Effects of Hypoxia on Proliferation and Signal Transduction Pathways in Pulmonary and Systemic Vascular Fibroblast cells

A thesis presented by

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To my Mother, Father and Sister

ABSTRACT

When pulmonary hypertension occurs in the face of hypoxia there is remodelling of all 3 layers of the pulmonary vessels, but in particular, there is an increase in number of adventitial fibroblasts. Hypoxia causes vasoconstriction in the pulmonary circulation and vasodilation in the systemic. We hypothesised that the remodelling process might be circulation specific and that there are fundamental differences in oxygen sensing and cell signalling between systemic and pulmonary artery cells in response to hypoxia. Both the mitogen-activated protein kinases (MAP kinase) and the stress-activated protein kinases (SAPK) have been shown to play an important role in cellular growth and proliferation in a number of cell systems. The aim of this present study was to examine the effects of acute and chronic hypoxia on proliferation and determine a role for MAP/SAP kinases in response to both G-protein linked receptor agonists and growth factors in fibroblasts from the pulmonary and systemic arteries of cows and rats.

In bovine pulmonary artery fibroblast (BPAF) cells short-term exposure (24h) to 2% oxygen (hypoxia) increased proliferation. In the presence of certain agonists hypoxia augmented this proliferation. This enhanced proliferation was not seen in fibroblasts from the mesenteric artery (BMAF). Hypoxia also gave rise to increases in the second messenger, inositol 1,4,5-trisphosphate (IP_3) mass in conjunction with the agonists responsible for increased proliferation in the BPAF cells. There was no increase in IP_3 mass in the BMAF cells. The effect of acute hypoxia on activity of MAP kinase isoforms was also studied. Hypoxia was found to increase the SAP kinase isoforms, p38 MAP kinase and c-jun N-terminal kinase (JNK) without effecting MAP kinase in BPAF cells. In contrast, hypoxia had no effect on the MAP kinase family of enzymes in BMAF cells.

Pulmonary artery fibroblasts from chronically hypoxic rats (HRPAF) displayed increased proliferation to serum and constitutive increases in p38 and MAP kinase, with no increase in JNK when compared to control cells even when maintained in normoxic conditions. This increase in proliferation was not observed in aortic fibroblasts from chronically hypoxic rats (HRAF). 5-HT, with the addition of low serum levels, increased proliferation in the rat cells and could further enhance the phosphorylation of p38 and MAP kinase in HRPAF cells. Specific 5-HT agonists and antagonists showed the $5\text{-}HT_{2A}$ receptor to be the relevant receptor responsible for these observations.

The results reported in this thesis show for the first time that hypoxia can increase fibroblast proliferation in cells from pulmonary arteries whilst having so such effect in those from the systemic arteries. In addition, specific signalling pathways have been highlighted which are activated in the pulmonary cells to hypoxia but not in those of the systemic arteries.

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Declaration

This thesis is entirely my own composition and the experimental work detailed within was undertaken wholly by myself, with the exception of figures 6.1 – 6.5 which were produced with the help of Dr. Pamela Scott.

Signed

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

Publications

Full papers

Welsh DJ, Harnett M, MacLean M and Peacock AJ. (2001) Chronic hypoxia induces constitutive p38 MAP kinase activity which correlates with enhanced cellular proliferation in fibroblasts from rat pulmonary but not systemic arteries. *Am. J. Resp. Crit. Care Med* ***in press***

Welsh DJ, Scott P, Plevin R, Wadsworth R and Peacock AJ. (1998) Hypoxia enhances cellular proliferation and inositol 1,4,5-trisphosphate generation in fibroblasts from bovine pulmonary artery but not from mesenteric artery. *Am. J. Resp. Crit. Care. Med.* **158:** 1757-1762

Scott P, Paul A, Belham C, Peacock AJ, Wadsworth R, Gould GW, **Welsh DJ** and Plevin R. (1998) Hypoxic stimulation of the stress-activated protein kinases in pulmonary artery fibroblasts. *Am. J. Resp. Crit. Care. Med.* **158/3:** 958-62

Abstracts

Welsh DJ, Harnett M, MacLean M and Peacock AJ. (2001) HIF-1 alpha is induced in pulmonary artery fibroblasts from chronically hypoxic rats but not in those from control rats *Am. J. Resp. Crit. Care. Med.* ***in press***

Welsh DJ, Harnett M, MacLean M and Peacock AJ. (2000) 5-HT stimulated p38 MAP kinase activity in pulmonary artery fibroblasts from chronically hypoxic rats is mediated through the 5-HT_{2A} receptor. *Am. J. Resp. Crit. Care. Med.* **161/3:** A641

Welsh DJ, Harnett M, MacLean M, Weir K and Peacock AJ. (1999) The effect of blocking K+ channels on proliferation of chronically hypoxic rat fibroblasts from the pulmonary and systemic circulation. *Am. J. Resp. Crit. Care. Med.* **159/3:** A347

Welsh DJ, Harnett M, MacLean M and Peacock AJ. (1999) The effect of chronic and acute hypoxia on proliferation and stimulation of stress activated protein kinases in rat fibroblasts from the pulmonary and systemic circulations. *Am. J. Resp. Crit. Care. Med.* **159/3:** A347

Welsh DJ, Harnett M, MacLean M and Peacock A. (1998) The specific p38 MAP Kinase inhibitor SB203580 abolishes the proliferative response of Bovine Pulmonary Artery Fibroblasts to Hypoxia. *Am. J. Resp. Crit. Care.Med.* **157/3:** A430

List of Abbreviations

AII – Angiotensin II

ATP – Adenosine Triphosphate

BMAF – Bovine Mesenteric Artery Fibroblasts

BPAF – Bovine Pulmonary Artery Fibroblasts

BSA – Bovine Serum Albumin

cdk – Cyclin-Dependent Kinase

CREB – cAMP Response Element Binding Protein

DAG – Diacylglycerol

DMEM – Dulbeccos' Modified Eagles Medium

DNA – Deoxyribonucleic Acid

DPM – Disintegration's Per Minute

ECM – Extracellular matrix

EGF – Epithelial Growth Factor

ERK – Extracellular Signal Regulated Kinase

ET-1 – Endothelin-1

FACS – Fluorescence-Activated Cell Sorter

FCS – Foetal Calf Serum

h – Hour

HIF – Hypoxia Inducible Factor

HO – Hemeoxygenase

HRP – Horseradish Peroxidase

5-HT – 5-hydroxytryptamine

5-HTT – 5-Hydroxytryptamine transporter

IL-1 – Interleukin – 1

iNos – Inducible Nitric Oxide

IP_3 – Inositol 1,4,5- trisphosphate

JNK – c-jun n-Terminal Kinase

LPS – Lipopolysaccharide

MAP Kinase – Mitogen Activated Protein Kinase

MAPKAPK-2 – Mitogen Activated Protein Kinase Activated Protein Kinase –2

MEK – Mitogen-Activated Protein Kinase Kinase

min – Minute

MKP – Mitogen Activated Protein Kinase Phosphatase

mmHg – Millimetres of Mercury

MMP – Matrix Metalloproteinases

PA EC – Pulmonary Artery Endothelial Cells

PAP – Pulmonary Arterial Pressure

PBS – Phosphate Buffered Saline

PC – Phosphatidyl-choline

PDGF – Platelet-Derived Growth Factor

PI3K – Phosphatidylinositol 3-Kinase

PIP_2 - Phosphatidyl-inositol –4,5-Bisphosphate

PKA – Protein Kinase A

PKC – Protein Kinase C

PLA_2 – Phospholipase A_2

PLC – Phosolipase C

PLD – Phospholipase D

PO_2 – Percentage Oxygen

p38 MAP Kinase – p38 Mitogen Activated Protein Kinase

pp38 – Phosphorylated p38

RAF – Rat Aortic Fibroblast

RPAF – Rat Pulmonary Artery Fibroblast

RTK – Receptor Tyrosine Kinase

s – Second

S.D. – Standard Deviation

SAPK – Stress Activated Protein Kinase

SH_2 – Src Homology Domain

SMC – Smooth Muscle Cells

SRF – Serum Response Factor

TGF-β - Transforming Growth factor - β

TNF – Tumour Necrosis Factor

VEGF – Vascular Endothelial Growth Factor

Chapter 1

Introduction

1 General Introduction

The acceleration of pulmonary artery fibroblast cell proliferation and the resultant remodelling is thought to be a primary event in the development of pulmonary hypertension (Reid and Davies, 1989). At present, no underlying mechanism for remodelling has been found. One question is the link between vasoconstriction and remodelling. Does vasoconstriction lead to remodelling? It is tempting to say that this is the case because many vasoconstrictors, including hypoxia, are also growth factors, that is, there is a link between cell replication and vascular control ("cell growth-vasomotor coupling") (Scott and Peacock, 1995). Understanding the cell signalling events involved in the regulation of normal and abnormal fibroblast cell proliferation due to hypoxia may provide an important insight into the degeneration of growth control during pulmonary hypertension. In some instances irregular proliferation may be the result of over activity or dysfunction of component(s) of the growth regulating, intracellular signalling pathway(s). Investigation of these pathways may point to possible sites of pharmacological intervention for the prevention of pulmonary hypertension.

1.1 Pulmonary Hypertension.

Pulmonary Hypertension is a condition of abnormally high pressure within the pulmonary circulation and develops in many species, including humans. It is important because it contributes to the morbidity of most heart and lung diseases. One cause of pulmonary hypertension is chronic exposure to normobaric and hypobaric hypoxia (Janssens *et al,* 1991). Exposure to hypoxia results in an acute increase in pulmonary arterial pressure (PAP), as a result of vasoconstriction which can be followed by sustained pulmonary hypertension resulting from a combination of

polycythemia and morphological alterations of the pulmonary vascular bed. This late increase in PAP is not immediately or totally correctable upon improvements in oxygen concentrations to normal values (Vender, 1994).

1.1.1 Epidemiology of pulmonary hypertension

There are several causes of pulmonary hypertension

Left to right shunt. This means that blood at high pressure (systemic circulation) passes directly to the pulmonary circulation (low pressure).

Pulmonary Thrombo-Embolism. A condition in which pulmonary arteries are blocked by a clot formed *in situ* or carried in the bloodstream from the site of remote formation.

Primary pulmonary hypertension. This is a rare disorder of unknown cause. Histological examination of the lung shows an increase in smooth muscle and fibroblast deposition in the small pulmonary arteries.

Chronic hypoxic lung disease. This includes:- Kyphoscoloiosis, chronic bronchitis and emphysema, cystic fibrosis and interstitial lung disease and is thought to cause pulmonary hypertension in two ways: alveolar hypoxia causes pulmonary vasoconstriction and structural damage in the lung reduces pulmonary vascular compliance.

1.1.2 Pathological processes seen in pulmonary hypertension

These fall in to three categories: -

Hypoxic pulmonary vasoconstriction.

Hypoxic pulmonary vasoconstriction has been known to exist since 1946 (von Euler and Liljestrand, 1946) but the mechanism is not yet fully understood. It is turned on

within 1-2 minutes after a sudden decrease in alveolar PO_2, is fully developed after 3-5 minutes, remains more or less stable thereafter according to the experimental conditions, and is generally reversed in less than 1 minute. Hypoxic vasoconstriction is observed in isolated lungs devoid of nervous connections. It is located mostly in small order arterioles but may extend to large branches of the pulmonary artery or to pulmonary capillaries. The biological mechanism of hypoxic pulmonary vasoconstriction remains unknown (Grovner, 1983).

Destruction of the pulmonary vascular bed.

Examples of this are loss of lung parenchyma- e.g. emphysema, and pulmonary vascular arteritis- e.g. polyarteritis nodosa.

Pulmonary vascular remodelling.

In addition to short-term autoregulatory changes in vascular tone, vessels can undergo profound fibrocellular changes. This is an active process termed vascular remodelling which results in an increase in the medial coat of the normally muscular arteries and an extension of muscle into smaller and more peripheral vessels than is normal. There are various clinical conditions in which these features are found. Hypoxia is one cause and is a useful model because it can be tested in the laboratory. These include Kyphoscoliosis (Naeije, 1961), chronic bronchitis and emphysema (Semmons and Reid, 1974) and cystic fibrosis (Ryland and Reid, 1975). Pulmonary vascular remodelling is important clinically because it renders the vessels relatively unresponsive to vasodilators.

1.2 Pulmonary Vascular Remodelling.

1.2.1 Pathological pulmonary vascular remodelling.

Pathologically, pulmonary vascular remodelling represents increased muscularisation of both the large and small pulmonary arteries, which results in a reduction of the area of the pulmonary arterial bed (Reid and Meyrick, 1982). One of the main causes of remodelling in humans is hypoxia.

Thickening of the muscular coat of small pulmonary arteries begins a few days after exposure to hypoxia (Hunter *et al*, 1974). Furthermore, fibroblasts migrate into arterioles and in some studies appear to undergo transformation into smooth muscle cells (Sobin *et al*, 1983) that reduce the size of the vessel lumen. In addition to this, the proximal pulmonary arteries respond to the rise in blood pressure by an increase in the thickness of the media and adventitia. Proliferation of adventitial fibroblasts occurs before the other cell types (Reid and Davies, 1989). High blood flow can also contribute to the remodelling process by increasing the wall shear stress which may directly damage endothelial cells (Esterly *et al*, 1968), and activate platelets to release growth factors leading to smooth muscle cell proliferation. There may also be release of endothelial-derived growth factors. There is experimental evidence that mechanical forces (e.g. stretch) directly stimulate the vascular wall to undergo remodelling in the absence of endothelial cell damage (Archer *et al*, 1988).

1.2.2 Morphological features of pulmonary vascular remodelling.

One of the main features of pulmonary vascular remodelling is the proliferation of fibroblast and smooth muscle cells in the pulmonary arteries. When this occurs, the proliferation of intima and media will encroach on the lumen of the vessel, which in

turn will alter the relationship between flow and pressure in the vessel (Owens and Schwartz, 1983; Murphy *et al,*1981). Structural changes within the vessel can also occur in the larger of the pulmonary arteries. Thickening of the vessel walls also involves an increase in the number of fibroblast cells, which may be a consequence of chemotaxis from a distant site, mitogenesis or both (Harris and Heath, 1986).

1.2.3 Mechanisms of pulmonary vascular remodelling.

The process of pulmonary vascular remodelling consists of some or all of the following events:-

1/ Altered physical and haemodynamic forces.

These include shear stress and transmural pressure. Endothelial cells are subjected to shear stress caused by the movement of blood over their surface. Transmural pressure is the increased atrial pressure causing an increase in stress and strain on all cell types across the vessel wall (Sumpio *et al*, 1988).

2/ Relay of signals to cells.

This may consist of signals / substances (Sharkey *et al,* 2000; Santilli *et al* 1991) secreted from one cell type or in the form of stress which either stimulates or suppresses the growth or activity of another cell type within the vessel.

3/ Alteration of vessel wall and extracellular matrix.

Marked increases in extracellular matrix protein synthesis by fibroblast and smooth muscle cells *in vivo* under conditions of chronic hypoxia have been well established (Saed *et al,* 1999; Crouch *et al,* 1989). Studies in chronically hypoxic adult and neonatal animals from several species demonstrate increases in the production and accumulation of collagen and elastin in the media of conducting pulmonary arteries (Novotna and Herget, 1998; Xu *et al,* 1995). In the adult rat, substantial and rapid

increases in the relative rates of connective tissue protein synthesis in the explants of main pulmonary arteries have been demonstrated (Poiani *et al*, 1990)

4/ Growth promoting substances.

There has been much work done on growth factors, which stimulate all forms of cells from within the pulmonary artery to proliferate. Several peptide growth factors are known to cause smooth muscle cells to proliferate (Schwartz and Reidy, 1987) and indeed the same is true for fibroblasts (Welsh *et al*, 1996). The response of cells to these factors may also be enhanced by the addition of environmental change, such as hypoxia (Welsh *et al*, 1996).

1.2.4 Cells and extracellular matrix in remodelling in response to hypoxia

Little is known of the mechanisms by which hypoxia mediates vascular remodelling. It is unclear whether abnormal cell proliferation and connective tissue deposition in pulmonary arteries is a direct effect of hypoxia itself or secondary to the rise in pulmonary artery pressure and blood flow caused by hypoxic vasoconstriction.

1.2.5 *In vivo* studies of pulmonary vascular remodelling

Ultrastructural and morphometric analysis of pulmonary arteries of animals exposed to chronic hypobaric hypoxia show a significant thickening of each of the three vessel wall layers and a narrowing of the vessel lumen. The nature of the hypoxic response in the media varies with distal progression along the vessel. Medial thickening in large diameter muscular pulmonary arteries seen in the rat (Rabinovitch *et al*, 1979, Walker *et al*, 1984), dog (Rorie *et al*, 1988), newborn domestic calf (Stenmark *et al*, 1988) and humans native to high altitudes (Heath *et al*, 1988) is caused principally by hypertrophy of pre-existing smooth muscle cells (SMCs). This, due mainly to an

increase in the organelles of protein synthesis, the rough sarcoplasmic reticulum and golgi apparatus (Meyrick & Reid, 1980). There is also an increase both in the thickness of the elastic laminae separating SMCs from endothelial cells and extracellular connective tissue deposition including elastin and collagen. Fibroblasts too are able to migrate into the media in response to hypoxia (Sobin *et al*, 1983) and express a dramatic change in phenotype, acquiring structural characteristics of contractile cells.

Adventitial thickening during the development of chronic pulmonary hypertension in rats (Meryick & Reid, 1980) and neonatal calves (Stenmark *et al*, 1988) has been shown to be caused by both an early and a prolonged intense fibroblast proliferative response greater than either the endothelial cell or SMCs (Meryick & Reid, 1979). In addition to an increase in the number of fibroblasts observed in the adventitia of hypoxic pulmonary arteries, marked increases in matrix protein deposition has been noted, especially that of collagen and elastin, as described in human and animal studies of pulmonary hypertension.

1.2.6 *In vitro* studies of pulmonary vascular remodelling

Smooth muscle cells isolated from muscular pulmonary arteries of newborn calves exposed to hypoxia produce more elastin than cell from normal calves (Mecham *et al*, 1987). Cultured human fibroblasts have been shown to exhibit a proliferative response to hypoxia (2.5% O_2) (Storch & Talley, 1988). Although there is compelling evidence that there is SMC replicative response to hypoxia *in vivo*, this observation has not been extended to cultured SMCs. In fact, authors have shown that hypoxia actually inhibits proliferation of cultured bovine foetal, neonatal and adult pulmonary artery SMCs (Benitz *et al*, 1990; Stenmark *et al*, 1991; Dempsey *et al*, 1991)

suggesting that reducing oxygen tension, at least by itself, is unlikely to be a direct stimulus accounting for the increased quantity of smooth muscle found in the pulmonary arteries in association with hypoxemia. However, a homogenous population of pulmonary arterial SMCs in a cultured cell system may not accurately reflect their behaviour *in situ*. They are removed from the influence of pressure flow and physical interactions with matrix components and other cell types usually found in close proximity within the vessel wall, which may influence their response to hypoxia. Other vascular cells including endothelial cells, fibroblasts and macrophages, or blood-borne cells such as the platelet or monocyte may be oxygen sensors themselves and facilitate SMC proliferation in response to hypoxia by their generation of extracellular growth-promoting factors that act in a paracrine manner. The stimulatory effect of reducing oxygen on cultured fibroblast proliferation may also be partially explained by the enhanced synthesis and secretion of autocrinic peptide mitogens. Several groups have used conditioned media from cultured cells exposed to hypoxic conditions to look at its effect on growth of cells and to identify and quantify the factors released (Ankoma-Sey *et al,* 2000).

1.2.7 Growth factors in remodelling

Hypoxia increases the expression of growth factors in whole lung and isolated vascular cells (Sodhi *et al,* 2000; Ankoma-Sey *et al,* 2000). It is not clear which are critical to the remodelling process, although endothelin–1 (ET-1), platelet-derived growth factor (PDGF), Angiotensin II (AII), Thrombin and 5-hydroxytryptamine (5-HT) are probably important.

Endothelin-1: Vender and colleagues (1987) first described a SMC macromolecular mitogen released from hypoxic pulmonary artery endothelial cells (PA ECs). Since then, ET-1 has been identified as an EC-derived G protein-coupled receptor agonist peptide shown to be mitogenic for cultured SMCs and fibroblasts from the systemic vasculature (Suzuki *et al,* 1999). Production of ET-1 by cultured human umbilical vein ECs is elevated by hypoxic exposure (Peacock *et al*, 1992). However, cultured bovine SMCs from the pulmonary artery exhibit only a modest increase in growth in response to maximal ET-1 concentrations. Indeed, bovine pulmonary artery fibroblasts as measured by cell counts and [^{3}H]thymidine incorporation, a reflection of DNA synthesis show no increase in proliferation to ET-1 (Hassoun *et al*, 1992, Welsh *et al*, 1996). Furthermore, bovine PA ECs show either no change (Hassoun *et al*, 1992) or a decrease (Wiebke *et al*, 1992) in ET-1 release in response to hypoxia of 48h duration. These discrepant and contradictory results have made it difficult to interpret the role of ET-1 as a factor regulating SMC and fibroblast proliferation and in how hypoxia modifies ET-1 production by ECs in the pulmonary circulation.

Platelet-derived growth factor: Platelets contain a mitogenic protein factor referred to as PDGF (Deuel, 1987). This protein is present in platelet α granules (Gerrard *et al*, 1980) and is released when platelets are stimulated to aggregate (Ross *et al*, 1986). It is a major mitogen in serum for many types of cells, including fibroblasts, glial cells, and vascular smooth muscle cells, (Heldin and Westermark, 1984; Deuel *et al*, 1983; Ross *et al* 1986) but not in endothelial cells (Davies *et al*, 1978). PDGF is a dimeric molecule that occurs as a homodimer (AA and BB) and a heterodimer (AB) of related polypeptide chains: PDGF –A and PDGF –B (Heldin and Westermark, 1984).

Platelets contain mainly PDGF-AB and some PDGF-BB. On the other hand, VSMC secretes PDGF-AA in a growth and differentiation-dependant manner, which may contribute to the growth-stimulating mechanisms of blood vessels. In contrast to fibroblasts, which show an equal growth response to PDGF-AA and –BB (Mehmet *et al*, 1990), the mitogenic responses of VSMC to the three PDGF isoforms are not uniform, e.g. PDGF –AA is a far weaker mitogen than PDGF –AB and –BB (Sachinidis *et al*, 1990). In fibroblast and smooth muscle cells, PDGF has been shown to increase the cellular replicative responses to hypoxia (Welsh *et al*, 1996; Stenmark *et al*, 1991).

5-Hydroxytryptamine (5-HT): 5-HT, also known as serotonin, is one of the best known examples of a neurotransmitter that mediates a wide variety of physiological effects, including peripheral and central actions, through the binding to multiple receptor subtypes (Wilkinson and Dourish, 1991). The large diversity of 5-HT functions is paralleled by the pharmacological complexity of 5-HT receptors. At least four classes of 5-HT receptors have been distinguished pharmacologically, based on the second messenger systems to which the receptor is coupled. The family including 5-HT_1 and 5-HT_5 subtypes of receptors interacts negatively with adenylyl cyclase, the 5-HT_2 subfamily of receptors is coupled to the activation of the phospholipase C-β, the 5-HT_3 receptor is a ligand-gated ion channel, and the family, including 5-HT_4, 5-HT_6, and 5-HT_7 subtypes of receptors, activates adenylyl cyclase (Peroutka, 1995).
Of the receptor classes distinguished, the 5-HT_2 receptor is the most likely to be involved in the hypoxic responses seen in the pulmonary vessels. It mediates many of the central and peripheral physiological functions of 5-HT, including contraction of blood vessels and cell growth and development (Tecott *et al*, 1995).

Angiotensin II (AII): AII stimulates a variety of physiological responses related to the regulation of blood pressure, salt and fluid homeostasis (Raizada *et al*, 1993). In addition, AII promotes growth responses in many cells, including cardiomyocytes, fibroblasts (Marshall *et al*, 2000), and vascular smooth muscle cells (Aceto and Baker, 1990; Sadoshima and Izumo, 1993). AII exerts effects through specific G-protein coupled receptors, predominantly the AT_1 receptor subtype. AT_1 receptors couple to intracellular calcium mobilisation, activation of tyrosine kinases such as $p125^{FAK}$ and induction of serine/threonine kinases, including protein kinase C (PKC) and mitogen-activated protein kinases (MAP kinases) (Lindpainter and Ganten, 1994; Sadoshima and Izumo, 1993). AII may act directly through these pathways or indirectly via the release of growth factors such as PDGF and TGF-β, as demonstrated for rat vascular smooth muscle cells (Sadoshima and Izumo, 1993). Like other growth factors, AII induces a rapid increase in the growth associated nuclear proto-oncogenes c-*myc*, c-*fos* and c-*jun* and several cellular genes including tenascin, fibronectin and collagen (Naftilan *et al*, 1990; Taubman *et al*, 1989). These studies indicate that AII can induce rapid changes in gene expression and function that may ultimately lead to increased cell growth (Dosal *et al*, 1991)

Thrombin: Thrombin may have a role in the pathogenesis of pulmonary vascular disease. It is well documented as causing pulmonary vasoconstriction (Horgan *et al*, 1987) and has been implicated in the development of intimal thickening after balloon angioplasty (Sarembock *et al*, 1991). The action of thrombin on vascular cells involves the proteolytic activation of G protein-coupled receptors that are subjected to rapid and irreversible de-sensitisation. Thrombin is mitogenic in neonatal rat vascular

smooth muscle (Huang and Ives, 1987) as well as various fibroblast cell lines (Vouret-Craviari *et al*, 1992; Carney *et al*, 1985). Furthermore, the mitogenic effects of thrombin can be mimicked by thrombin receptor-derived peptides that activate the thrombin receptor (McNamara *et al*, 1993).

Growth factors mediate their effect in general by binding to the cell surface via specific receptors and initiating a cascade of intracellular signalling events (Chapter 1.8).

1.3 Hypoxia.

1.3.1 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. The effect of hypoxia on an animal is characterised by tachycardia, hypertension, peripheral vasoconstriction, and mental confusion.

1.3.2 Causes of hypoxia

1.3.2.1 Altitude

The first clue that altitude adversely affected the lung circulation of the altitude resident was the recognition of 'brisket disease' in cattle. In 1913, ranchers in Colorado (10,000 feet) asked veterinary scientists at Colorado State University to investigate why their herds were dying with swelling ventral to the sternum in the area

of the brisket of beef, hence the name 'brisket disease' (Glover & Newsom, 1915). At the turn of the century when cattle from Kansas and Texas were being introduced into Colorado high pastures, mortality approached 40%. Investigations, which began then, revealed that the swelling was due to accumulation of heart failure fluid and that cattle recovered when taken to lower altitude.

After von Euler and Liljestrand (1946) reported that acute hypoxia induced pulmonary vasoconstriction, investigators in Colorado confirmed that pulmonary hypertension in the cattle was the result of sustained vasoconstriction from chronic hypoxia (Alexander & Jenson, 1959).

1.3.2.2 Lung disease

Many lung diseases are associated with hypoxia which stimulate pulmonary vasoconstriction and cause pulmonary vascular remodelling. Examples of these are chronic bronchitis, emphysema and interstitial lung disease. Chronic bronchitis leads to airway narrowing and obstruction and in some cases polycythemia results from chronic hypoxemia (Hasleton *et al*, 1968). Emphysema is the result of destructive changes in the alveolar wall resulting in loss of lung elasticity and gas exchange. Interstitial lung disease may be idiopathic but may also follow industrial exposure to inorganic dusts, such as asbestos or silica. There is scarring of the lung tissue and hence reduction of the lungs ability to allow oxygen transport to the blood.

1.3.3 Fffects of hypoxia

In response to hypoxia, mammals exhibit systemic responses that decrease cellular oxygen need and dependence and increase tissue oxygen supply. Alterations in oxygen tension elicit important responses at the organ and tissue level. Exposure to

chronic hypoxia causes pulmonary hypertension in most mammalian species. The 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) (Stenmark and Mecham, 1997). 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 biological messengers. 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.

1.3.3.1 Endothelial cells

Large amounts of work have been carried out in endothelial cells. PA ECs are continually exposed to low levels of oxygen due to the low oxygen content of mixed venous blood. This level of oxygen can become even lower during hypoxic exposure. In response to hypoxia, a number of genes have been shown to be induced. These include vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β). Current evidence suggests that most of the presumably essential adaptive responses to hypoxia exhibited by endothelial cells are mediated through the transcription factor called hypoxia inducible factor (HIF) (Bunn and Poyton, 1996, Bunn *et al*, 1998). Changes in endothelial gene expression regulated by the HIF transcription factor include gene glycolytic enzyme, the glucose transporter GLUT-1, VEGF-receptor expression, hemeoxygenase (HO), endothelin and PDGF (Ankoma-Sey *et al,* 2000; Sodhi *et al,* 2000; Blancher *et al,* 2000; Richard *et al,* 2000). Yan *et al* (1998) demonstrated that in contrast to hypoxia-induced adaptive responses that

appear to be the subject mostly to regulation by HIF-1, those responses that could be considered maladaptive (inflammation) appear to be under the control of other transcription factors, most notably early growth response gene product (Egr-1). Thus hypoxia appears to activate several distinct types of host response mechanisms in endothelial cells.

1.3.3.2 Smooth muscle cells

Research into the effect of hypoxia on smooth muscle cells has led to a substantial body of evidence which suggests that there are specific subsets of smooth muscle cells which display unique differences depending on the location of the cell along the axis of the arterial tree, as well as in distinct layers. These differences would appear to play an important role in the cells reaction to hypoxia (Frid *et al,* 1994). Cells exhibiting unique morphological and biochemical characteristics also differ significantly with respect to their proliferative responses to growth promoting stimuli. In general the 'non-muscle-like' cell sub-populations exhibit markedly enhanced growth capabilities under serum-stimulated conditions compared to 'differentiated' SMC populations (Frid *et al*, 1997a; Frid *et al*, 1997b).

1.3.3.3 Fibroblasts

In animal models, the earliest and most dramatic structural changes following hypoxic exposure are found in the adventitial compartment of the vessel wall (Jones and Reid, 1995). Fibroblast cells from the adventitia exhibit increases in proliferation that exceed those observed in other cell types (Belknap *et al*, 1997). The fibroproliferative changes in the adventitia are ultimately associated with luminal narrowing and

progressive decreases in the ability of the vessel wall to respond to vasodilating stimuli (Durmowicz *et al*, 1993).

1.3.3.4 Intracellular targets of hypoxia

As noted previously, the activity of several different transcription factors are known to be influenced by low oxygen tensions. Protein phosphorylation appears to be crucial for expression of many hypoxic-inducible genes. Inhibition of tyrosine kinase pathways has been shown to inhibit many HIF-inducible genes. One important target is the mitogen-activated protein kinase (MAPK) family of enzymes. Many studies have demonstrated that the MAPK's p44 (Erk 1) and p42 (Erk 2) are crucial to proliferation in response to growth factors in a variety of cell types (Chapter 1.16; Segal and Greenberg, 1996)

In addition to the "classic" forms of MAP kinase, families of related protein serine/threonine kinases exist in cells, and are referred to as the stress-activated protein (SAP) kinases (Chapter 1.17). These consist of p38 MAP kinase and JNK homologues and have also been shown to be activated in adventitial fibroblasts to acute hypoxia (Scott *et al,* 1998).

1.4 Fibroblast physiology

Within the developing vasculature, fibroblasts influence the growth and development of blood vessels. The normal arterial wall is predominantly composed of three layers; intima, media and adventitia. Smooth muscle cells are the principle cells found within the media in association with a matrix of connective tissue components (collagen and elastin) and provide mechanical and structural support to the vessel. The intima consists of a single layer of endothelial cells resting on a thin basal lamina. The

adventitia is comprised of connective tissue proteins secreted by locally resident fibroblasts. These fibroblasts play a significant role in arterial repair.

Recent insights into arterial remodelling have implicated the adventitial layer as an important modulator of remodelling through its interactions with the media and intima. In pulmonary hypertension, adventitial thickening with increased cellularity and extra cellular matrix (ECM) deposition are most prominent in the small, muscular pulmonary arteries (Stenmark and Mecham, 1997; Riley *et al*, 1986), which typically share a number of structural features in common with systemic muscular arteries. There are several factors that contribute to significant structural changes in both the adventitia and media. These include fibroblast proliferation, fibroblast apoptosis, and fibroblast migration into the intima, adventitial fibrosis and expression of matrix metalloproteinases (MMPs). It is important to assess evidence for these activities in the pathogenesis of remodelling in the pulmonary and systemic circulation because hypoxia has opposite and contrasting effects in the 2 populations.

1.4.1 Fibroblasts from the pulmonary and systemic circulations

As mentioned previously, whereas hypoxia causes vasoconstriction in the pulmonary arteries, it has a vasodilatory effect in the systemic arteries (Heisted and Abbound, 1980; Wagner and Mitzner, 1988). Teleologically, this vasodilation increases oxygen delivery to hypoxic tissues; this can be problematic when treating pulmonary hypertension as pulmonary vasodilators can give rise to systemic hypotension. Although metabolic intermediaries such as ATP depletion or the formation of adenosine have been suggested as likely mechanisms, how systemic vasodilation is effected remains obscure. There has been further evidence of this tantalising discrepancy between the pulmonary and systemic circulations by the observations that

administration of ET-1 to intact-chest cats constricts pulmonary vessels and dilates systemic vessels (Lippton *et al,* 1989a; Lippton *et al,* 1989b). The role played by endothelium-derived mediator(s) in the opposite effects of hypoxia on the pulmonary and systemic circulation awaits clarification.

Although many properties are shared between adventitial fibroblasts in the muscular arteries of the pulmonary and systemic circulation, differences also exist that need further elucidation, particularly in response to hypoxia. It is possible that fibroblasts from the pulmonary artery exhibit increases in intracellular signalling pathways when exposed to hypoxia that are not seen in those from the systemic circulation. By studying the intracellular mechanisms from normal and hypoxic fibroblasts from both the pulmonary and systemic arteries, possible targets may be found for treating hypoxic pulmonary hypertension without causing adverse effects in the systemic circulation.

1.5 Signalling events controlling cell proliferation

1.5.1 The Cell Cycle

The cell cycle is a chain of events which is necessary for successful cell division; this period varies depending on the type of cell but generally takes from between 24-30 hours. The cell cycle contains a number of phases; G_1 (the first gap before DNA replication), S phase (when chromosomes are replicated), G_2 (the gap after DNA replication) and M phase (the period when the replicated chromosomes are split into two daughter cells (Figure 1.1). Many non-dividing cells in tissues (e.g. all quiescent fibroblasts) suspend the cycle after mitosis and just prior to DNA synthesis; such "resting" cells are said to have exited from the cell cycle and to be in the G_0 state. It is

possible to identify cells when they are in one of the three interphase stages of the cell cycle, by using a fluorescence-activated cell sorter (FACS) to measure their relative DNA content: a cell that is in G_1 (before DNA synthesis) has a defined x amount of DNA; during S (DNA replication), it has between x and 2x; and when in G_2 (or M), it has 2x of DNA. Extracellular stimuli in the form of growth factors, mitogens or stress determine whether a quiescent cell will begin to proliferate and also whether a normal proliferating cell in G_1 will continue to traverse the cell cycle or revert to quiescence (reviewed in Pardee, 1989). Once the S phase is entered, mainly intracellular triggers compose regulatory events. In fact, the control regulating the onset of the M phase is now believed to be via levels of various cyclins and their partner cyclin-dependent kinases (cdks) as well as cdk inhibitors such as p16 and $P27^{kip1}$. The use of clonal cell lines has greatly facilitated the analysis of the early events induced by growth factors. These cell lines can be made quiescent by either withdrawing serum growth factors from the culture medium or by allowing the cells to reach confluency so that all of the available growth factors are depleted and the cells contact inhibited. By re-adding serum or defined growth factors, quiescent cells are stimulated to re-enter the cell cycle and thus early biochemical events in response to the growth factor can be investigated.

Figure 1.1

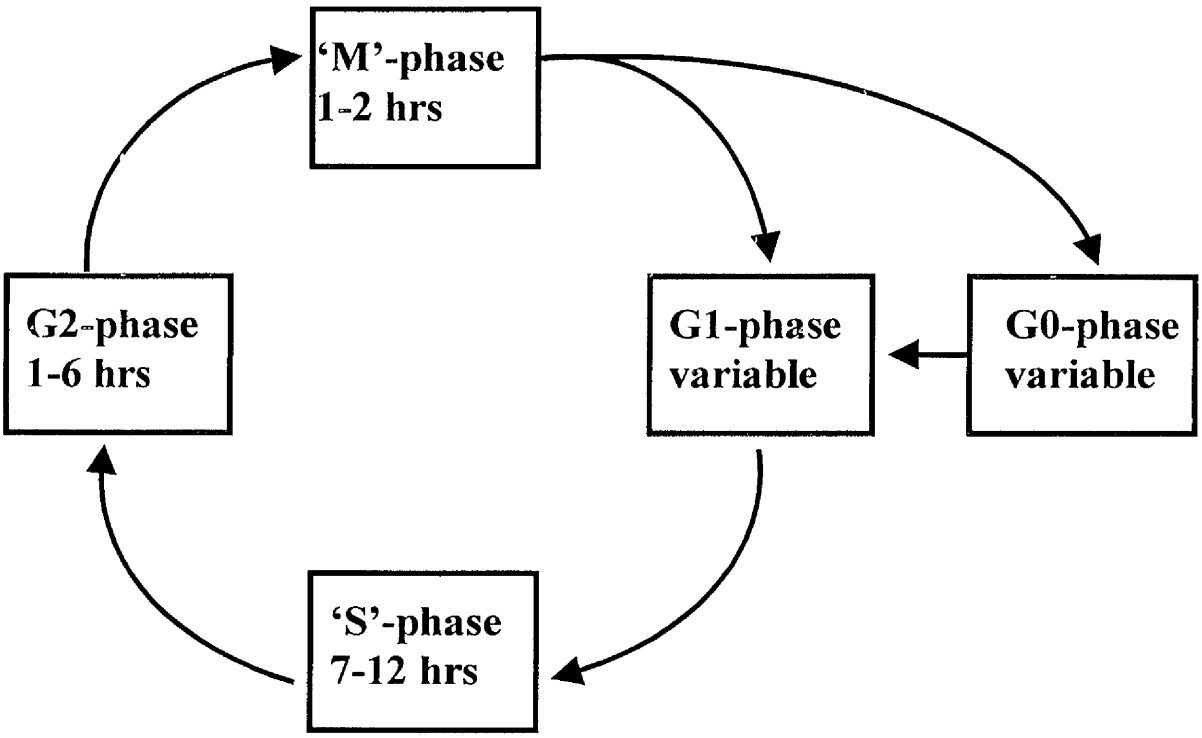


Figure 1.1 The cell cycle

Cell growth can be conceptualised as occurring in distinct phases; the mitotic M phase and the synthetic S phase, each separated by a Gap phase (G_1 or G_2). Cells may temporarily occupy an additional G_0, or "resting phase".

1.5.2 Transcriptional regulation of cell cycle dependent genes.

In order for a cell to respond to extracellular signals and progress through the cell cycle, the co-ordinate regulation of the transcription of cell cycle dependent genes is essential. To activate or repress transcription, transcription factors must be activated in the nucleus, bind to specific regions of DNA (palindromic response elements) and interact with the transcription apparatus (e.g. RNA polymerase). Regulation of transcription is achieved at a number of levels:

(1) Nuclear translocation, where the location of transcriptional factors is determined by the activity of either nuclear localisation signals or cytoplasmic retention signals. For example, translocation of NFκB to the nucleus is prevented by its association with an inhibitory subunit, IκB, in the cytoplasm (Beg and Baldwin, 1993).

(2) Transcriptional activation, where phosphorylation and acetylation events directly facilitate interaction with transcription machinery. For example, cAMP/Ca^{2+} response element binding protein (CREB) activity is dependent on regulated phosphorylation of Ser^{133} (Gonzales, 1991).

(3) Alteration of DNA binding properties. For example, some phosphosphorylation events directly regulate transcription factor binding to response elements, as is the case for serum response factor (SRF) (Rivera *et al*, 1993). These mechanisms have been reviewed by Hill and Treisman (1995).

Transcriptional regulation in response to extracellular signals is both cell type and developmentally specific. This level of control is determined by a number of interacting factors. Principally, the specificity of transcriptional activation is due to the presence of a host of transcription factors that specifically bind to response elements (Table 1.1) by two mechanisms (Hill and Treisman, 1995):

Transcription factor family	Response elements /complex formation	Properties regulated by stimulus	Activating signal
Ternary complex factor (TCF) eg Elk-1 and SAP-1	Binds serum response elements (SRE) as ternary complex with serum response factor (SRF)	Transactivation, DNA binding	Growth factors via MAP or SAP kinase cascades
cAMP responsive element-binding proteins (CREB)	Binds cAMP response elements (CRE) as homodimer or as heterodimer with other ATF family members.	Transactivation; Association with coactivator CBP	PKA pathway; Ca^{2+} /calmodulin
AP-1 eg Jun and Fos	Binds to 12-O-Tetradecanoylphorbol-13-acetate (TPA) responsive elements as homodimer or heterodimer with other AP-1 family members	Transactivation; DNA binding; Association with coactivator CBP?	UV irradiation; SAP kinase Cascade; PKC activation
Signal Transducer and activator of transcription (STAT)	Binds to GAS elements and related sites as homodimer or heterodimer, or to interferon-stimulated regulatory elements (ISRE) as part of a heterotrimeric complex (ISGF3); STAT1, STAT2 and p48	Dimerisation; Nuclear localisation	Cytokines; Growth factors
Nuclear factor/Necrosis factor κB (NFκB)	Binds to κB sites as homodimer or heterodimer with other family members	Nuclear localisation; Release from the inhibitor IκB	Lymphokines, TNFα

Table 1.1 Transcription factors

Examples of some transcription factors, their response elements, mechanisms of activation and activation signal (modified from Hill and Treisman 1995)

(a) Promoter activity is dependent upon the binding of a specific set of transcription factors at multiple response elements immediately on the 5' side of the promoter. Transcription factor binding is specific for both the agonist and signal transduction pathway activated.

(b) The activity of certain promoters is dependent on the binding of ternary complexes composed of different families of transcription factors that, in some cases, require the activation of a number of different specific signalling pathways.

In addition, the level of transcriptional activation or 'promoter strength' may also be regulated by both the duration and magnitude of signal. For example, the ability of the response element(s) to induce the same promoter with different activities may be dependent on the number of activated transcription factors bound to the response elements, which will ultimately be dependent upon the activity of the signalling pathway.

