate is an obligatory signalling molecule upstream of p38 MAP kinase. Though time did not permit direct demonstration of hypoxia-induced Rac1 activation, the consistent observation that a Rac1 inhibitor duplicated the effects of low dose statin and p38 MAP kinase inhibitor provides strong circumstantial evidence the Rac1 is this signalling protein.
- The sensitivity of this hypoxic signalling pathway to statin inhibition is relatively novel. The majority of reported beneficial effects of statins in cell culture experiments have required doses around 10μM. Our findings of inhibitory effects on hypoxic PAF proliferation and mitogen release with a statin dose of 1uM indicate a signalling system which requires a consistent supply of GGPP,. This suggests that the protein involved is not prenylated prior to hypoxic stimulation. Possibilities are that activation of the protein by hypoxia facilitates prenylation, that there are no intracellular stores of GGPP to draw on and that ongoing *de novo* synthesis of GGPP is required. Alternatively prenylation of this protein could be a dynamic / unstable situation where GGPP is required for constant replenishment of the modified protein. Recognition of this has implications for our understanding of hypoxic signalling and Rho family protein function. It is considered relatively likely that hypoxia-induced activation of the protein involved (Rac1 or GTPase?) involves an prenylation-facilitated alteration of cellular localisation from the cytosol to an intracellular membrane. Also, the fact that we have found that some cell signalling systems are more sensitive to statin inhibition than others may be a mechanism explaining the successful translation of statin therapy in some clinical fields, but lack of effect at standard dosage in other clinical trials (despite preceding observations about disease pathophysiology and pre-clinical cell biology studies suggesting statins would be beneficial).
- We have shown that the proposed hypoxia-Rac1-p38 MAP kinase pathway is responsible not only for PAF proliferation but also for mitogen release. Though mitogen release by acutely hypoxic PAFs had been shown to be

dependant on HIF-1α stabilisation, this is the first study to directly demonstrate a common molecular mechanism for these cellular processes (proliferation and mitogen release) which likely contribute significantly to hypoxic pulmonary vascular remodelling. Also, the identical patterns of inhibition on these separate cellular process with low dose statin, Rac1 inhibitor, geranylgeranyl transferase inhibitor and p38 MAP kinase inhibitor suggest that these compounds may have a broad spectrum inhibitory effect on hypoxic PAFs, which may be therapeutically more useful in comparison to an agent which only possess anti-proliferative properties.

- Previous observations about the "phenotypic switch" (persistent hyperproliferation of PAFs from chronic hypoxic rats despite re-oxygenation, with constitutive phophorylation of p38 MAPK) had been considered to reflect a potentially irreversible change in these cells. Positive effects with low dose statin and the other inhibitors utilised not only demonstrate that the phenotypic switch is reversible; they also imply that the signalling pathway involved is dynamic. The requirement for a ready supply of GGPP suggests an aspect of this signalling pathway must be continuously in flux, rather than constitutively altered.

Our results are consistent with the majority of the literature in this field, as noted in the relevant chapter discussions. One notable contradiction - with the data in this thesis and our previous results - is with the work of Stenmark's group in Denver, Colorado. As previously briefly discussed (Chapter 1.8), rather than attributing hypoxia-induced proliferation of PAFs to p38 MAP kinase phosphrylation, they find hypoxia-induced proliferation to be dependant on activation of both a phosphatidylinositol-3-kinase - AKT – mTOR signalling pathway (which we have not assessed in our cell model) and ERK1/2 (which we also find to be phosphorylated in our cells, but not required for proliferation). These results are not completely inconsistent: the Stenmark group also show hypoxia-induced biphasic p38 MAP kinase phosphorylation in hypoxic PAFs (but find it not to be required for hypoxic proliferation). Also, Rac1 – which we are implicating upstream of p38 MAPK in our hypothetical cell signalling model in PAFs – has been shown in other cell types to associate with AKT[248]: we may be looking at different aspects of the same pathway, or parallel signalling pathways. That both our group and the

Stenmark group find complete / selective inhibition of hypoxic PAF proliferation with inhibition of our competing pathways is, however, confounding. The most likely explanation for these discrepancies lies in the nature of the cell types studied. We experiment on PAFs from of adult rats, cows and (in previous work but not in this thesis) humans whilst Stenmark's group report on PAFs from neonatal or fetal calves[67]. The neonatal pulmonary circulation in all species studied is known to respond more briskly to hypoxia than the mature circulation, with a tendency to increased constriction and more rapid remodelling. The differences in hypoxic PAF cell signalling shown by our group and Stenmark's group may reflect maturation and may explain the different responses of the pulmonary circulation, at different ages.