1.6 Early growth factor-stimulated events

Since the initiation of DNA synthesis is a late event in the cell cycle attention has focused on the initial cellular responses associated with a mitogenic stimulus and their importance in the later proliferative response. As discussed previously there is an increasing list of growth promoting agents acting on different types of cells that elicit a common pleiotypic response. These initial cellular responses to mitogens include an intrinsic rise in intracellular pH, a transient rise in cytoplasmic Ca^{2+}, the phosphorylation of a common set of proteins and an increase in c-fos and c-myc mRNA.

Stimulation of a rise in cytoplasmic pH has been reported to be a common response in the activation of many quiescent cell types. Growth promoting agents such as serum, endothelin, EGF, α-thrombin, insulin, bombesin, PDGF and vasopressin have all been shown to induce a rapid rise in pH by controlling Na^+/H^+ exchange. The Na^+/H^+ exchanger is a cell membrane protein that exchanges Na^+ ions for H^+ ions in an electorneural fashion. (Hesketh *et al*, 1985; Simonson *et al*, 1989; Grinstein *et al*, 1989).

In addition to pH changes a characteristic feature of many growth stimuli is that they induce increases in the intracellular free Ca^{2+} concentrations ($[Ca^{2+}]i$). Hormone-receptor-mediated increases in the cytosolic $[Ca^{2+}]_i$ often result from both mobilisation of Ca^{2+} from intracellular stores and transmembrane Ca^{2+} influx (Tsien and Tsein, 1990; Berridge, 1993a). In individual cells, the hormone-stimulated elevation of intracellular Ca^{2+} has a complex temporal and spatial regulation, as the rise in intracellular Ca^{2+} is often observed as a series of repetitive oscillations, or spikes (Berridge and Galione, 1988; Meyer and Stryer, 1991). The spatial counterpart of a $[Ca^{2+}]_i$ spike is a wave, where an initial local $[Ca^{2+}]_i$ elevation ultimately spreads throughout the cell in a regenerative manner (Roony *et al*, 1991; Lechleiter *et al*, 1991). The link between hormone-receptor activation and Ca^{2+} release is the intracellular messenger inositol 1, 4, 5-trisphosphate (IP_3) which mobilises Ca^{2+} by binding to specific intracellular receptors. These receptors also form the channels through which stored Ca^{2+} is released (Taylor and Richardson ,1991; Ferris and Snyder, 1992). In non-excitable cells the Ca^{2+} spikes and waves, described above, are due to the cyclical and co-ordinated activation of IP_3 receptors (IP_3R).

$[Ca^{2+}]_i$ is clearly very important for the control of many essential cellular responses and therefore the increase in $[Ca^{2+}]_i$ observed with many growth factors and mitogenic peptides may play a role in their mechanism of action.

In addition to the events in the membrane and cytosol, as described above, growth factors rapidly and transiently induce the expression of the cellular oncogenes *c-fos* and *c-myc* in quiescent fibroblasts (Kelly *et al*, 1983). The enhanced expression of *c-fos* messenger RNA (mRNA) occurs within minutes of, for example, PDGF addition followed by increased expression of *c-myc*. The induction of *c-fos* mRNA is one of the earliest nuclear events that follow the addition of PDGF. Since these cellular oncogenes encode nuclear proteins (Abrams *et al*, 1982) their transient expression may play a role in the transduction of the mitogenic signal in the nucleus.

One of the best characterised pathways, the Ras→ Raf→ MEK→ ERK cascade (where ERK is extracellular-signal-regulated kinase and MEK is mitogen-activated protein (MAP) kinase (ERK) kinase) is stimulated by many mitogens and growth factors. MAP kinase is phosphorylated by a unique dual-specificity kinase on both tyrosine and threonine in one of three motifs (Thr-Glu-Tyr, Thr-Phe-Tyr or Thr-Gly-Tyr), depending on the pathway. In addition to activating one or more protein cascades, the initiating stimulus may also mobilise a variety of other signalling molecules (e.g. PKC isoforms, phosphatidylinositol-3 kinase (PI3K), phospholipid kinases, G-protein α and $\beta\gamma$ subunits, phospholipases, intracellular Ca^{2+}). These various signals impact to a greater or lesser extent on multiple downstream effectors. Important concepts are that signal transmission often entails the targeted relocation of specific proteins in the cell, and the reversible formation of protein complexes by means of regulated protein phosphorylation. The signalling circuits may be completed

by the phosphorylation of upstream effectors by downstream kinases, resulting in a modulation of the signal.

1.7 Protein phosphorylation

Protein phosphorylation is a regulatory covalent modification of a protein, in which the phosphate group is transferred from ATP to a side chain of the protein, catalysed by a protein kinase. A particularly large amplification is possible if two or more protein kinases are arranged in series – a system known as a protein kinase cascade. There are at least two purposes of phosphorylation cycles in cells:

1. They are the major mechanism by which extracellular signal molecules acting at the cell surface receptors produce their ultimate intracellular effect.
2. They are the major mechanism through which events that occur discontinuously in the cell cycle are initiated and timed.

Regulatory protein phosphorylation normally occurs on serine or threonine residues (about two thirds) but it can also occur on tyrosine residues.

An important class of protein kinases are activated by the binding of second messengers. This class includes cAMP-dependent protein kinase (protein kinase A), cGMP-dependent protein kinase, and PKC. Another class of protein kinases are those activated by extracellular signals (receptor protein kinases). In these cases the protein kinase catalytic domain is present as the cytoplasmic domain of a transmembrane receptor in which the external domain binds an extracellular messenger. This binding activates the internal protein kinase, probably by causing aggregation of receptors. The most well-studied examples are receptors for polypeptide hormones or growth factors in which the protein kinase is tyrosine specific, such as PDGF. However, a few recent examples have been found where the protein kinase domain is more

closely related to serine/threonine kinases, for example the TGF-β receptor. As stated previously, some protein kinases are themselves regulated by phosphorylation of other protein kinases.

1.8 Extracellular receptors.

Cells are sensitive to a large number of chemical signals that control cell and tissue behaviour. The 'target cells' for each signal are the cells that bear receptors for that agent. Each extracellular stimulus acts at a unique receptor, but onward transmission of information into the cells is channelled through signalling pathways built into the plasma membrane. As a result, one signalling pathway may mediate the effects of many extracellular agents on many intracellular responses in many target cells. The way in which this information is processed from the outside of the cell to the nucleus is summarised in Figure 1.2.

The genome of every cell encodes many more receptors than the number of different chemical signals that any individual cell ever has to recognise. A small subset of these genes is expressed in a differentiated cell at a particular time. This allows each cell to respond only to the limited range of stimuli for which it is a target, and each cell's receptor repertoire is constantly undergoing environmental regulation rather than remaining static.

There are several ways in which a cell can process information from the extracellular to the intracellular and these will be discussed below.

1.9 G – protein coupled receptors.

Some cell surface receptors signal through relay systems in which three components act in sequence:

Receptor → G protein → effector protein

G proteins are guanine-nucleotide dependent coupling proteins and are the largest most versatile class of cell surface signal transducing proteins known. The effector protein is usually an enzyme or an ion channel (Iismaa & Shine, 1992). The receptors that function in this way seem to be members of a single structural family of polypeptides termed 7-span receptors. These '7-span' receptors are embedded within the plasma membrane and have cytoplasmic domains. G proteins are heterotrimeric and consist of an α subunit that binds GDP/GTP, and two intimately associated subunits β and γ. The βγ subunits are common between different G proteins, and it is mainly the α subunits that confer specificity for downstream signalling although the βγ subunits are the effectors in a few cases.

Receptor activation of adenylate cyclase is mediated through the α subunit of one particular G protein, G_s. The GTPase activity of α_s is such that several thousand molecules of cAMP are produced through activation of a single receptor. Over 20 different α subunits of G proteins have been identified by purification or by cDNA cloning.

G proteins are known to regulate hydrolysis of plasma membrane phospholipids such as phosphatidylinositides (PI) and phosphatidylcholine (PC). G_q regulates PI hydrolysis through phospholipase C_β (PLC_β). Other G proteins are involved in the regulation of ion channels such as those for K^+ and Ca^{2+}.

Another large family of 7-span receptors feeds its input to the cell through a signalling system based on the hydrolysis of the membrane phospholipid phosphatidyl-inositol 4,5-bisphosphate (PIP_2) by PLC (Berridge, 1993b). By recruiting different isozymes of PLC, it can be activated either through receptor tyrosine kinases (RTK) (via PLCγ) or by G protein coupled receptors (GPCRs).

Figure 1.2

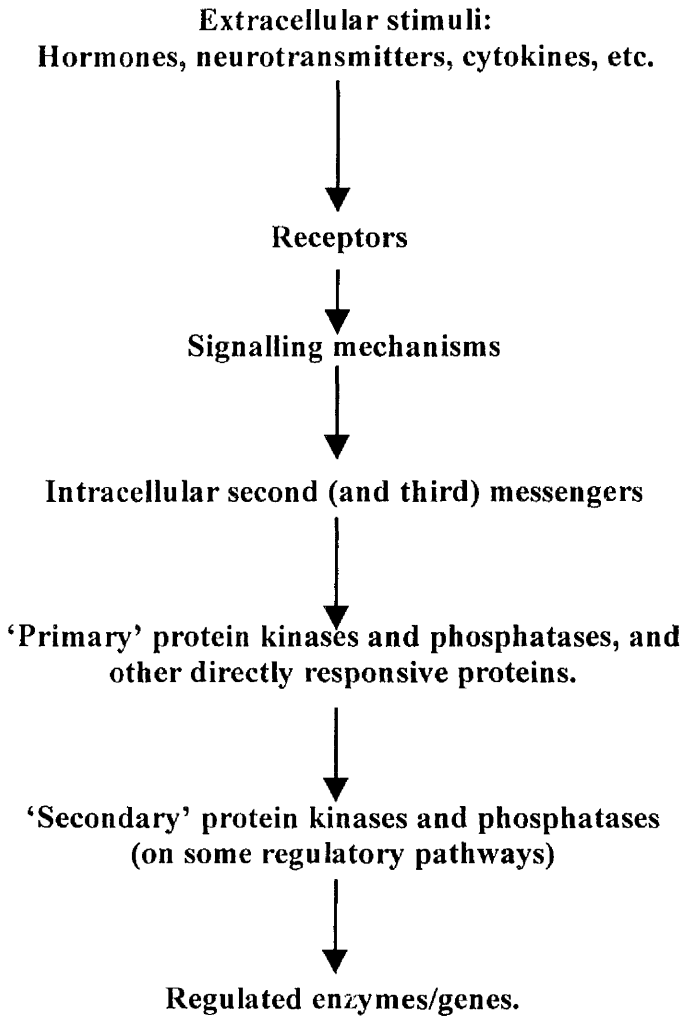


Figure 1.2 Overview of extracellular signalling into the cell

A schematic summary of the manner in which cells use a limited number of signal transduction pathways to transmit to the cell interior the diverse regulatory information that is brought by a very large number of stimuli which act at an even larger number of receptors. This summary also gives an approximate idea of the magnitude of the genetic resources that cells invest in machinery for the receipt and interpretation of extracellular information.

1.10 Agonist-stimulated phospholipid hydrolysis in fibroblast cells

The majority of agonists which evoke proliferation of fibroblasts are believed to act via stimulation of phospholipase C (PLC) (Lee and Rhee, 1995). Stimulation of PLC results in the rapid breakdown of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the generation of the second messenger inositol 1,4,5-tris phosphate (IP_3) and diacylglycerol (DAG).

Following the hypothesis of Berridge and Irvine (1984) that IP_3 is the "missing link" between the plasma membrane receptors and internal Ca^{2+} stores, several papers demonstrated that IP_3 acts by mobilising Ca^{2+} from intracellular pools (Berridge and Irvine, 1989; Carsten & Miller, 1990; Coburn & Baron, 1990; Somlyo & Somlyo, 1992). It has been shown that IP_3 is rapidly generated in fibroblasts and smooth muscle cells after agonist stimulation (Berridge, 1987; Cook *et al,* 1990) and the finding that IP_3 can lead to release of intracellular calcium stores are compelling evidence that IP_3 production is directly linked to the initiation of fibroblast and smooth muscle cell proliferation.

As noted previously, hydrolysis of PIP_2 generates IP_3 and another second messenger, DAG, which remains associated with the membrane. The principal function of DAG is to activate a family of plasma-membrane protein kinases collectively termed protein kinase C (PKC). In the absence of hormone stimulation, PKC is present as a soluble cytosolic protein that is catalytically inactive. A rise in the cytosolic Ca^{2+} level causes PKC to bind to the cytoplasmic leaflet of the plasma membrane, where it can be activated by the membrane-associated DAG. Thus activation of certain PKC isoforms depends on both Ca^{2+} ions and DAG, suggesting an interaction between the two branches of the inositol-signalling pathway.

The activation of PKC in different cells results in a varied array of cellular responses, indicating that it plays a key role in many aspects of cellular growth and metabolism. PKC represents a family of related kinases, which are of central importance in intracellular signalling pathways. At least 11 distinct isozymes of PKC have been identified, several of which exist in pulmonary artery fibroblasts (Pai *et al,* 1991).

1.11 Phospholipase D

Regulation of phospholipase D (PLD) has received considerable attention as hydrolysis of the membrane phospholipid, phosphatidylcholine (PC), by this enzyme produces phosphatidic acid, a potential second messenger in many cell types (Exton, 1994). The activity of PLD in a wide variety of cells is influenced by many hormones, neurotransmitters, growth factors and other agonists linked to heterotrimeric G proteins or tyrosine kinases (Exton, 1994; Exton, 1997). The mechanisms by which G protein-coupled receptors and receptor tyrosine kinases (RTKs) are linked to PLD are not well understood but PKC activation appears to play a major role in many cell types. (Elder *et al*, 1993; Balboa *et al*, 1994). Since the majority of agonists that promote PLD activation also stimulate PLC, a logical mechanism is that PLD activation is secondary to PKC stimulation due to production of DAG from PIP_2 (Exton, 1997). At present, the mechanism(s) by which PKC activates PLD is undefined, indeed the role for PLD in early mitogenic signalling is controversial, with both supporting and conflicting evidence (Boarder, 1994).

1.12 Phospholipase A_2

The hydrolysis of PC by phospholipase A_2 (PLA_2) was one of the earliest phospholipase pathways to be described. The 'classical' role of PLA_2 activation is to

generate arachidonic acid, which is the rate limiting precursor of prostaglandin and leukotriene biosynthesis (Lark *et al*, 1995). It is possible that in inflammatory states this is the major function of arachidonate, however in mitogenic signal transduction, other pathways may be important. These alternative pathways include the activation of certain isoforms of PKC (Asaoka *et al*, 1992a) as well as PLD stimulation (Wang *et al* 1992). Although PLA_2 is activated by many agonists linked to heterotrimeric G proteins, there is no compelling evidence that G proteins interact directly with this enzyme (Clark *et al*, 1995). RTK growth factors such as PDGF and EGF can stimulate PLA_2 activity (Goldberg *et al*, 1990), indeed it has been demonstrated that PLA_2 can be phosphorylated and activated by MAP kinase (Lin *et al*, 1993). Since growth factors stimulate MAP kinase, it is possible that both RTK-linked growth factors and G protein-coupled receptor agonists control the activity of PLA_2 through MAP kinase activation.

1.13 Other lipid/lipid-derived second messengers

The mechanisms discussed previously account for the major routes of phospholipid signalling. However, other modes of signalling for phospholipid second messengers are known to exist.

Arachidonic acid (apart from being a precursor for eicosanoids) and lysophosphatidylcholine may function as lipid second messengers through the activation of effector enzymes such as PKC (Khan *et al*, 1993; Naor *et al*, 1988). Lysophosphatidylcholine has been shown to have several biological actions such as stimulation of chemotaxis and smooth muscle contraction (Asaoka *et al*, 1992b), whilst arachidonic acid stimulates expression of early growth responsive genes such as *c-fos* and *c-jun* in VSMC (Rao *et al*, 1994; Rao *et al*, 1993).

The structure, regulation and function of PI3K has been extensively reviewed by Downes & Carter (1992) and Fry (1994). This kinase is a receptor-controlled enzyme capable of phosphorylating the 3-OH group of the inositol ring of PIP_2, leading to the formation of IP_3. It is activated by many receptor tyrosine kinases in response to growth factors by recruiting p85/p110 heterodimer to the membrane where they associate via the SH2 domains in p85 with phosphorylated YXXM motifs within the cytoplasmic receptor domain of the receptor itself (Carpenter *et al*, 1993). The ability of GPCRs to regulate PI3K was first indicated by observations that stimulation of platelets with thrombin resulted in the rapid accumulation of 3-phosphorylated phosphoinositide species (Traynor-Kaplan *et al*, 1988; Kazlauskas & Cooper, 1989; Escobedo *et al*, 1991). PI3K has been proposed to mediate a number of intracellular events involved in mitogenesis such as activation of PKC (Nakanishi *et al*, 1993; Liu, 1996), stimulation of a ribosomal kinase (Lane *et al*, 1993) and activation of the MAP kinase pathway (Rodriguez-Viciana *et al*, 1994).

1.14 Protein kinase C

1.14.1 Protein kinase C structure

The effects of phospholipid-derived DAG are mediated through the activation of PKC. However, PKC exists as a growing family of serine/threonine protein kinase isoforms that are differentially expressed in various cells and subcellular localities and can be subdivided into three groups based on their structural and biochemical properties. Members of the PKC family so far examined are dependent on phosphatidylserine, but show clearly different requirements of Ca^{2+} and phospholipid metabolites for their activation. The classical or conventional PKC isoforms (cPKC)

include, β1, β11 and γ are regulated by Ca^{2+} and DAG. The novel or non-classical isoforms (nPKC) δ, ε, η and θ are insensitive to Ca^{2+} as they lack the Ca^{2+} -binding C2 domain although they respond to DAG. The atypical PKC subgroup (aPKC) λ and ζ, isoforms are insensitive to Ca^{2+} and DAG or phorbol ester (Reviewed by Musashi *et al,* 2000).

The specific regulatory requirements of the different members of the PKC family produce distinct PKC activation patterns with respect to the subspecies of PKC that becomes activated, the extent and duration of the response and perhaps their intracellular localisation. In particular, a number of reports have demonstrated differential functions of DAG derived from PIP_2 and PC in the activation of PKC. In IIC9 fibroblasts, translocation of the Ca^{2+} -dependent PKCα isoform from the cytosol to the plasma membrane by thrombin, regarded as a parameter of enzyme activation, parallels the initial peak in DAG formed from phosphoinositide, but not from PC hydrolysis (Leach *et al*, 1991; Ha and Exton, 1993). Conversely, translocation of the Ca^{2+} -independent isoform PKCε, also occured rapidly but was maintained during the persistant phase of DAG production derived from PC (Ha and Exton, 1993). The presence of Ca^{2+} accompanying the initial DAG peak, rather than different molecular species of DAG derived from PIP_2 and PC, varying in their fatty acid composition, accounted for the differential activation of the PKC isoforms (Ha and Exton, 1993).

1.14.2 Role of PKC in mitogenic signal transduction.

PKC has been implicated as a major component of cellular signalling networks regulating a variety of cell functions including cell growth and differentiation (reviewed by Nishizuka, 1992; Dempsey *et al,* 1997). The role of PKC in mitogenesis has received much attention ever since the discovery that the enzyme was the major

receptor for tumour promoters of the phorbol ester family, cell permeable analogues of DAG. Exogenous application of phorbol esters has been reported to mimic the effect of growth factors, and down regulation of PKC by prolonged PMA pre-treatment has been shown to inhibit the mitogenic effects of a number of agonists in different cell types. While the tissue-specific distribution and distinct modes of activation suggests functional diversity of the PKC family members, information on their specific biological roles including the control of proliferation, however, is still limited as few of the physiological substrates of PKC have been identified, and of those that have, no clear difference in substrate specificity among the members has been demonstrated. Furthermore, phorbol esters are not selective in the PKC species that they activate or downregulate, potentially exerting pleiotropic effects. The lack of specific inhibitors has prevented the dissection of responses attributable to particular isoforms. Despite this, studies in which distinct PKC isoenzymes have been overexpressed have revealed that PKC β1 and ε exhibit transforming characteristics (Housey *et al*, 1988). Other studies using similar approaches complimented by data with a selective pseudosubstrate peptide inhibitor indicated that the atypical PKC isoform ζ is involved in mitogenic signalling (Berra *et al*, 1993). The precise role of PKC in regulating cellular proliferation has not been clarified. However, PKC can either directly or indirectly control the expression of certain genes by inducing transcriptional activation through TRE, serum-response element (SRE) (Hata *et al*, 1993) and NFκB (Hirano *et al*, 1995) –responsive promoters in an isoenzyme specific manner. These effects may be mediated by the integration of PKC in growth factor-initiated phosphorylation cascades, by phosphorylating and activating kinases that are intrinsic components of such pathways, including c-Raf-1 of the MAP kinase cascade

(Chapter 1.16.3; Sozeri *et al*, 1992; Kolch *et al*, 1993) and elements upstream of the $p70^{s6k}$ pathway (Susa *et al*, 1992).

1.15 Receptors with intrinsic tyrosine kinase activity

The role of tyrosine kinase-linked signal transduction pathways has been examined largely in the context of long-term responses such as cell proliferation. RTKs form a large and important class of cell surface receptors. Several sub-classes of RTK exist, but all share a similar molecular structure consisting of a large extracellular ligand-binding region, a single hydrophobic transmembrane segment, a cytoplasmic portion containing the tyrosine kinase catalytic domain and lastly, a carboxy-terminal regulatory region (see Cardenn & Gill, 1992; Panettieri, 1996). Activation of the RTK is necessary for transduction of the growth factor-mediated response. Once a receptor is phosphorylated, its kinase site opens to provide access for the phosphorylation by other proteins (Cardenn & Gill, 1992). The tyrosine phosphorylated receptors and other tyrosine-phosphorylated proteins then associate with a number of target proteins that transfer to the cell interior the signals received by the receptors. These proteins that associate with some or all activated RTKs include:

1 RasGAP, a GTPase-activating protein which modulates the activity of the Ras proteins (a trio of small GTP-dependent switch proteins encoded by c-*ras* proto-oncogenes).

2 Raf-1, a serine-threonine protein kinase encoded by the c-*raf* proto-oncogene.

3 The cytoplasmic protein tyrosine kinase Src which is encoded by the c-src proto-oncogene.

4 The phospholipase Cγ (PLCγ) and PI3K, which hydrolyse and phosphorylate inositol-containing phospholipids.

5 Shc protein, the product of the *shc* gene (Pazin & Williams, 1992).

Of these, RasGAP, c-src, PLCγ, the M_r regulatory subunit of PI3K, and Shc all contain one or more copies of a shared polypeptide sequence motif, known as the Src homology domain type 2 (SH2 domain).

Substrates with SH2 domains are responsible for coupling activated growth factor receptors to intracellular pathways involved in the control of a variety of cellular functions such as proliferation and gene expression. Proteins with one or more SH2 domains include PLC, PI3K (PI 3-kinase), Ras and other cytoplasmic kinases (Carpenter, 1992). Many of the proteins that have SH2 domains also contain a distinct sequence of approximately 50 amino acid residues termed the SH3 domain. SH3 domains modulate protein-protein interactions through the recognition of short peptide sequences that do not require phosphorylation. Signalling complexes based on the formation of SH2/SH3 interactions are then followed by activation of downstream effector proteins which involve, for example, non-receptor tyrosine kinases and MAP kinases (Satoh & Kaziro, 1992; L'Alleman *et al*, 1991; Meloche *et al*, 1992) (Figure 1.3).

Figure 1.3

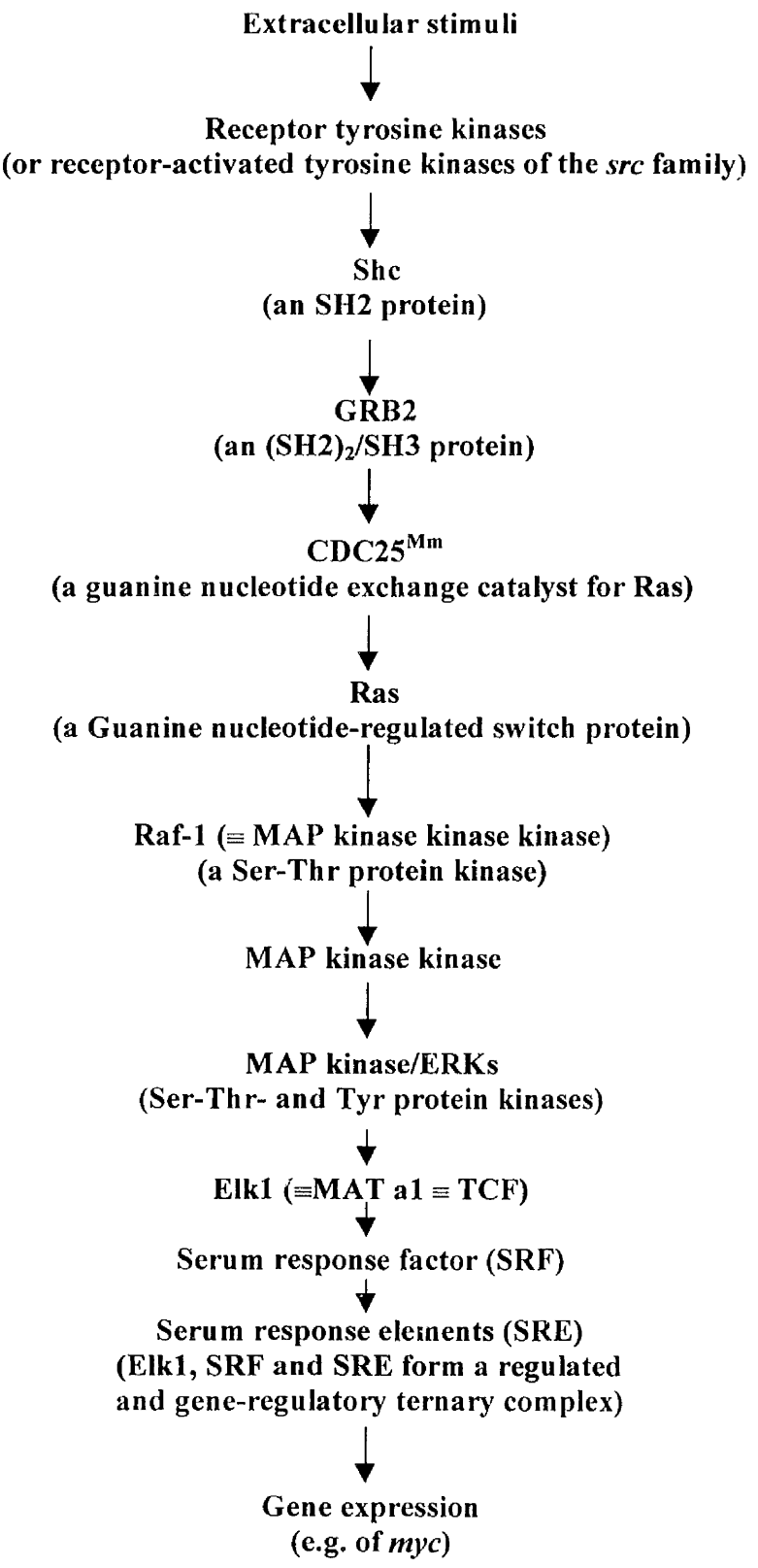


Figure 1.3 Tyrosine kinase cell signalling overview

1.16 The Mitogen Activated Protein Kinase Pathway

Activation of the MAP kinase pathway, also known as extracellular regulated kinase (ERK), is believed to play an important role in cell growth and proliferation (Milanini *et al,* 1998). MAP kinase is initially located within the cytoplasm (Northwood *et al*, 1991), however following activation, MAP kinase translocates to the nucleus (Chen *et al*, 1992; Sanghera *et al*, 1992) and initiates the activation of transcription factors such as Elk-1. Thus, MAP kinase provides a physical link in the signal transduction pathway from the cytoplasm to the nucleus.

Two forms of MAP kinase has been purified from fibroblasts with molecular weights of 42 and 44 kDa and referred to as ERK1 and ERK2, respectively. Expression of both the 42 and 44-kDa isoforms appears to be essential for agonist-induced mitogenesis in various cell types (Pages *et al*, 1993; Cowley *et al*, 1994; Mansour *et al*, 1994). The activation of MAP kinase requires phosphorylation of both tyrosine and threonine residues (Anderson *et al*, 1990) by dual specificity tyrosine/threonine kinase MAP kinase kinases, MEK1 and MEK2 (MacDonald *et al*, 1993; Yan & Tempelton, 1994). Raf-1 is currently the only activator of MEK, although there is evidence for the existence of other MEK kinases (Lange-Carter, 1993). Raf is in turn activated by the membrane-bound small guanine-nucleotide binding protein Ras (McCormick, 1993) which is linked to RTKs by intermediate guanine nucleotide exchange factors (GEFs) such as SOS and Grb (Bonfini *et al*, 1992; Bowtell *et al*, 1992; Egan *et al*, 1993) (Figure 1.4). The SH3 domain of Grb binds to SOS, which is a GEF for Ras and facilitates the replacement of GDP to GTP (Rebello and Martinez, 1999). When Ras becomes GTP-loaded, Raf binds to Ras and becomes activated.

Figure 1.4

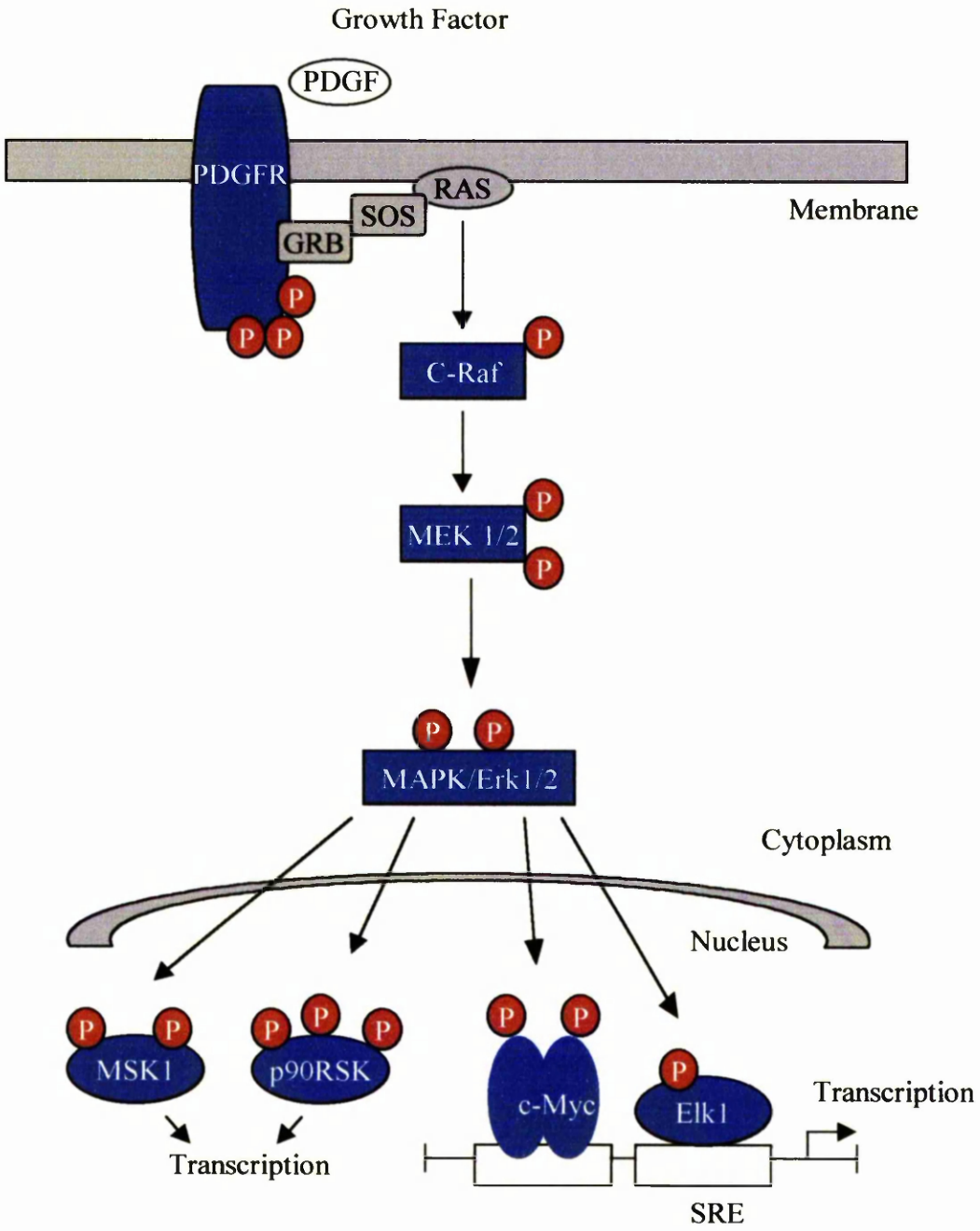


Figure 1.4 Overview of p42/p44 MAP kinase cell signalling

The MAP kinase pathway has been associated with growth factor receptors which display intrinsic tyrosine kinase activity (Fantl *et al*, 1993; Egan *et al*, 1993) however, it is now clear that signals from GPCRs can also impact on the Ras/MAP kinase signal transduction cascade (DeVivo & Iyengar, 1994).

The intracellular pathways that link GPCRs to MAP kinase activation are poorly understood, but for many agonists, stimulation of PKC appears to be a critical step (Granot *et al*, 1993; Bogoyevitch *et al*, 1994). Evidence suggests that the βγ subunit from individual G proteins may be able to stimulate Ras (Faure *et al*, 1994; Crespo *et al*, 1994), thus leading to MAP kinase activation.

In pulmonary artery fibroblasts, MAP kinase is activated by a number of mitogenic factors including PDGF, ET-1, thrombin and 5-HT (Trejo *et al,* 1996; Lee *et al,* 1999; Kivinen and Laiho, 1999). Sustained activation of MAP kinase is associated with mitogenesis, whereas agonists, which stimulate only a transient activity of MAP kinase, fail to induce a proliferative response (Malarkey *et al*, 1995a; Kelleher *et al*, 1995). The identification of additional MAP kinase members (including JNK and p38 MAP kinase homologues as described in Chapter 1.17) implies this family of kinases is extensive and some cross-talk between the family does exist (Figure 1.5).

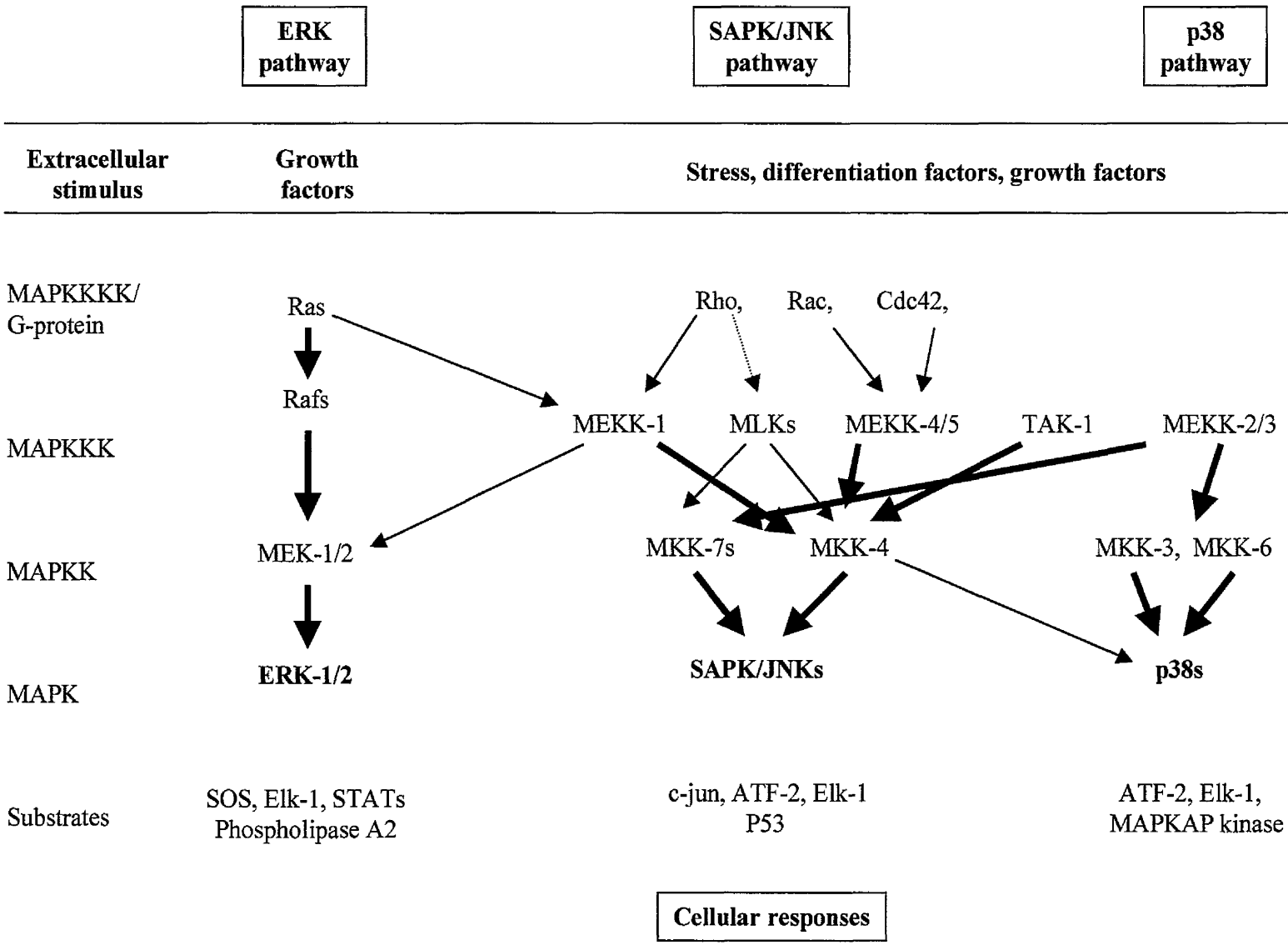
1.16.1 Regulation of p42/44 MAP kinase by MEK

MAP kinase becomes activated by a single protein kinase which is unusual in exhibiting dual specificity and capable of phosphorylating both the regulatory threonine (Thr183) and tyrosine (Tyr185) residues and is termed MAP kinase kinase or MEK. In addition to MEK1, a second cDNA encoding closely related MAP kinase activator has been identified in humans and designated MAK2 (Zheng & Guan,

Figure 1.5 Overview of the ERK pathway, SAPK/JNK pathway, and the p38 pathway

The MAP kinase cascades consist of a MAPKKK, a MAPKK and a MAPK. MAPKKKs are activated through a large variety of extracellular signals such as growth factors, differentiation factors and stress. The activated MAPKKK can phosphorylate and activate 1 or several MAPKKs, which, in turn, phosphorylate and activate a specific MAPK. Activated MAPK phosphorylates and activates various substrates in the cytoplasm and the nucleus of the cell, including transcription factors. These downstream targets control cellular responses (eg, apoptosis, proliferation, and differentiation). Thick arrows connect the signalling proteins with their preferred substrates (effectors). Note the complexity and the potential for cross-talk between the pathways.

Figure 1.5


ERK pathway
SAPK/JNK pathway
p38 pathway
Extracellular stimulus
Growth factors
Stress, differentiation factors, growth factors
MAPKKKK/ G-protein
MAPKKK
MAPKK
MAPK
Substrates
Ras
Rafs
MEK-1/2
ERK-1/2
Rho,
Rac,
Cdc42,
MEKK-1
MLKs
MEKK-4/5
TAK-1
MEKK-2/3
MKK-7s
MKK-4
MKK-3, MKK-6
SAPK/JNKs
p38s
SOS, Elk-1, STATs
Phospholipase A2
c-jun, ATF-2, Elk-1
P53
ATF-2, Elk-1,
MAPKAP kinase
Cellular responses

1993). Overexpression of MEK in cultured cells increases MAP kinase activity (Seger *et al*, 1992)

1.16.2 Regulation of MEK by Raf-1

A 74 kDa serine/threonine protein kinase known to be involved in mitogenic signalling is c-Raf-1 (Morrison *et al*, 1988). A vast amount of evidence has shown that c-Raf-1 is at least one of the intermediates directly upstream of MEK in a linear cascade leading to activation of MAP kinase (Dent *et al*, 1992; Howe *et al*, 1992; Kyriakis *et al*, 1993). Cells transformed with c-Raf, or overexpression of oncogenic mutants of c-Raf-1, display elevated MEK and MAP kinase activities (Dent *et al*, 1992). Furthermore, c-Raf-1 immunoprecipitated from mitogen-stimulated cells can directly phosphorylate the active MEK *in vitro* (Dent *et al*, 1992; Howe *et al*, 1992; Kyriakis *et al*, 1993). Other studies have demonstrated that Raf-1 forms a stable complex with MEK1 *in vivo* and *in vitro* (Huang *et al*, 1993) and phosphorylates MEK1 exclusively on serine residues (Yan and Tempelton, 1994, Zheng and Guan, 1994).

1.16.3 Regulation of MEK independent of Raf-1

It has been demonstrated that other serine/threonine kinases can serve as direct regulators of MEK, which include other members of the Raf family of proto-oncogene products. B-Raf has been identified as an activator of MEK in NIH3T3 fibroblasts (Catling *et al*, 1994) and A-Raf in Hela cells has been shown to activate MEK1 but not MEK2 (Wu *et al*, 1996).

Other kinases that activate MEK include MEK1 kinase (MEKK1). Initially it was thought that MEKK may be responsible for MEK activation by heterotrimeric GPCR

agonists, however, recent evidence suggests that this kinase is more likely to act upstream of another MEK family member responsible for activating the MAP kinase homologue c-jun N-terminal kinase in the parallel stress-activated protein kinase pathway (Chapter 1.17) (Minden *et al*, 1994 and Yan *et al*, 1994).

1.16.4 Raf-1 activation by Ras

The proto-oncogene gene product Ras plays a central role in signal transduction pathways involved in cellular processes including cellular proliferation (Mulcahy *et al*, 1985). Ras becomes active then GTP binding takes the place of the inactive state when GDP is bound. This can be stimulated by the addition of growth factors. The mechanism by which Ras aids in the activation of Raf-1 is by causing c-Raf-1 translocation to the plasma membrane where additional protein-protein or protein-lipid interactions regulate Raf-1 activity (Chapter 1.16 and Reuter *et al*, 2000).

1.16.5 Ras-independent activation of MAP kinase

It has been well documented in the literature that phorbol esters stimulate MAP kinase activation. Furthermore, depletion of PKC by prolonged pre-treatment with phorbol esters has been shown to block the activation of MAP kinases following stimulation with agonists (de Vries-Smits *et al*, 1992). This indicates the existence of at least two distinct pathways regulating MAP kinase that can operate in one cell type and also implicates PKC in this role. PKC is thought to activate the MAP kinase pathway at the level of Raf-1 since chronic phorbol ester pre-treatment prevents agonist-stimulated c-Raf-1 activation (Kolch *et al*, 1993).

1.16.6 Cellular targets of p42 and p44 MAP kinase

Downstream of MAP kinase, several proteins have been identified as substrates. These include lipases, transcription factors and other protein kinases (reviewed by Reuter *et al,* 2000). MAP kinase can activate the dephosphorylated 90 kDa ribosomal protein S6 kinase II ($p90^{rsk}$) (Sturgill *et al*, 1988). It can also directly regulate gene expression by phosphorylating the ternary complex transcription factor Elk-1/$p62^{TCF}$ (Gille *et al*, 1992) which in turn facilitates the transcription of *c-fos*; one of the immediate early genes induced by many stimuli. MAP kinase also phosphorylates and activates the cytosolic PLA_2 (PLA_2) *in vitro* and in intact cells (Lin *et al*, 1993). A role for PLA_2 as a substrate of MAP kinase *in vivo* is supported by the finding that expression of a recombinant $cPLA_2$ in which the MAP kinase phosphorylation site Ser505 is mutated, does not become activated on agonist-stimulation (Lin *et al*, 1993).

1.17 Stress-Activated Protein Kinases

The last few years have marked the arrival of novel members of what now appears to be a MAP kinase superfamily. Two protein kinases that are highly related to the classical p42/p44 MAP kinases have been identified, and characterised as integral components of parallel protein kinase cascades activated by proinflammatory cytokines and cellular stresses. It has now become apparent that these MAP kinase homologues may play a role in the cellular effects of an increasingly large number of extracellular stimuli including GPCR agonists (Coso *et al*, 1995)

As their name suggests, stress-activated protein kinases (SAPK) are activated by agents which induce cellular stress, such as hypoxia, UV light and the cytokines, tumour necrosis factor α and interleukin-1 (Scott *et al,* 1997; Kolesnick & Goldie, 1994). An investigation of kinases responsible for phosphorylating the transcription

factor c-Jun on Ser^{63} and Ser^{73} within its amino terminal activation domain in cells exposed to UV irradiation identified that this activity was attributable to two protein kinases of 46 and 55 kDa named c-Jun N-terminal kinases 1 and 2 (JNK1 and JNK2), respectively (Hibi *et al*, 1993). Subsequent cloning of JNK1 revealed that this protein kinase shared sequence homology to the p42/p44 MAP kinases (Figure 1.6; Derijard *et al*, 1994).

In addition to JNKs, a stress-activated MAP kinase homologue has also been identified, termed p38, that led to the activation of MAPKAP kinase-2, a serine kinase responsible for phosphorylating one of a family of small heat shock proteins (Hsp 25) in response to chemical stress or heat shock (Figure 1.7; Rouse *et al*, 1994). These are thought to exist in signal transduction cascades distinct from MAP kinase. There is evidence, however, for the existence of cross-talk between the two pathways since RTK-linked growth factors (Minden *et al*, 1994) and GPCR agonists (Dalton & Treisman, 1992) can also activate the SAPK. In recent studies SAPKs were shown to be upregulated in cells from the pulmonary vasculature exposed to hypoxia (Welsh *et al* 1998; Stenmark *et al* 1999).

Figure 1.6

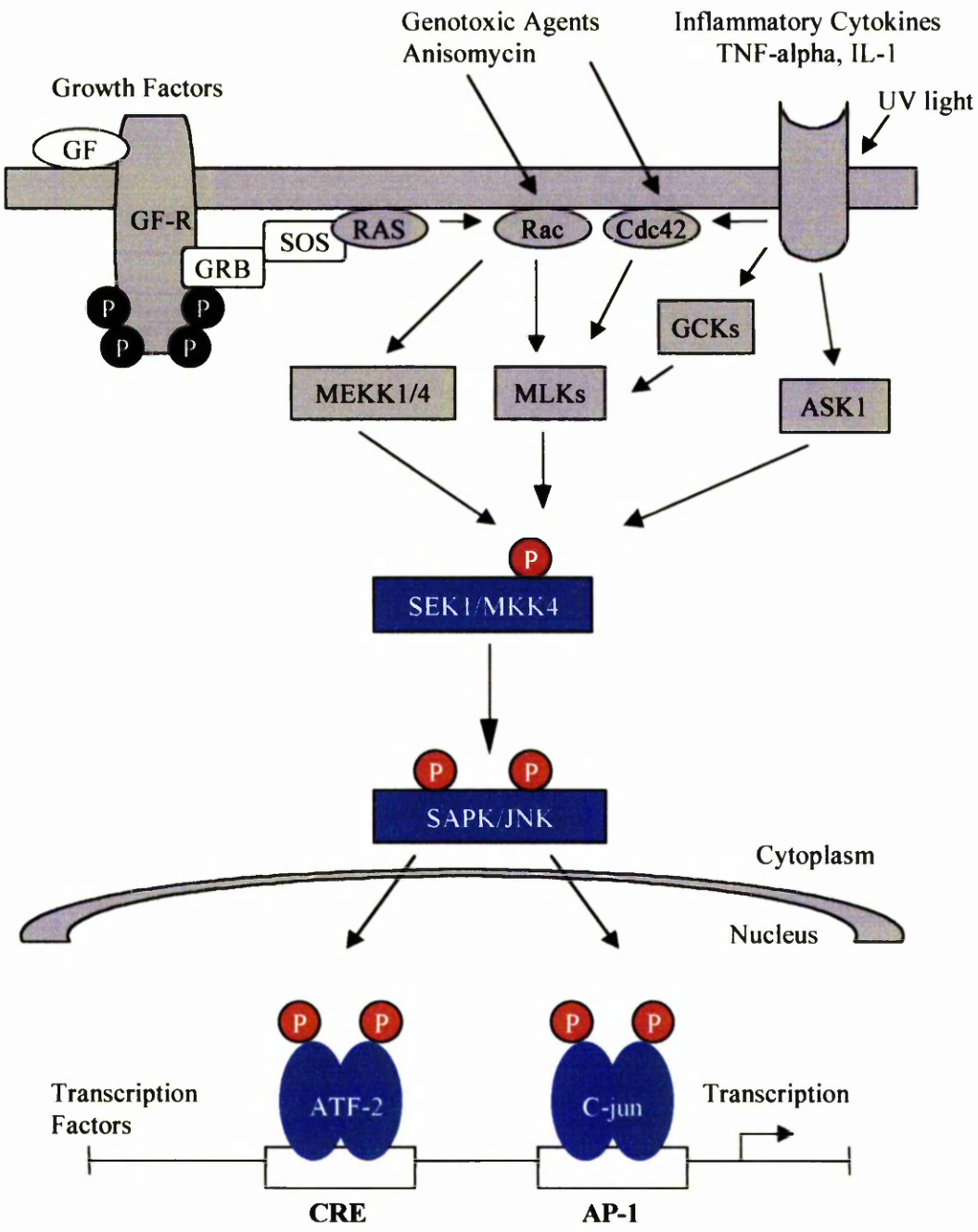


Figure 1.6 Overview of JNK MAP Kinase cell signalling

Figure 1.7

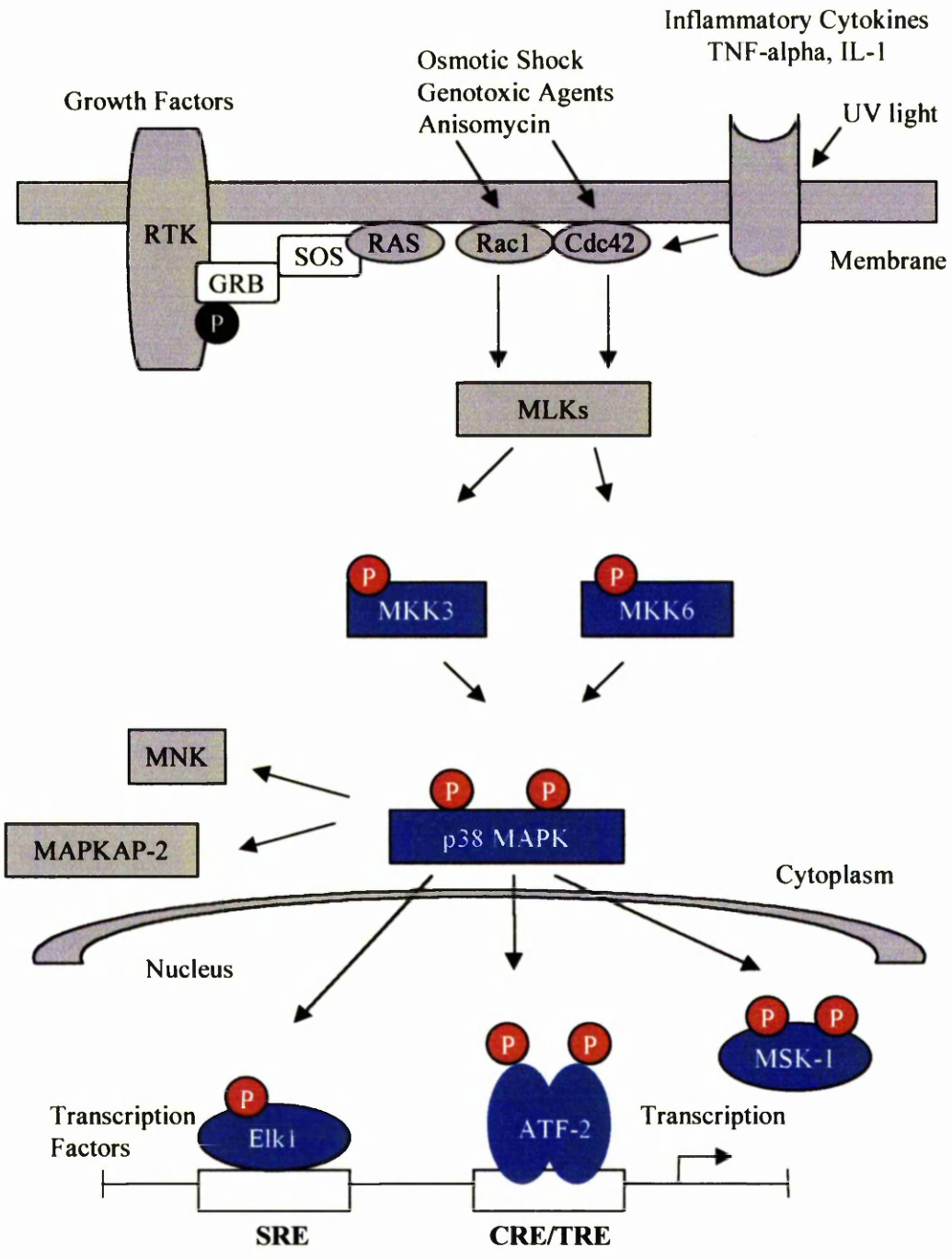


Figure 1.7 Overview of p38 MAP kinase cell signalling

1.17.1 MKK homologues involved in JNK and p38 activation.

The pathways leading to the activation of the SAPKs are not as well understood as those of the classical p42/p44 MAP kinases. However, like the p42/p44 pathway, the SAPK pathways consists of several protein kinases acting in sequence: a MAP kinase homologue (or SAPK/JNK/p38), MAP kinase kinase homologue (or MKK/MEK/SEK/JNKK) and a MAP kinase kinase kinase (or MEKK). Again, like MAP kinase, the activation of the SAPKs relies on their phosphorylation at specific dual phosphorylation motifs, although in this case the sequences are defined by TPY for JNK (Derijard *et al*, 1994) and TGY for p38 MAP kinase (Rouse *et al*, 1994). These residues are specifically phosphorylated by MKK/MEK homologues (also termed SAPK kinases (SAPKK)) distinct from MKK/MEKs 1 and 2 that are responsible for the activation of the classical p42/p44 MAP kinase isoforms. Another MAP kinase kinase identified in humans and designated MKK3, has been shown to selectively activate p38 MAP kinase (Derijard *et al*, 1995).

1.17.2 Activation of MKK homologues by MEKKs

Activation of the relevant MKK/MEK/SAPKK activity requires its phosphorylation by an upstream kinase analogous to the Raf kinase isoforms that function upstream in the classical p42/p44 MAP kinase pathway. The novel serine/threonine kinase MEKK1, which was originally identified as a kinase able to phosphorylate MEK-1 *in vitro* (Lange-Carter *et al*, 1993), has been subsequently shown to be a more efficient activator of MEKK/MEK4/SEK-1/JNKK in *in vitro* and co-infection studies (Yan *et al*, 1994). In this role, MEKK1, but not Raf-1 was also found to participate in both growth factor and TNF-stimulated activation of JNK.

The identity of the equivalent MEKK-like protein upstream of MKK3/6 in the p38 pathway is less well understood. However, TAK-1 which has been isolated from TGF-β-activated kinase and which has been related to MEKK (Yamaguchi *et al*, 1995) has been shown to directly activate both MKK6 and MKK3 *in vitro*. Thus, activation of p38 MAP kinase may occur via a kinase pathway involving TAK-1, MEKK 3/6 and p38 MAP kinase.

1.17.3 Possible role of p38 and JNK activation in cell growth

There have been a number of papers which link p38 MAP kinase and JNK to apoptosis and cell cycle arrest (Verheij *et al*, 1996; Xia *et al*, 1995). However evidence suggests that SAPKs may have a positive role to play in cell growth (Auer *et al*, 1998). Clerk and colleagues (1998) found that ET-1 and phenylephrine, both mitogens, stimulated p38 MAP kinase phosphorylation 12-fold. It has also been shown that thrombin stimulation of JNK in airway smooth muscle cells correlates with DNA synthesis (Shapiro *et al*, 1996). It is also true that these SAPK have the ability to activate transcription factors, which regulate genes that are induced on mitogenic stimulation.

1.18 Hypothesis

Characterisation of the cellular signalling events that occur during cell proliferation by hypoxia is an important issue from both biological and clinical perspectives. From a biological view, this will further our understanding of the processes by which hypoxia mediates cellular effects during proliferation. From a clinical view, this will provide a model for the pathways that operate in response to acute and chronic hypoxia, such as during the pathogenesis of pulmonary hypertension characterised by

blood vessel cell wall remodelling. In addition, intracellular targets may be identified for the development of novel pharmaceuticals to alleviate such disease.

The review of the literature indicates that while hypoxia causes proliferation of a number of cell types, the mechanisms by which this is achieved is unknown. It is possible that clues to finding more about these mechanisms may lie in the differences seen between pulmonary arteries, which constrict to hypoxia, and systemic arteries which dilate in response to hypoxia *in vivo*. It is not known whether isolated pulmonary artery fibroblast cells would proliferate *in vitro* to cellular stresses such as hypoxia and if so whether these cells may differ with regards to the potency of agonists, activity of intracellular pathways as well as growth characteristics to hypoxia. It is also unknown whether hypoxia would have a differential effect on fibroblast cells isolated from the systemic circulations. Furthermore, there is very little information on the involvement of the MAP kinase signalling pathway or the SAPKs in hypoxia-stimulated mitogenesis of pulmonary and systemic artery fibroblasts.

Currently defined signalling phenomena associated with stimulation of cell growth have been exposed by studies, which have routinely used immortalised cell lines and therefore their growth regulatory mechanisms have been fundamentally altered. This may be problematic particularly when examining pathways involved in proliferation. Caution must therefore be taken in predicting how cells will behave physiologically by extrapolating from signalling events characterised in these cells. The investigations in this thesis have been carried out in fibroblasts obtained from primary cultures. We believe that these represent a better model of mammalian cells under physiological or pathophysiological conditions.

We have hypothesised that hypoxia may cause increased proliferation of pulmonary artery fibroblast cells by an intracellular mechanism(s) present in the pulmonary vasculature which is not present in systemic fibroblasts.

1.19 Aims

The aims of this thesis were as follows:

1 To establish a cell model for studying proliferation of fibroblasts from the pulmonary and systemic arteries in response to acute hypoxia.

2 To study acute hypoxia in mitogenic proliferation of bovine pulmonary artery and bovine mesenteric artery fibroblasts.

3 To look at early signalling events involved in acute hypoxic proliferation of pulmonary and mesenteric artery fibroblasts.

4 To study the effects of acute hypoxia on classical and stress-activated protein kinases in bovine pulmonary artery fibroblasts.

We also aimed to compare the effects of acute hypoxia on fibroblasts with fibroblasts obtained from rats exposed to chronic hypoxia by:

5 Studying the proliferation and generation of MAP kinases in pulmonary and aortic fibroblasts harvested from chronically hypoxic rats.

6 Examining the effects of 5-HT on proliferation and generation of MAP kinases in pulmonary artery fibroblasts harvested from chronically hypoxic rats.

Chapter 2

Materials & Methods

2. MATERIALS & METHODS

2.1 Materials

All general chemicals were of Analar grade and were supplied by Merck (Thornliebank, Glasgow) or Sigma (Poole, Dorset) unless otherwise stated. All tissue culture plastics were from Greiner Labortechnick Ltd (Gloucestershire.UK). All components of tissue culture medium were purchased from Gibco Life Technologies (Paisley, Scotland). [γ-^{32}P]ATP and [^{3}H]thymidine were from Amersham (Little Chalfont, Bucks). Antibodies used for Western Blot analysis were from New England Biolabs (Hertfordshire, UK). Gel electrophoresis equipment was purchased from Bio-Rad Laboratories (Herts, UK). Incubators were supplied by LEEC (Nottingham, UK). I would like to thank Dr R. Plevin (University of Strathclyde) for the gift of GST-c-jun and GST-MAPKAP kinase 2 used as substrates for measuring activity of JNK and p38 MAP kinase.

2.2 Animal Models

2.2.1 Bovine

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 were removed from 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).

2.2.2 Rat

Cells derived from the pulmonary artery and aorta of adult Wistar rats from the "In House" breeding stock of the University of Glasgow's Central Animal Facility were also used. Thirty to thirty-three day old rats were maintained in either normoxic conditions or hypoxic conditions in a hypobaric chamber (PO2= 70mmHg) for 2 weeks (Chapter 2.5). Animals were maintained on a 12 hour (h) light/dark cycle and allowed free access to standard diet and water.

2.3 Primary Cell Culture

Fibroblasts used throughout these studies were obtained from primary culture by explant using tissues derived either from bovine or rat.

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

2.3.1 Routine Cell Maintenance

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

The cultures were kept in a humidified atmosphere of 5% CO_2 in air at 37°C. Cell lines were checked for mycoplasma contamination on a monthly basis using a commercially available mycoplasma detection kit (Gen-Probe, San Diego, USA). Culture medium was changed every 2 days and cells were passaged just prior to confluency.

Cell passage was performed by removing the culture medium and washing the cells with 5ml of filter-steriled (2μm membrane) pre-warmed phosphate buffered saline (PBS; NaCl 137mM, KCl 2.7mM, KH_2PO_4 1.5mM, $Na_2H_2PO_4$ 8.1mM, CaCl 0.9mM, MgCl 0.5mM, pH 7.4). Trypsin solution (0.05% trypsin / EDTA 0.02%) was added to the layer of cells (2ml) 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°C until they had began to detach from the surface of the flask. This was observed under a light microscope (Olympus CK2). Gentle tapping of the dish was used to dislodge the cells and 10ml of DMEM containing 10% FCS was added to the flask to re-suspend the cells. A portion of this cell suspension (3ml) was then aliquoted into new flasks containing another 7mls of fresh medium. At this stage cells could be plated out onto dishes as required for experimentation. Cells were used for experimentation between passage 3-10.

Figure 2.1

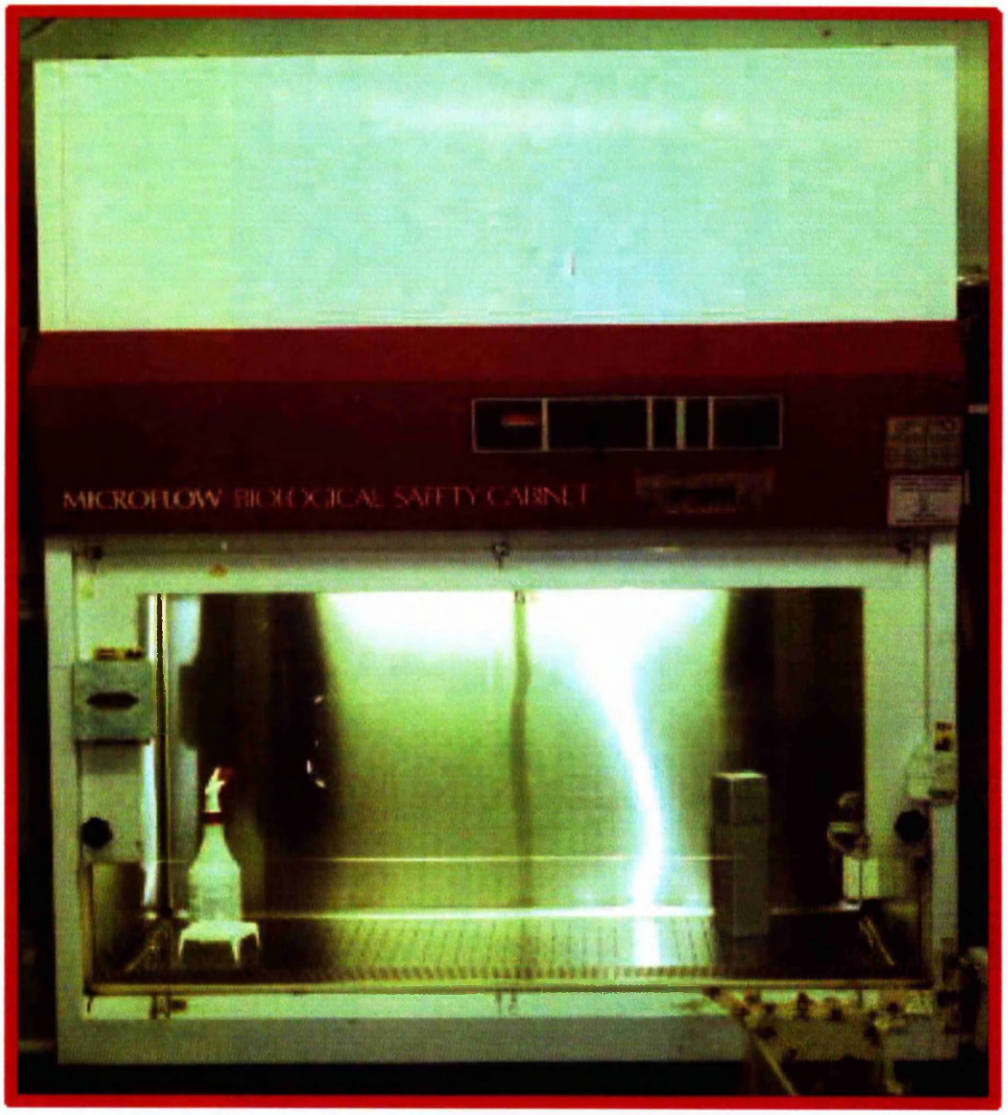


Figure 2.1 Microflow Biological Laminar Safety Cabinet.

2.3.2 Primary 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 (diameter of 5mm and 15cm in length) located towards the apex (Figure 2.3) of the lobe was cut longitudinally and opened into a flat sheet (Figure 2.4 and 2.5). Pulmonary artery fibroblasts were prepared using the technique of Freshney (1983), with some modifications. Muscular tissue and endothelial cell layers were removed by gentle abrasion of the vessel using a sterile razor blade (Figure 2.6). The remaining tissue (adventitia) was then dissected into $5mm^2$ portions (Figure 2.7). Approximately 25 portions of tissue were evenly distributed over the base of a $25cm^2$ culture flask containing 2ml of DMEM with 20% FCS, supplemented with penicillin/streptomycin (400iu/ml and 400μg/ml) and amphotericin B (5μg/ml) (Figure 2.8). The explants were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. Within a few days cells were observed growing out from the tissue fragments (Figure 2.9). Once a monolayer of cells had partially covered the flask, cells were lifted from the flask by trypsinisation as described in Chapter 2.3.1. Tissue fragments were removed by aspiration.

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. The method used to culture primary cells was the same as described above.