7.3 Acute and Chronic Hypoxic Cell Models: Strengths and Weaknesses

The results obtained (effects of statins on PAFs and hypoxic cell signalling pathways illustrated) are only directly related to the cells on which they have been obtained. Problems with the definition of hypoxia, animal cell origin and different subpopulations of resident cells within different territories of the pulmonary artery have been commented on previously.

5% hypoxia, used in the acute hypoxia experiments has been previously shown to achieve a supernatant PO2 of 35mmHg, which approximates typical alveolar PO2 in chronic hypoxic disorders. Also, previous work has reassured that changes in pH in acute hypoxia for this time period are insignificant[233]. Perhaps most important is the observation that maintenance of PAFs for 24 hours in 5% normobaric hypoxia leads to proliferative and cell signalling responses which mimic exactly those seen in PAFs from chronic hypoxic animals. It is considered a strength of the experimental program presented in this thesis that experiments were repeated in the acute and chronic hypoxic models, with consistent results. The consistent results obtained with statins, cholesterol intermediates and inhibitors in these models also provides further reassurance about the relevance of our acute hypoxic PAF cell model. An advantage of the acute hypoxic model are that it offers the potential to rapidly screen dose ranges of a large number of compounds, allowing for future experiments on less

readily available cells (eg chronic hypoxic or human) to be rationalised, as was achieved in this thesis.

Whether 24 hours of 5% hypoxia is relevant to pulmonary artery smooth muscle cells is, as previously discussed, less certain. Where PASMCs have been previously found to proliferate to acute hypoxia, it has been at oxygen fractions of 1-5%. Our results therefore cannot be taken as conclusive that isolated PASMCs do not proliferate to hypoxia, however, our observations are consistent with the majority of the literature. Whilst it seems likely that whilst there may be a population of PASMCs which proliferate in direct response to hypoxia in the native pulmonary artery, proliferation of PASMCs as a consequence of hypoxia-induced mitogen release by other cells is likely important in pulmonary vascular remodelling.

We derived both our PAFs and PASMCs from proximal vessels and this may be a weakness when considering the application of our results to *in vivo* pulmonary vascular remodelling, where the dominant changes are in the distal vessels. We also did not distinguish between different subpopulations of fibroblasts or SMCs: PAFs and PASMCs with differing hypoxia / proliferative responses have been previously demonstrated to reside in both proximal and distal vessels[61, 176]. Again, similar results in this thesis with the acute and chronic hypoxic PAFs offer some reassurance that the population of PAFs obtained for the acute hypoxic studies is of relevance. It is, however, possible that subpopulations of PAFs and PASMCs, whose hypoxic proliferative and cell signalling responses differ from those that were cultured in this project are important *in vivo* but this is not being appreciated in our experiments.

An important limitiation of this project is that the majority of work was carried out on rat cells. The applicability of any of these findings to human disease is uncertain. As previously outlined it has, however, previously been shown that rat and human PAFs have a number of shared responses to acute hypoxia (increased proliferation and p38 MAP kinase phosphorylation). Some additional reassurance can be taken from the fact that results with acutely hypoxic bovine PAFs duplicated those obtained with statins and other mediators on the rat cells: bovine PAF proliferative and MAPK responses have previously shown the greatest homology to human cell responses. There is difficulty in obtaining and interpreting results on PAFs derived