Figure 2.2

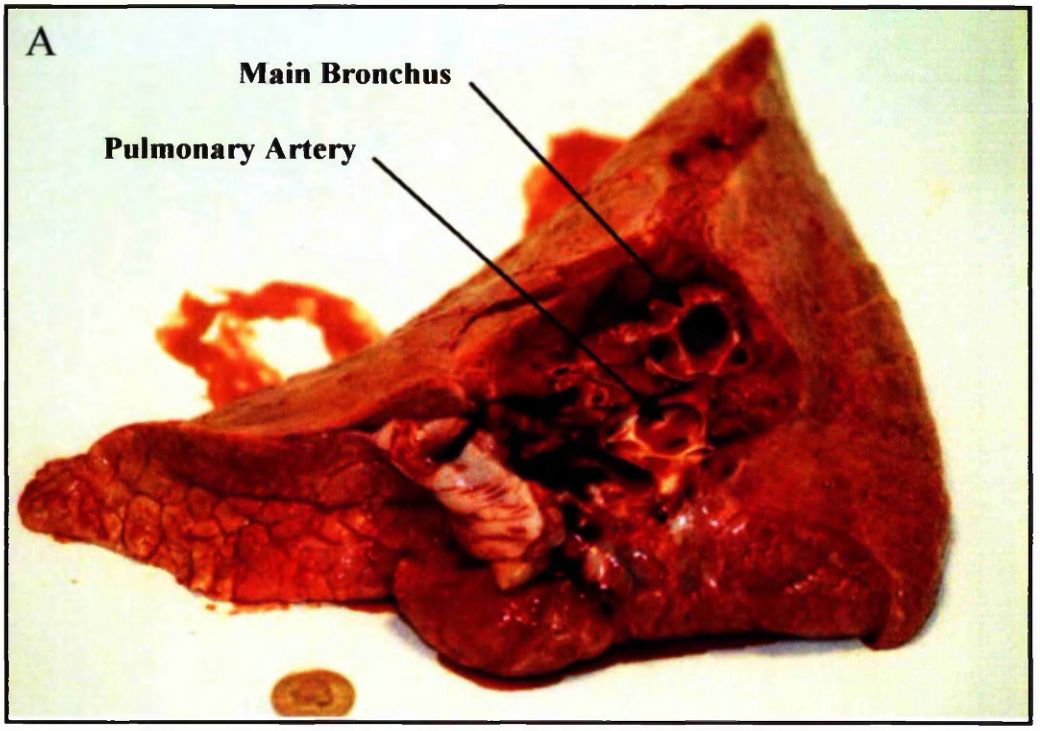


Figure 2.2 Location of Pulmonary Artery from Freshly Excised Bovine Lung

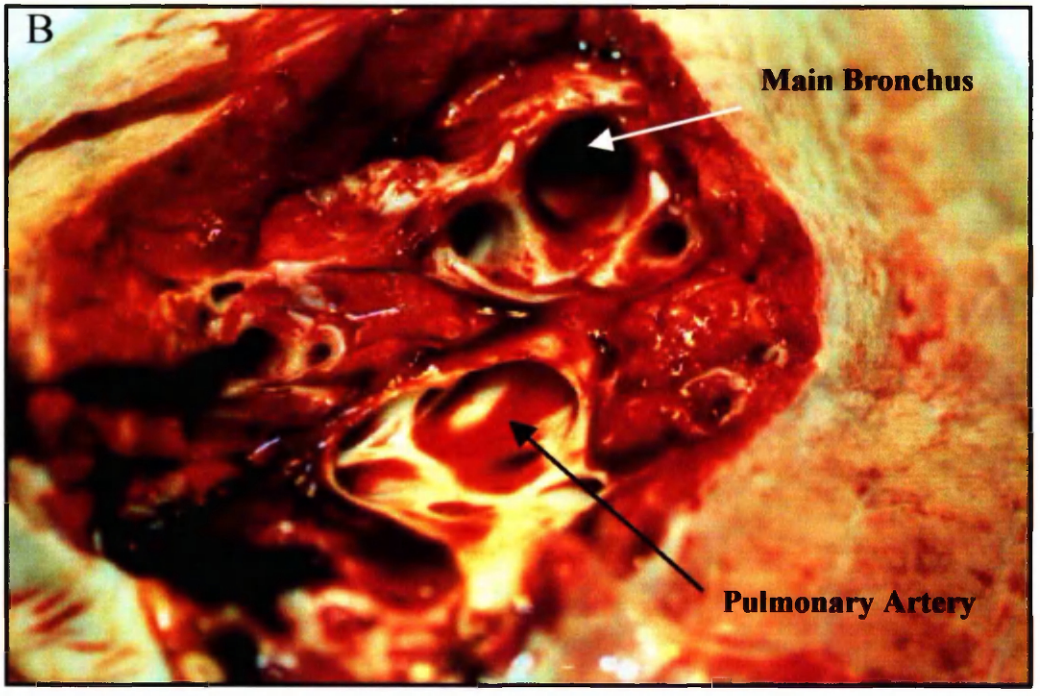


Figure 2.3

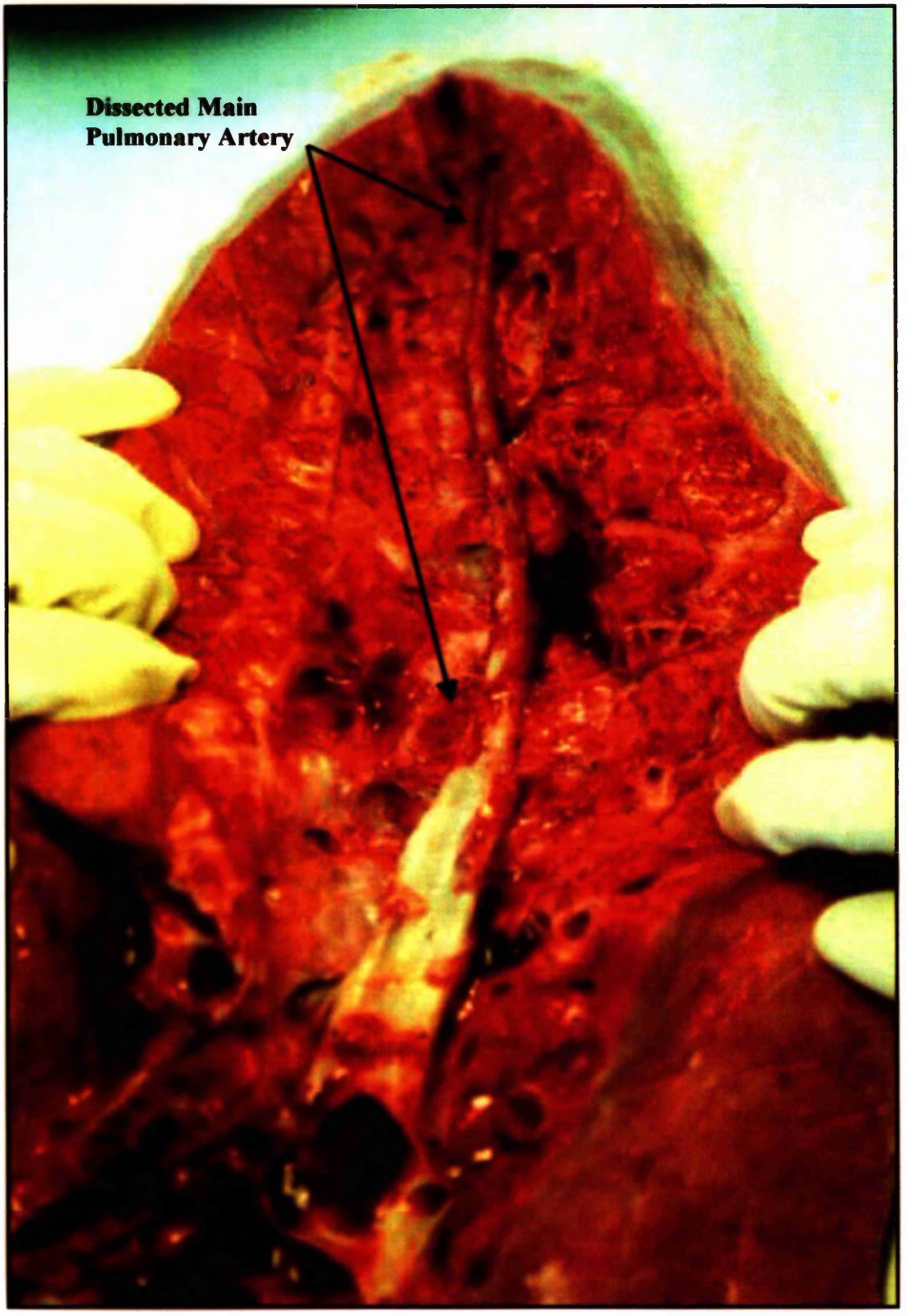


Figure 2.3 Pulmonary Artery from Freshly Excised Lung

Figure 2.4

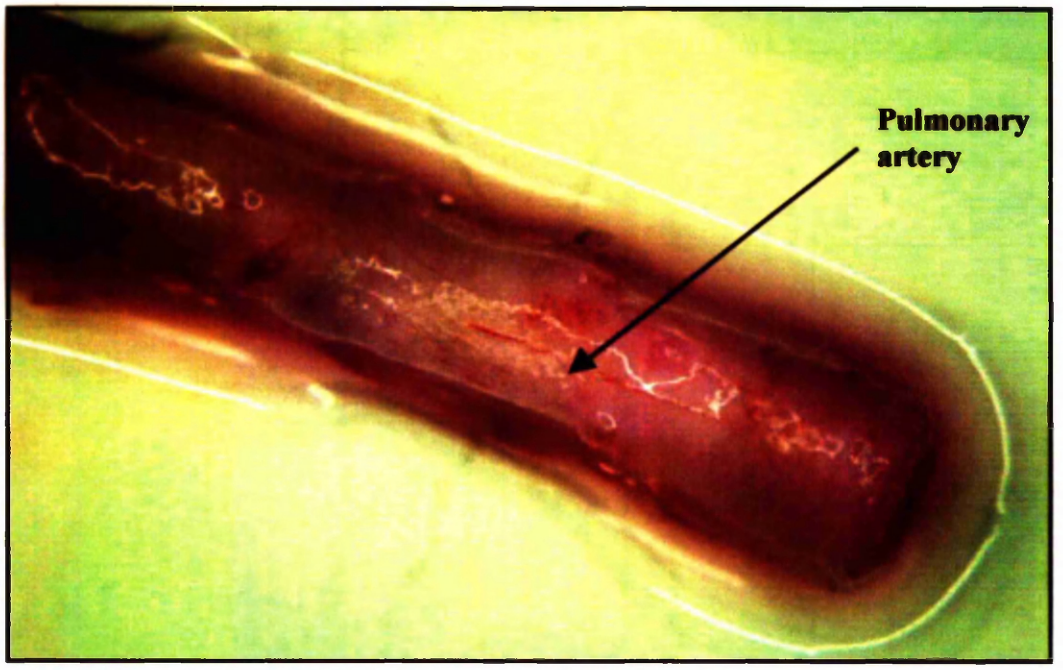


Figure 2.4 Pulmonary Artery Prior to Cleaning

Figure 2.5

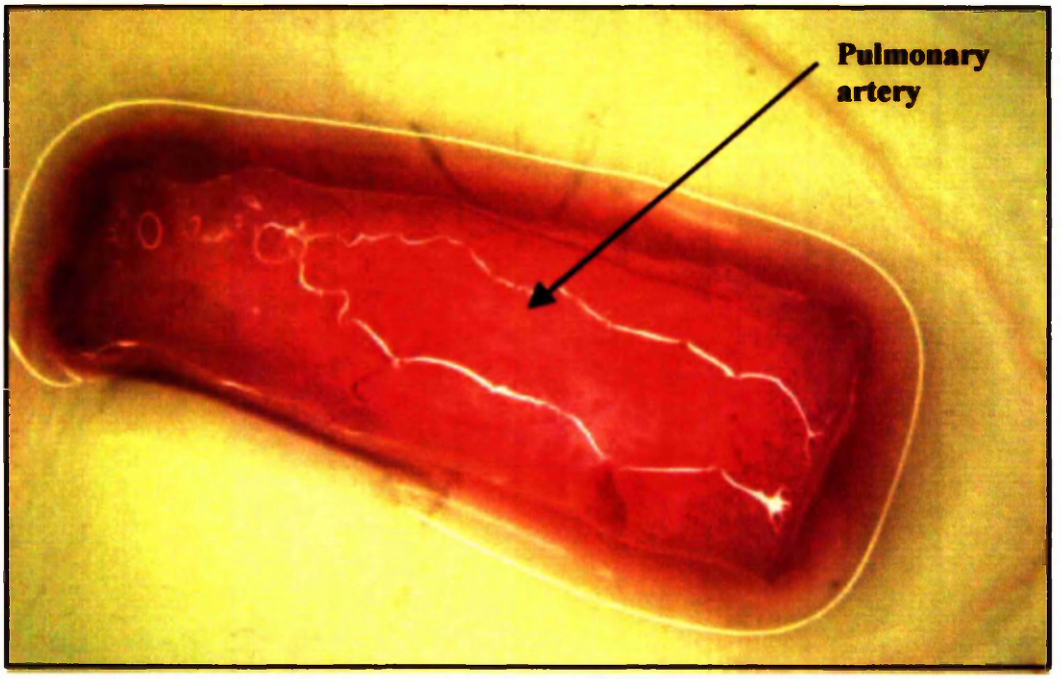


Figure 2.5 Pulmonary Artery after cleaning

Figure 2.6

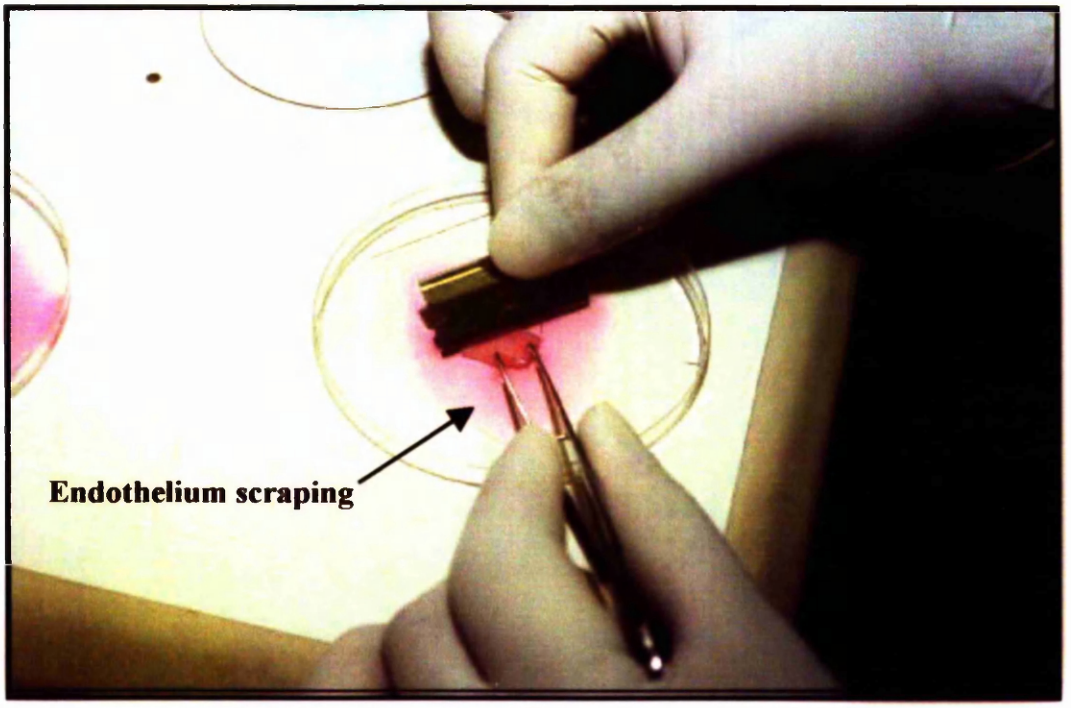


Figure 2.6 Removal of Endothelial layer

Figure 2.7

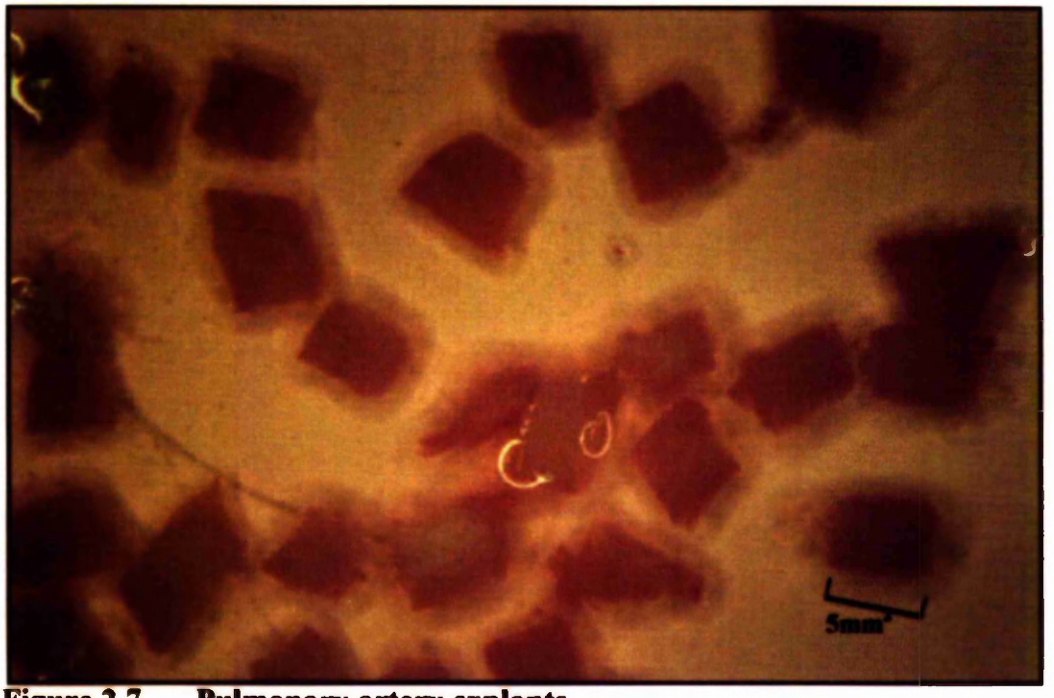


Figure 2.7 Pulmonary artery explants

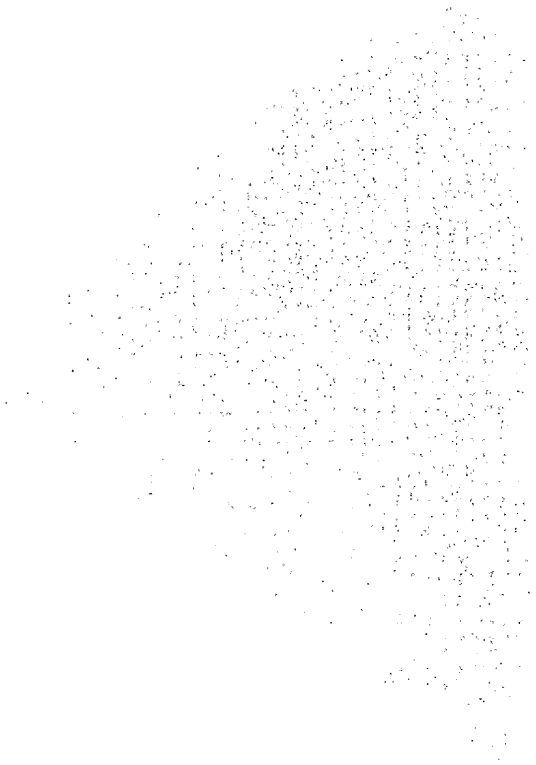


Figure 2.8

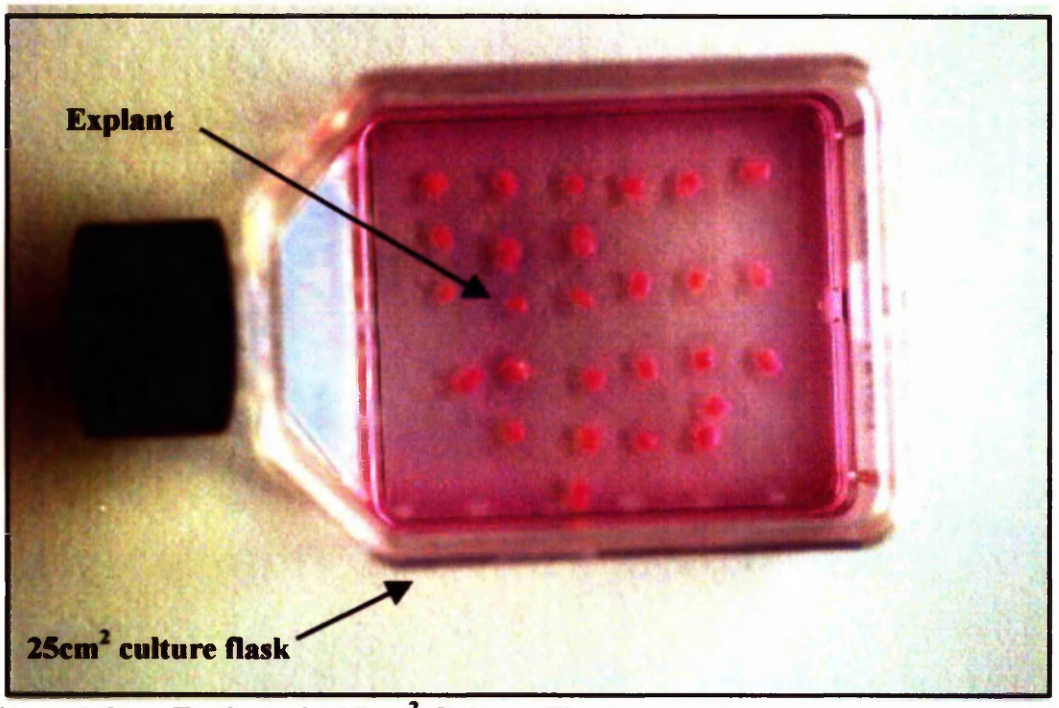


Figure 2.8 Explants in 25cm^2 Culture Flasks

Figure 2.9

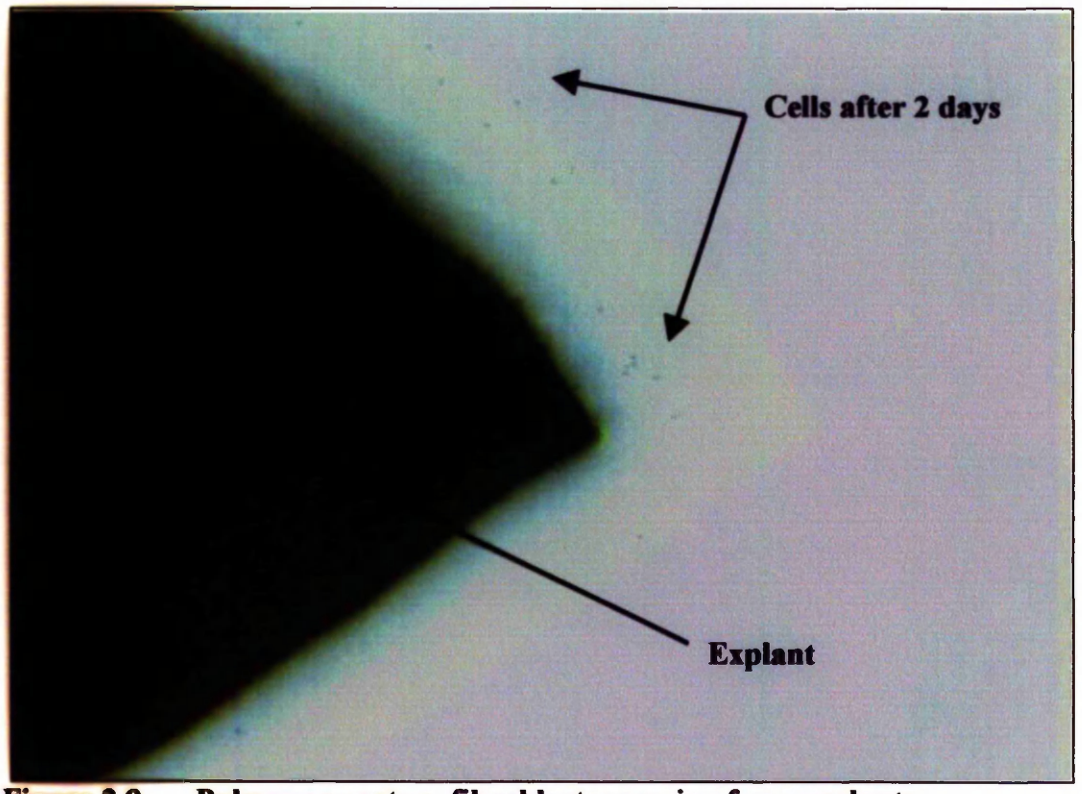


Figure 2.9 Pulmonary artery fibroblasts growing from explants

2.3.3 Immunohistochemical Characterisation of Primary Fibroblast Cultures

All primary cell cultures were established as having a fibroblast-like origin by determining whether they expressed smooth muscle α-actin. Primary fibroblast cultures were allowed to grow to confluence on sterile acid-etched coverslips grown in 35mm^2 culture flasks. These were then fixed using 70% methanol / 10ml 30% hydrogen peroxide (Sigma, UK) for 30 mins at room temperature. The coverslips were then washed in sterile PBS. 50μl PBS containing 3% (w/v) bovine serum albumin (BSA, Sigma, UK) was placed onto each coverslip, for 30 minutes, in a humidified incubator at 37°C. Without rinsing, 10% goat serum (Sigma, UK) in PBS was added and the coverslips were then incubated for a further 30 mins. The coverslips were then rinsed once in PBS and the primary monoclonal antibody against smooth muscle α-actin (Tsukada *et al,* 1987) (Sigma, UK) was added to each coverslip (diluted in PBS at 1:1000). The coverslips were then left for 1h at 37°C without agitation.

Each coverslip was then rinsed 3x in PBS and a biotinylated anti-mouse IgG (1:500 in PBS) was added. The coverslips were then incubated for a further hour at 37°C, after which they were rinsed 3x in PBS. Strepavidin-peroxidase (Sigma, UK) diluted 1:1000 in PBS was then added to each coverslip. The coverslips were then incubated for an hour at 37°C, and then rinsed 3x in Tris Buffer (0.05M, pH 7.4). Coverslips were then placed in Tris Buffer containing diaminobenzidine tetrahydrochloride (0.5mg/ml) (Sigma, UK) and 10.5 % (v/v) hydrogen peroxide (30% solution). They were incubated for 10 minutes and then washed 3x in PBS. Coverslips were then mounted onto microscope slides using DPEX mountant (Sigma, UK) and observed under a light microscope (Olympus) (Figure 2.10). A distinct brown coloration determined the localisation of smooth muscle α-actin. Primary fibroblasts derived

from bovine or rat pulmonary and systemic arteries were compared to smooth muscle cells cultured from bovine trachea and Rat 2 fibroblast cell lines that acted as positive and negative controls, respectively.

2.3.4 Cell Freezing/Thawing

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

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

Figure 2.10

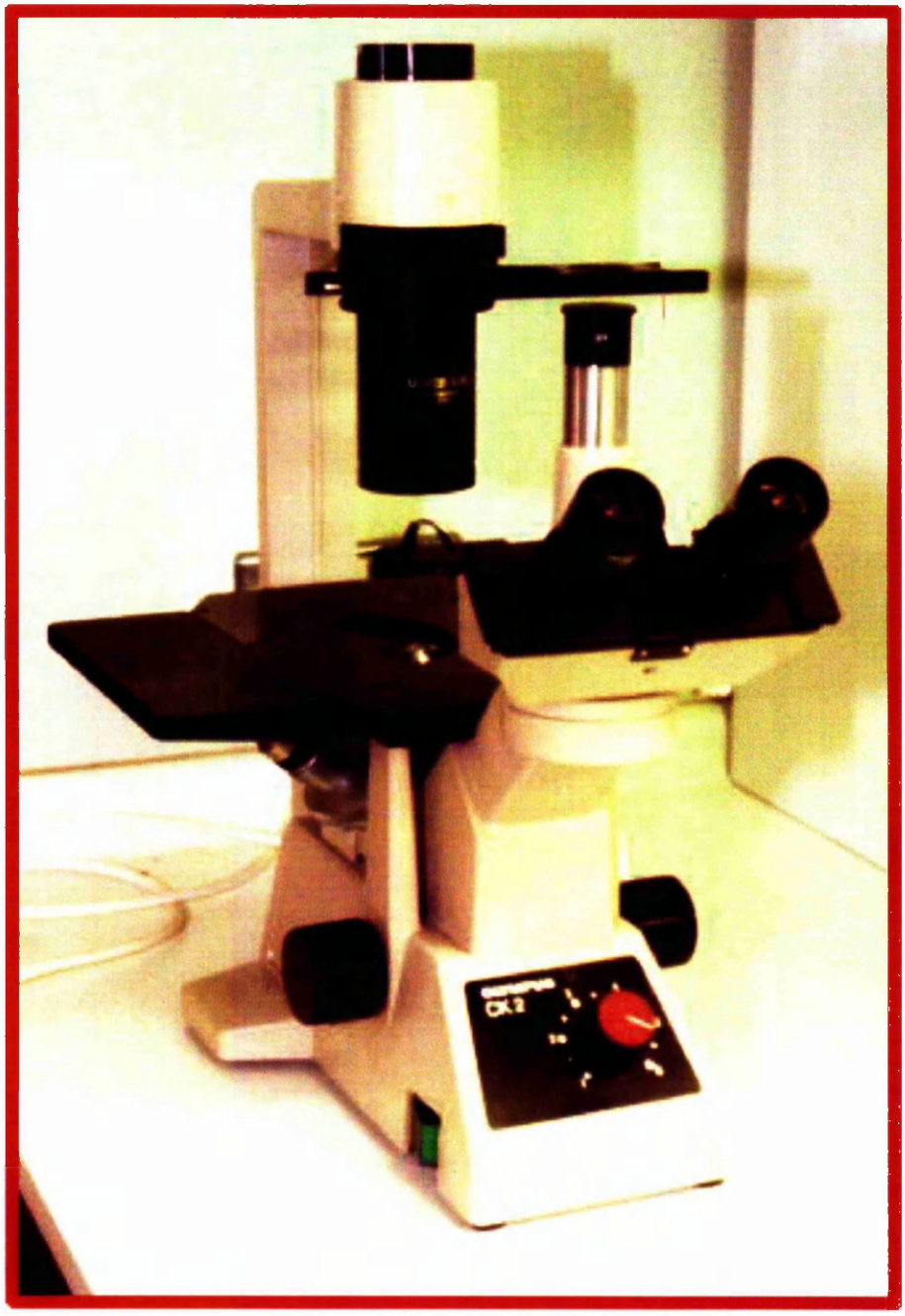


Figure 2.10 Olympus CK2 Light Microscope

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

2.4.1 Generation of Hypoxic Environment

A humidified temperature controlled incubator (Model GA156; LEEC, Colwick, Nottingham, UK) (Figure 2.11) was used to produce a hypoxic environment. This incubator allows control of internal oxygen levels between 0 and 21% using medical grade nitrogen, while the CO_2 level is simultaneously controlled at 5%. Due to the large volume of nitrogen required to sustain a suitable degree of hypoxia, nitrogen cylinders were linked using a Pneuchange automatic gas cylinder change over unit (NTC, Woulton, Liverpool, UK) which activated a fresh supply of nitrogen as required.

2.4.2 Measurement of Hypoxia

The levels of hypoxia achieved within the environment of the incubator could be monitored with the oxygen probe that was an integral part of the unit. Measurements from within the bathing medium of the cells were also analysed with a portable oxygen probe (Jenway: 9015. Dunmon, Essex, UK) to determine the rate of gaseous diffusion, and the pH of the medium analysed throughout the course of hypoxic exposure with the use of a portable pH probe (Mettler Delta 340, Hanstead, UK). Cells were routinely maintained for acute hypoxia in an atmosphere of 2% O_2 and 5% CO_2 at 37°C.

Figure 2.11

Figure 2.11 Hypoxic Chamber: LEEC O_2/CO_2 humidified temperature controlled incubator

2.5 Hypoxia: Methods for studying cells from chronically hypoxic animals

2.5.1 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.

2.5.2 Maintenance of animals

The chamber was housed in a specially designed environmental room which maintains temperature at approximately 21°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 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 split into two groups of four. One group of animals were placed in the chamber and the other group remained in normal atmospheric conditions to act as age-matched controls.

2.5.3 Maintenance of Chronic Hypoxic Rats

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°C, animals were not showing signs of distress and that the pump was in good operating condition. At weekends a member of the Central Animal Facility staff (University of Glasgow) checked the chamber. 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, two of the rats were removed and the remaining two rats taken back to the experimental chamber for a further two days. Of the two rats removed, one was immediately sacrificed and studied on that day along with an aged matched control. The second rat was left in room air to be studied no longer than 24h after removal from the chamber. The same procedure occurred on day 16 for the remaining two rats.

2.6 Assessment of Cell Proliferation

Cell proliferation was measured by determining the uptake of [^{3}H]thymidine into DNA. Cells were seeded at a density of $5x10^3$ cells / well into 24-well plates in 500µl of culture medium. Cells were grown to 60% confluency in 24-well plates and then

growth-arrested for 48h by replacing the medium with 500µl serum-free DMEM. Cells were then stimulated with appropriate agonists and incubated for 24h, either in a normal CO_2 incubator (5% CO_2) or in the hypoxic incubator to obtain an acute hypoxic exposure (Chapter 2.4). In the latter case, the O_2 content of the atmosphere was reduced from 21% to 2% by flooding with N_2.

For the final 4h of agonist stimulation, cells were labelled with [^{3}H]thymidine (0.1µCi/well). The reaction was stopped by washing the cells twice in ice-cold PBS (500µl/well). Proteins were precipitated by washing three times with 500µl/well 5% trichloroacetic acid (TCA) and lipid fractions were solubilised by washing twice with 90% ethanol (500µl/well) for 30mins. The remaining cell contents were incubated in 0.3M NaOH (500µl/well) for 30mins. The contents of each well was transferred to scintillation vials, to each of which was added 3mls of Ecoscint A (Ecoscint, Atlanta, Georgia, USA) scintillation fluid. Vials were vortexed for 1min before radioactivity was measured by scintillation counting using a Wallac scintillation counter. Results are expressed as disintegrations per minute (DPM).

2.7 Inositol 1,4,5-Trisphosphate Mass Assay

2.7.1 Production of Binding Protein

Approximately 20 bovine adrenal glands were obtained from the local abattoir, immediately placed in ice, and brought back to the laboratory. Fat was removed from the glands and they were dissected longitudinally. The Central straw-coloured medulla was scraped off and disposed of leaving the red/brown cortex, which was homogenised and blended. The homogenate was then centrifuged at 5000g for 15min at 4°C and the resulting supernatant centrifuged at 20,000g for 23mins at 4°C. The

final pellet was then resuspended in Homogenisation Buffer (20mM $NaHCO_3$ and 1mM DTT, pH 7.5, 4°C) and centrifuged again at 20,000g for 23mins. The binding protein was stored in 10ml portions at –80°C and was stable for at least 6 months. The binding protein could tolerate approximately four freeze-thaw cycles without loss of binding activity. 20 adrenal glands produced approximately 100ml of binding protein normally giving 40mg protein/ml which was sufficient for ~ 4000 individual assay samples.

2.7.2 Cell Preparation

Cells were seeded at a density of $5x10^3$ cells/well into 24-well dishes and grown for a further 3 days until confluent. Cells were then growth-arrested for 24h by culturing in serum-free DMEM. The reaction was started by the addition of agonists and then stopped by the addition of 25µl ice-cold 10% perchloric acid (PCA, v/v). 30-40µl of Neutralisation Buffer (60mM Hepes pH 8.9, 1.5M KOH) and 3µl aliquots of Universal Indicator was then added until the bathing media had turned green. Cells were mechanically disrupted by scraping using a cell scraper and the contents placed in microfuge tubes on ice. The samples were centrifuged in a bench top microfuge at 5000g at 4°C for 5mins. The resulting supernatant was stored at –20°C until time of assay.

2.7.3 The binding assay (for Standard Curve)

A stock of unlabelled Ins (1,4,5)P_3 (4µM) was diluted into Eppendorf tubes in dH_2O as shown in Table 2.1 in a final volume of 25µl. The following solutions were then added to these standards (25µl):

(1) Neutralisation Buffer (100µl)

(2) Incubation Buffer (Tris-Base 0.04M, pH9.0, EDTA 4mM, EGTA 4mM, 0.4% BSA).

(3) [^{3}H]Ins(1,4,5)P_3 (5µCi) diluted in dH_2O (150µl).

(4) Binding protein (40mg/ml stock) diluted in Homogenisation Buffer (125µl) to a final concentration of 8mg/ml.

The binding protein was always added last.

Samples were then mixed by vortexing and left on ice for 30mins. After this time the samples were centrifuged at 3660g for 15mins at 4°C. The supernatant was removed and 1ml of scintillation fluid was then added to each pellet and mixed by vortexing to dissolve. Radioactivity was measured in a scintillation counter.

2.7.4 The binding assay (for Samples)

This procedure was the same as the assay for the standard curve except 25µl of sample (prepared as in Chapter 2.6.2) replaced the standards.

Table 2.1

[Stock Solution]	Dilution	pmoles Ins (1,4,5)P_3
4μM	1	100
1μM	1/4	25
0.5μM	1/8	12.5
0.15μM	1/16	6.25
0.125μM	1/32	3.125
62.5nM	1/64	1.56
31.25nM	1/128	0.78
15.6nM	1/256	0.39
7.8nM	1/512	~0.2
3.9nM	1/1024	~0.1
H_2O Only	0	0

Table 2.1 Dilution of stock IP_3 for use with standard curve

2.7.5 Calculation of Ins(1,4,5)P_3 mass.

% B/ Bo was calculated where:

B = Specific binding (of unknown or of standard)

= DPM in pellet – DPM in non-specific binding sample

Bo = maximum specific

= DPM in pellet of 0 pmol IP_3 tubes – DPM in non-specific binding sample.

%B/Bo vs. pmol Ins(1,4,5)P_3 was then plotted as a standard curve on semi-log paper. (See Figure 5.1)

The %B/Bo values of unknowns from the standard curve = pmoles / sample (i.e. not a concentration).

Concentration of IP_3 in assay was dependent upon the assay volume used.

i.e. 5pmol in 500µl assay = 10nM.

2.8 Detection and Analysis of Proteins

2.8.1 Preparation of samples for SDS-PAGE and immunoblotting

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

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

2.8.2 Assay of protein concentration

Quantification of protein concentration was determined using a Bio-Rad™ DC Protein Assay Kit based on a colorimetric assay devised by Lowry *et al* (1951). Protein samples were prepared in appropriate lysis buffer. For each assay performed, a standard curve was prepared using five dilutions of BSA as a protein standard diluted in RIPA buffer over the concentration range of 1mg-16µg/ml protein. Appropriate dilutions of both samples and standards were made in H_2O to a volume of 200µl. Reagent A (an alkaline copper tartrate solution supplemented with 5% SDS) (100µl) was added to each of the standards and samples. These were vortexed and 800µls

Reagent B (a dilute Folin Reagent) added and immediately vortexed. Samples were left for 15min for colour development from pink to green as a result of reduction of Folin reagent by copper-protein complex. Colour development was quantified by absorbance spectroscopy at 750nm within 1h. Estimates of protein content in samples were made from the standard curve.

2.8.3 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were analysed using a SDS-PAGE system

2.8.3.1 Preparation of acrylamide gels

The casting apparatus was Bio-Rad™ mini gel electrophoresis units assembled according to manufacturer instructions. The separating and stacking gels were made as follows:

Resolving polyacrylamide gels: Normally 10% resolving gels were used which were prepared using the following composition; 37% acrylamide / 0.8% bisacrylamide (to a final 10%) (w/v); 0.375M Tris-HCl pH 8.5, 0.1% SDS (w/v), 0.05% ammonium persulphate (w/v), 0.05% TEMED (v/v). Polymerisation was initiated by addition of the ammonium persulphate and TEMED. The solution was mixed and poured into the assembled glass plates, leaving sufficient space at the top for the stacking gel. Gels were overlaid with 200-400µl of 0.1% SDS and allowed to polymerise for 30-45 mins.

Stacking polyacrylamide gels: Following polymerisation of the resolving gels, the overlay was removed and the surface of the gel rinsed with water to remove any unpolymerised acrylamide. The stacking polyacrylamide gels used were of the following composition: 5% acrylamide/bisacrylamide (w/v), 0.125M Tris-HCl pH

8.5, 0.1% SDS (w/v), 0.05% ammonium persulphate (w/v) and 0.1% TEMED (v/v). Stacking polyacrylamide gel components were overlaid on the polymerised resolving gel and combs added immediately. After polymerisation of the stacking gel, combs were removed and the wells rinsed with Electrophoresis Buffer (Tris-base 25mM, glycine 192mM and SDS 0.1%).

2.8.3.2 Electrophoresis conditions

All protein samples to be examined 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)). DTT was added fresh, immediately before use. 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 polyacrylamine gel subjected to electrophoresis for approximately 1.5-2h at a constant current of 240mA.

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).

2.8.3.3 Fixing and drying gels

Gels containing radioactive proteins were fixed in 20% methanol (v/v)/10% acetic acid (v/v) for 30 min and rinsed in distilled water. The gel was sandwiched between cellophane sheets in a drying frame and dried under vacuum for 60 min at 80°C by a Hoeffer Eazy-Breeze™ gel dryer. The radiolabelled protein was detected by autoradiography. The dried gel was exposed to Kodak X-OMAT LS X-ray film for 4-24h at –20°C in a spring loaded metal cassette and developed by a KODAK M35-M X-OMAT processor.

2.8.4 Western blot analysis.

2.8.4.1 Transblotting to nitrocellulose.

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.

2.8.4.2 Immunoblotting

Samples (30-50μg of protein) were subjected to SDS-PAGE (Chapter 2.8.3) and the proteins transferred to nitrocellulose by Western blotting (Chapter 2.8.4.1). The nitrocellulose blots were washed in PBS/T (PBS containing 0.02% Tween-20 (v/v)) and blocked for non-specific binding for 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 the appropriate dilution of primary antibody. The primary antibody dilutions used for detection of specific antigens is detailed in Table 2.2. The nitrocellulose blots were rinsed in PBS/T, and washed 4x over an hour with PBS/T. The blots were then incubated for a further 60mins in PBS/T containing 5% Marvel (w/v) containing donkey anti-rabbit IgG antibody conjugated to HRP (NEB; 1:15000). The blots were then washed as before. Following completion of washing procedure proteins were detected using a method of Encanced Chemilumenescence (ECL). Blots were incubated in ECL solution (Amersham) for 30s and sandwiched between acetate film. Care was taken to ensure all air bubbles were removed. Blots were then placed in an X-ray cassette and light emission from the HRP enzymatic action on its substrate contained within the ECL solution was detected following exposure (1-10mins) of nitrocellulose blots to a piece of X-ray film using a KODAK M35-M X-OMAT processor.

Table 2.2

Analysed Protein	Antibody Type	Dilution
phospho p38	rabbit monoclonal anti-phospho p38 IgG antibody	1:1000
whole p38	rabbit monoclonal anti- whole p38 IgG antibody	1:1000
phospho p42/44	rabbit monoclonal anti- phospho p42/44 IgG antibody	1:1000
whole p42/44	rabbit monoclonal anti- whole p42/44 IgG antibody	1:1000
phospho Jnk	rabbit monoclonal anti- phospho Jnk IgG antibody	1:1000
whole Jnk	rabbit monoclonal anti- whole Jnk IgG antibody	1:1000

Table 2.2 Primary antibody dilutions utilised for Western blotting

2.8.4.3 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°C with agitation. Blots were then rinsed with PBS/T 4x over 40 mins before the immunodetection protocol was repeated (Chapter 2.8.4.2)

2.8.5 Solid phase JNK activity assay

JNK activity was measured using recombinant truncated N-terminus of c-jun (c-Jun_{5-89}) immobilised on glutathione (GSH)-sepharose beads (GST-c-jun) (Dai *et al,* 1995). Cells were grown to subconfluence on 6-well plates and treated as required. To stop the reaction, cells were washed 2x with ice-cold PBS, then stimulated cells were scraped on ice in 500µl Solubilisation Buffer (20mM HEPES pH7.7, 50mM NaCl, 0.1mM EDTA, 0.2mM PMSF, 0.5mg/ml leupeptin, 0.5mg/ml aprotinin and 1% (v/v) Triton X-100). Lysates were centrifuged at 14,000g for 15min at 4°C. Protein concentration was measured in the supernatant as in chapter 2.8.2. Aliquots (150 µg) of solubilised cell extracts were added to 20µl of GST-c-jun and mixed for 3h on a rotary wheel at 4°C to allow binding of active JNK in the cell extracts. The sepharose beads were centrifuged at 10,000g for 1min at 4°C and the supernatant removed. The beads were washed 3x in 500µl Solubilisation Buffer and 1x in 500µl Kinase Buffer (25mM HEPES pH 7.6, 20mM $MgCl_2$, 5mM β-glycerophosphate, 0.1mM Na_3VO_4 and 2mM DTT). The beads were resuspended in 30µl of Kinase Buffer [γ-^{32}P]ATP (20µM, 2µCi) and the tubes were incubated for 30min at 30°C. The reaction was terminated by adding 10µl 4x Laemmli SDS sample buffer. Samples were boiled for 5

minutes and proteins resolved by SDS-PAGE (Chapter 2.8.3) on 10% polyacrylamide gels. Gels were fixed and dried as in Chapter 2.8.3.3 and incorporation of [γ-^{32}P] into GST-c-Jun detected by autoradiography.

2.8.6 Solid phase p38 MAP kinase assay

The activity of p38 MAP kinase was assayed *in vitro* by a procedure based on Bogoyevich, *et al* (1996). Cell extracts were prepared in a p38 MAP kinase Solubilisation Buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 1% Triton X-100, 10% glycerol, 2mM EDTA, 20mM NaF, 2.5mM β-glycerophosphate, 0.2mM PMSF, 0.5mg/ml leupeptin and 0.5 mg/ml aprotinin) and p38 affinity-purified by incubating lysates with MAPKAP kinase-2 immobilised on GSH-sepharose beads (GST-MAPKAP kinase-2). The kinase reaction was carried out in the same buffer as that used for the c-jun activity assay (Chapter 2.8.5). The incorporation of [γ-^{32}P]ATP into MAPKAP kinase-2 was carried out using a similar method to that for GST-c-jun.

2.8.7 p42/44MAP kinase activity assay

In vitro MAP kinase activity was assessed using a BIOTRAK MAP kinase assay kit to measure the incorporation of radiolabelled phosphate into a specific MAP kinase substrate peptide (KRELVEPLT^{669}PAGEAPNALLAR) derived from a portion of the EGF receptor (EGFR$^{661-680}$). This method was carried out according to the manufacturers instructions.

After treatment, cells in 6-well plates were rinsed 2x in ice-cold PBS and scraped on ice in 500µl Lysis Buffer (10mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 0.2mM Na_3VO_4, 1% Triton X-100, 0.5% NP-40, 0.2mM PMSF, 0.5 µg/ml leupeptin and 0.5µg/ml). Lysates were transferred to eppendorf tubes then left on ice

for 30mins followed by were centrifugation at 13000g for 5mins at 4°C. Protein concentration was measured in the supernatant as in Chapter 2.8.2. Equal quantities (approximately 50μg protein) of supernatant were transferred to fresh tubes in a volume of 15μl to which was added 10μM $EGFR^{661-680}$ (600μM). The reactions were initiated by addition of 5μl [γ-^{32}P]ATP (50μM, 1μCi) in 75mM HEPES buffer (pH 7.4) containing 1.2mM $MgCl_2$. The reactions were incubated at 37°C for 15 min. Reactions were stopped by the addition of 25μl of 300mM H_3PO_4 and aliquots (25μl) were spotted onto 3x3cm P-81 phosphocellulose paper. Filters were washed 2x for 5min with 75mM H_3PO_4 and 2x for 5min with dH_2O, then transferred to 6ml plastic scintillation vials to which 3mls Ecoscint A. was added. Radioactive phosphopeptide bound to the filter was quantified by liquid scintillation counting in a Wallac scintillation counter. In every assay, background activity was measured from a sample of kinase buffer reaction mixture in the absence of lysate.

2.9 Densitometric analysis of blots

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

2.10 Data analysis

Data are expressed as mean ± S.D. for replicate plates from the same experiment. Experiments were repeated in cells ranging from 4-8 different animals. The statistical significance of differences between mean values from control and treated groups were determined by Student's t-tests, unpaired and paired where applicable, using OXTAT software. Two-tailed probability values of less than 0.05 ($P<0.05$) were considered to be significant.

Chapter 3

Establishment of a Cell Model for Studying Bovine Pulmonary Artery Fibroblast Cell Proliferation in Acute Hypoxia

3.1 INTRODUCTION

Pulmonary hypertension causes remodelling of the fibroblast, smooth muscle and endothelial cell layers of the pulmonary artery (Meryrick and Reid, 1979). The hypoxic environment results in increased proliferation of all three cell layers (Meryrick and Reid, 1979). The cause of this increased cell replication is not understood. Hypoxia causes different effects in various cell types in cell culture. For example, acute hypoxia causes an increase in proliferation of smooth muscle and endothelial cells (Lou *et al*, 1996; Lou *et al*, 1997). However, in other cell types such as PC12 cells, hypoxia causes a decrease in cell proliferation (Yamakawa *et al*, 2000). Work has been carried out to study proliferation of endothelial and VSMC derived from the pulmonary artery (Stenmark *et al*, 1997; Feng and Cai, 1996), however, the effect of hypoxia on primary fibroblast cells derived from this tissue is unknown. A model to study pulmonary artery-derived fibroblasts in culture has been established in this chapter which provided the basis for experiments in the future chapters.

Some of the earliest and most dramatic structural changes following hypoxic exposure occur in the adventitial compartment of the vessel wall (Stenmark and Mecham, 1997; Stenmark *et al*, 1988). In animal models of hypertension, the resident adventitial fibroblasts have been shown to exhibit early (within 24h) and sustained increases in proliferation and exhibit dramatic increases in extracellular matrix protein synthesis (Meryrick and Reid, 1979; Rabinovitch *et al*, 1987; Reeves and Herret, 1984). These proliferative changes in the fibroblasts are associated with luminal narrowing and a progressive decrease in the ability of the vessel wall to respond to vasodilating stimuli (Morin and Stenmark, 1995). The mechanisms contributing to the excessive fibroblast

growth in the pulmonary adventitia under conditions of acute and chronic hypoxia remain poorly understood.

3.2 METHODS

3.2.1 Assessment of fibroblast determination.

All primary cell lines used in this thesis were obtained by the explant procedure outlined in Chapter 2.3.2, and tested for smooth muscle α-actin. The morphology of the cells was visually assessed to discount the presence of endothelial cells.

3.2.2 Determination of [^{3}H]thymidine Uptake

For measurement of DNA synthesis, [^{3}H]thymidine was added at a time when it would be incorporated into DNA. To determine the optimum time for incorporation of [^{3}H]thymidine, this was added for various times prior to stopping the reaction and measuring incorporated [^{3}H]thymidine (Chapter 2.6).

3.2.3 Assessment of PO_2 and pH

Cells were grown to approximately 60% confluency in 24-well plates, serum-starved for 48h, and then transferred to an environment of 2% O_2. At 0, 1, 3, 6, 12 and 24h a portable oxygen probe was used to determine the PO_2 and a portable pH meter used to determine the pH in the cell growth medium. [^{3}H]Thymidine uptake was used to assess the effect of the pH change on the growth of bovine pulmonary artery fibroblast (BPAF) cells. To alter the pH of the growth medium, 1M HCL (prepared in 1M Hepes Buffer) was added until the desired pH was reached. The medium was re-sterilised by filtration through a 0.2μm filter.

3.2.4 Assessment of Proliferation of BPAF cells Grown in Hypoxic Conditions

Cells were grown to approximately 60% confluency in 24-well plates, serum-starved for 48h, then either maintained in normoxia (21%) or transferred to an environment of 2% O_2. After preincubation, as described in Chapter 3.2.3, the cells were maintained in the absence of serum, for various lengths of time under normoxic or hypoxic (2% O_2) conditions. [^{3}H]Thymidine was added 4h prior to the end of the assay period at which time [^{3}H]thymidine uptake was measured (Chapter 2.6). Experiments, which used various preincubation times to assess maximum proliferation, were also studied.

3.2.5 Statistics

Results are expressed as the mean ± S.D. Statistical analysis was carried out as in Chapter 2.10.

3.3 RESULTS

3.3.1 Assessment of the fibroblast cell type

α-Actin staining was used routinely to assess the presence of smooth muscle cell contamination in cells derived from the pulmonary artery. A dark brown colouration is a positive stain for smooth muscle α-actin. Figure 3.1A shows fibroblasts derived from the pulmonary artery, which were found to be contaminated with smooth muscle cells and would not have been used for further experimentation. Figure 3.1B show fibroblasts derived from the pulmonary artery after hypoxic exposure. The shape of the cell also provided an identification of the cell type. The fibroblast were sub-confluent by which they assume multipolar or bipolar shapes and are well spaced on the culture surface, but at confluence they are bipolar and less well spread (Freshney, 1994).

Figure 3.1

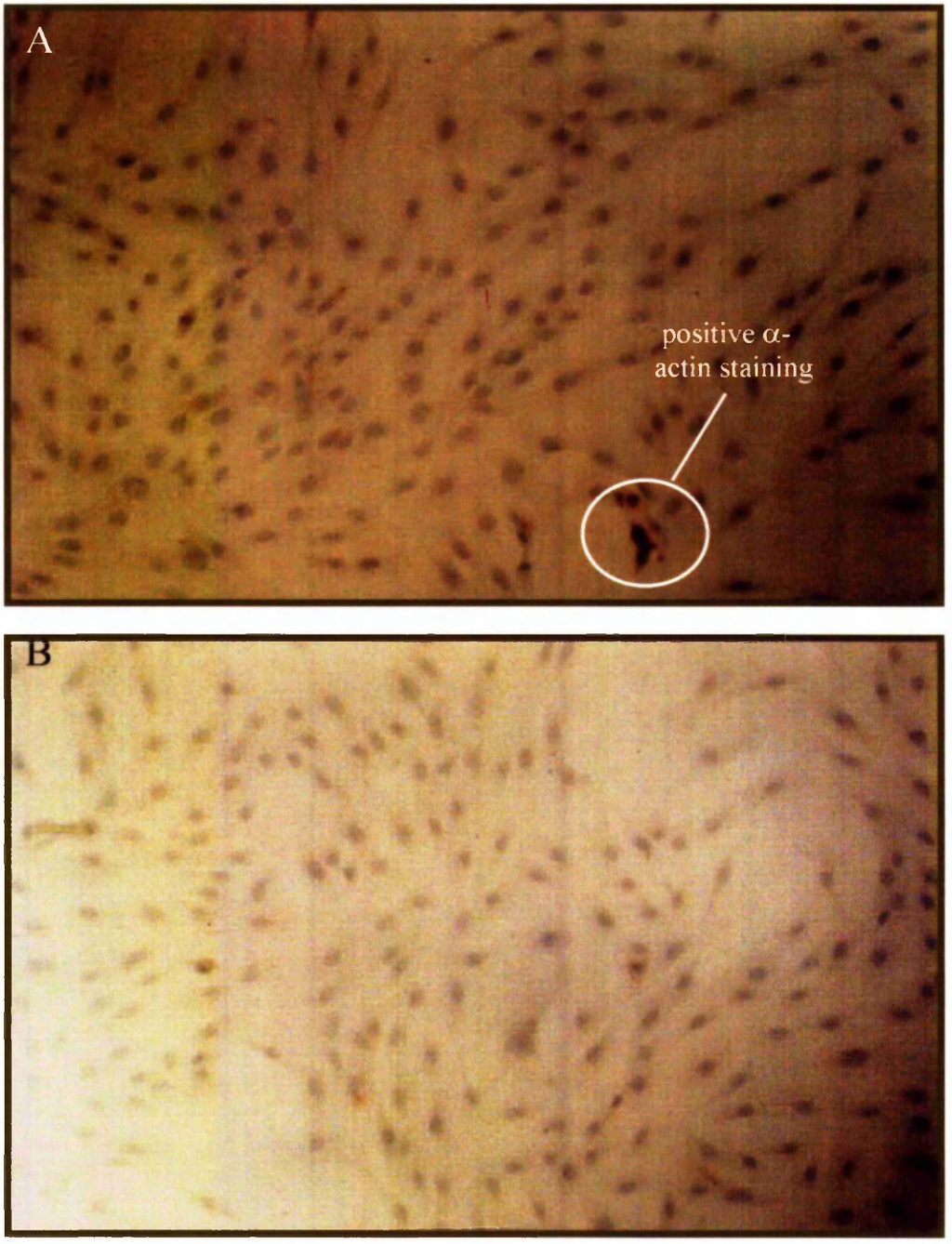


Figure 3.1 α-Actin Staining of Normoxic and Hypoxic Pulmonary Artery Fibroblasts.

Fibroblasts from the pulmonary artery were stained for α-Actin as described in Chapter 2.3.3.

3.3.2 Determination of maximal [^{3}H]thymidine incorporation

To assess the growth of primary fibroblast cell cultures it was first necessary to optimise the method for determining [^{3}H]thymidine uptake in these cells. Figure 3.2 demonstrates the time of maximal [^{3}H]thymidine incorporation in pulmonary artery fibroblast cells. As thymidine is incorporated whilst the cells are synthesising DNA this gives a good estimation of whether cells are proliferating or not. The addition of [^{3}H]thymidine for time points > 4h prior to the end of a 24h-proliferation assay resulted in maximum incorporation. It was decided to add the thymidine as late as possible as to reduce background incorporation.

Figure 3.2

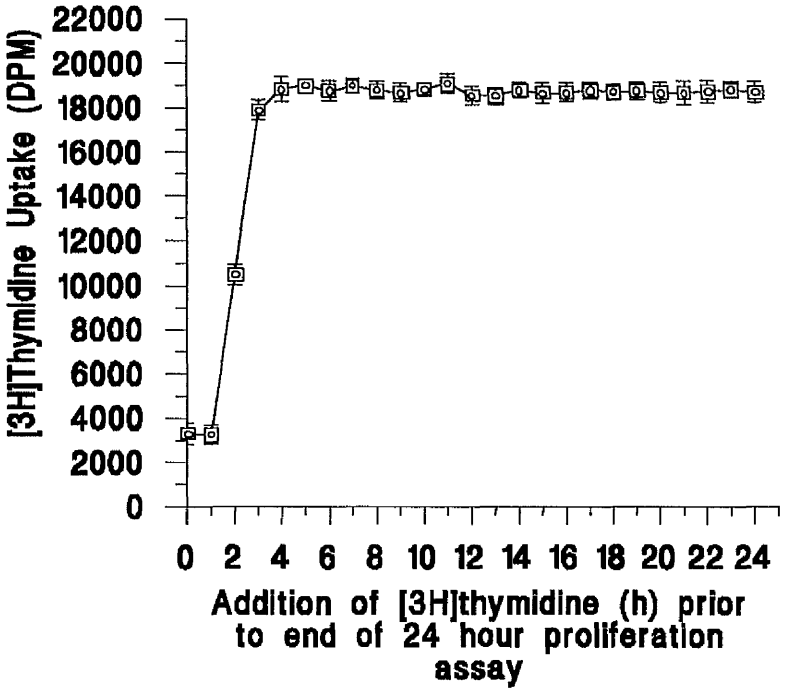


Figure 3.2 Determination of Maximum [^{3}H]Thymidine Uptake in Pulmonary Artery Fibroblast Cells

BPAF cells were grown to 60% confluency in normoxic conditions in the presence of 10% serum in 24-well plates. The cells were serum-starved for 24h before stimulation with 5% serum. The cells were then allowed to grow in normoxic conditions for a period of 24h. [^{3}H]Thymidine was added to different wells for every hour of the 24h growth assay. Values shown are the mean ± S.D. for four replicate wells from one experiment. The experiment was repeated four times using cells derived from different animals.

3.3.3 Determination of time taken to reach an adequate hypoxic environment.

In order to determine the time required to achieve a hypoxic environment in the growth medium of cells cultured in 24-well plates, cells were placed in a hypoxic incubator at 2% O_2 and O_2 levels in the growth medium measured over time. A 6h-incubation period was required for the growth medium to reach the desired 35mmHg which represents the physiological level that is found in the pulmonary artery of patients with pulmonary hypertension (Souhami & Moxham, 1998) (Table 3.1). The hypoxic environment in the medium was maintained for the times > 6h (Table 3.1). A similar length of time was necessary to reach 35mmHg in different sizes of tissue culture dishes used in other experiments (data not shown).

To determine the effect of hypoxia on the pH of the growth medium, a portable pH meter was used to measure pH during the time the cells were grown in the hypoxic incubator. The pH of the medium decreased from pH 7.5 as the PO_2 of the medium decreased and stabilised at pH 7.1 ± 0.2 (average of several experiments) (Table 3.2).

To determine the effect of this pH change on the proliferation of fibroblasts, [^{3}H] thymidine uptake was used to measure the effect of slight changes in pH of the growth medium. The pH of the cell culture medium was altered to that exhibited in hypoxic conditions by growing the cells in media at a pH range of 7.0-7.5. The pH remained constant throughout the assay period (results not shown). Normoxic BPAF cells subjected to this pH change did not exhibit changes in their ability to proliferate (Figure 3.3) and hence, a change in pH of the growth medium does not alter cell proliferation.

Table 3.1

Hours of preincubation in 2% O_2	PO_2 in Bathing Medium (mmHg)
0	147
1	126
3	63
6	35
12	35
24	35

Table 3.1. Generation of a Hypoxic Environment for BPAF Cells

BPAF cells were grown to 60% confluency in 24-well plates before being placed in the hypoxic incubator set at an oxygen level of 2%. At the times indicated a portable oxygen probe was used to determine the pO_2 in the cell growth medium. Values shown are representative of one individual well per time point. The experiment was repeated four times and results shown are typical of those obtained.

Table 3.2

Hours of preincubation in 2% O_2	pH
0	7.5
1	7.2
3	7.2
6	7.1
12	7.0
24	7.1

Table 3.2 Effect of Hypoxia on pH of Growth Medium

BPAF cells were grown to 60% confluency in 24-well plates before being placed in a hypoxic incubator set at an oxygen level of 2%. At the various times indicated a portable pH probe was used to determine the pH in the cell growth media. Values shown are representative of one individual well per time point. The experiment was repeated four times and results shown are typical of those obtained.

Figure 3.3

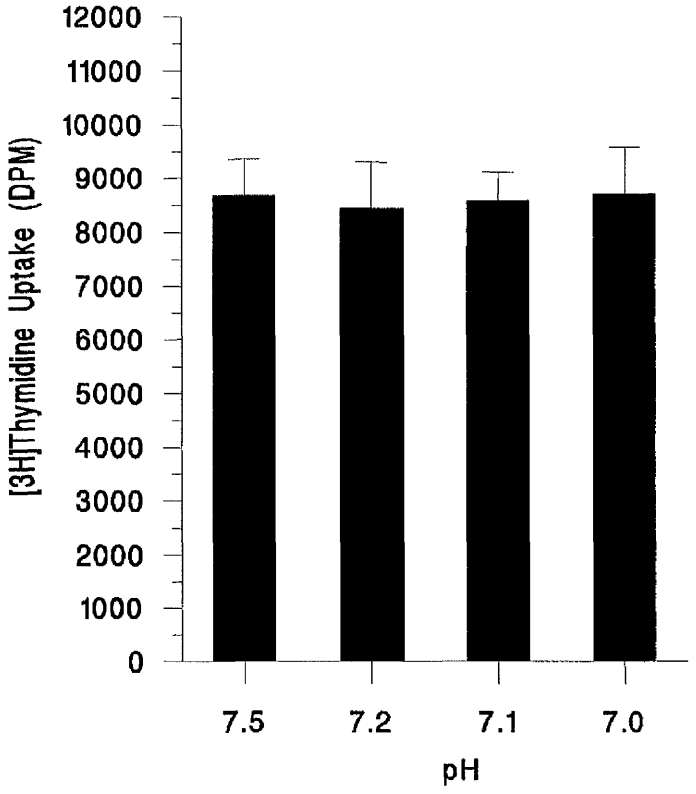


Figure 3.3 Effect of pH on [^{3}H]Thymidine Uptake in Normoxic Pulmonary Artery Fibroblasts

BPAF cells were grown to 60% confluency in 24-well plates and then serum-starved for 48h. At this point the medium was replaced in different wells with media at a range of pHs. The pH had been altered to 7.5, 7.2, 7.1 and 7.0 (Chapter 3.2.3). The cells were then left for a further 24h with [^{3}H]thymidine added for the final 4h. Values shown are the mean ± S.D. for four replicate wells from one experiment. The experiment was repeated in cells derived from four different animals.

3.3.4 Effects of hypoxia on proliferation of Bovine Pulmonary Artery Fibroblasts

To determine the effect of hypoxia on the growth of BPAF's, serum-starved cells were pre-incubated for various lengths of time prior to a 24h proliferation assay as described in Chapter 3.2.3. In the absence of a 6h pre-incubation period in hypoxia, which was shown in Table 3.1 to be required for the cell growth medium to become hypoxic, no hypoxic growth of the fibroblast cells was noted over a 24h period (288 ± 43.5 DPM for hypoxic cells which was not significantly different from control cell levels at 255 ± 33.7 DPM). Figure 3.4 demonstrates that following a 6h preincubation in hypoxic conditions BPAF cells proliferated at an increased level (1753.3 ± 69.6 DPM) which was significantly ($P<0.001$ for data sets) greater than those of control cells (286.9 ± 19.4 DPM). Further pre-incubation in hypoxic conditions did not enhance the growth of these cells. The graph suggests that further preincubation may cause the cells to either die or stop proliferating, however, the apparent reduction in growth was due to the cells becoming over confluent and lifting from the base of the cell culture plate.

To determine whether a longer growth assay period following the 6h pre-incubation would result in greater [^{3}H]thymidine uptake, the effects of varied lengths of growth time was examined. Figure 3.5 demonstrates that there would appear to be no advantage to allowing the cells a longer period of growth over 24h to enhance the hypoxic response.

Visual confirmation of the hypoxic proliferation of these cells over a 24h period when compared with those maintained in normoxia can be seen in Figure 3.6. Figure 3.6A and B shows serum-starved normoxic fibroblast cells at T_0. Figure 3.6C and D shows fibroblast cells 24h later after 5% serum stimulation. Figure 3.6C shows fibroblasts

maintained in normoxia and figure 3.6D fibroblasts kept in hypoxic conditions. Even visually, increased proliferation of the hypoxic cells it is quite apparent.

FIGURE 3.4a

Pre-incubation Time (h)	Normoxic BPAF	S.D.	Hypoxic BPAF	S.D.	p value
0	**255**	**33**	**288**	**44**	**> 0.05**
	290	54	318	76	> 0.05
	301	72	327	97	> 0.05
6	**287**	**19**	**1753**	**70**	**< 0.001**
	319	43	1810	102	< 0.001
	331	54	1796	72	< 0.001
12	**325**	**58**	**1018**	**92**	**< 0.001**
	352	71	1412	96	< 0.001
	362	107	1019	141	< 0.001
24	**413**	**98**	**438**	**44**	**> 0.05**
	441	107	429	76	> 0.05
	481	135	477	87	> 0.05

Figure 3.4a Effects of Hypoxic Pre-Incubation on BPAF Cell Proliferation

Figure 3.4a summarises data from 3 different animals. Data in bold is represented in Figure 3.4. Each data point represents the mean of 4 individual wells from the same experiment.

Figure 3.4

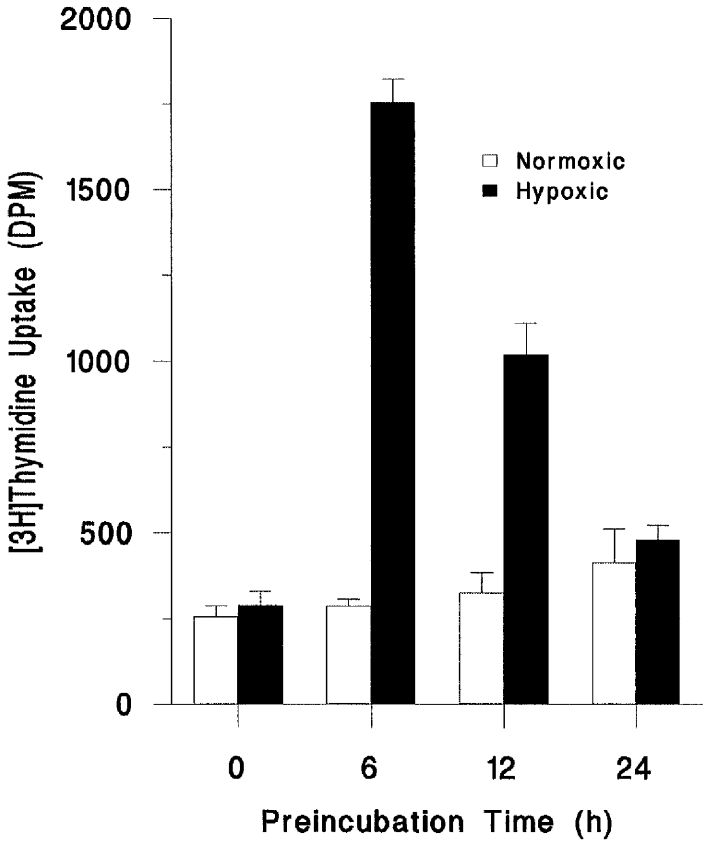


Figure 3.4 Effect of Hypoxic Pre-Incubation on BPAF Cell Proliferation

Uptake of [^{3}H]thymidine in BPAF cells in normoxia (white) and hypoxia (black). Cells to be made hypoxic were pre-incubated in 2% 0_2 for 0, 6, 12 and 24h prior to a 24h proliferation assay. Each data point represents the mean ± S.D. of 4 individual wells from three separate experiments.

Figure 3.5

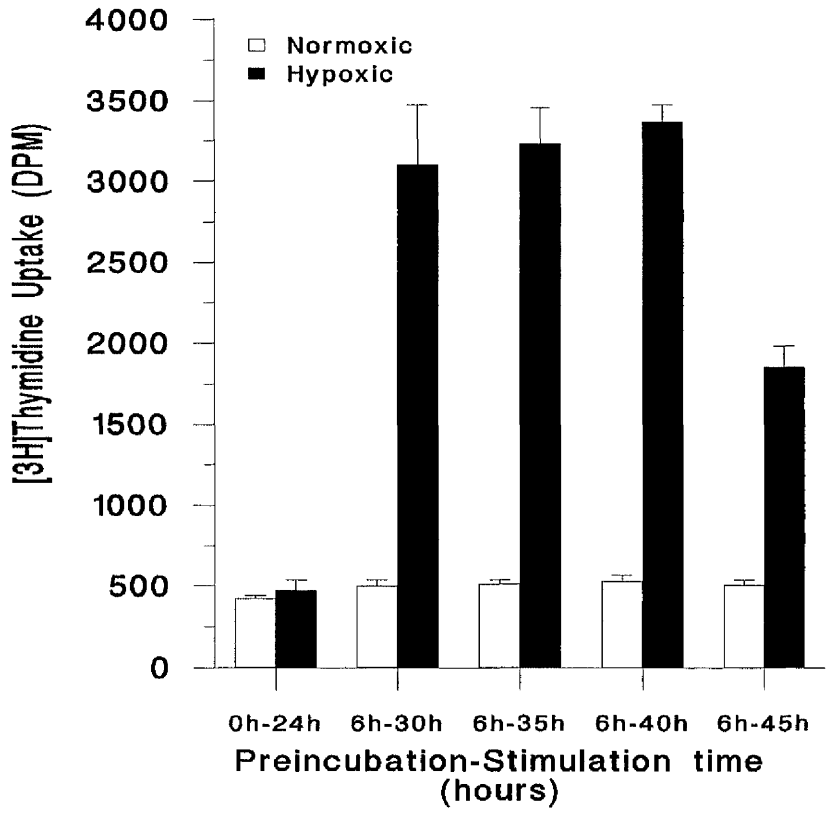


Figure 3.5 Effect of a 6h Hypoxic Pre-Incubation with Varied Lengths of Growth Time on the Proliferation of BPAF Cells

Uptake of [^{3}H]thymidine in BPAF cells in normoxia (white) and hypoxia (black). Cells to be made hypoxic were pre-incubated in 2% 0_2 for 6h prior to addition of 10% serum for 24, 30, 35, 40 and 45h. Each data point represents the mean ± S.D. of 4 individual wells from three separate experiments. Each data point represents the mean ± S.D. of 4 individual wells from three separate experiments.

Figure 3.6

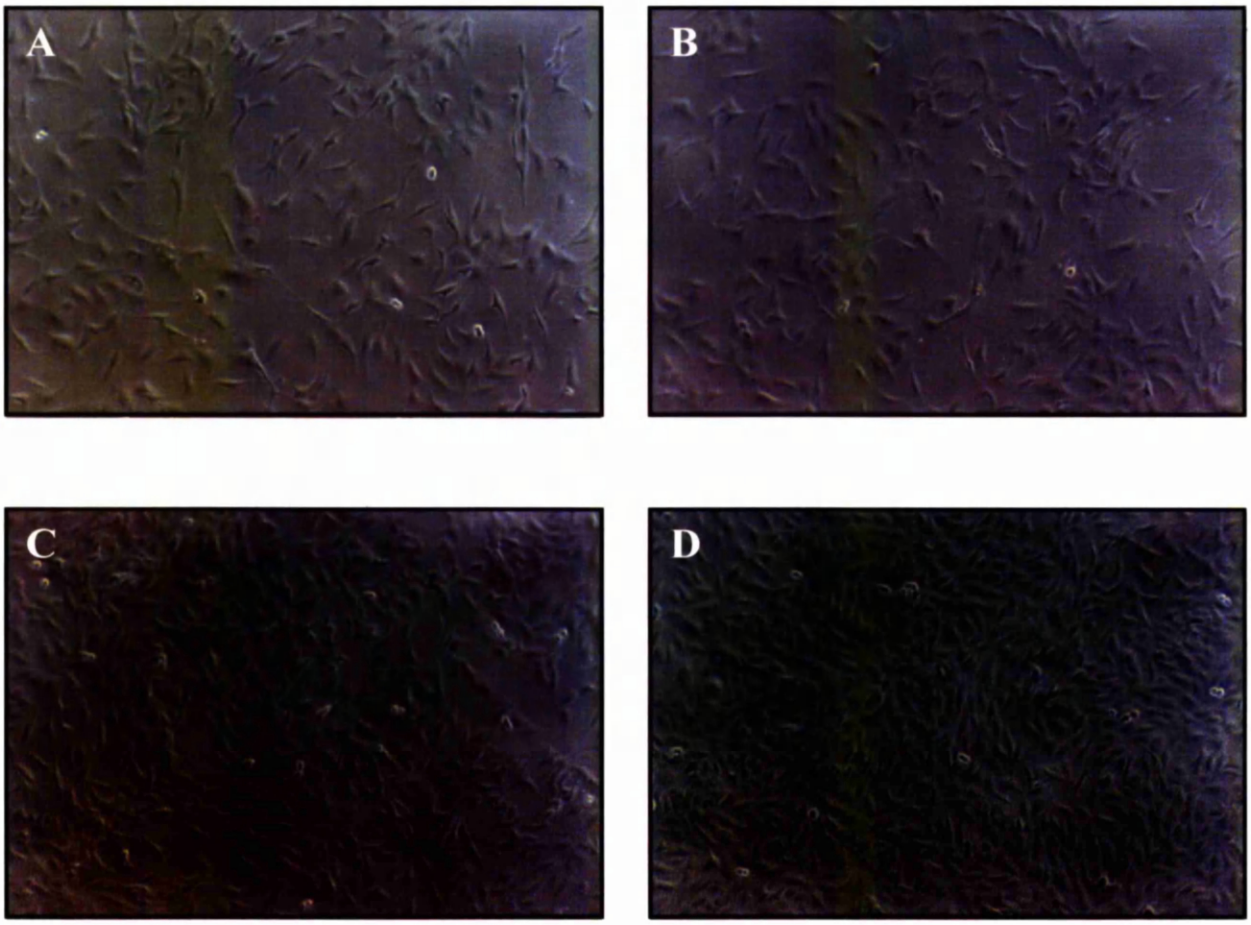


Figure 3.6 Visual Assessment of BPAF Cell Proliferation.

BPAF cells (A and B) were grown to 50% confluency in 75cm^2 flasks and serum-starved for 48h. 5% serum was then added and the cells allowed to proliferate in normoxic (C) and hypoxic (D) conditions for a further 24h.

3.4 Discussion

In this chapter a cell model has been established for looking at changes in the proliferative response of primary cultures of fibroblast cells derived from the pulmonary artery in response to acute hypoxia. It was established that the cell culture medium required a hypoxic pre-incubation of 6h to achieve a suitable level of hypoxia in the growth medium. During this time there was a slight drop in pH, but we have shown that this change in pH did not effect the proliferative response of the fibroblast cells. It was consistently found (that is, in fibroblast cells from at least four animals) that the cells derived from the pulmonary artery exhibited an enhanced growth response when cells were cultured in hypoxic conditions.

Changes in oxygen concentration within the physiologic range have a profound effect on the proliferation of mammalian cells. Reducing the oxygen concentration from normal levels of 21% to 2.5% enhances the growth of fibroblasts and lymphocytes in tissue culture in conjunction with growth factors and/or serum (Packer and Fuehr, 1977; Taylor *et al*, 1978; Storch and Talley, 1988). Feng *et al*, (1996) showed that acute hypoxia (2%) significantly increased [^{3}H]thymidine uptake in pulmonary artery smooth muscle cells which were grown in the presence of 2% serum. Similarly, Lou *et al*, (1997) showed the same was true for retinal microvessel endothelial cells (133% increase in 24h). However, the proliferative response of hypoxia alone without the addition of growth factors has not been reported in fibroblast cells. The results presented in this chapter show hypoxia alone can stimulate the proliferation of BPAF and this is the first report to show this effect in this particular cell type. The results presented here are also comparable to work carried out on fibroblast cells derived from neonatal calves exposed to chronic hypoxia after birth (Das *et al*, 1999). In that

study, cells from chronically hypoxic animals showed increased growth characteristics to purified mitogens when compared to those from control animals.

In general, techniques used to assess proliferation measure the assimilation of radiolabelled nucleotides such as [^{3}H]thymidine into newly synthesised DNA. Thymidine is commonly used for this purpose. Most tissue cultures comprise cells at various stages of the cell cycle, so cells are typically growth-arrested in serum-free media. This synchronises cells to the G_0/G_1 phase of the cell cycle in which cells minimally incorporate [^{3}H]thymidine (Panettieri *et al*, 1989).

There are a number of potential drawbacks in relating [^{3}H]thymidine uptake to DNA synthesis. Firstly, although most DNA synthesis occurs during cell replication, some of the DNA synthesis may be for ongoing reparative processes rather than for proliferation. Secondly, [^{3}H]thymidine may be incorporated into cellular macromolecules other than DNA, such as RNA and lipids. Despite these potential artefacts, measuring uptake of [^{3}H]thymidine remains one of the best and most widely used methods for assessing cell proliferation.

These studies have established that pulmonary artery fibroblasts exposed to acute hypoxia are associated with a significant change in the proliferative phenotype. It is now important to understand the mechanisms contributing to the excessive fibroblast proliferation under these conditions. The results presented in this chapter are the foundations for the remaining chapters, which study further the responses of pulmonary artery fibroblasts with respect to acute and chronic hypoxia and compare these responses to those of cells from the systemic circulation.

Chapter 4

Effect of Acute Hypoxia on Mitogenic Stimulation of Bovine Pulmonary Artery and Bovine Mesenteric Artery Fibroblast cells.

4.1 INTRODUCTION

In the previous chapter a cell model was established and characterised for measuring acute hypoxic effects on the growth of primary cultures of fibroblast cells. Results from that chapter showed that hypoxia increased [^{3}H]thymidine uptake in the fibroblast cells derived from bovine pulmonary artery.

It has been known for some time that hypoxia causes vasoconstriction in the pulmonary circulation and vasodilation in the systemic circulation (von Euler and Liljestrand, 1946). We hypothesised that there are fundamental differences in oxygen sensing and cell signalling between systemic and pulmonary artery cells in response to hypoxia, that is there is a link between the observed physiological vasoconstriction and vascular remodelling. Growth factors are implicated in a wide variety of physiological and pathological processes. These include growth and development and cell survival and tissue repair (Evered *et al*, 1985). An important link between growth factors or their receptors and oncogene products has also been established (Heldin and Westermark, 1984; Bishop, 1985; Weinberg, 1985). Thus, the elucidation of the mechanism of action of growth factors has emerged as one of the fundamental problems in biological sciences and may prove a crucial prerequisite for understanding the cause or causes underlying the unrestrained proliferation of pulmonary cells to hypoxia in disease states like pulmonary hypertension or of other hypoxic lung diseases (Lafont, 1995). In fact, it has been shown that there may be some synergistic effects between hypoxia and mitogens on the proliferation of cells. For example, Feng and Cai (1996) demonstrated a synergistic effect of hypoxia on smooth muscle cell proliferation with ET-1, whilst others (Das *et al*, 1999) recently

showed synergism of hypoxia on proliferation of neonatal bovine pulmonary artery fibroblasts to PDGF, basic fibroblast growth factor and insulin-like growth factor-1. Previous work has shown that in pulmonary hypertension there is an increase in the levels of several mitogens such as ET-1, PDGF, thrombin, angiotensin II and 5-HT (Rose *et al,* 1986; Ono and Voelkel, 1991; Giaid *et al*, 1993). In this chapter, we have determined the effect of hypoxia in combination with these mitogens to determine the proliferative response of BPAF and BMAF. We chose to compare the response with that of systemic vascular cells from the mesentery since previous work has suggested that hypoxia affects ion exchange (potassium currents) differently in mesenteric and pulmonary artery cells (Weir and Archer, 1995).

4.2 METHODS

BPAF and BMAF were grown in 24-well plates as described in Chapter 2.3.1. Following growth arrest, cells were either transferred to a hypoxic environment of 2% O_2 or left in normoxic 21% O_2 conditions. After a 6h preincubation to allow the cell culture medium to become hypoxic, cells were stimulated with various agonists for 24h, [^{3}H]thymidine being added for the final 4h. The uptake of [^{3}H]thymidine was measured as in Chapter 2.6.

4.3 RESULTS

4.3.1 Effect of hypoxia on serum-stimulated proliferation of BPAF and BMAF cells.

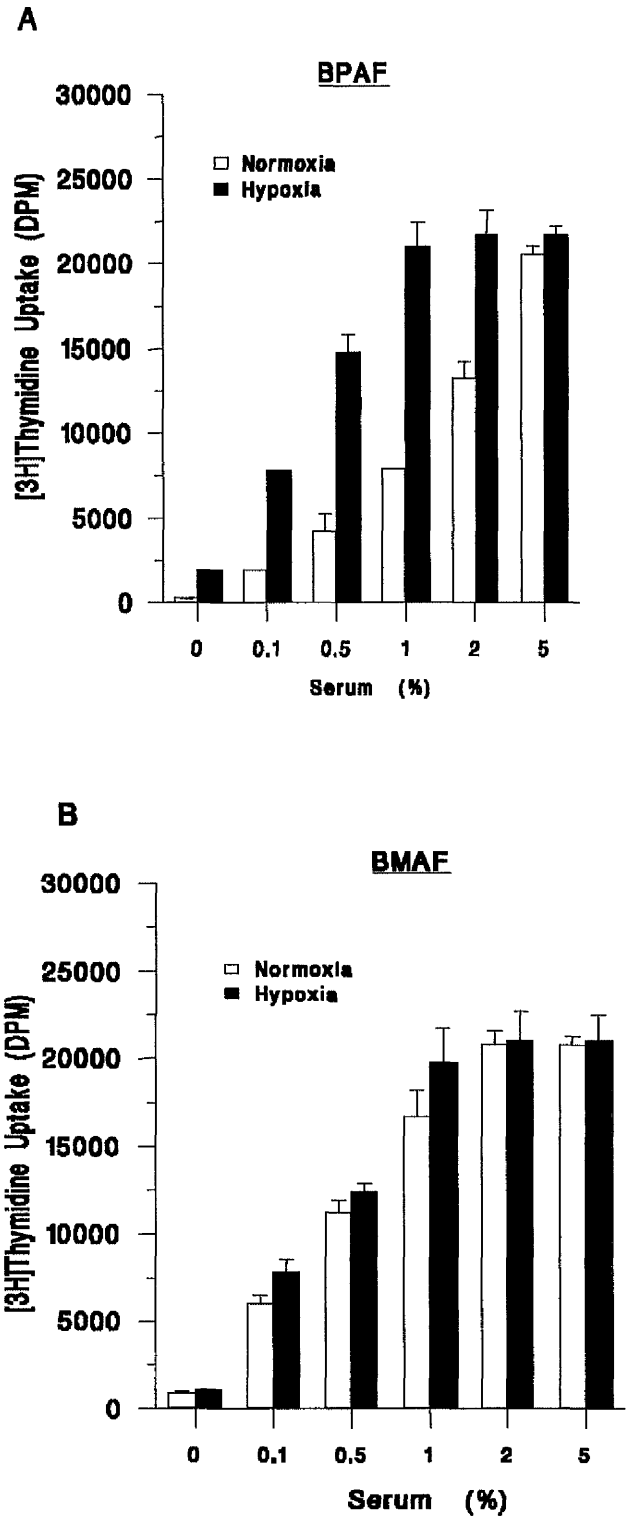
In the previous chapter it was shown that growth arrested BPAF cells proliferated in response to acute hypoxia in the absence of mitogens (Chapter 3.4). To test the effects of hypoxia on proliferating bovine fibroblasts, growth-arrested BPAF were exposed to 2% O_2 for 6h to make the cells hypoxic and then stimulated with 5% serum for 24h. [^{3}H]Thymidine uptake was used as a measure of proliferation. These cells were compared with those grown under normoxic conditions. In addition, growth of fibroblasts derived from the pulmonary artery were compared to those from the mesenteric artery. Serum concentrations as low as 0.1% were enough to stimulate proliferation of both BPAF and BMAF (Figure 4.1) by 6.6-fold and 5.5-fold respectively. Hypoxia significantly enhanced the effect of serum in BPAF cells by

about 6-fold ($p<0.001$). In contrast, there was no effect of hypoxia in growth arrested or serum-stimulated BMAF cells (Figure 4.1B).

Figure 4.1 Effect of Hypoxia on Serum-Induced Proliferation of BPAF and BMAF cells.

BPAF (A) and BMAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. Cells were either maintained in a normoxic environment for a further 6h or were placed in a hypoxic incubator (2% O_2) for 6h. The cells were then stimulated for 24h with a range of serum concentrations as indicated in the graph. The cells were then allowed to grow in either normoxic or hypoxic conditions for a period of 24h. Cell growth was assessed by [^{3}H]thymidine uptake. Values shown are the mean ± S.D. for four replicate wells from the same experiment. The experiment was repeated in cells derived from four different animals.

FIGURE 4.1


A
BPAF
30000
25000
20000
15000
10000
5000
0
[3H]Thymidine Uptake (DPM)
Normoxia
Hypoxia
0
0.1
0.5
1
2
5
Serum (%)
B
BMAF
30000
25000
20000
15000
10000
5000
0
[3H]Thymidine Uptake (DPM)
Normoxia
Hypoxia
0
0.1
0.5
1
2
5
Serum (%)

4.3.2 Effect of hypoxia on PDGF-stimulated proliferation of BPAF and BMAF cells

To study the effect of a specific growth factor on the proliferation of BPAF and BMAF cells in culture, similar experiments were carried out as described in the previous section. PDGF concentrations as low as 3ng/ml was enough to stimulate proliferation of both BPAF and BMAF cells (Figure 4.2) by 5.5-fold and 6-fold, respectively. Hypoxia significantly enhanced the effect of PDGF in BPAF cells by 2-fold ($p<0.001$). In contrast, there was no effect of hypoxia in growth-arrested or PDGF-stimulated BMAF cells (Figure 4.2B). There appeared to be no effect of hypoxia at 30ng/ml PDGF. This was due to the cells being 100% confluent in the plates and by the end of the experiments the cells had become contact inhibited.

Figure 4.2 Effect of Hypoxia on PDGF-Stimulated Proliferation of BPAF and BMAF Cells.

BPAF (A) and BMAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. Cells were either maintained in a normoxic environment for a further 6h or were placed in a hypoxic incubator (2% O_2) for 6h. The cells were then stimulated for 24h with a range of PDGF concentrations as indicated in the graph. The cells were then allowed to grow in either normoxic or hypoxic conditions for a period of 24h. Cell growth was assessed by [^{3}H]thymidine uptake. Values shown are the mean ± S.D. for four replicate wells from the same experiment. The experiment was repeated in cells derived from four different animals.

FIGURE 4.2

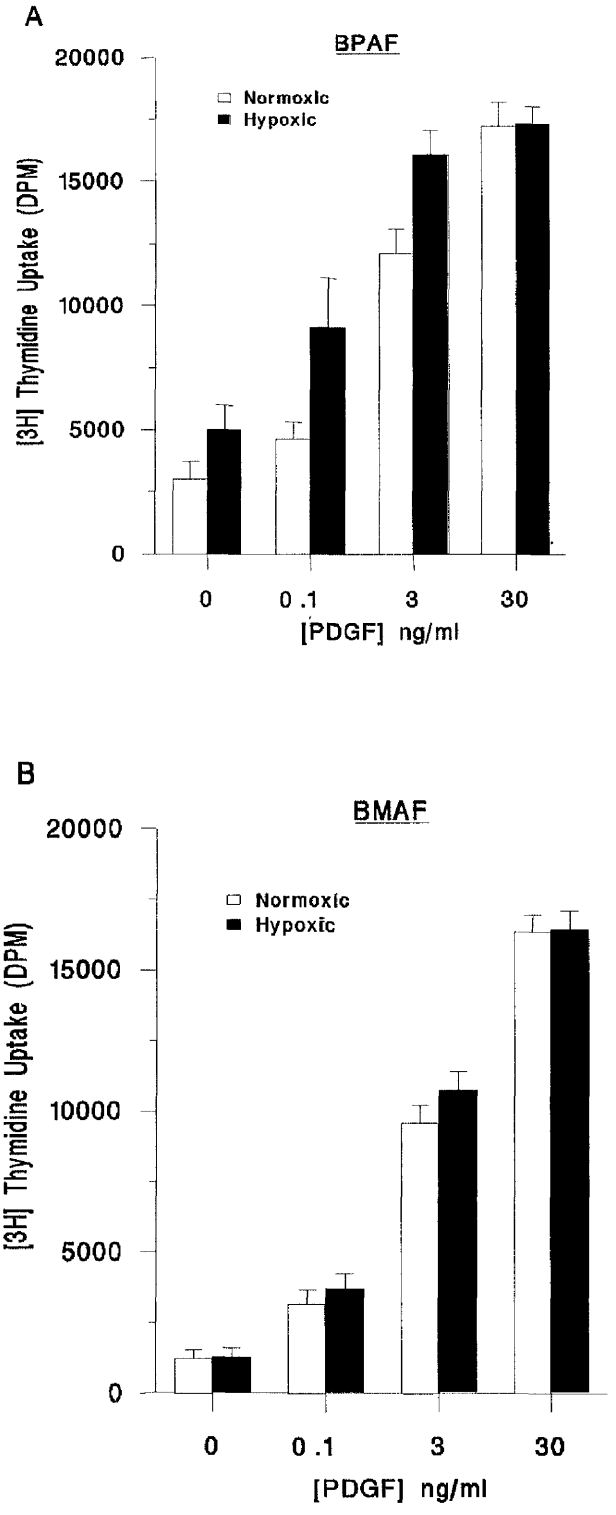

A
BPAF
Normoxic
Hypoxic
[3H] Thymidine Uptake (DPM)
20000
15000
10000
5000
0
0
0.1
3
30
[PDGF] ng/ml
B
BMAF
Normoxic
Hypoxic
[3H] Thymidine Uptake (DPM)
20000
15000
10000
5000
0
0
0.1
3
30
[PDGF] ng/ml

FIGURE 4.2a

[PDGF] ng/ml	Normoxic BPAF	S.D.	Hypoxic BPAF	S.D.	p value
0	**3127**	**703**	**4979**	**1063**	**<0.01**
	1627	362	2542	540	<0.01
	2595	296	4131	879	<0.01
	4692	1036	7471	1612	<0.01
0.1	**4615.8**	**697**	**9113**	**1979**	**<0.001**
	2327	326	4611	989	<0.001
	3853	1072	7572	811	<0.001
	6926	460	13670	2971	<0.001
3	**12089**	**1102**	**16058**	**982**	**< 0.01**
	6188	521	8239	521	<0.01
	10053	942	13370	831	<0.01
	16172	1552	20523	1465	<0.01
30	**17186**	**893**	**17287**	**679**	**>0.05**
	8823	466	8872	371	>0.05
	14372	739	14421	559	>0.05
	25742	1371	25931	1011	>0.05

[PDGF] ng/ml	Normoxic BMAF	S.D.	Hypoxic BMAF	S.D.	p value
0	**1233**	**311**	**1256**	**359**	**>0.05**
	641	161	637	179	>0.05
	1021	249	1049	302	>0.05
	1827	461	1891	402	>0.05
0.1	**3176**	**563**	**3704**	**549**	**>0.05**
	1619	256	1897	279	>0.05
	2676	471	3112	436	>0.05
	4687	813	5446	823	>0.05
3	**9577**	**621**	**10729**	**657**	**>0.05**
	4893	326	5101	332	>0.05
	8977	522	8726	466	>0.05
	14401	911	15336	863	>0.05
30	**16324**	**632**	**16419**	**643**	**>0.05**
	8261	319	8319	319	>0.05
	13611	531	13692	576	>0.05
	24491	952	24618	896	>0.05

Figure 4.2a Effects of Hypoxia on PDGF-Stimulated Proliferation of BPAF and BMAF Cells

Figure 4.2a summarises data from 4 different animals. Data in bold is represented in Figure 4.2. Each data point represents the mean of 4 individual wells from the same experiment.

4.3.3 Effect of hypoxia on G-protein coupled receptor (GPCR) agonist-stimulation of BPAF and BMAF cells.

There are several GPCR's that may be involved in re-modelling of the pulmonary artery exposed to hypoxic conditions (Sudhir *et al,* 1993; Malek and Izumo, 1992). The effect of these mitogens on growth of cells maintained in a hypoxic environment was investigated as described in Chapter 2.4. To test the effects of hypoxia on proliferating bovine fibroblasts using selected GPCR agonists, growth-arrested hypoxic and normoxic cells were stimulated with varied concentrations of ET-1, AII, 5-HT and Thrombin. [^{3}H]Thymidine uptake was used to measure DNA synthesis.

ET-1 did not significantly stimulate either cell type above basal levels in normoxic conditions ($p>0.05$). The stimulation of BPAF cells in hypoxic conditions was no greater than that seen with hypoxia alone (Figure 4.3). Similarly, AII did not significantly stimulate either cell type above basal levels in normoxic conditions ($p>0.05$). The stimulation of BPAF cells in hypoxic conditions was no greater than that seen with hypoxia alone (Figure 4.4). In contrast, 5-HT caused a significant increase in [^{3}H]thymidine uptake under both normoxic and hypoxic conditions reaching a maximum effect at 10μM where the increase was by 13.5-fold and 20-fold, respectively (Figure 4.5) ($p<0.001$). The effect was greater in conditions of hypoxia for the BPAF cells than for the BMAF cells. 5-HT caused a dose-dependent increase in cell replication for both types of cell, but whereas hypoxia shifted the dose response curve (but not maximal response) to the left on the BPAF cells, this effect was not seen in the BMAF cells. Thrombin caused a marked increase in replication under both normoxic and hypoxic conditions by 4-fold and 7-fold, respectively (Figure 4.6) ($p<0.001$). The effect was greater in hypoxic conditions for the BPAF

cells than for the BMAF cells by about 1.7-fold. Thrombin caused a dose-dependent increase in cell replication for both types of cell, but whereas hypoxia shifted the dose response curve (but not maximal response) to the left on the BPAF cells, this effect was not seen in the BMAF cells.

Figure 4.3 Effect of Hypoxia on ET-1-Stimulated Proliferation of BPAF and BMAF Cells.

BPAF (A) and BMAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. Cells were either maintained in a normoxic environment for a further 6h or were placed in a hypoxic incubator (2% O_2) for 6h. The cells were then stimulated for 24h with a range of ET-1 concentrations as indicated in the graph. The cells were then allowed to grow in either normoxic or hypoxic conditions for a period of 24h. Cell growth was assessed by [^{3}H]thymidine uptake. Values shown are the mean ± S.D. for four replicate wells from the same experiment. The experiment was repeated in cells derived from four different animals.

FIGURE 4.3

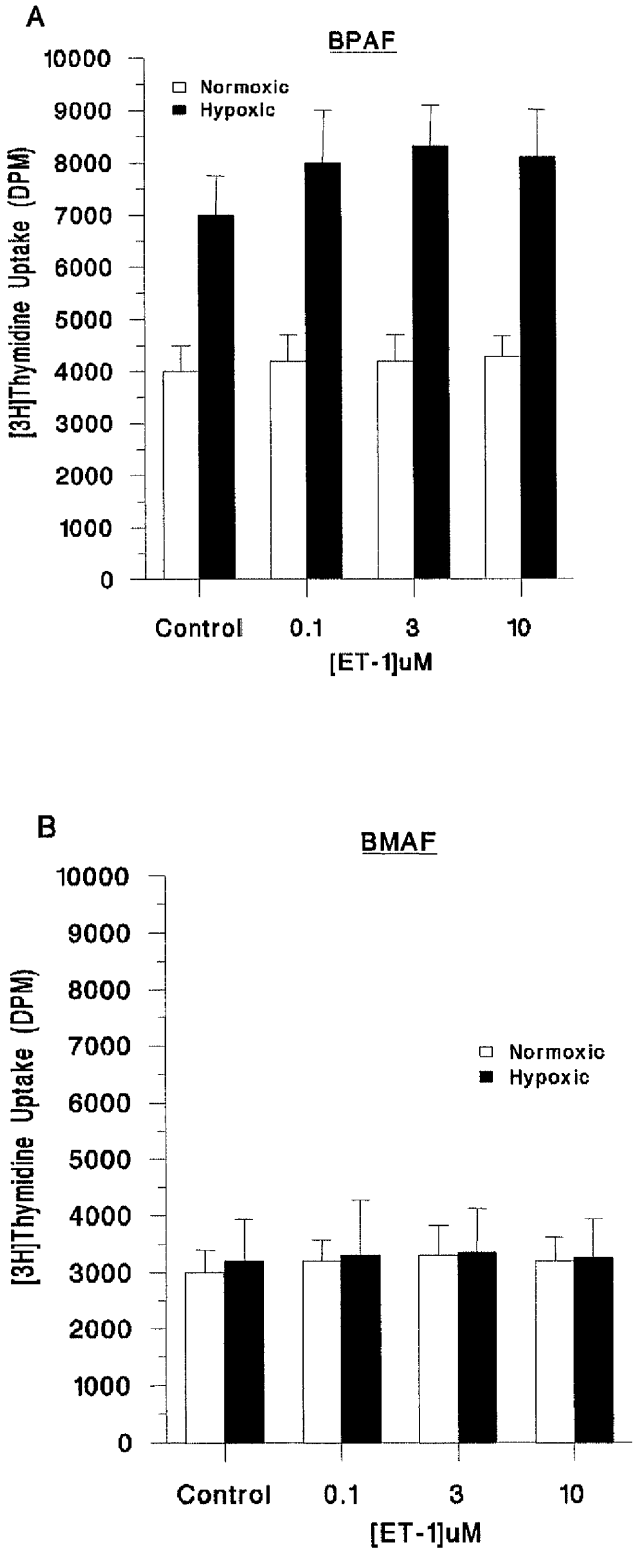

A
BPAF
Normoxic
Hypoxic
[3H]Thymidine Uptake (DPM)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
0.1
3
10
[ET-1]uM
B
BMAF
Normoxic
Hypoxic
[3H]Thymidine Uptake (DPM)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
0.1
3
10
[ET-1]uM

Figure 4.4 Effect of Hypoxia on AII-Stimulated Proliferation of BPAF and BMAF cells.

BPAF (A) and BMAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. Cells were either maintained in a normoxic environment for a further 6h or were placed in a hypoxic incubator (2% O_2) for 6h. The cells were then stimulated for 24h with a range of AII concentrations as indicated in the graph. The cells were then allowed to grow in either normoxic or hypoxic conditions for a period of 24h. Cell growth was assessed by [^{3}H]thymidine uptake. Values shown are the mean ± S.D. for four replicate wells from the same experiment. The experiment was repeated in cells derived from four different animals.

FIGURE 4.4

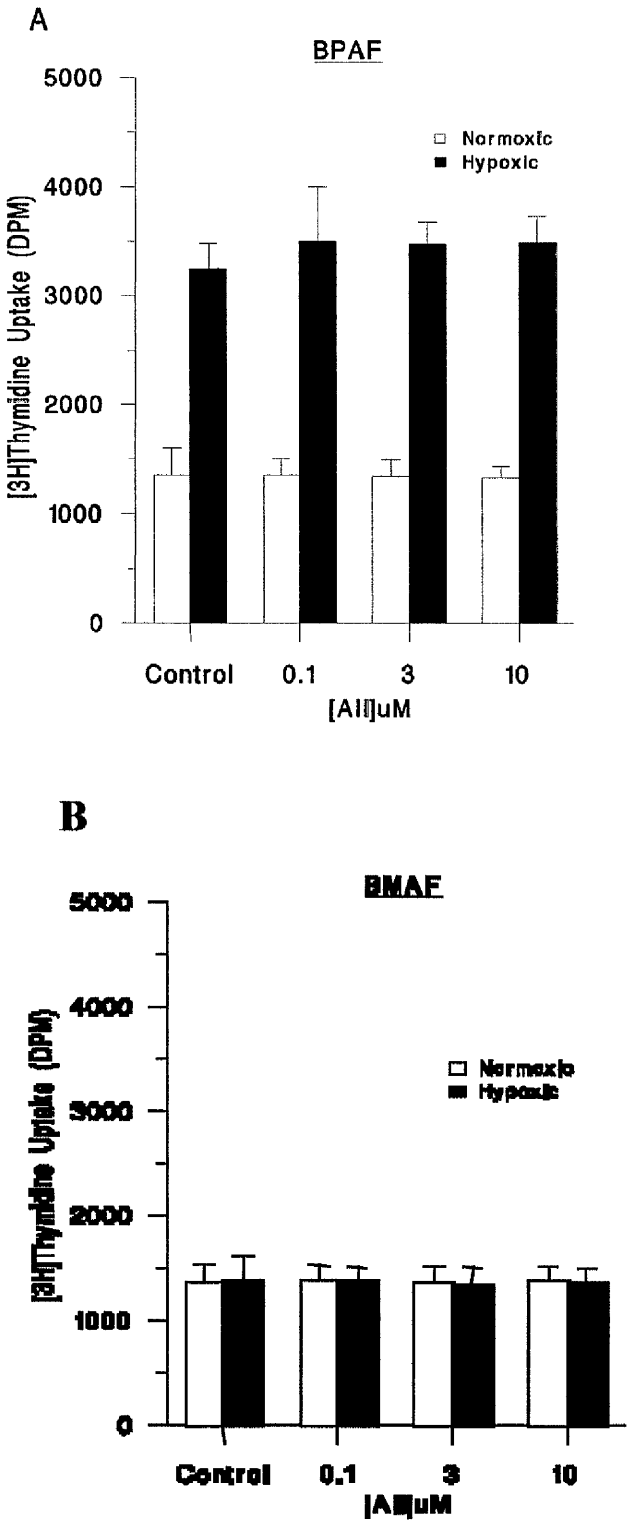

A
BPAF
5000
4000
3000
2000
1000
0
[3H]Thymidine Uptake (DPM)
Normoxic
Hypoxic
Control
0.1
3
10
[All]uM
B
BMAF
5000
4000
3000
2000
1000
0
[3H]Thymidine Uptake (DPM)
Normoxic
Hypoxic
Control
0.1
3
10
[All]uM

Figure 4.5 Effect of Hypoxia on 5-HT-Stimulated Proliferation of BPAF and BMAF Cells.