from human tissue. PAFs from “normal” patients are typically acquired from elective lung resections, usually carried out for lung cancer surgery. These patients will typically have been exposed to tobacco smoke – which has direct effects on the pulmonary circulation - and it is therefore difficult to regard these PAFs as “normal”. Pulmonary arteries are occasionally available from unused lungs acquired from organ donors but there will usually have been a significant pre-morbid injury and a period of invasive ventilation and, again, it is difficult to regard these cells as truly “normal”. PAFs from hypertensive pulmonary arteries are typically sourced from explanted lungs at the time of transplant surgery. Again there are problems with these: there is considerable heterogeneity in the underlying primary lung disease, the cells will have been exposed to various pulmonary vascular therapeutics prior to explant and the pulmonary arteries are at the end stage of the disease (rather than at the early stage, when we would be hoping to treat the condition). The relevance of any results obtained solely on PAFs from either normal patients or pulmonary hypertensive patients is therefore considered to be as questionable as those obtained on animal cells. Taking all of this together, it is considered that human and animal cell models are complementary. The results from this project provide a necessary foundation for performing a limited profile of complementary statin experiments on human PAFs and this is proposed as future work arising from this thesis.

7.4 Relevance of Observed Effects of Statins on PAF Behaviour to Hypoxia-Induced Pulmonary Vascular Remodelling

The initial premise to this research project was the observed reversal of experimental PH with statin drugs. Determining the mechanism of action of the statins in these animal models is difficult: differentiating cellular and molecular events which are a direct consequence of statin therapy from those which are simply a secondary consequence of reduced remodelling is not possible. Observed effects of statins in these experimental models include a reduction in vessel wall thickness, reduction in medial hypertrophy, smooth muscle cell apoptosis, reduced Rho kinase activity and upregulation of eNOS and BMPR1a transcription (see chapter 1.10.6). All of these observations, however, were based on experiments on the animals at the end of a period of statin treatment. Early effects of statins were not studied and, notably, any

effects of either experimental conditions (chronic hypoxia, monocrotaline etc) or statin on the vessel adventitia or the adventitial fibroblasts have not been reported.

Given that the earliest changes – possibly the initiating changes - in chronic hypoxic pulmonary vascular remodelling occur in the adventitia, it may be that the efficacy of statin drugs at preventing CH rat PH are a consequence of the inhibition of hypoxia-induced PAF proliferation and mitogen release that we have demonstrated here. Also, the demonstration that low doses of statins can completely reverse the proliferative phenotypic switch in PAFs may be a mechanism responsible for the reversal of CH rat PH achieved by statins: restoration of normal fibroblast behaviour may allow normal pulmonary vascular homeostasis to re-establish. Alternatively, there may be additional cellular effects of statins in the remodelled pulmonary circulation (eg pro-apoptotic, anti-inflammatory) which contribute. We have not yet studied the effects of statins on other fibroblast behaviours influenced by hypoxia (eg migration, transdifferentation, collagen, elastin and matrix synthesis): these may be under control of the same hypoxic signalling pathway as proliferation and mitogen release, and they may be additionally important cellular targets for any potential pulmonary vascular remodelling therapy.

Based on previous observations about the role of the resident pulmonary adventitial fibroblasts to hypoxia-induced pulmonary vascular remodelling and all of the data presented in this thesis, Figure 7.1 illustrates a hypothetical model of this aspect of pulmonary vascular remodelling and indicates the likely effects of fluvastatin and the other drugs used in this project on this.

The relevance of any experiments conducted on isolated cell culture to the *in vivo* situation (where cells are in contact with one another, with the extracellar matrix, the extracellular fluid, vascular mediators etc) is uncertain. For all of the reasons previously noted experiments to elucidate basic mechanisms or compare different therapies in whole animal experiments are difficult and cell culture models offer the only available compromise. The work described here with PAF-PASMC co-culture is an early aspect of a proposed future major research direction, whereby we hope to better model the *in vivo* pulmonary artery with cell culture techniques. Statins look

likely to remain a useful experimental tool to interrogate the different cell-cell and cell-matrix interactions that we hope to explore.