BPAF (A) and BMAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. Cells were either maintained in a normoxic environment for a further 6h or were placed in a hypoxic incubator (2% O_2) for 6h. The cells were then stimulated for 24h with a range of 5-HT concentrations as indicated in the graph. The cells were then allowed to grow in either normoxic or hypoxic conditions for a period of 24h. Cell growth was assessed by [^{3}H]thymidine uptake. Values shown are the mean ± S.D. for four replicate wells from the same experiment. The experiment was repeated in cells derived from four different animals.

FIGURE 4.5

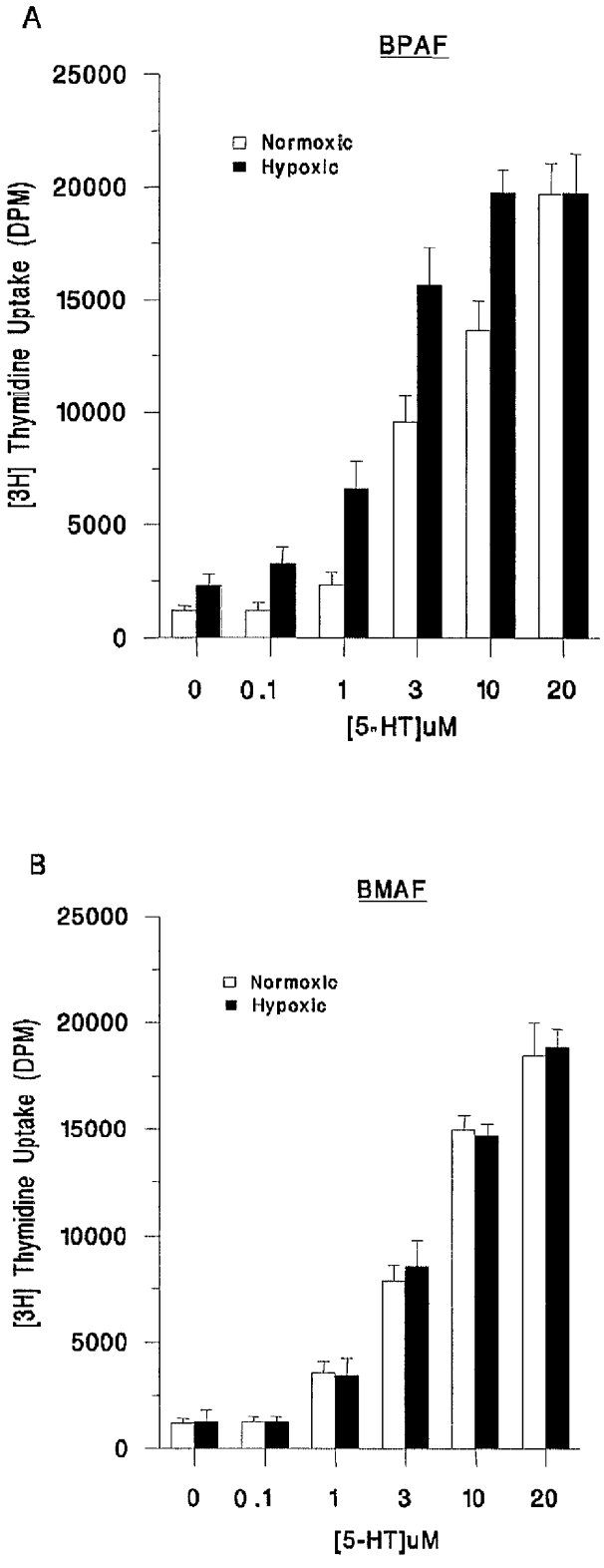
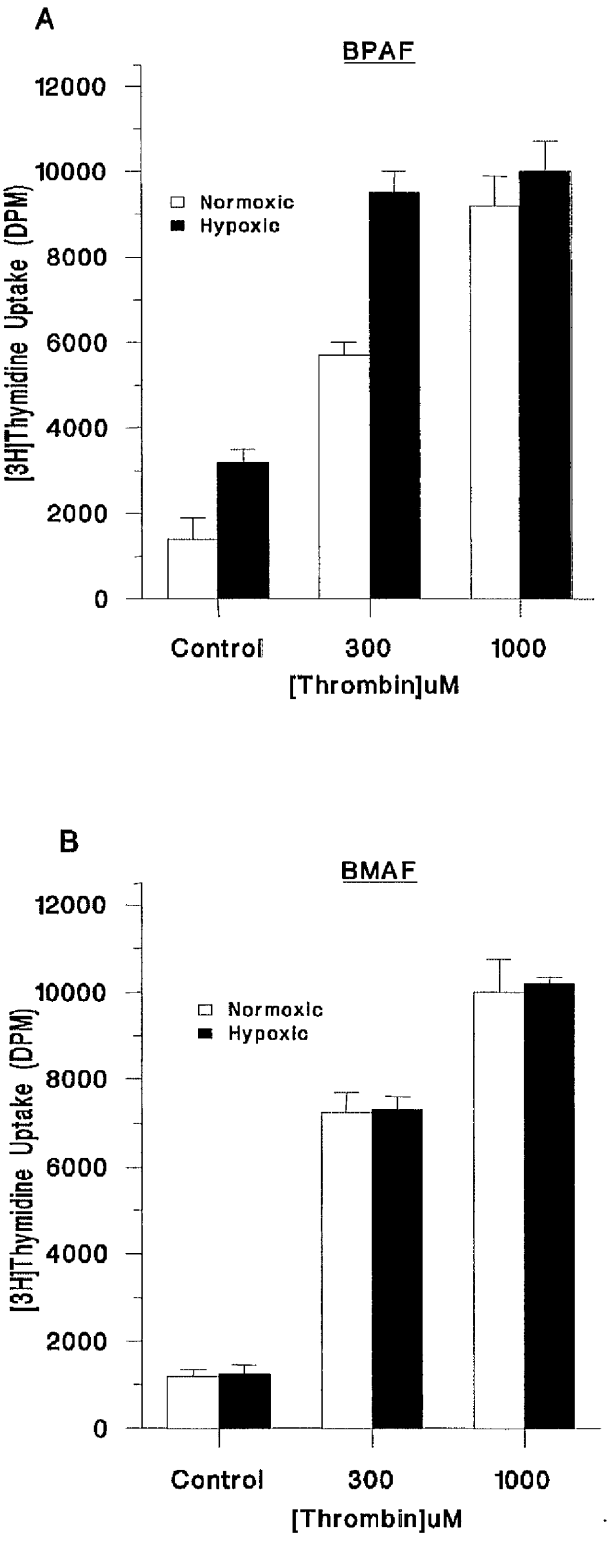

A
BPAF
25000
20000
15000
10000
5000
0
[3H] Thymidine Uptake (DPM)
Normoxic
Hypoxic
0
0.1
1
3
10
20
[5-HT]uM
B
BMAF
25000
20000
15000
10000
5000
0
[3H] Thymidine Uptake (DPM)
Normoxic
Hypoxic
0
0.1
1
3
10
20
[5-HT]uM

Figure 4.6 Effect of Hypoxia on Thrombin-Stimulated Proliferation of BPAF and BMAF Cells.

BPAF (A) and BMAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. Cells were either maintained in a normoxic environment for a further 6h or were placed in a hypoxic incubator (2% O_2) for 6h. The cells were then stimulated for 24h with a range of thrombin concentrations as indicated in the graph. The cells were then allowed to grow in either normoxic or hypoxic conditions for a period of 24h. Cell growth was assessed by [^{3}H]thymidine uptake. Values shown are the mean ± S.D. for four replicate wells from the same experiment. The experiment was repeated in cells derived from four different animals.

FIGURE 4.6


A
BPAF
12000
10000
8000
6000
4000
2000
0
[3H]Thymidine Uptake (DPM)
Normoxic
Hypoxic
Control
300
1000
[Thrombin]uM
B
BMAF
12000
10000
8000
6000
4000
2000
0
[3H]Thymidine Uptake (DPM)
Normoxic
Hypoxic
Control
300
1000
[Thrombin]uM

4.4 Discussion

This chapter demonstrates that oxygen concentrations at the physiological concentration of 5%, which is similar to that observed in pulmonary hypertensive patients (Souhami & Moxham, 1998), increases the proliferation of BPAFs over and above the response of these cells to serum and specific growth factors and GPCR's. DNA synthesis was increased in BPAF's in response to serum, as well as to PDGF, 5-HT and thrombin and this was enhanced further under hypoxic conditions. This effect was specific to cells derived from the pulmonary artery since cells derived from the systemic circulation were non-responsive to an acute hypoxic challenge. We have previously shown ET-1 to be a potent agonist on pulmonary artery fibroblasts in the rat (Peacock *et al*, 1992), but this did not affect bovine pulmonary artery cells in either hypoxic or normoxic conditions. This is consistent with our previous studies where we showed that ET-1 does not promote replication in bovine cells (Peacock and Aidulis, 1994). We have been unable to find any other study where BMAF cell replication has been studied.

As well as changes observed here in hypoxia-stimulated proliferation between BPAF and BMAF cells, other workers have observed physiological differences between the two types of cell. For example, there is also a differential effect of hypoxia on K^+ channel activation in isolated pulmonary and mesenteric artery cells (Weir and Archer 1995).

Increased pulmonary artery fibroblast proliferation in cells derived from the cow in response to hypoxia was accompanied by changes in specific protein kinase C (PKC) isozymes, suggesting a signal transduction pathway linking hypoxia with

proliferation in the adventitial fibroblast (Stenmark and Mecham, 1997). These results agree with the findings by others that oxygen concentrations within the physiological range may control proliferation indirectly by altering the activity of a signalling pathway that regulates the response to growth factors.

It is possible that the additive effect of hypoxia over and above normal growth responses leading to increased proliferation triggers a novel signalling pathway, or enhances the activation of an existing pathway leading to the increased proliferation. Hypoxia may also cause the increase in transcription of proteins involved in cell growth, for example, VEGF or iNOS. The gene for this has already been show to be turned on in hypoxic conditions (Feldkamp *et al,* 1999) There is evidence in other cell types of specific genes switched on by hypoxia such as EPO (Firth *et al*, 1994). It is possible that pulmonary artery cells contain genes not present in BMAF cells allowing both contraction and replication to hypoxia and that these phenomena are coupled (Scott and Peacock, 1995).

Chapter 5

Effects of Acute Hypoxia on IP_3 Generation in BPAF and BMAF cells.

5.1 INTRODUCTION

In the previous chapter, it was shown that exposing quiescent cells to an oxygen concentration at a physiological level of hypoxia could increase proliferation. In addition, it also enhances the effect of some growth factors and GPCR agonists. This effect appeared to be specific for pulmonary derived fibroblast cells since this did not occur in the same cell type derived from the mesenteric artery. If low oxygen concentrations can control cell proliferation then it might be expected that the activity of signalling enzymes, and pathways, might be altered in the cellular response to hypoxia. The aim of the studies described in this chapter was to investigate the possible importance of the second messenger IP_3 to the hypoxic response of cells.

The actions of many GPCR agonists and growth factors are mediated by the hydrolysis of PIP_2 catalysed by PLC (Berridge and Irvine, 1989). This hydrolysis releases IP_3, which mobilises Ca^{2+} from the endoplasmic reticulum, and DAG, which activates some PKC isoforms (Hug and Sarre, 1993).

Previous work has shown that hypoxia increases cytosolic $[Ca^{2+}]_i$ in pulmonary artery smooth muscle cells. It was not known whether or not the source of this increased Ca^{2+} was intracellular or extracellular, since previous reports have not specifically looked at the effect of hypoxia in IP_3 mediated generation of Ca^{2+} (Cornfield *et al*, 1993). The Ca^{2+} may be derived from intracellular stores or by Ca^{2+} influx mediated by voltage-dependent or receptor-operated Ca^{2+} channels (Berridge, 1993b). In general, transient increases in $[Ca^{2+}]_i$ are mediated by IP_3 release of Ca^{2+} from intracellular stores whilst sustained increases in $[Ca^{2+}]_i$ are mediated by Ca^{2+} influx through activation of the Ca^{2+} channels (Chadwick *et al,* 1990). Minami *et al* (1991) examined AII induced changes in IP_3 levels in VSMC from hypertensive rats and

found a rapid increase in IP_3 levels peaking at 5 seconds. Similar results were demonstrated by Yokokawa *et al* (1995), who studied the response of endothelial cells from hypertensive rats to ET-1. Transient increases in IP_3 levels were also reported when frogs were subjected to hypoxic/anoxic conditions (Holden and Storey, 1997).

In the present study, IP_3 levels were measured in quiescent BPAF and BMAF cells exposed acutely to hypoxic conditions in the presence and absence of various agonists, which have been shown to be increased in patients with pulmonary hypertension.

5.2 METHODS

BPAF and BMAF cells were plated out into 24-well plates and quiesced as described in chapter 2.3.1. Cells were then either maintained in normoxic conditions or exposed to 2% O_2 for 6h prior to addition of agonists. IP_3 mass was measured as described in 2.6.

5.3 RESULTS

5.3.1 Typical standard curve for IP_3 generation.

Figure 5.1 shows a typical standard curve generated using a competitive binding assay with bovine adrenal glands which was used to find the unknown levels of IP_3 in stimulated cell extracts.

Figure 5.1

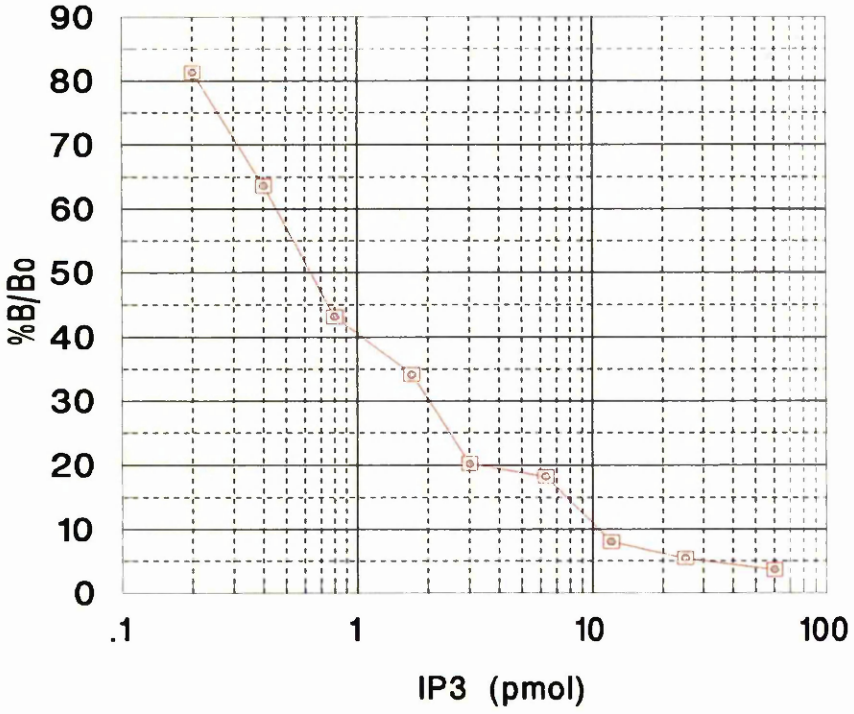


Figure 5.1 Typical Standard Curve used to Measure IP_3 Generation

5.3.2 Effect of agonists and hypoxia on IP_3 generation

The effect of hypoxia on agonist-stimulated IP_3 generation was investigated. Figure 5.2 demonstrates the effect of PDGF and hypoxia on IP_3 generation. Under normoxic conditions PDGF (30ng/ml) did not significantly alter IP_3 mass in BPAF (A) or in BMAF (B) cells. Hypoxia alone had no significant effect on IP3 mass in either BPAF of BMAF cells. However, when BPAF cells were incubated under hypoxic conditions, there was a sharp increase in IP_3 mass in the presence of PDGF (maximal at 10s) by 2-fold ($p<0.001$) which fell to basal levels at 30s (Figure 5.2A). In contrast, there was no change in IP_3 mass when the BMAF cells were grown in hypoxia (Figure 5.2B). Figure 5.3 demonstrates the effect of ET-1 and hypoxia on IP_3 generation. Unlike that of PDGF, ET-1 (10μM) caused a rise in IP_3 mass in the BPAF cells (Figure 5.3A) in normoxic conditions (also maximal at 10s). However, hypoxia did not significantly increase this stimulation of IP_3 above normoxic values ($p>0.05$). In the BMAF cells (Figure 5.3B), ET-1 did not give rise to IP_3 generation in either normoxic or hypoxic conditions. Figure 5.4 demonstrates the effect of thrombin and hypoxia on IP_3 generation. Under normoxic conditions thrombin (300nM) did not significantly alter IP_3 mass in BPAF (Figure 5.4A) or in BMAF (Figure 5.4B) cells. When BPAF cells were incubated under hypoxic conditions, there was a sharp increase in IP_3 mass in the presence of thrombin (maximal at 10s) by 2.6-fold ($p<0.001$). In contrast, there was no change in IP_3 mass when the BMAF cells were grown in a hypoxic environment.

Figure 5.2

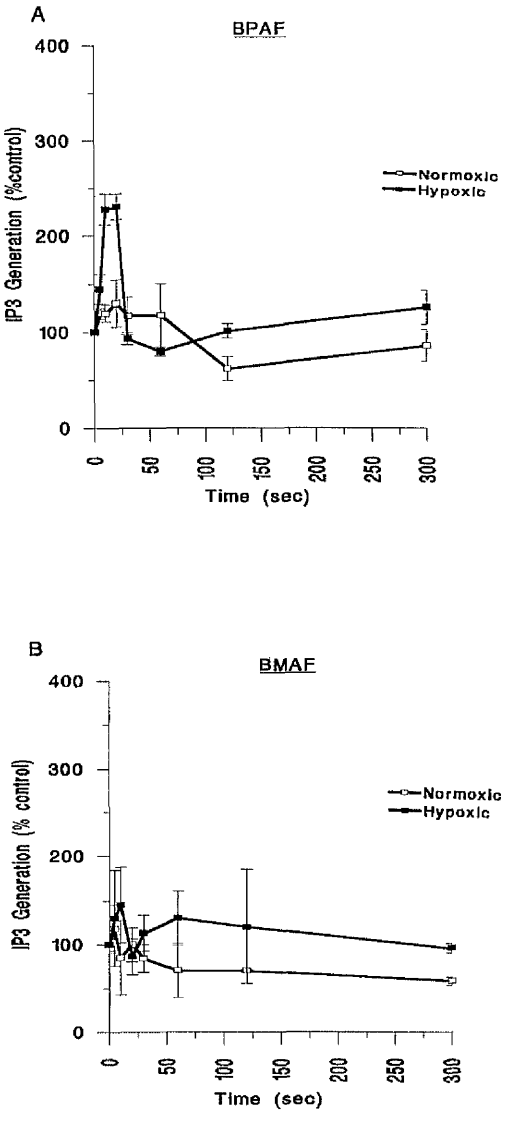


Figure 5.2 Effect of PDGF and Hypoxia on IP_3 Generation in BPAF and BMAF Cells.

BPAF (A) and BMAF (B) cells were grown to 90% confluency in 24-well plates and then serum-starved for a further 48h. Cells to be made hypoxic were placed in the hypoxic incubator for 6h and normoxic controls kept in a normal CO_2 incubator. The reaction was started by the addition of PDGF (30ng/ml). The reaction was then stopped by the addition of 25µl ice-cold 10% PCA (w/v) at 0, 5, 10, 20, 30, 60, 120 and 300s time points. After lysis, the resulting supernatants were assayed for IP_3 content as described in Chapter 2.7.4. Values shown are mean±S.D. for four replicate wells from the same experiment. The experiment was repeated in cells from four different animals.

Figure 5.3

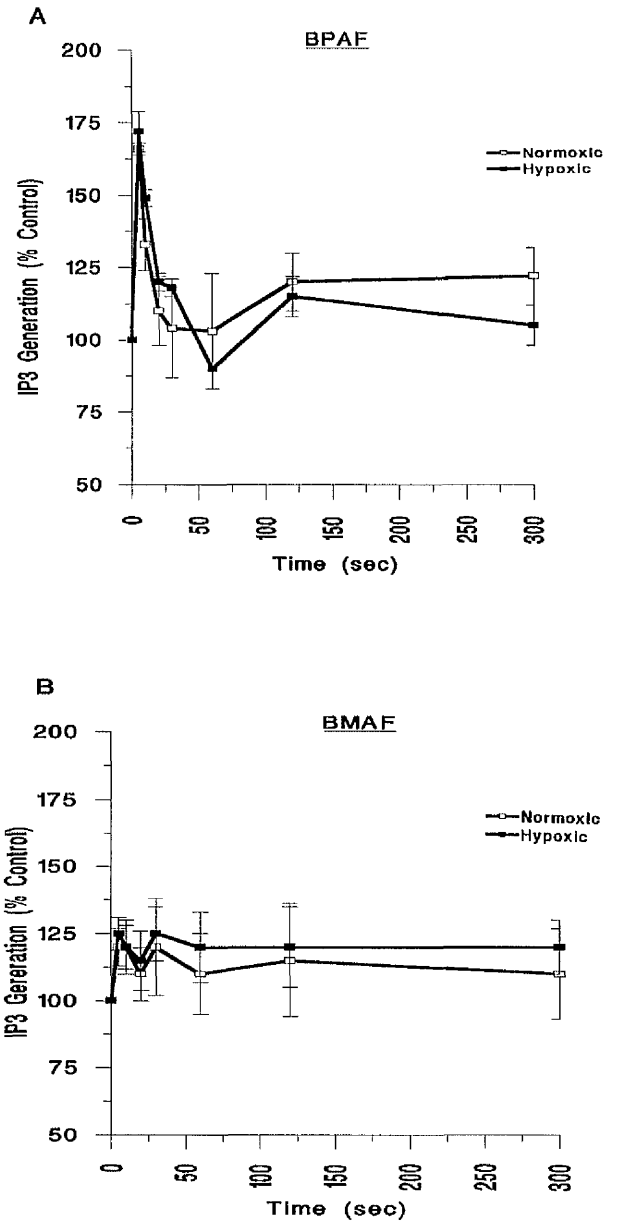


Figure 5.3 Effect of ET-1 and Hypoxia in IP_3 Generation in BPAF and BMAF Cells.

BPAF (A) and BMAF (B) cells were grown to 90% confluency in 24-well plates and then serum-starved for a further 48h. Cells to be made hypoxic were placed in the hypoxic incubator for 6h and normoxic controls kept in a normal CO_2 incubator. The reaction was started by the addition of ET-1 (10μM). The reaction was then stopped by the addition of 25μl ice-cold 10% PCA (w/v) at 0, 5, 10, 20, 30, 60, 120 and 300s time points. After lysis, the resulting supernatants were assayed for IP_3 content as described in Chapter 2.7.4. Values shown are mean±S.D. for four replicate wells from the same experiment. The experiment was repeated in cells from four different animals.

Figure 5.4

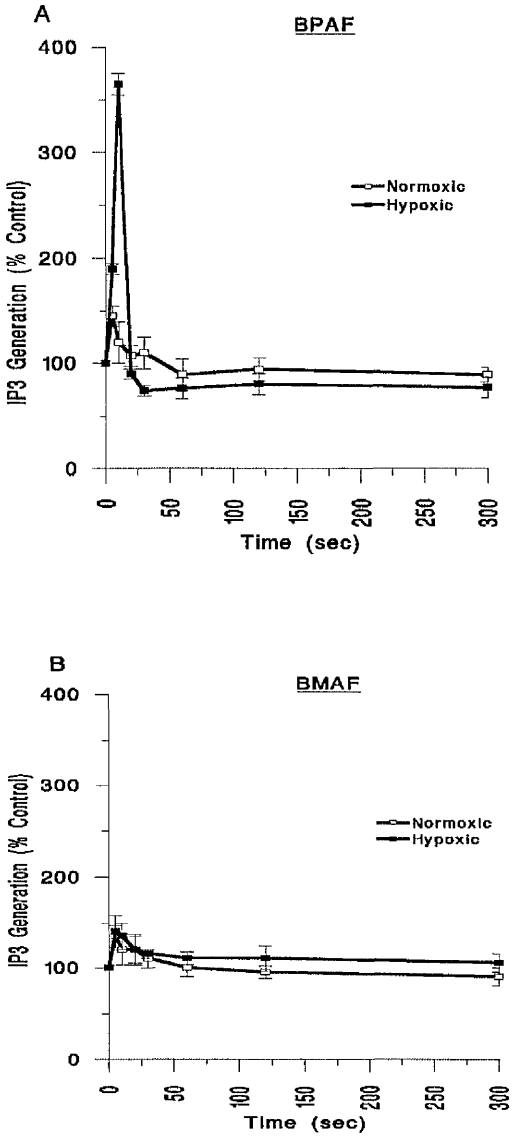


Figure 5.4 Effect of Thrombin and Hypoxia in IP_3 Generation in BPAF and BMAF Cells.

BPAF (A) and BMAF (B) cells were grown to 90% confluency in 24-well plates and then serum-starved for a further 48h. Cells to be made hypoxic were placed in the hypoxic incubator for 6h and normoxic controls kept in a normal CO_2 incubator. The reaction was started by the addition of thrombin (300nM). The reaction was then stopped by the addition of 25µl ice-cold 10% PCA (w/v) at 0, 5, 10, 20, 30, 60, 120 and 300s time points. After lysis, the resulting supernatants were assayed for IP_3 content as described in Chapter 2.7.4. Values shown are mean±S.D. for four replicate wells from the same experiment. The experiment was repeated in cells from four different animals.

5.4 DISCUSSION

In this chapter, IP_3 mass was analysed in both BPAF and BMAF cells in response to various agonists in cells grown under normoxic or hypoxic conditions. Hypoxia alone did not increase basal IP_3 mass but stimulated an early rise in IP_3 mass when cells were stimulated with PDGF or thrombin. IP_3 mass was found to be increased in response to ET-1 in the BPAF cells irrespective of the oxygen environment. BMAF cells did not display this IP_3 peak in response to hypoxia with any of the agonists used, and basal levels of IP_3 in the cells made hypoxic were not altered.

In the previous chapter, hypoxia was found to increase cell proliferation over and above that of agonists alone, for example, in response to PDGF and thrombin. We have shown for the first time that there is a rapid rise in IP_3 mass when hypoxic cells are stimulated with agonists, which also stimulate proliferation, such as thrombin. In the case of ET-1, which does not stimulate BPAF proliferation, there is no additional increase in IP_3 mass with hypoxia. In contrast to the pulmonary cells, fibroblast cells from the mesenteric artery did not have an increase in IP_3 mass generation under hypoxic conditions.

The difference in response to hypoxia between pulmonary cells and mesenteric cells may be due to an hypoxia-activated pathway which is either present only in pulmonary artery cells, or is repressed in mesenteric artery cells. Activation of cell-surface receptors and their associated tyrosine kinase and phospholipases (C-γ, A_2 or D) leads to the generation of the lipid second messengers IP_3 and DAG (Kohno *et al*, 1992; Nishizuka, 1992). IP_3 promotes mobilisation of intracellular Ca^{2+} stores (Short *et al*, 1993) whereas DAG is an endogenous activator of PKC (Nishizuka 1992). Hence, an increase in IP_3, caused in BPAF by hypoxia, may accompany an increase in

DAG or PKC activity. Although DAG was not examined in this chapter, it has been reported that hypoxia can increase PKC activity in PASMC (Xu *et al*, 1994). Thus, PKC activity could be enhanced in two ways; firstly, by increasing DAG and, secondly, by IP_3 induced release of Ca^{2+} from intracellular Ca^{2+} stores. Increases in intracellular Ca^{2+} can directly activate some isoforms of PKC (Ho *et al*, 1988). Interestingly, activators of PKC have been shown to promote many of the same cellular responses (contraction, hypertrophy and proliferation) attributed to hypoxia (Orton *et al*, 1990; Starksen *et al*, 1986; Dempsey *et al*, 1991). In addition, induction and phosphorylation of PKCα has been observed in response to hypoxia in lung fibroblast cells (Hasen *et al*, 1996). This isoform is involved in the PKC-dependent regulation of the erythropoetin (EPO) gene, which is induced by hypoxia (Fandrey *et al*, 1994). If this explanation of the activation of hypoxia is true it is not clear why the same pathway is not activated in mesenteric cells. The differences within the two cell types studied here do, however, show the same trends as seen in the circulation of humans *in vivo* when given hypoxic challenges (Von Euler and Liljestrand, 1946). That is, the pulmonary arteries contract and cellular proliferation ensues, whereas in the systemic circulations this does not happen.

It is likely that pulmonary artery cells contain genes allowing both contraction and replication to hypoxia and that these phenomena are coupled (Scott and Peacock, 1995). It is tempting to speculate that the phosphoinositide pathway might be involved downstream from any hypoxic sensor and responsible for vasomotor - cell growth coupling, that is, IP_3 causes increased intracellular Ca^{2+} resulting in cellular contractions but also augmentations of DAG-induced cell replication.

Chapter 6

Effects of Acute Hypoxia on the Stimulation of the Stress-Activated protein kinases in Pulmonary Artery Fibroblasts

6.1 INTRODUCTION

As described in previous chapters, serum-deprived BPAF cells proliferate under hypoxic conditions, and the second messenger, IP_3, is increased in these hypoxic cells. However, the intracellular signalling mechanisms by which hypoxia stimulates cell proliferation in pulmonary artery fibroblasts need to be further characterised.

Of particular importance in growth factor signalling is the activation of a series of kinase cascades involving multiple serine/threonine and tyrosine phosphorylation events (Malarkey *et al*, 1995). One such pathway is the MAP kinase cascade (Davis, 1993) which stimulates the phosphorylation of several intracellular substrates, such as $p90^{rsk}$ and Elk-1, believed to play a role in initiating mitogenesis (Ballou *et al*, 1991; Gille *et al*, 1995). MAP kinases are proline-directed protein kinases that mediate the effects of numerous extracellular stimuli on a wide array of biological processes, such as cellular proliferation, differentiation and death (Datta *et al*, 1999). MAP kinase is also regulated in a cell cycle-dependent fashion and is required for cells to enter S-phase (Tamemoto *et al*, 1992). For example, in Chinese hamster lung fibroblasts, Pages and co-workers (1993) found that activation of both p42 and p44 isoforms of the 'classical' p42/p44 Map kinases were essential for proliferation. Similarly, using human airway smooth muscle cells (HASM), Orsini and co-workers (1999) found that proliferation in this cell type required prolonged activation of p42/p44 MAP kinase. Not only have these forms of MAP kinase been shown to be activated in response to mitogens, but also have been shown to be activated in response to hypoxia. For example, in rat cardiac myocytes, components of the MAP kinase cascade, including Raf-1, MEK and MAP kinase, have been shown to be activated in response to low oxygen conditions (Seko *et al*, 1996). However, these responses are very small when

compared to mitogen stimulation of these pathways. Other workers (Richard *et al*, 1999), again using Chinese hamster lung fibroblasts, found that p42/p44 MAP kinase phosphorylated hypoxia-inducible Factor 1α (HIF-1α) and enhanced the transcriptional activity of HIF-1.

In recent years, protein serine/threonine kinases related to the 'classical' forms of MAP kinase have been identified, and are referred to as the stress-activated protein kinases (SAPK) (Kyriakis, *et al*, 1994). These kinases, consisting of at least two homologues, c-Jun N-terminal kinase (JNK) and p38 MAP kinase (Han *et al*, 1994; Rouse *et al*, 1994; Raingeaud *et al*, 1995), are strongly activated by environmental stress including heat shock and UV irradiation, cytokines such as tumour necrosis factor (TNF), interleukin-1 (IL-1), toxins such as lipopolysaccharide (LPS) and hypoxia (Han *et al*, 1994; Rouse *et al*, 1994; Raingeaud *et al*, 1995; Verheij *et al*, 1996; Lee *et al*, 1994b). Others have shown that the JNK cascade is a key pathway by which agonists stimulate DNA synthesis in primary cultures of rat hepatocytes (Auer *et al*, 1998) whilst a role for p38 has been shown to increase myocardial cell growth and gene expression (Zechner *et al*, 1997), and also is involved in the proliferation of adventitial fibroblasts from chronically hypoxic calves (Das *et al*, 1999). Since these kinase pathways play a critical role in responding to cellular stress and promoting cell growth and survival (Widmann *et al*, 1999), we have investigated the effect of hypoxia upon the p38 KAP kinase, JNK and p42/p44 MAP kinase signalling enzymes.

6.2 METHODS

6.2.1 Cell Culture

Bovine lungs from adult cows were obtained from the local abattoir and pulmonary artery fibroblasts from the freshly excised lung tissue were harvested as described in Chapter 2.3.2. The identity of fibroblast cells was confirmed by immunohistochemical stains as described in Chapter 2.3.3.

6.2.2 Growth of cells in a hypoxic environment

A humidified temperature controlled incubator (LEEC model GA156 Colwick, Nottingham, UK) was used as a hypoxic chamber as described in Chapter 2.4.1.

6.2.3 Solid-Phase JNK Assay

JNK activity was measured by a solid-state kinase assay with GST-c-Jun as the substrate (Chapter 2.8.5). Phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography.

6.2.4 Solid-Phase p38 MAP kinase Assay

p38 MAP kinase activity was measured by a solid-state kinase assay utilising GST-MAPKAP kinase-2 as the substrate (Chapter 2.8.6).

6.2.5 p42/44 MAP kinase phosphorylation and activation

MAP kinase phosphorylation status was assessed by Western blotting using retardation on SDS-PAGE gels as a marker of phosphorylation (Chapter 2.8.4). Activity was assayed in solubilised cell lysates using the EGF receptor peptide $EGF^{660-681}$ as substrate (Chapter 2.8.7). The phosphorylated peptide was separated from other products by ion-exchange chromatography on Whatman p81 paper and quantified by liquid scintillation counting.

6.2.6 Statistics

Results are expressed as the mean ± S.D. Statistical analysis was undertaken as reported in Chapter 2.10.

6.3 RESULTS

6.3.1 Effect of hypoxia on JNK and p38 MAP kinases

Hypoxia is an extremely common physiological stressor. To investigate the effects of hypoxia on the stress-activated signalling pathways, BPAF cells were exposed to 2% oxygen for various times. Cell lysates were used to study the phosphorylation of GST-c-jun (JNK activity) or GST-MAPKAPK-2 (p38 activity).

Figure 6.1 shows the effect of hypoxia on JNK activity over a 24 h period. After a lag period of up to 3h a large increase in JNK activity was observed in response to hypoxia which peaked at approximately 6h before decreasing slowly towards basal values for the remainder of the time course. Stimulation of JNK activity under hypoxic conditions at the 6h time point was 10.5-fold increased over basal activity, and was at least as great as that induced by 0.5M sorbitol, a known activator of JNK.

Figure 6.2 shows the effect of hypoxia on p38 MAP kinase activity over a 30h time period. It was found that hypoxia could also stimulate p38 MAP kinase activity. Like JNK, maximum activity was reached by 6h before returning to basal between by 12h (Figure 6.2A). However, in contrast to JNK activity, a second peak of p38 MAP kinase activity was observed which was maximal between 18-24 h before returning to basal levels again by 30h (Figure 6.2B).

Figure 6.1

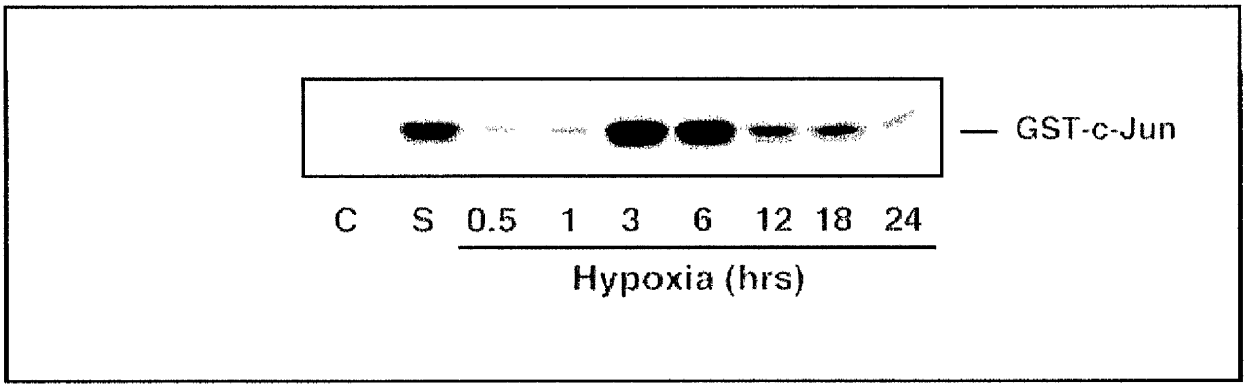


Figure 6.1 Acute Hypoxic Stimulation of JNK activity in BPAF Cells.

JNK activity as assessed by a solid state kinase assay as described in Chapter 2.8.5. Cells were incubated in hypoxic conditions for 0 (control, C), 0.5, 1, 3, 6, 12, 18 or 24h or with sorbitol (S) (0.5M for 30mins). Numbers represent time in h. The blot is a representative example from at least 4 independent experiments.

Figure 6.2

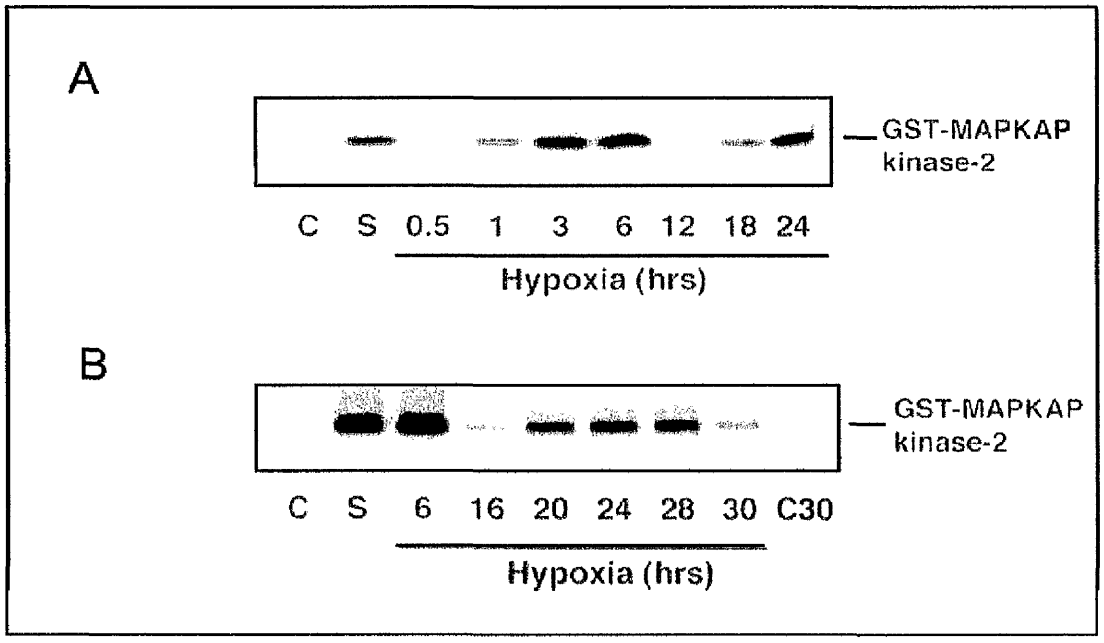


Figure 6.2 Acute Hypoxic Stimulation of p38 MAP Kinase Activity in BPAF Cells

p38 activity as assessed by a solid state kinase assay as described in Chapter 2.8.6. Cells were incubated in hypoxic conditions in the first instance (A) for 0, (Control, C), 0.5, 1, 3, 6, 12, 18 and 24h, or in the second instance 0, (Control, C), 6, 16, 20, 24, 28 and 30h or with sorbitol (0.5M for 30 mins). Numbers represent the time in hours. The blot is a representative example from at least 4 independent experiments.

6.3.2 Effect of hypoxia on p42/44 MAP kinase phosphorylation and activity

To determine whether the observed increases in p38 MAP kinase and JNK activities were components of a general response to hypoxic conditions by all MAP kinase family members, we analysed the effect of lowered O_2 on p42/44 MAP kinase activity. Figure 6.3 shows the effect of acute hypoxia upon the phosphorylation and activity of p42/44 MAP kinase. A decrease in the electrophoretic mobility of a protein was indicative of its phosphorylation. Cells incubated over a time course of up to 28h in 2% O_2 showed no change in electrophoretic mobility of p42 or p44 MAP kinase isoforms representative of their phosphorylation (Figure 6.3A). In contrast, stimulation of cells with 10% serum for 30min resulted in a decrease in the electrophoretic mobility of both p42 and p44 MAP kinase isoforms, representative of their phosphorylation (Figure 6.3A). In addition, there was a weak activation of MAP kinase activity (Figure 6.3B) in response to lowered O_2 with a maximum 1.8-fold increase in activity after 3h of hypoxic challenge. In contrast, 10% serum stimulated a substantial 6-8 fold increase in activity of MAP kinase.

Figure 6.3

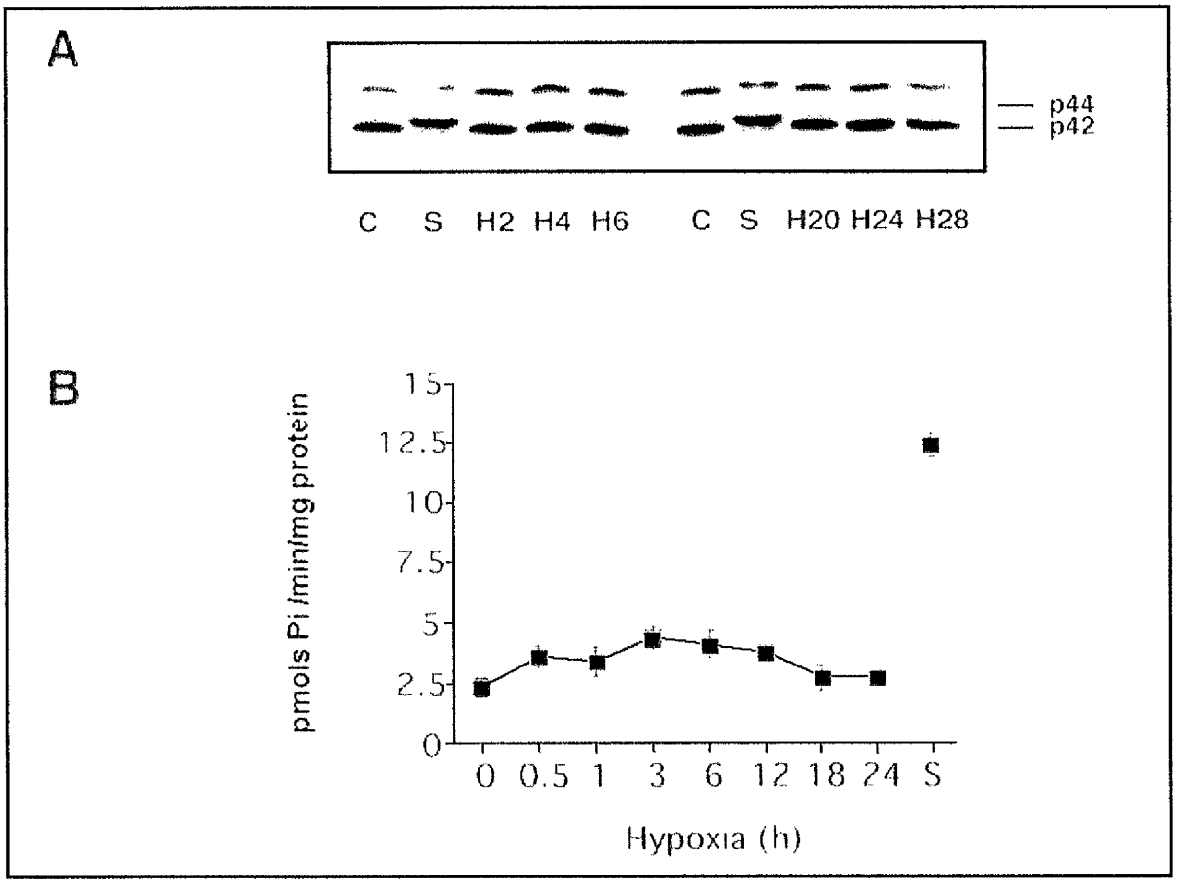


Figure 6.3 Acute Hypoxic Stimulation of p42/p44 MAP Kinase Activity in BPAF Cells

MAP kinase activity as assessed by western blot analysis (A) and an *in vitro* kinase assay (B) as outlined in Chapters 2.8.4 and 2.8.7, respectively. In A, cells were incubated in hypoxia (H) for 0 (Control, C), 2, 4, 6, 20, 24 and 48h or with Serum (S) (10%) for 30mins. Cell extracts were prepared and equal protein amounts subjected to SDS-PAGE and western blot analysis of p42/p44 MAP kinase isoforms. In B, cells were incubated in hypoxia for 0, 0.5, 1, 3, 6, 12, 18 and 24h or with serum (S) (10%) for 30mins. Cell extracts were prepared and used in an assay to measure MAP kinase activity. Numbers represent time in hours. Each experiment is representative of at least 3 independent experiments.

6.3.3 Inhibition of Hypoxic Stimulation of p38 MAP Kinase Activity by SB203580

Since whole cell lysates were utilised as a source of p38 MAP kinase in these activity assays, we utilised a specific inhibitor if this enzyme to ascertain whether p38 MAP kinase was the enzyme that phosphorylated MAPKAPK-2 in these assays. Following 6h of hypoxic challenge, a 9.9 ± 3.8 fold (n=3) phosphorylation of MAPKAPK-2 was observed (Figure 6.4). This response was approximately 50% of the phosphorylation of this substrate in response to sorbitol (18.2-fold ± 4.5; n=3). At the 24h time point, a 5-fold increase in phosphorylation of MAPKAPK-2 was observed in response to hypoxia (4.9 fold ± 2.8; n=3). SB 203580 (0.1μM), a specific inhibitor of p38 MAP kinase (Cuenda *et al*, 1995), prevented the rise in p38 MAP kinase activity caused by hypoxia and sorbitol (Figure 6.4).

Figure 6.4

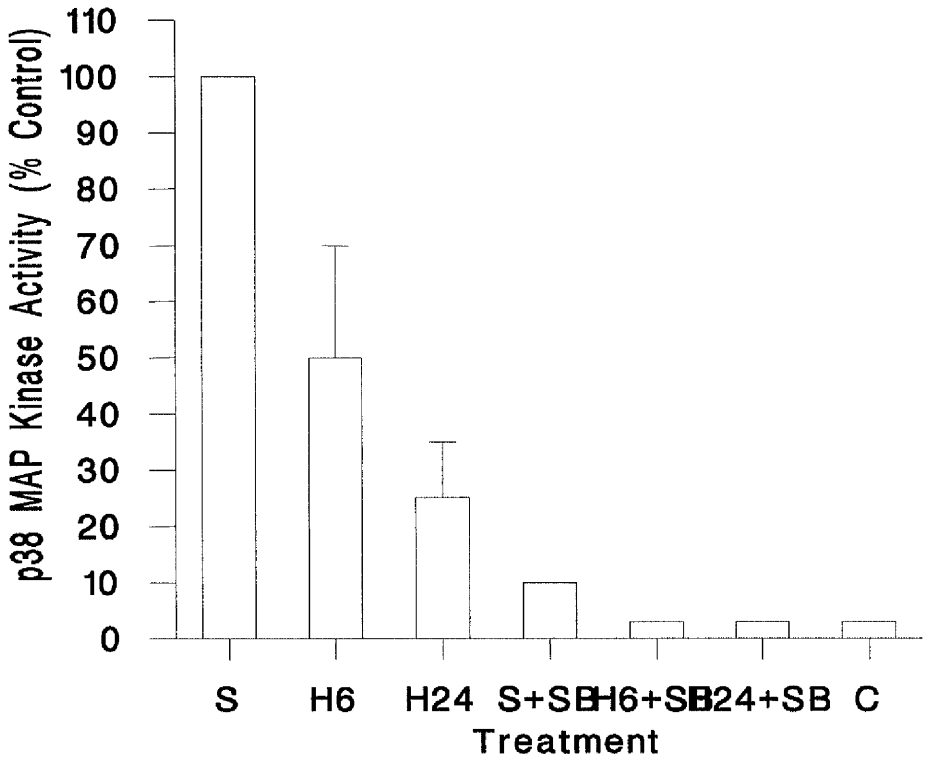


Figure 6.4 Inhibition of Hypoxic Stimulation of p38 MAP kinase by SB203580

p38 MAP kinase activity as assessed by an *in vitro* kinase assay as outlined in Chapter 2.8.6. Cells were incubated in hypoxic conditions (H) for 6 and 24h or with sorbitol (S; 0.5M) for 30mins. Whole cell lysates were prepared which were then used to measure MAPKAP-kinase 2 phosphorylation. The experiments were repeated following pre-incubation with 0.1μM SB203580 (SB), a specific p38 MAP kinase inhibitor, for 60min. Each value is the mean±S.D. of 3 independent experiments.

6.3.4 Reversal of the late phase of hypoxic-mediated p38 MAP kinase activity by re-oxygenation.

In order to determine if continuous hypoxia was required for the late phase (24h) of p38 MAP kinase activity observed in Figure 6.2, cells were re-oxygenated at different times after the initial stimulation period. Re-oxygenation prevented the hypoxic stimulated p38 MAP kinase activity after 6 and 12h (H6, H12) but not after 18 and 24h (H18, H24) (Figure 6.5), suggesting the requirement of a continued hypoxic environment for the initiation of the second phase of the response.

Figure 6.5

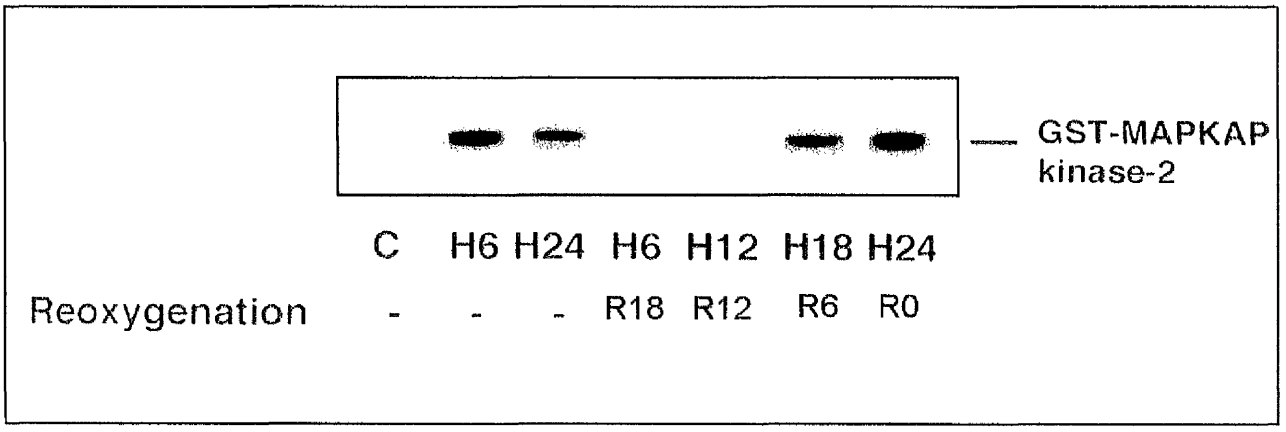


Figure 6.5 Reversal of the Late Phase of Hypoxic-Mediated p38 MAP Kinase Activity by Re-Oxygenation.

Effect of re-oxygenation on reversal of hypoxic mediated p38 MAP kinase activity by re-oxygenation. Cells were either exposed to hypoxic conditions for various times (H6 and H24) without subsequent re-oxygenation (R) or were exposed to hypoxia for 6, 12 and 18h followed by 18, 12 and 6h of re-oxygenation, respectively. Following this time, cell extracts were prepared and used to measure p38 MAP kinase activity as in Chapter 2.8.6. Each value is representative of at least three experiments (R = re-oxygenation).

6.4 DISCUSSION

In Chapter 4 we showed that acute hypoxia stimulated a significant increase in incorporation of [^{3}H]thymidine into DNA in fibroblast cells derived from the pulmonary artery. This suggests that hypoxia could increase the proliferation of these cells. The present chapter has focussed on the effect of acute hypoxia on the MAP kinase family of enzymes that have been implicated as playing an important role in cell growth and proliferation (Milanini *et al,* 1998). A study by Seko *et al* (1996), showed that the classical p42/p44 MAP kinase isoforms were involved in the initiation of hypoxia-mediated increases in cell growth and division in rat cardiac myocytes. In that study only a small stimulation of p42/44 MAP kinase activity was observed which agrees with the findings of this chapter. Whilst some studies have implicated stress-activated MAP kinases in the stress responses of cardiac myocytes to ischaemic damage and reperfusion (Morooka *et al*, 1995; Bogoyevitch *et al*, 1996; Bogoyevitch *et al*, 1995), this is the first study which implicates a role for stress-activated protein kinase activation in the response of cells derived from the pulmonary vasculature to hypoxia.

An initial peak of JNK and p38 MAP kinase activity was observed between 3 and 6 hours of hypoxic challenge which was comparable in magnitude to sorbitol stimulation, a well-recognised activator of the SAP kinases. This result was similar to that described by Conrad *et al* (1999) who observed maximal p38 MAP kinase phosphorylation after 6h exposure by hypoxia in PC12 cells. This is unlike the p38 MAP kinase and JNK response to other environmental stress agents and cytokines, where peak activation occurs between 30 and 60 min (Han *et al*, 1994; Rouse *et al*, 1994; Raingeaud *et al*, 1995; Verheij *et al*, 1996; Lee *et al*, 1994b). One possible

reason for this may be the time required to equilibrate the media to low oxygen conditions in this study. However, this time course of activation is similar to that observed in PC12 cells following serum-deprivation (Xia *et al*, 1995) and so it is possible that this is a physiologically relevant pattern of cellular activation of stress-activated protein kinases following certain stimuli, including hypoxia. We also found that the classical p42/p44 MAP kinases were stimulated in response to hypoxia, however, this was a weak stimulation in comparison to that seen with the stress-activated forms.

A second peak of p38 MAP kinase activity was observed under hypoxic conditions in pulmonary artery fibroblasts, occurring between 18 and 24h at a time when JNK activity was low in these cells. Significantly, this second phase of p38 MAP kinase activity coincided with the time required for the initiation of DNA synthesis in this cell type (Belham *et al*, 1996). This second phase also required the continued presence of a hypoxic environment suggesting a clear specificity in the signals required to initiate this response. The effect was blocked by the specific p38 MAP kinase inhibitor, SB 203580. To date only one other study has indicated activation of p38 MAP kinase in the absence of JNK and this is following ischemic challenge of cardiac myocytes (Bogoyevitch *et al*, 1996). Thus, under certain conditions related to the oxygen environment, p38 MAP kinase may be activated specifically.

Following the early peak in JNK and p38 MAP kinase activity in response to hypoxia, the activation of these enzymes decreases. It is possible that these are being inactivated by phosphatases. Interestingly, recent publications have shown specific mitogen-activated protein kinase phosphatases (MKP) which are induced by low oxygen conditions and are specific for SAPKs (Laderoute *et al*, 1999; Keyse*l*, 1999;

Thoedosiou *et al*, 1999). Such phosphatases may be responsible for the observed decreases in JNK and p38 MAP kinase activities.

Following the initial peak in p38 MAP kinase, there was a second peak in activity and it may be that the activity of this enzyme is regulated during the cell cycle in response to hypoxia. Since pulmonary artery cells are stimulated to contract and proliferate in response to hypoxia, it is possible that p38 MAP kinase plays a positive role in cell division by regulating cell cycle progression (Lavoie *et al,* 1996).

These studies have established that both JNK and p38 MAP kinase are activated in response to acute hypoxic stimulation of pulmonary artery-derived fibroblasts. Since the effects of pulmonary hypertension are a result of continuous, chronic exposure of cells to hypoxia, it is of interest to establish if these enzymes are also activated by long-term hypoxic exposure.

Chapter 7

Effects of Serum on Proliferation and Generation of MAP Kinases in Fibroblasts Harvested from Pulmonary Arteries from Chronically Hypoxic Rats

7.1 INTRODUCTION

In the previous chapters it was shown that acute hypoxia was capable of causing BPAFs to proliferate, either on its own or in conjunction with mitogens. In addition, it was also shown that hypoxia gave rise to increases in SAPK activity, p38 MAP kinase and JNK. Although hypoxia induces structural remodelling and vasoconstriction of the pulmonary arteries *in vivo*, the effects of hypoxia on systemic vascular cells are quite different (Welsh *et al,* 1998). As hypoxia does not result in vasoconstriction but rather induces vasodilation of systemic vessels (von Euler and Liljestrand, 1946), we have proposed that there are fundamental differences in oxygen sensing and cell signalling between systemic and pulmonary artery cells. Indeed, our own work has shown that hypoxia stimulates proliferation and second messenger production in pulmonary artery fibroblasts but does not affect fibroblasts derived from the mesenteric artery.

For the first time our studies have shown a link between vasoconstriction and remodelling since acute hypoxia was found to increase the rate of replication of pulmonary artery derived fibroblasts but not fibroblasts from the systemic circulation (Welsh *et al,* 1998). The biochemical and molecular mechanisms by which hypoxia stimulates proliferation of pulmonary artery fibroblasts are unknown. There is however, considerable evidence in the literature that p42/p44 MAP kinases, and the related stress-activated kinases, JNK and p38 MAP kinase, which have been implicated as key regulators of cell proliferation (Scott *et al,* 1998), can be activated in response to hypoxic stress (Robinson and Cobb, 1997; Takahashi and Berk, 1998). These modulate the phosphorylation, and hence activation status of transcription factors, and link transmembrane signalling with gene induction events in the nucleus (Robinson and Cobb, 1997).

In this current study we have investigated whether fibroblasts derived from the pulmonary and systemic circulations of chronically hypoxic rats, in which irreversible pulmonary vascular re-modelling has taken place (Hunter *et al,* 1974), exhibit differential replicative properties. In addition, we compared some of the cell signalling enzymes that were activated in pulmonary artery fibroblasts derived from chronically hypoxic rats with those derived from control rats kept under normoxic conditions. In particular, we investigated the roles of p42/p44 MAP kinase and stress-activated MAP kinases in hypoxia-induced pulmonary artery fibroblast proliferation and pulmonary vascular remodelling following chronic exposure to hypoxia.

7.1.1 Normobaric versus Hypobaric hypoxia

Hypoxic animal models (mainly rats and mice) have been used since the 1920's to study various environmental conditions and disease states. The animals were exposed to hypoxic environments by the use of environmental chambers (normobaric hypoxia; a decreased inspired O_2 at normal atmospheric pressure, or hypobaric hypoxia; a decreased inspired O_2 due to decreased atmospheric pressure) or by actual relocation of the animals to altitude where the inspired O_2 levels are lower than at sea level (Campbell, 1927a,b,c; Timiras *et al.*, 1957). These early investigations studied the acclimatisation of animals to altitude, or the O_2 tensions that would be experienced at altitude. Animals exposed to these hypoxic environments exhibited initial weight loss and alterations in certain organ weights (Camprell, 1935; Timiras *et al.*, 1957). The basic principles of normobaric and hypobaric hypoxia are described below.

7.1.2 Normobaric hypoxia

The normobaric method of chronic hypoxia used by most investigators has been adapted from a chamber described by Cryer and Bartley (1974). The O_2 concentration within the chamber is reduced from the normal 21% to ~10% (160mmHg to ~80mmHg O_2) by intermittent gaseous infusion of N_2. To prevent the build up of CO_2, humidity and other gases, the air is circulated through specific chemical absorbers.

7.1.3 Hypobaric hypoxia

Hypobaric hypoxia reduces the inspired O_2 content of the environment by reducing the atmospheric pressure within the chamber. This is the equivalent of taking the animals to high altitude. As the atmospheric pressure decreases, the partial pressure of the gaseous components of air decreases. Therefore, while the percentage of the gaseous components of the air remains the same (O_2 ~ 21% and N_2 ~ 78%), the effective partial pressure of inspired O_2 declines. Hypobaric hypoxia is achieved by withdrawing air from the chamber by use of a pump until the pressure within the chamber is equivalent to ~0.5 atmospheres, which reduces the inspired O_2 pressure from 160mmHg to 80 mmHg. The chamber is continuously flushed with room air to maintain conditions of low humidity and CO_2.

While normobaric and hypobaric chambers are both commonly used (mainly for the study of pulmonary hypertension), the hypoxic hypobaric chamber has proved to be more econimical and it is the type which was used in these studies. The Royal Hallamshire Hospital (Sheffield) designed and manufactured the hypoxic hypobaric chamber for Dr Margaret MacLean and her pulmonary research group based in the Institute of Biomedical and Life Sciences at the University of Glasgow. The chamber conforms to the high safety standards required by the Home Office.

Cells were derived from animals maintained in the hypobaric chamber and compared to those from animals from a normoxic environment. Differences in proliferation and signalling mechanisms between these cells have been studied.

7.2 METHODS

7.2.1 Chronic hypoxic rat model of pulmonary hypertension.

Pulmonary hypertensive rats were made hypoxic using the technique of MacLean *et al* (1995) (Chapter 2.5).

7.2.2 Pulmonary artery fibroblast cell culture

Rat pulmonary artery fibroblast (RPAF) and rat aortic fibroblast (RAF) cells were harvested from freshly excised lung tissue of control and chronically hypoxic animals as described in Chapter 2.3. All tissue was obtained from the laboratory of Dr. M. McLean (Dept. of Physiology and Pharmacology, University of Glasgow) and age-matched animals were always used as controls. The identity of the fibroblast cells was confirmed by immunocytochemical staining as described in Chapter 2.3.3.

7.2.3 Measurement of Serum-Stimulated [^{3}H]Thymidine Uptake in RPAF and RAF cells.

For these experiments, primary cultures of RPAF or RAF cells derived from normoxic or chronically hypoxic rats were transferred to 24-well plates. For acute hypoxic conditions we used an atmosphere of 2% O_2 (Chapter 3.2.3). Our previous studies (Chapter 3.2.3) have established that a 6h preincubation under these conditions of hypoxia is required to achieve the desired PO_2 levels (35mmHg) in the culture medium. Once established, we found the PO_2 levels to remain constant at 35mmHg (Welsh *et al,* 1998).

Proliferation of rat pulmonary artery fibroblasts was assessed by measuring the uptake of [^{3}H]thymidine (Chapter 2.6). Cells were grown to approximately 60% confluency in 24-well plates at 37^{o}C and then serum-starved for 24h. After this time 5% serum was added for a further 24h in the presence and absence of the p38 MAP kinase inhibitor, SB203580 (0.1μM) or of the MAP kinase inhibitor, U0126 (1μM). [^{3}H]Thymidine was added for the final 4h of the incubation. The reaction as stopped and assessed as in Chapter 2.6.

7.2.4 Assessment of Phosphorylation of p42/p44 MAP kinase and members of the Stress-Activated MAP kinase family

Cells were grown to approximately 90% confluency in 6-well plates at 37^{o}C and then serum starved for 24h. After this time the following additions were made as described below.

To determine the effect of serum on p38 MAP kinase, p42/p44 MAP kinase and JNK activity on RPAF and RAF cells from normoxic and chronically hypoxic cells, 5% serum was added to the cells for 0, 1, 2, 4, 8, 16, 24, 32 and 48h.

To determine the effect of SB203580 on p38 MAP kinase activity, RPAF and RAF cells from normoxic and chronically hypoxic animals, 0.1μM SB203580 was added to the cells for 1h prior to serum-stimulation for 2h.

To determine the effect of U0126 on p42/p44 activity on RPAF and RAF cells from normoxic and chronically hypoxic animals, 1μM U0126 was added to the cells for 1h prior to serum stimulation for 2h.

Assessment of the phosphorylation status of these enzymes was by SDS-PAGE gel electrophoresis and western blot analysis using phospho-specific antibodies (Chapter 2.8). In each case, the primary rabbit anti-dual phosphorylated, activated MAP kinase antibody for the protein of interest (p42/44, p38 and JNK) were utilised.

7.2.5 Statistics

Results are expressed as the mean ± S.D. Statistical analysis was undertaken as reported in Chapter 2.10.

7.3 RESULTS

7.3.1 Effect of Acute Hypoxia on RPAF and RAF cells Derived from Normoxic and Chronically Hypoxic Rats.

In Chapter 3 it was shown that BPAFs proliferate in response to hypoxia in the absence of any mitogens. To test the effect of acute hypoxia on rat cells, growth-arrested and serum-stimulated pulmonary artery-derived rat fibroblasts (RPAF) were exposed to 2% O_2 or left under normoxic conditions. The effect of acute hypoxia on the same cells derived from rats exposed to chronic hypoxia (2 weeks 18% O_2) was also investigated. Figure 7.1 shows the effects of acute hypoxia on proliferation of RPAF and RAF cells from rats either kept in normoxic conditions or kept in hypoxia for 2 weeks.

There was a 9.3-fold increase in [^{3}H]thymidine uptake when RPAF cells were cultured in the presence of 5% serum following serum-starvation for 24 h ($p<0.01$) (Figure 7.1A). When RPAF cells were simultaneously exposed to 5% serum and acute hypoxia, there was a 19.2-fold increase in the resultant level of DNA synthesis observed ($p<0.01$) in comparison to control, unstimulated cells (Figure 7.1A). Similarly, RAF cells showed a 9-fold increase in [^{3}H]thymidine uptake in response to serum (Figure 7.1B). In contrast to RPAF cells, however, RAF cells do not exhibit an enhanced level of DNA synthesis in response to acute hypoxia ($p>0.05$) (Figure 7.1B). Unlike pulmonary artery fibroblasts derived from cows, the same cells derived from rats do not respond to acute hypoxia alone (Figure 7.1A).

RPAF cells from chronically hypoxic rats exhibited an 18-fold increase in the level of DNA synthesis relative to cells from control rats ($p<0.01$) (Figure 7.1A and 7.2A). Further increase above the enhanced level of DNA synthesis observed in RPAF cells

from chronically hypoxic rats could not be achieved by exposing these cells to acutely hypoxic conditions ($p>0.05$) (Figure 7.2A). RAF cells showed similar levels of replication regardless of whether the explants were derived from normoxic or chronically hypoxic rats (639±47) (Figures 7.1B & 7.2B) (also Table 1: stimulation indices for pooled data for 8 different animals).

Table 7.1

	Control		5% Serum	
RPAF	**Normoxia**	**Acute Hypoxia**	**Normoxic**	**Acute Hypoxia**
Control	1	0.996 ± 0.09	7.39 ± 0.39	14.39 ± 0.76
Chronic Hypoxic	1	1.1 ± 0.97	14.76 ± 0.91	14.95 ± 0.87
RAF				
Control	1	0.97 ± 0.05	7.57 ± 0.56	7.62 ± 0.48
Chronic Hypoxic	1	0.96 ± 0.7	7.49 ± 0.95	7.51 ± 0.21

***Definition of abbreviations*: RPAF = Rat pulmonary artery fibroblasts, RAF = Rat aortic fibroblasts.**

Table 1 Stimulation Indices of DNA Synthesis for Control and Chronic Hypoxic Rat Fibroblast cells from the Pulmonary and Aortic Circulation Following Acute Hypoxic Challenge.

RPAF and RAF cells from control and chronically hypoxic animals were grown in normoxia to 60% confluency in 24-well plates then quiesced in serum-free medium for 24h before stimulation with 5% serum. The cells were then allowed to grow in either normoxia or transferred to an environment of 2% oxygen (PO_2 of 35mmHg) for a period of 24h. Cell growth was assessed by [^{3}H]thymidine uptake. Values shown are the stimulation indices (mean ± S.D.) for pooled data for 8 different animals for the 'typical' results shown in Figures 7.1 & 7.2.

Figure 7.1

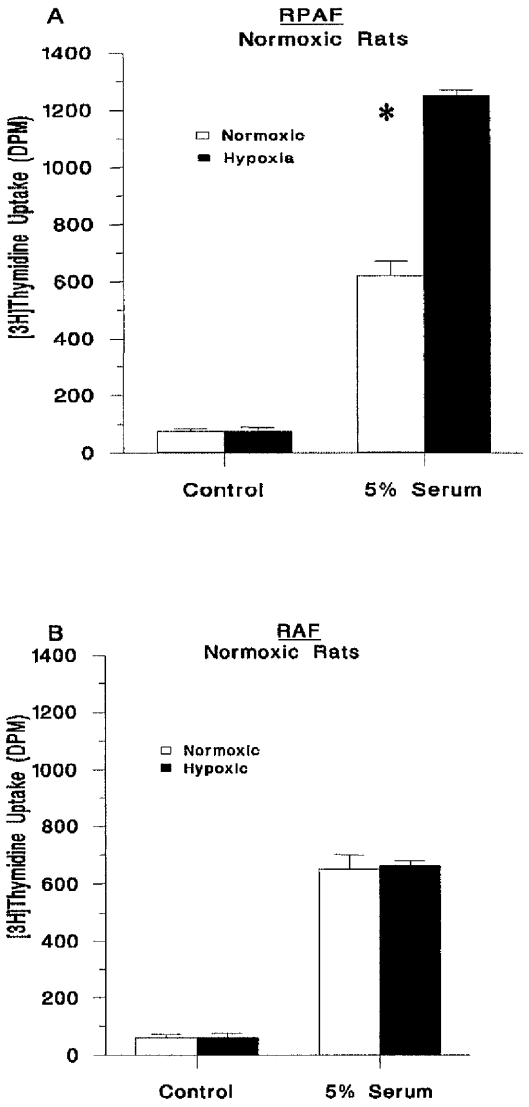


Figure 7.1 The Effect of Serum on [^{3}H]thymidine Uptake from Normoxic (□) and Acutely Hypoxic (■) RPAF and RAF cells.

RPAF (A) and RAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. Cells were either maintained in a normoxic environment for a further 6h or were placed in a hypoxic incubator (2% O_2, 35mmHg) for 6h. The cells were then stimulated with 5% serum as indicated in the graph and allowed to grow in either normoxic or hypoxic conditions for a period of 24h. The experiment was repeated with cells from 8 different animals and the results shown ate typical of those obtained. See table 7.1 for pooled results from all animal data. (DPM = Disintegrations per minute, * = values for hypoxia significantly greater than normoxia).

Figure 7.2

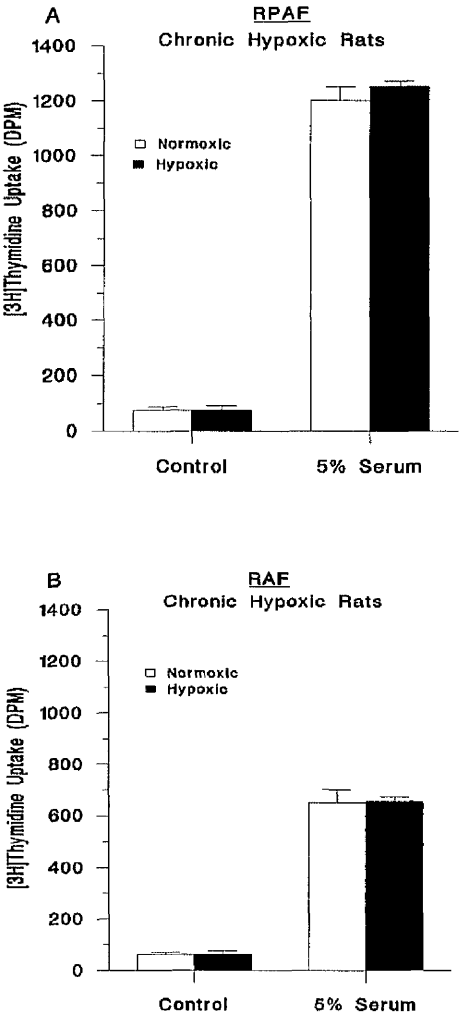


Figure 7.2 Effect of Serum on [^{3}H]thymidine Uptake in RPAF cells from Chronically Hypoxic Rats (□) and Fibroblasts from Chronically Hypoxic Rats exposed to Acute Hypoxia (■) and in RAF cells

RPAF (A) and RAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. Cells were either maintained in a normoxic environment for a further 6h or were placed in a hypoxic incubator (2% O_2, 35mmHg) for 6h. The cells were then stimulated with 5% serum as indicated in the graph and allowed to grow in either normoxic or hypoxic conditions for a period of 24h. The experiment was repeated with cells from 8 different animals and the results shown are typical of those obtained. See Table 7.1 for pooled results from all animal data. (DPM = Disintegrations per minute, * = values for hypoxia significantly greater than normoxia.

7.3.2 Differential p38 and p42/p44 MAP kinase phosphorylation in RPAF and RAF cells from normoxic and chronically hypoxic rats.

We have previously shown that exposure of BPAFs to acute hypoxia induces the activation of the SAPKs, JNK and p38 MAP kinase. We therefore investigated whether these signalling enzymes played a role in the enhanced proliferation and remodelling of fibroblasts observed after prolonged (14 days) exposure to chronic hypoxia. To do this, we have examined the activation of members of the MAP kinase family by detecting their dually phosphorylated (tyr/thr) forms by Western blotting using specific anti-active phosphokinase antibodies (Feng *et al,* 1999). Serum-stimulation of RPAF from control rats incubated under normoxic conditions showed a weak activation of p38 MAP kinase as indicated by its dual phosphorylation of its regulatory thr/tyr motif which peaked at between 1 and 4 h stimulation with serum (Figure 7.3A, upper panel). In contrast, p38 MAP kinase was found to be strongly constitutively active in RPAF cells derived from rats subjected to chronically hypoxic conditions, and this activity was further enhanced in the presence of serum, particularly in the period 16-48h post-stimulation (Figure 7.3A, upper panel). This increase in p38 MAP kinase phosphorylation was not due to an upregulation of the p38 protein expression, as Western Blot analysis using an antibody which detects total amounts of p38, revealed comparable levels of p38 MAP kinase expression in RPAF cells from chronically hypoxic and normoxic rats (Figure 7.3A, lower panel). RAF cells, however, despite expressing substantial levels of p38, did not show significant change in the phosphorylation of p38 MAP kinase in response to serum, nor was this activity modulated following chronic exposure of the rats to hypoxia (Figure 7.3B).

A similar pattern was observed for p42/p44 MAP kinase. Serum-induced p42/p44 MAP kinase (particularly the p42 MAP kinase isoform) activity in normoxic RPAF cells in a multiphasic manner (peaks at 1h and 24h), and RPAF from chronically hypoxic rats showed strong constitutive activity of p42/p44 MAP kinase (Figure 7.4A). Although, there appeared to be a slight upregulation in p42/p44 MAP kinase expression in these cells, this upregulation was not sufficient to explain the very high levels of kinase activity observed (Figure 7.4A). As with p38 MAP kinase, chronic exposure to hypoxia did not modulate p42/p44 MAP kinase activation in RAF cells, which already appeared to exhibit a considerable level of constitutive p42/p44 MAP kinase activity which was not significantly modulated by addition of serum (Figure 7.4B). There was a small increase in phosphorylation of JNK on the addition of serum to normoxic and hypoxic RPAF cells, however, there was no significant difference in the amount of phosphorylated JNK or amount of total JNK protein following serum-stimulation of hypoxic RPAF relative to that observed in RPAF from control normoxic rats (Figure 7.5A). There was no significant change in the phosphorylation of JNK in response to serum in RAF cells derived from either normoxic or hypoxic rats (Figure 7.5B).

Figure 7.3

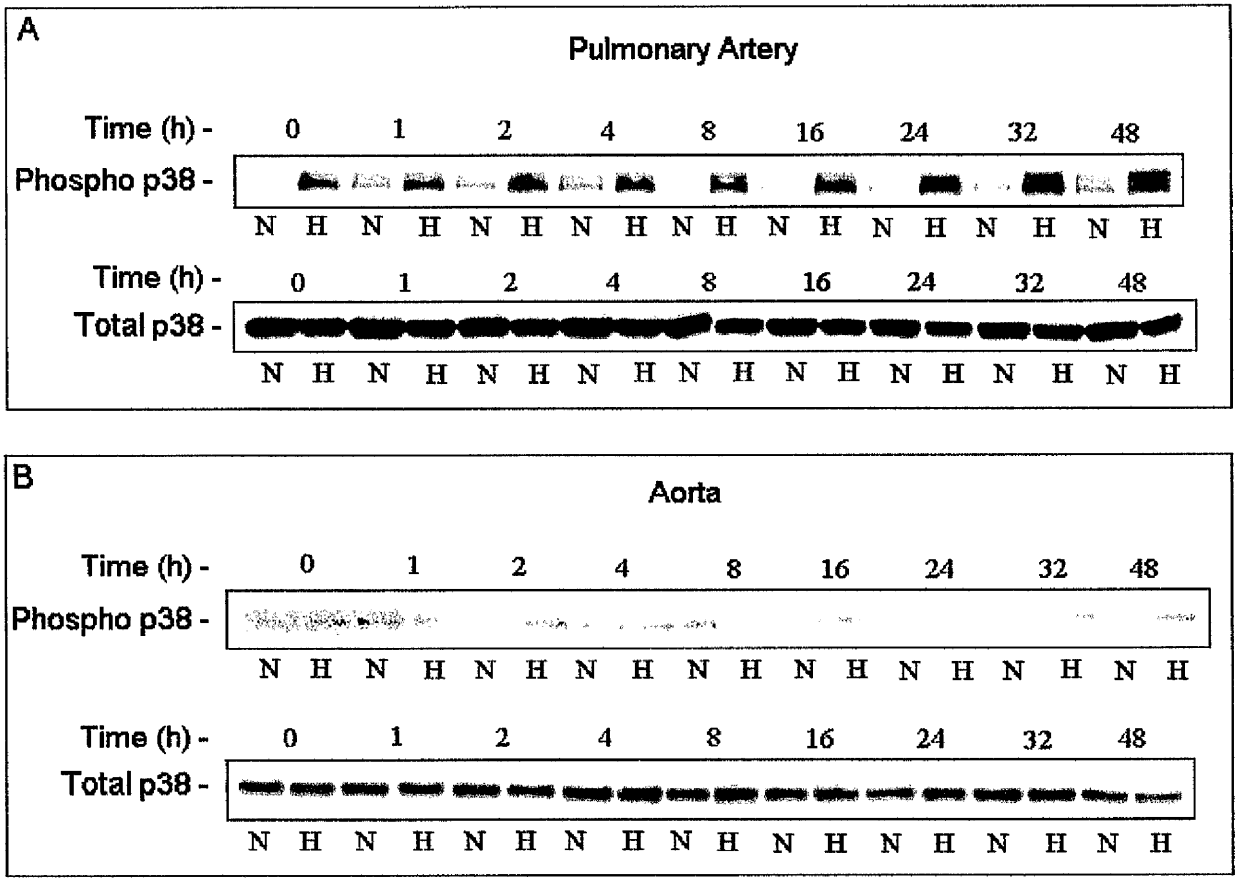


Figure 7.3 p38 MAP kinase Phosphorylation in Pulmonary Artery and Aortic Fibroblast cells from Normoxic and Chronically Hypoxic Rats.

RPAF (A) and RAF (B) cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were then stimulated with 5% serum for a range of times as indicated in the graph. Assessment of phosphorylation was by SDS-PAGE gel electrophoresis and western blot analysis using phospho-specific antibodies (Chapter 2.8.4.2). (N = cells from normoxic rats: H = Cells from chronically hypoxic rats). The experiment shown is one of 4 replicate experiments and is typical of those obtained.

Figure 7.4

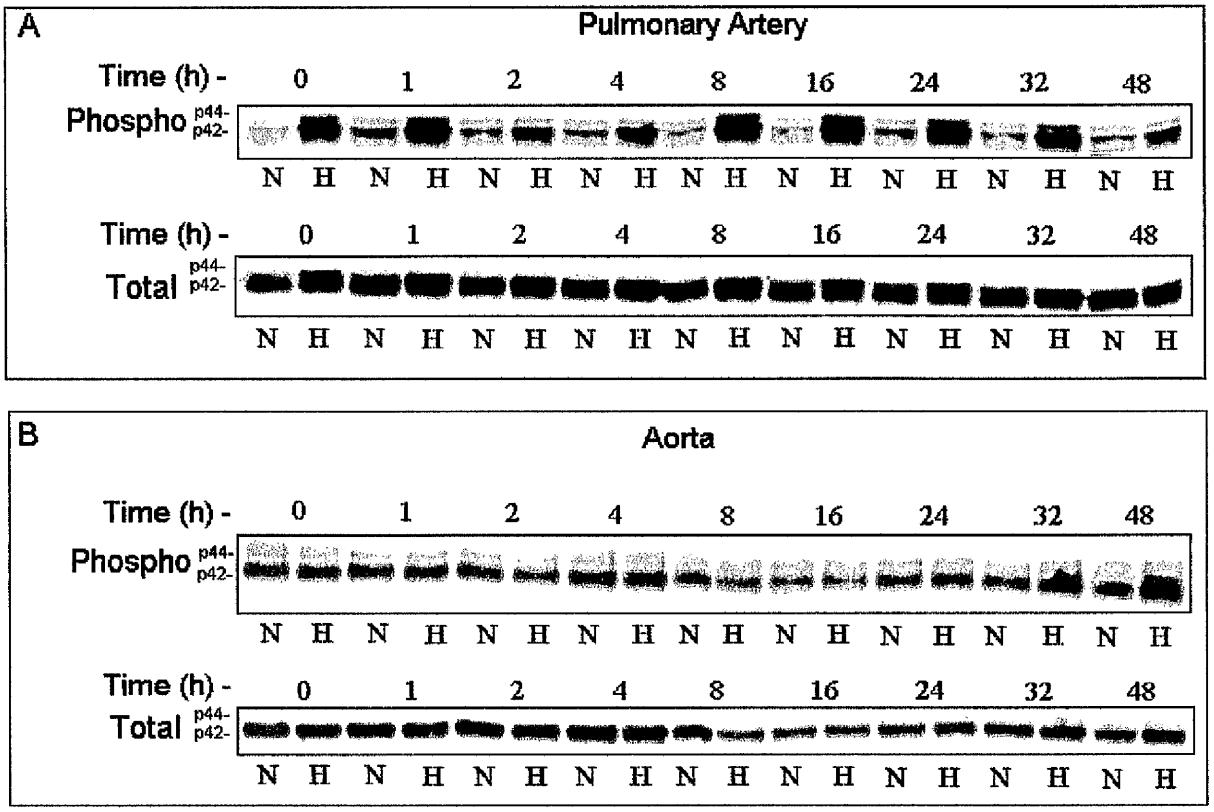


Figure 7.4 p42/p44 MAP kinase phosphorylation in Pulmonary Artery and Aortic Fibroblast Cells from Normoxic and Chronically Hypoxic Rats.

RPAF (A) and RAF (B) cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were then stimulated with 5% serum for a range of times as indicated in the graph. Assessment of phosphorylation was by SDS-PAGE gel electrophoresis and western blot analysis using phospho-specific antibodies (chapter 2.8.4.2). (N = cells from normoxic rats: H = Cells from chronically hypoxic animals). The experiment shown is one of 4 replicate experiments and is typical of those obtained.

Figure 7.5

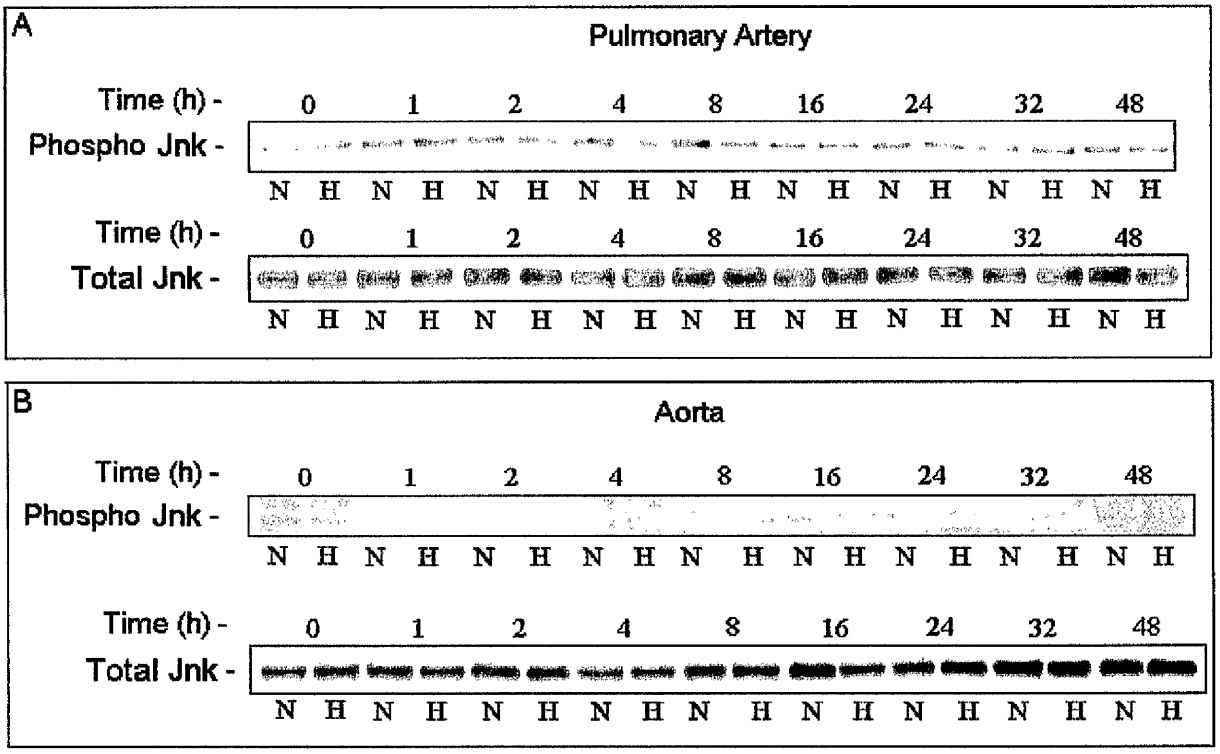


FIGURE 7.5 JNK Phosphorylation in Pulmonary Artery and Aortic Fibroblast Cells from Normoxic and Chronically Hypoxic Rats.