7.5 Potential Clinical Applications

A major obstacle in the treatment of pulmonary hypertension in the past has been an inability to selectively affect the pulmonary circulation: this was a particular problem with vasodilator therapies. The results obtained in this thesis once again underscore the fundamental differences between the pulmonary and systemic circulations and suggest that—with appropriate dosing— statins may have a stimulus (hypoxia) and circulation (pulmonary) selective anti-fibroproliferative effect.

There are substantial limitations in applying any of the results obtained here to pulmonary hypertension in humans, or its treatment. These were experiments on animal cells, in artificial conditions, with drugs dosed directly onto cells. Failure of results obtained at the bench to translate to benefits at the bedside is, in general, more common than success. With all of these provisos, however, some speculation does seem appropriate, particularly as clinical trials of statins for pulmonary hypertension have already been commenced, with only the limited animal experiments previously commented on (chapter 1.10.6) as their foundation.

The observations that low doses of statins can inhibit PAF proliferation and mitogen release in acute hypoxia, and reverse the proliferative phenotypic switch in chronic hypoxic PAFs, suggest that statins may have broad spectrum inhibitory effects on a cell type which is likely to be an important contributor to the initiation, maintenance and progression of chronic hypoxic pulmonary vascular remodelling. The proliferative PAF phenotypic switch, which persists despite cellular re-oxygenation, may be a factor in the failure of chronic hypoxic pulmonary hypertension to consistently improve with oxygen therapy and the experimental results presented here may indicate a potential for statin therapy to augment the beneficial effects of oxygen therapy in patients with chronic hypoxic lung disease and related pulmonary hypertension.

In the cell models, we found positive effects on hypoxic PAFs identical to those achieved with fluvastatin, with a geranylgeranyl transferase inhbitor, a Rac1 inhibitor and a p38 MAP kinase inhibitor. Geranylgeranyl transferase inhibitors and the Rac1 inhibitor are recently described compounds and use of these in animals or humans has not yet been described. p38 MAP kinase inhibitors are in a more advanced state of drug development but there are concerns about toxicity and progression of these beyond phase II clinical trials appears to have stalled. Whilst alternative strategies to achieve p38 inhibition may come to fruition (eg siRNA, inhalation of drug rather than systemic treatment), the ready availability, low cost and established safety profile of statins makes them more attractive than these inhibitors for any proposed clinical study. Also, statins have theoretical beneficial effects in the pulmonary circulation (eg anti-inflammatory, enhance eNOS) which exceed those of these other inhibitors.

With the exception of inhibiton of hypoxic PAF-conditioned media induced PASMC proliferation by bosentan, the identified beneficial effects of statins on hypoxic PAFs were not duplicated by a drug from each class established in pulmonary hypertension therapy. This is an important observation as the majority of patients with severe pulmonary hypertension (in resourced parts of the world) are currently treated with one or more of these drugs. The beneficial effects of statins on PAF behaviours may therefore complement the mechanisms of action of these established drugs, rather than simply duplicate it.

When we consider the current therapeutic scenario for PAH in general, the PH community is moving towards combination therapy, and currently treating all forms of PH similarly (on a relatively empirical basis). Given patient heterogeneity and the difficulties of comparative clinical trials, establishing the most appropriate drug treatment(s) for the different forms of severe PH and establishing the most rational combinations (with complementary rather than duplicate or contradictory mechanisms) requires basic science work. This approach has been successfully employed in oncology, where laboratory research has established the most rational combinations from the large complement of chemotherapeutics and biological agents now available, for different tumour types, with notable (though qualified) clinical success. It would seem sensible to duplicate these translational strategies in

pulmonary hypertension research, studying the effects of established and experimental therapies on different cell types, in experiments aiming to model the different forms of pulmonary hypertension. As combination and comparison experiments in animal models are impossible (the effects of a single drug are usually too marked to allow any differentiation) we consider the experimental approach utilised here – comparing effects of a new drug with established drugs in complementary cellular modles – one which should be adopted for future experimental programs studying disease pathophysiology and potential therapeutic strategies in pulmonary hypertension.

Considering statin therapy for PH more specifically, we compared different lipophilic statins, in the acute hypoxic rat PAF model, and found them to be equipotent. Comparisons of different statins in the experimental PH animal models are incomplete, but no clear cut differences in efficacy have been shown. There are notable differences in the pharmacokinetics of these statins in humans (see Table 1.2). Simvastatin and atorvastatin undergo substantial hepatic first pass metabolism and, whilst there may be circulating active metabolites of these drugs, only very small peak circulating concentrations of these drugs are achieved in humans. In contrast, fluvastatin undergoes relatively little first pass metabolism and achieves much higher concentrations in the post-hepatic (ie systemic and pulmonary) circulations) after standard oral dosing. It is notable that standard oral dosage of fluvastatin achieves peak circulating concentrations of ~1μM, a dose that had a positive and selective effect in our experiments. Our study is therefore the first to demonstrate that statins - specifically fluvastatin - may have an inhibitory effect on pulmonary vascular cell proliferation at cell culture doses that are within the concentration range achievable in the human pulmonary circulation, with oral statin dosing.

The clinical trials of statins for pulmonary hypertension currently in progress have selected simvastatin in their protocols. Our results and observations suggest that this drug may be problematic: it may not achieve adequate concentrations in the human pulmonary circulation to achieve the beneficial effects seen in the animal models. Also, simvastatin metabolism is enchanced by bosentan (which a substantial number of enrolled patients will be taking) which will potentially confuse interpretation of

results. In relation to this, perhaps the most important message which can be derived from this thesis is that if these initial clinical trials of simvastatin for PAH are negative, we should not dismiss statins as potential agents for selected forms of severe PH. Hypoxia-associated PH may be particularly susceptible to statin therapy and fluvastatin may be a more appropriate choice of statin. It is concluded, however, that substantial additional preclinical work – determining the differential actions of statins and other drugs in complementary cell and animal PH models - is required if we are to avoid potentially confounding and wasteful negative clinical trials.

7.6 Future Work

The work in this thesis has extended our understanding of hypoxic signalling in pulmonary artery fibroblasts and identified potentially therapeutically relevant effects of fluvastatin on this. Some of the gaps in our experimental program have been acknowledged in the chapter discussions. Appropriate immediate future directions would include clarifying Rac1 prenylation / turnover in acute hypoxia, clarifying Rac1 involvement in proliferative hypoxic signalling (eg via siRNA studies) and extending the experimental protocol used in Chapters 4 and 5 to human cells.

The pattern of experiments conducted here – comparing effects of a potential new therapy with established therapies – is considered a relevant template for future work on cellular and molecular pulmonary arterial pathophysiology and drug exploration. As indicated, the success of this approach in the oncology field suggests this template should be more widely adopted and extended. Specifically, utilisation of other proposed PH therapeutics (eg PDGF inhibitors, vasoactive intestinal polypeptide) in our hypoxic cell models may facilitate new insights into cellular mechanisms, therapeutic potential and allow deduction of rational combination therapies for clinical study.

The single cell type cell culture model will always be problematic in translational medicine but novel approaches to better model the normal and diseased pulmonary vessels in the cell culture lab are proposed. The fibroblast-smooth muscle cell co-

culture and conditioned media experiments that we conducted would seem to be a small step towards better modelling the complete pulmonary artery *in vitro.* Improving on this, initially by including other cell types (eg endothelial cells and distal rather than proximal vascular cells) seems relevant.

Lastly, it is clear that pulmonary vascular remodelling and pulmonary hypertension is not a process involving simply a segment of the pulmonary artery: circulating cells, pulmonary veins and the right heart are critical in the overall clinical manifestation. Whilst it remains likely (though still contentious) that the primary problem is an arteriopathy, the contribution of these other cell and tissue types to the disease state, and the effects of experimental stimuli (eg hypoxia) and investigational therapies (eg statins) on these need to be characterised.

In conclusion, the data and interpretations presented in this thesis are considered a small, but significant aspect of an increasingly active worldwide research program into this important group of diseases. In addition to the ongoing research in the SPVU laboratory which this thesis has prompted it is hoped that the conclusions made will prove to be interesting and stimulating for the pulmonary vascular community in general.

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