RPAF (A) and RAF (B) cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were then stimulated with 5% serum for a range of times as indicated in the graph. Assessment of phosphorylation was by SDS-PAGE gel electrophoresis and western blot analysis using phospho-specific antibodies (Chapter 2.8.4.2). (N = cells from normoxic rats: H = Cells from chronically hypoxic animals). The experiment shown is one of 4 replicate experiments and is typical of those obtained.

7.3.3 Differential Roles for p38 and p42/p44 MAP kinase in RPAF and RAF DNA synthesis from Normal and Chronically Hypoxic Rat Cells.

As p38 and p42/p44MAP kinase were highly phosphorylated in RPAF cells derived from hypoxic but not normoxic rats, we decided to investigate, using specific pharmacological inhibitors, the role of these kinases in the hypoxia-enhanced proliferation of RPAF cells. A selective and potent inhibitor of the p42/p44 MAP kinase cascade, U0126, mediates its effects by binding to and inactivating MEK, whereas it has no effect on any of the components of the JNK or p38 MAP kinase cascades (Feng *et al,* 1999). Similarly, the compound SB203580 is a selective and potent inhibitor of p38 MAP kinase that does not affect either p42/p44 MAP kinase or JNK (Cuenda *et al,* 1995). These reagents are useful pharmacological tools in identifying the functional activities mediated by p38 and p42/p44 MAP kinases. Pre-incubation for 1h with SB203580 (0.1μM) inhibited p38 phosphorylation (Figure 7.6A) in RPAF and RAF cells. In addition, pre-incubation for 1h with U0126 (1μM) profoundly inhibited both the hypoxia- and serum-stimulated p42/p44 MAP kinase activation in these cells (Figure 7.6B).

Having demonstrated the activity of these kinase inhibitors, we used them to test the importance of p42/44 and p38 MAP kinase activity observed in RPAF, but not RAF cells derived from chronically hypoxic rats on [^{3}H]thymidine uptake (Figure 7.7). These results showed that SB203580 and U0126 had no effect on the DNA synthesis resulting from basal (results not shown) or serum-stimulation of RPAF or PAF cells derived from normoxic rats (Figures 7.7A & 7.7B). In contrast, the p38 inhibitor SB203580 completely abrogated the enhanced serum-stimulated DNA synthesis observed in RPAF (701±72 with the inhibitor compared for 1217±118 without $p<0.01$), but not RAF cells from chronically hypoxic rats (981±117 with the inhibitor

compared for 952±96 without $p>0.05$) (Figures 7.7A & 7.7B), such that the response was now equivalent to that observed in cells derived from control normoxic rats (652±51). The p42/p44 MAP kinase cascade inhibitor, U0126 had no effect on this hypoxia-induced enhancement of DNA synthesis (1171±120 with U0126 compared with 1217±118 without $p>0.05$).

Figure 7.6

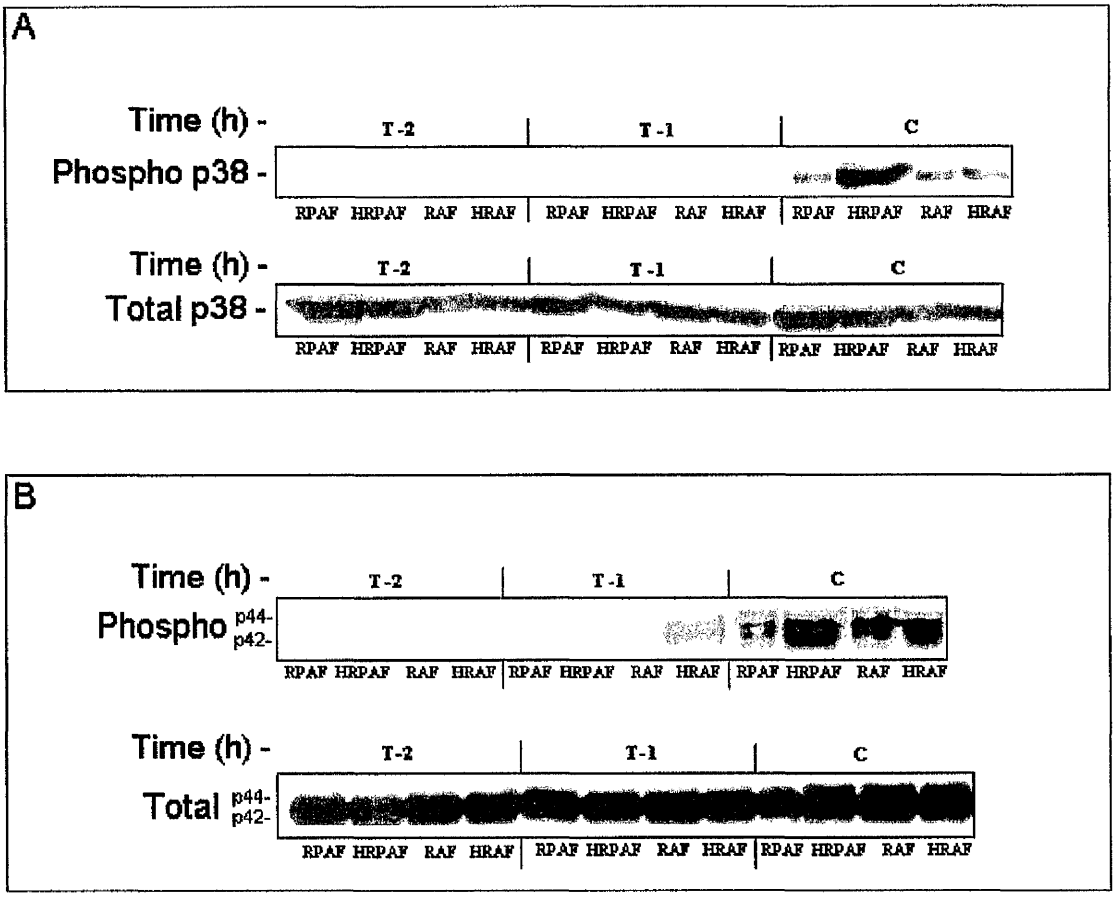


FIGURE 7.6 Effect of Specific Kinase Inhibitors on Phosphorylation of p38 and p42/p44 MAP kinases in Pulmonary Artery and Aortic Fibroblast Cells from Normoxic and Chronically Hypoxic Rats.

RPAF cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were then pre-incubated with 0.1μM SB203580 (A; lanes 1-8) or 1μM U0126 (B; lanes 1-8) for either 1h (T-1) or 2h (T-2) prior to stimulation with 5% serum for 2h. Assessment of phosphorylation was by SDS-PAGE gel electrophoresis and western blot analysis using phospho-specific antibodies (Chapter 2.8.4.2). The experiment shown is one of 4 replicate experiments and is typical of those obtained.

Figure 7.7

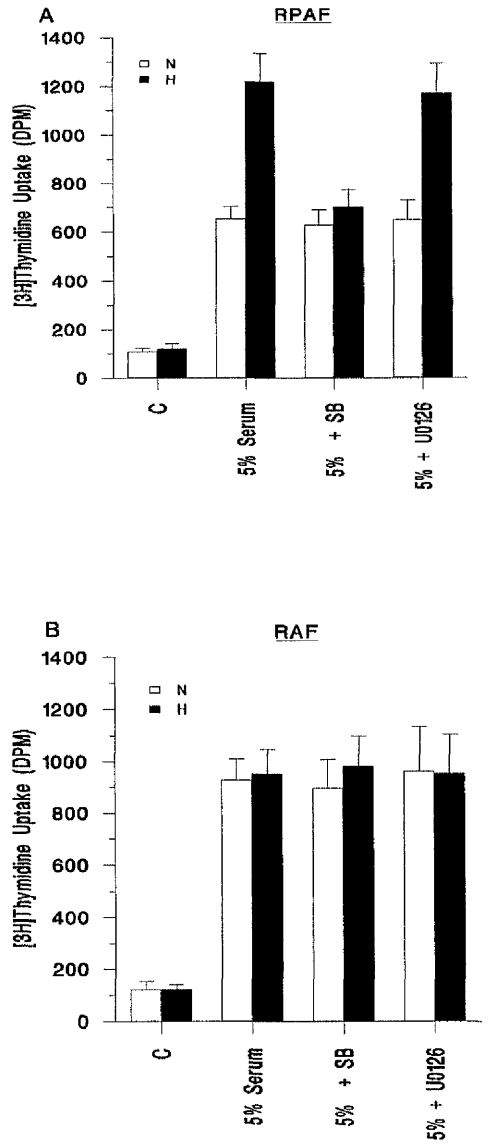


FIGURE 7.7 Effect of SB203580 and U0126 on the Enhancement of [^{3}H]Thymidine Uptake Observed Following Serum-Stimulated RPAF and RAF Cells from Chronically Hypoxic Rats.

Uptake of [^{3}H]thymidine from normoxic (□) and chronically hypoxic rats (■) in RPAF and RAF cells. RPAF (A) and RAF (B) cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. The cells were pre-incubated for 2h with either 1μM SB203580 or with 0.1μM U0126 and then stimulated with 5% serum. Cells were then allowed to grow in either normoxic or hypoxic conditions for a period of 24h. The experiment was repeated with cells from 8 different animals and the results shown are typical of those obtained.

7.4 DISCUSSION

In this chapter we have shown that pulmonary artery fibroblasts from chronically hypoxic rats exhibit enhanced proliferative responses to serum relative to those observed with RPAF derived from normoxic animals. This hypoxia-enhanced proliferation was maintained following multiple passages of these primary cultures of cells, which were maintained under normoxic conditions following their culture from the artery. This mirrors the changes observed *in vivo* in patients with hypoxic pulmonary hypertension, where remodelling of the pulmonary arteries due to increased cellular proliferation remains even after oxygen therapy (Traver *et al,* 1979). Sustained enhanced proliferative capacities have recently also been reported for fibroblasts isolated from the pulmonary artery of neonatal calves with the severe fibroproliferative changes characteristic of hypoxic neonatal pulmonary hypertension (Das *et al,* 1999). This latter study showed that the proliferative responses of such fibroblasts could be further enhanced following subsequent exposure to acute hypoxia. However, in our system, the augmented responses to serum observed in RPAF could not be further enhanced by re-exposure to acute hypoxia. This suggests that the mechanisms regulating pulmonary vascular remodelling in neonates and adults may be different and may provide a rationale for the widely established finding that the effects of hypoxia are more marked in neonates relative to adults (Das *et al,* 1999). Interestingly, no remodelling of the systemic circulation is observed in the patients with hypoxic pulmonary hypertension (von Euler and Liljestrand, 1946) and this is reflected in our rat model as fibroblasts from rat aorta did not show enhanced proliferative capacities following chronic exposure to hypoxia.

The molecular mechanisms by which hypoxia stimulates proliferation of pulmonary artery fibroblasts, but not fibroblasts from the systemic circulation, are unknown. There is considerable evidence in the literature, however, that p42/p44 MAP kinases,

As well as JNK and p38 MAP kinase can be activated in response to hypoxic stress (Robinson and Cobb, 1997; Takahashi and Berk, 1998). Indeed, we have shown in our model of acute bovine pulmonary artery hypoxia, that the SAPKs JNK and p38 MAP kinase, but not the classical p42/p44 MAP kinase isoforms, are activated in response to acute hypoxia (Scott *et al,* 1998). We therefore addressed the role of all three classes of these MAP kinases by investigating whether there was differential activation of these kinases in response to serum in fibroblasts derived from the pulmonary and systemic circulation of normoxic versus chronically hypoxic rats.

Although JNK was strongly activated by hypoxia in the acute model (Chapter 6; Scott *et al,* 1998), we could find no evidence of JNK phosphorylation in RPAF derived from rats subjected to chronic hypoxia (Figure 7.5). Moreover, we could not detect any differential phosphorylation of JNK in response to serum in RPAF derived from chronically hypoxic versus normoxic rats (Figure 7.5). However, as the JNK activation observed in response to hypoxia in the acute model was transient, peaking between 3-6 h following hypoxic challenge, these results may indicate that whereas a JNK kinase signal may be required for the initiation of hypoxia-mediated events, it is not involved in the remodelling process. In contrast, we found strong and constitutive activation of p42/p44 and p38 MAP kinases in RPAF from chronically hypoxic rats but not normoxic rats (Figures 3 and 4). In addition, this induction of constitutive p42/p44 and p38 MAP kinase activity was not observed in RAF from either chronically hypoxic rats or normoxic rats. Taken together, these results suggested that p42/p44 and/or p38 MAP kinase played a role in the enhanced proliferative responses observed in remodelled fibroblasts from chronically hypoxic rats.

To address the relative roles of p42/p44 and p38 MAP kinases in serum and/or hypoxia-induced proliferation of RPAF and RAF cells, we performed our

proliferation assays in the presence of specific inhibitors of the p42/p44 (U0126) and p38 (SB203580) MAP kinase cascades. Given the widely established roles for p42/p44 MAP kinases in promoting cell proliferation (Lew *et al,* 1999; Belham *et al,* 1996; Pages *et al,* 1993), and the strong constitutive activation of this MAP kinase in RPAF cells by chronic hypoxia (Figure 4), it could be considered surprising that the p42/p44 cascade inhibitor U0126 had no effect on the enhancement of serum-stimulated proliferation observed in RPAF from chronically hypoxic rats. However, examination of the levels of p42/p44 MAP kinase activity in RAF cells showed that these cells also exhibited considerable levels of constitutive p42/p44 MAP kinase activity despite an inability to display enhanced proliferative capacities following exposure to chronic hypoxia. Indeed, p42/p44 MAP kinase activity was found to be strongly and constitutively activated in RAF cells regardless of the oxygen status of the rats they were derived from. Taken together with the finding that p42/p44 activity could be stimulated in RPAF cells from normoxic rats following stimulation with serum, these results could suggest that p42/p44 MAP kinase activity serves to render cells permissive for growth factor mediated-stimulation of proliferation. Thus, the enhanced p42/p44 MAP kinase signals observed in hypoxic RPAF cells could contribute to the augmented growth factor-stimulated responses observed in response to chronic hypoxia reported in the bovine neonatal study (Das *et al,* 1999).

In contrast, and consistent with the differential patterns of p38 MAP kinase activity observed in RPAF and RAF from chronically hypoxic versus normoxic rats (Figure 3), we found that although p38 MAP kinase activity appeared to be essential for the enhanced proliferative response observed in RPAF from chronically hypoxic rats, it did not appear to play an important role in transducing serum-induced proliferation in either RPAF or RAF cells. This important role for p38 MAP kinase in transducing proliferative signals in response to hypoxia reflects our earlier studies on the bovine

acute hypoxia model which showed that p38 MAP kinase activation was dependent on the presence of a maintained hypoxic environment as sustained p38 MAP kinase activity was blocked by re-oxygenation. Moreover, although p38 MAP kinase has generally been considered to play a role in the transduction of apoptotic (Takahashi and Berk, 1998) rather than proliferative signals as evidenced by negative regulation of cyclin D expression (Lavoie *et al,* 1996) and its activation under conditions of cellular stress (Xia *et al,* 1995; Chen *et al,* 1996) including ischemic damage and reperfusion of cardiac myocytes (Morooka *et al,* 1995; Bogoyevitch *et al,* 1995), there is some evidence not only that crosstalk between JNK and p38 MAP kinase may be required for apoptosis (Wilson *et al,* 1996; Ichijo *et al,* 1997) but also that activation of p38 MAP kinase can promote cellular activation, proliferation and differentiation (Zhang *et al,* 1999). Whereas most of the physiological and chemical stresses which can induce apoptosis generally activate both JNK and p38 MAP kinase (Wilson *et al,* 1996; Ichijo *et al,* 1997), we find that under these stress conditions of chronic hypoxia, JNK does not appear to be activated and p38 MAP kinase acts to promote cellular proliferation.

Finally, there is an urgent need for drugs that could reverse or prevent the pulmonary vascular remodelling which occurs in nearly all forms of cardiac and respiratory disease. At present, however, most vasodilating drugs used to counteract pulmonary hypertension result in systemic hypotension. However, since systemic fibroblasts do not replicate in the presence of hypoxia and p38 MAP kinase does not appear to be necessary for the normal replication of pulmonary systemic vascular fibroblasts, it is possible that therapies targeting RPAF p38 MAP kinase could lead to the control of pulmonary vascular remodelling without affecting the systemic circulation.

Chapter 8

Effect of 5-HT on Proliferation and Generation of MAP kinases in Fibroblasts Harvested from Pulmonary Arteries from Chronically Hypoxic Rats

8.1 INTRODUCTION

As described in Chapter 1, numerous groups have studied changes that take place in the pulmonary arteries as a result of pulmonary hypertension. As a result of these studies, numerous pro-inflammatory factors have been identified which can influence these changes (Bitterman and Henke, 1991). The aim of this chapter was to examine the possible role of one such factor, serotonin, on fibroblast cell proliferation and intracellular signalling from the pulmonary arteries of control and chronically hypoxic animals.

Serotonin, also known as 5-hydroxytryptamine (5-HT), is secreted from neuroendocrine cells in the gut, and tumours of these cells, called carcinoid tumours, are a source of increased production of 5-HT (Hart and Block, 1989; Perry and Vinik, 1996). Pulmonary neuroendocrine cells secrete vasoactive substances in response to airway hypoxia and hypercapnia (Johnson and Georgieff, 1989; Lauweryns *et al*, 1983). These cells commonly proliferate in patients with pulmonary hypertension, producing a variety of peptides in addition to large amounts of 5-HT (Gosney *et al*, 1989). In lung transplant recipients with end stage primary pulmonary hypertension the degree of hyperplasia of these cells was found to correlate with the extent of proliferation of myofibroblasts in the pulmonary arteries (Madden *et al*, 1994). On a molar basis, 5-HT is the most potent pulmonary vasoconstrictor identified to date in humans (Heffner *et al*, 1987) but in the systemic vasculature it causes profound vasodilation (Comroe *et al*, 1953).

Because of the prevalence of chronic lung disease, hypoxia is probably the commonest of all causes of pulmonary hypertension. As stated previously, the mechanisms of hypoxic vasoconstriction have been reviewed, but much remains unknown (Voelkel, 1986). In healthy subjects hypoxia per se does not appear to increase circulating 5-HT levels (Rahda

et al 1976), but levels are increased in patients with several types of chronic lung disease and associated pulmonary hypertension (Bobrov *et al*, 1985; Pribilova, 1976).

5-HT is a mitogen causing hyperplastic and hypertrophic changes in smooth muscles (Pakala *et al*, 1994). At least ten classes of 5-HT receptors have been identified, these include 5-HT_{1A-F}, 5-HT_{2A-C}, 5-HT_3 and 5-HT_4 (Hoyer *et al*, 1994), and different receptors have been implicated in the pathogenesis of a number of vascular disorders. For example, 5-HT_1 and 5-HT_2 are the principal receptors relevant to the pulmonary arteries (Frishman *et al*, 1996). Under most experimental conditions, stimulation of the 5-HT_1 receptor causes vasodilation and the 5-HT_2 receptor often mediates vasospasm (Frishman *et al*, 1996).

Use of several drugs has indicated a strong link between pulmonary hypertension and levels of 5-HT. Pulmonary hypertension can be induced in most mammals by numerous serotonergic drugs, some of which are sympathomimetic anorexients. One such drug is dexfenfluramine (Brenot *et al*, 1993). In dogs, intravenous dexfenfluramine augments hypoxic pulmonary vasoconstriction and, with long term oral use, pulmonary vascular resistance is increased (Naeije, 1996). How this drug can induce pulmonary hypertension is unknown. In contrast to this, drugs that block 5-HT receptors reduce pulmonary hypertension. For example, the specific 5-HT_{2A} receptor antagonist ketanserin is the best studied agent of this type for reducing pulmonary hypertension or vascular resistance (Brogden and Sorkin, 1990). Like most vasodilators this drug has varying efficacy, reflecting the complex pathophysiology of the disease. In the treatment of primary pulmonary hypertension, McGoon and Vliestra (1984) detected an average reduction of pulmonary vascular resistance of 18% with intravenous use in 10 patients. The effects of ketanserin on pulmonary hypertension suggest that serotonergic mechanisms may be contributing to the problem.

The aim of this chapter is to study the effects of 5-HT on the proliferation and generation of MAP kinases in rat pulmonary fibroblasts from control and chronically hypoxic animals. At the same time, the effects of specific 5-HT receptor antagonists, such as the 5-HT_{2A} receptor antagonist, ketanserin, will be used to help identify if a specific 5-HT receptor is involved in the remodelling of pulmonary arteries during pulmonary hypertension.

8.2 METHODS

8.2.1 Chronic hypoxic rat model of pulmonary hypertension.

Pulmonary hypertensive rats were prepared using the technique of MacLean *et al* (1995) (Chapter 2.5)

8.2.2 Pulmonary artery fibroblast cell culture

Rat pulmonary artery fibroblast (RPAF) cells were harvested from freshly excised lung tissue of control and chronically hypoxic animals as described in Chapter 2.3.2.
All tissue was obtained from the laboratory of Dr. M. McLean and age-matched animals were always used as controls.

The identity of the fibroblast cells was confirmed by immunocytochemical staining as described in Chapter 2.3.3

8.2.3 Assessment of Proliferation

Proliferation of rat pulmonary artery fibroblasts was assessed by measuring the uptake of [^{3}H]thymidine (Chapter 2.6). Cells were grown to approximately 60% confluency in 24-well plates at 37^{o}C and then serum-starved for 24h. After this time the following additions were made as described below.

To determine the effect of serum and 5-HT on proliferation in the normoxic and chronically hypoxic cells, 0.2% serum and 10μM 5-HT were added to the cells for 24h. 10μM of specific 5-HT agonists, α-methyl-5-HT, BW723C86 and MK212 were also studied. In addition, the incubations were also carried out in the presence and absence of 5-HT receptor antagonists, 0.1μM Ketanserin and 1μM SDZ SER 082 and RS102221. All drugs were added 2h prior to addition of growth factors for 24h.

In all experiments, for the last 4h of agonist stimulation, cells were labelled with [^{3}H]thymidine (0.1μCi/ml) before the reaction was stopped by washing in PBS (Chapter 2.6). Radioactivity was determined by liquid scintilation counting and results expressed as disintegrations per minute (DPM).

8.2.4 Assessment of MAP kinase activity.

Cells were grown to approximately 90% confluency in 6-well plates at 37^{o}C and then serum-starved for 24h. After this time, the following additions were made as described below. To determine the effect of serum and 5-HT on p42/44, p38 and JNK activity in the normoxic and chronically hypoxic cells, 0.2% serum and 10μM 5-HT were added to the cells for 24h. To determine the effects of the specific 5-HT antagonists on p42/44 MAP kinase, p38 MAP kinase and JNK activity in the normoxic and chronically hypoxic cells, 0.1μM Ketanserin and 1μM GR55562 were added 2h prior to addition of growth factors for 24h.

Assessment of phosphorylation of these MAP kinase family of proteins was by SDS-PAGE gel electrophoresis and western blot analysis (Chapter 2.8.3). In each case, the primary

rabbit anti-dual phosphorylated, activated MAP kinase antibody for the protein of interest (p42/44, p38 and JNK) was incubated with the blot for at least 1h at room temperature. The blots were then washed in PBS/Tween before incubating with sheep anti-rabbit Ig HRP in 5% non-fat dried milk for one hour with constant agitation.

8.2.5 Statistics

Results are expressed as the mean ± S.D. Statistical analysis was undertaken as reported in Chapter 2.10.

8.3 RESULTS

8.3.1 Effects of 5-HT Agonists and Antagonists on proliferation of Fibroblast cells from normoxic and chronically hypoxic rats.

The effect of 5-HT on the proliferation of pulmonary artery fibroblasts was determined by measuring its effect on the uptake of [^{3}H]thymidine into cells. Figure 8.1 shows the effect of 5-HT on the replication of pulmonary artery fibroblast cells from control and chronically hypoxic animals. The addition of 0.2% serum, both 3μM and 10μM 5-HT alone did not significantly enhance the proliferation of fibroblast cells from either normoxic or those from chronically hypoxic animals in comparison with control untreated cells ($p>0.05$). However, in the presence of 0.2% serum, both 3μM and 10μM 5-HT caused an 1.4-fold and 3.5-fold increase, respectively, in the [^{3}H]thymidine uptake in cells from normoxic animals. The effect was even greater in the cells from hypoxic animals where there was a 3.3-fold increase over control cells with the addition of 0.2% serum and 3μM 5-HT, and a 7.2-fold increase with the addition of 10μM 5-HT (Figure 8.1).

Figure 8.2 shows the effects of 10μM 5-HT on the proliferation of RPAF cells from control and chronically hypoxic animals in the presence of increasing concentrations of serum. There was an enhanced proliferative response in the presence of 5-HT and serum in the cells from chronically hypoxic animals ($p<0.001$) in comparison to serum alone. This was particularly true at lower concentrations of serum less than 1%. For example, the presence of 0.5% serum had no effect on the proliferation of RPAF cells from chronic hypoxic animals ($p>0.05$). However, in the presence of 10μM 5-HT there was an 11.7-fold increase over control levels.

Figure 8.3 shows the effects of specific 5-HT agonists and antagonists on the replication of pulmonary artery fibroblasts from control and chronically hypoxic animals. Unstimulated RPAF cells from control and hypoxic animals showed no difference in [^{3}H]thymidine uptake ($p>0.05$). In addition 0.2% serum alone and 5-HT (10μM) had no significant effect. However, as described for Figure 8.2, the addition of 5-HT (10μM) in the presence of 0.2% serum resulted in a 2.7-fold increase in proliferation in normoxic cells ($p<0.005$). This response was further enhanced in cells from chronically hypoxic animals where there was an 6-fold increase in comparison to control, untreated cells.

To examine this effect more closely, we studied a range of more specific 5-HT agonists. The addition of a 5-HT_2 agonist, α-methyl-5-HT (10μM) enhanced the proliferation of cells from hypoxic rats to the same degree as that of 10μM 5-HT alone (1750±251 DPM and 854±196 DPM respectively). In contrast, the 5-HT_{2B} agonist BW723C86 (10μM) did not increase proliferation in either cell type (332±52 DPM for the hypoxic cells and 325±110 DPM for the normoxic cells which was not significantly different from control cells). This was also true for the 5-HT_{2C} agonist, MK212 (10μM) which did not increase proliferation in either cell type (324±225 DPM for the hypoxic cells and 339±211 DPM for the normoxic cells which was not significantly different from control cells).

To study this further, we also looked at the effect of a range of 5-HT antagonists on the 5-HT-induced [^{3}H]thymidine uptake. The addition of the 5-HT_{2A} antagonist, ketanserin (0.1μM), abrogated the enhanced growth response observed in the cells from chronically hypoxic animals induced with 5-HT and 0.2% serum (926±115 DPM for the hypoxic cells which was not significantly different from normoxic cells 849±310 DPM). In contrast, neither the 5-HT_{2B+C} inhibitor SDZ SER 082 (1μM) nor the 5-HT_{2B} inhibitor RS102221 (1μM) had any effect on reducing proliferation of cells in the presence of 5-HT and 0.2%

serum (1796±121 and 1847±314 DPM for the hypoxic cells which was significantly different ($p<0.05$) from normoxic cells 882±265 and 886±172 DPM, respectively).

Figure 8.4 shows the effect of 5-HT with 0.2% serum and specific inhibitors for 5-HT (Ketanserin and GR55562) and MAP kinases (SB203580 and U0126) on the replication of pulmonary artery fibroblast cells from control and chronically hypoxic animals. Basal levels of replication in normoxic cells (220±15 DPM) and in cells from chronically hypoxic animals (230±20 DPM) were not significantly different ($p>0.05$). 10μM 5-HT with the addition of 0.2% serum gave rise to an 4-fold increase in proliferation in the normoxic cells and a further increase (6.2-fold) in the cells from chronically hypoxic animals ($p<0.005$). The addition of 1μM SB203580 (a specific p38 inhibitor) abolished this enhanced 5-HT growth response observed in the cells from chronically hypoxic animals (923±42 DPM). In the presence of this compound the increase in [^{3}H]thymidine uptake in cells from hypoxic rats was not significantly different to that from normoxic animals (793±102 DPM) ($p>0.05$). The addition of 0.1μM of the MEK inhibitor U0126 completely abolished the proliferative response of normoxic cells (312±28 DPM) and those from chronically hypoxic animals (331±38 DPM) which was not significantly different from control levels ($p>0.05$).

The effect of 5-HT antagonists were also investigated (Figure 8.4). The addition of the 5-HT_{2A} antagonist Ketanserin (0.1μM) abolished the enhanced 5-HT growth response seen in the cells from chronically hypoxic animals (913±50 DPM). Like SB203580, the enhanced response was reduced to the level of that observed for cells from normoxic animals (832±78 DPM) ($p>0.05$). In contrast, the 5-HT_{1B-1D} antagonist GR55562 (1μM) had no effect on the proliferation of cells from either the normoxic or hypoxic animals.

Figure 8.1

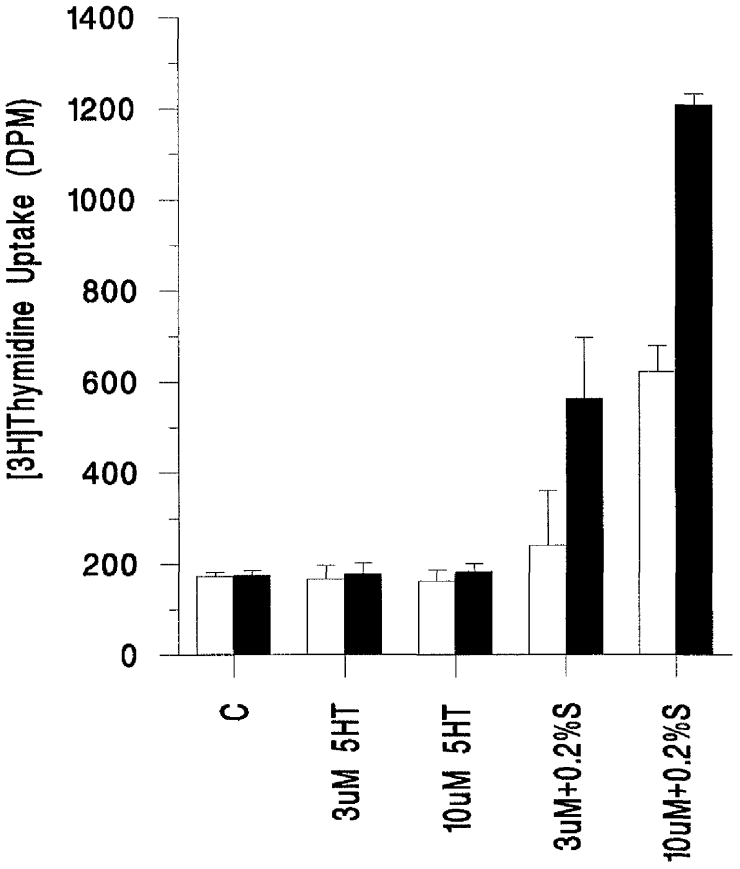


Figure 8.1 Effect of 5-HT and Serum on [^{3}H]Thymidine Uptake in RPAF cells from Normoxic and Chronically Hypoxic Rats.

Uptake of [^{3}H]thymidine from normoxic (□) and chronically hypoxic (■) rats. RPAF cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. The cells were stimulated with either 3μM or 10μM 5-HT alone or in conjunction with 0.2% serum as indicated in the graph. Cells were then allowed to grow in normoxic conditions for a period of 24h. The experiment was repeated with cells from 8 different animals and the results shown are typical of those obtained.

Figure 8.2

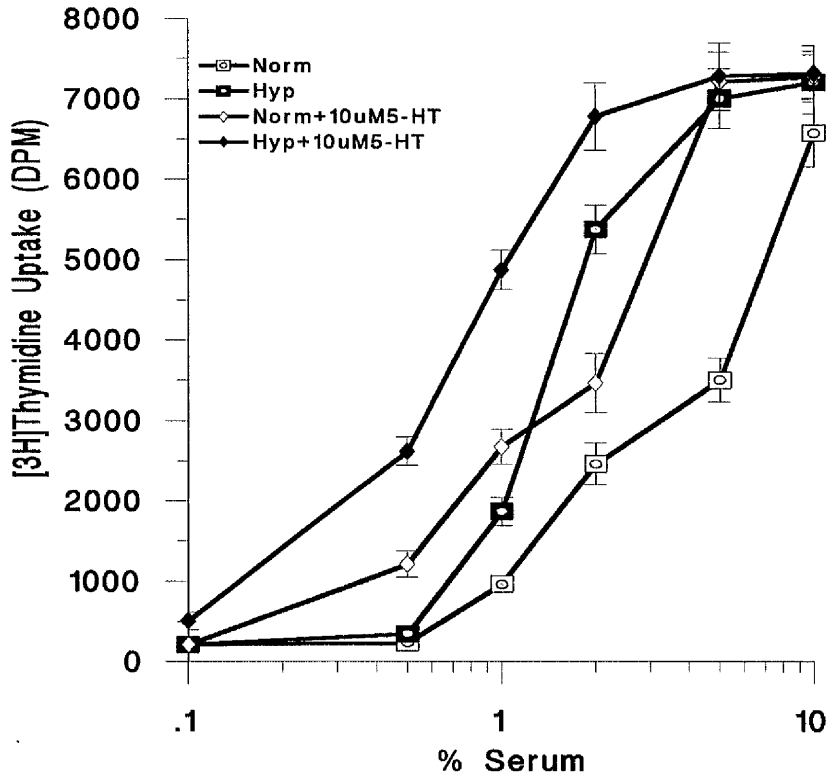


Figure 8.2 Effects of 10μM 5-HT and Serum on the Uptake of [^{3}H]Thymidine in Pulmonary Artery Fibroblasts from Control and Chronically Hypoxic Animals.

Uptake of [^{3}H] thymidine from normoxic (□) and chronically hypoxic rats (■). RPAF cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. The cells were stimulated with a range of serum concentrations either alone or in conjunction with 10μM 5-HT as indicated in the graph. Cells were then allowed to grow in normoxic conditions for a period of 24h. The experiment was repeated with cells from 8 different animals and the results shown are typical of those obtained.

Figure 8.3

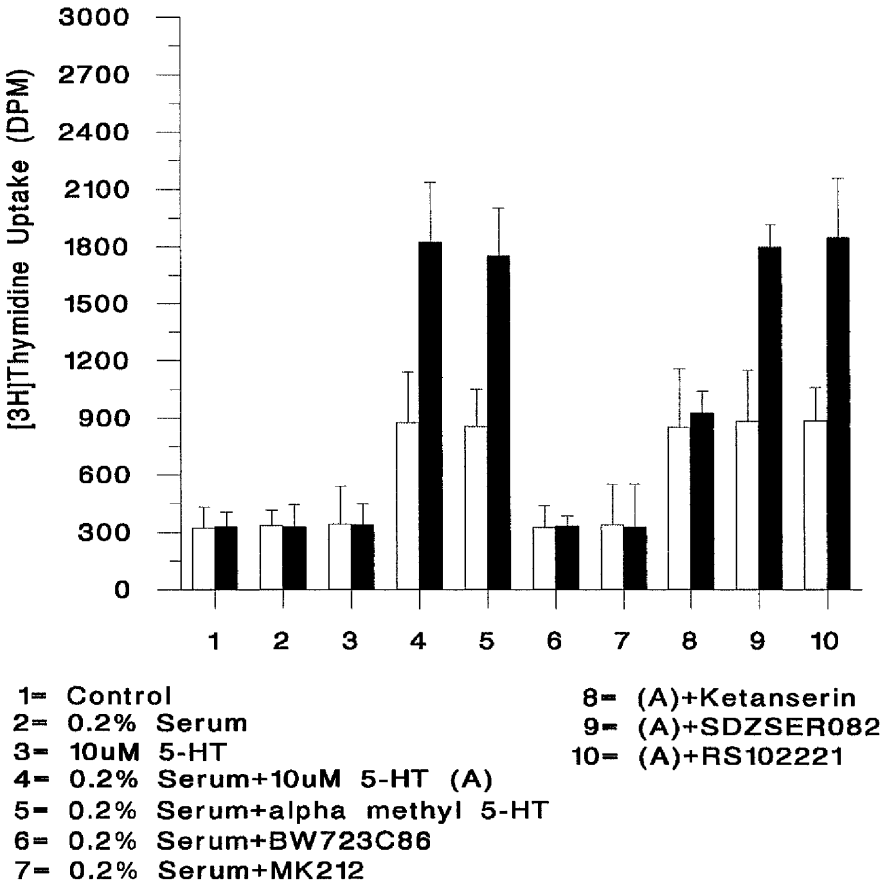


Figure 8.3 Effects of Specific 5-HT Agonists and Antagonists on the Replication of Pulmonary Artery Fibroblasts from Control and Chronically Hypoxic Animals.

Uptake of [^{3}H]thymidine from normoxic (□) and chronically hypoxic rats (■). RPAF cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. The cells were stimulated with serum (0.2%) either alone or in conjunction with 5-HT (10μM) in the presence or absence of α-methyl-5-HT (10μM), BW723C86 (10μM), MK212 (10μM), Ketanserin (0.1μM), SDZSER082 (1μM) and RS102221 (1μM) as indicated in the graph. Cells were then allowed to grow in normoxic conditions for a period of 24h. The experiment was repeated with cells from 8 different animals and the results shown are typical of those obtained.

Figure 8.4

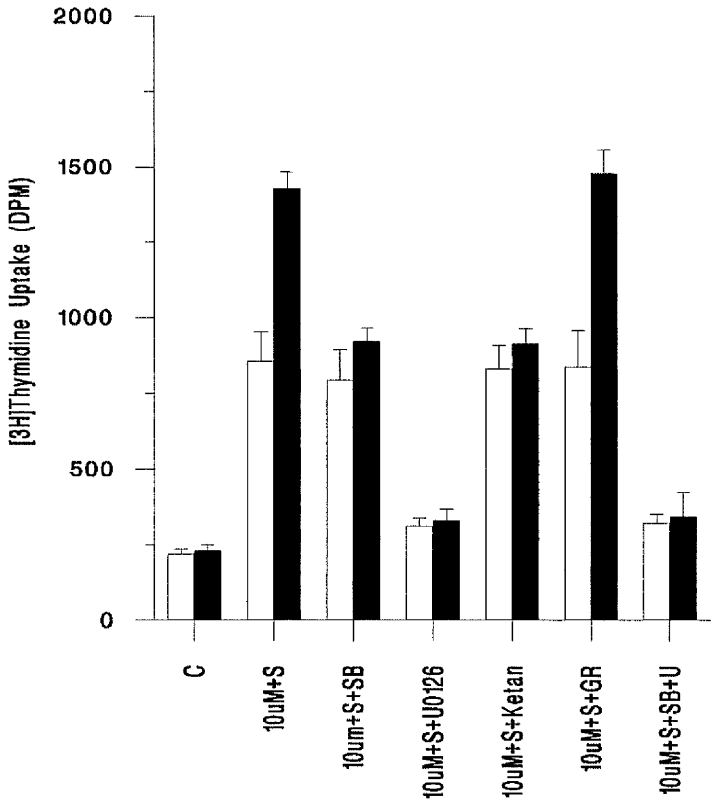


Figure 8.4 Effects of specific 5-HT and MAP kinase inhibitors on 5-HT-induced [^{3}H]thymidine uptake of pulmonary artery fibroblasts from control and chronically hypoxic animals.

Uptake of [^{3}H] thymidine from normoxic (□) and chronically hypoxic rats (■). RPAF cells were grown to 60% confluency in 24-well plates and then quiesced in serum-free medium. The cells were stimulated with 10μM 5-HT (10μM) in the presence of 0.2% serum (S) in the presence and absence of 0.1μM SB203580 (SB), 1μM U0126, 0.1μM Ketanserin (Ketan) or 1μM GR55562 (GR) as indicated in the graph. Cells were then allowed to grow in normoxic conditions for a period of 24h. The experiment was repeated with cells from 8 different animals and the results shown are typical of those obtained.

8.3.2 Effect of 5-HT and 5-HT Antagonists on p38 phosphorylation in Fibroblast cells from normoxic and chronically hypoxic rats.

Watts (1998) have shown that 5-HT can lead to the phosphorylation of MAP kinase. We were interested to see if 5-HT could further enhance p38 MAP kinase phosphorylation already observed (Chapter 7.32, Figure 7.3) in pulmonary artery fibroblasts from hypoxic rats.

Figure 8.5 shows the effect of increasing doses of 5-HT on phosphorylated p38 MAP kinase (pp38) in pulmonary artery fibroblast cells from control and chronically hypoxic animals. In the unstimulated control cells from chronically hypoxic rats, a clear increase in pp38 can be seen which is not apparent in the cells from normoxic animals (Figure 8.5 compare upper panel, lane 1 with lane 7). In the cells from chronically hypoxic animals pp38 MAP kinase was increased in a 5-HT dose-dependent manner with maximum phosphorylation occurring at 10μM 5-HT. In contrast, no increase was seen in the cells from normoxic animals. The total amount of p38 MAP kinase was examined (lower panel) to ensure equal loading of protein on the polyacrylamide gel.

Figure 8.6 shows the effect of 5-HT on pp38 MAP kinase in pulmonary artery fibroblast cells from control and chronically hypoxic animals. The upper panel shows that, 3μM and 10μM 5-HT gave rise to a substantial increase in pp38 MAP kinase in the cells from chronically hypoxic animals in comparison to control untreated cell extracts. There was no increase in pp38 MAP kinase in extracts from normoxic cells. The increase in pp38 MAP kinase in the cells from chronically hypoxic animals could be abrogated with the addition of 0.1μM Ketanserin, a specific 5-HT_{2A} antagonist. In contrast, addition of 1μM GR55562,

a 5-HT_{1B-1D} antagonist had much less of an effect. The total amount of p38 was examined (lower panel) to ensure equal loading of protein on the polyacrylamide gel.

Figure 8.5

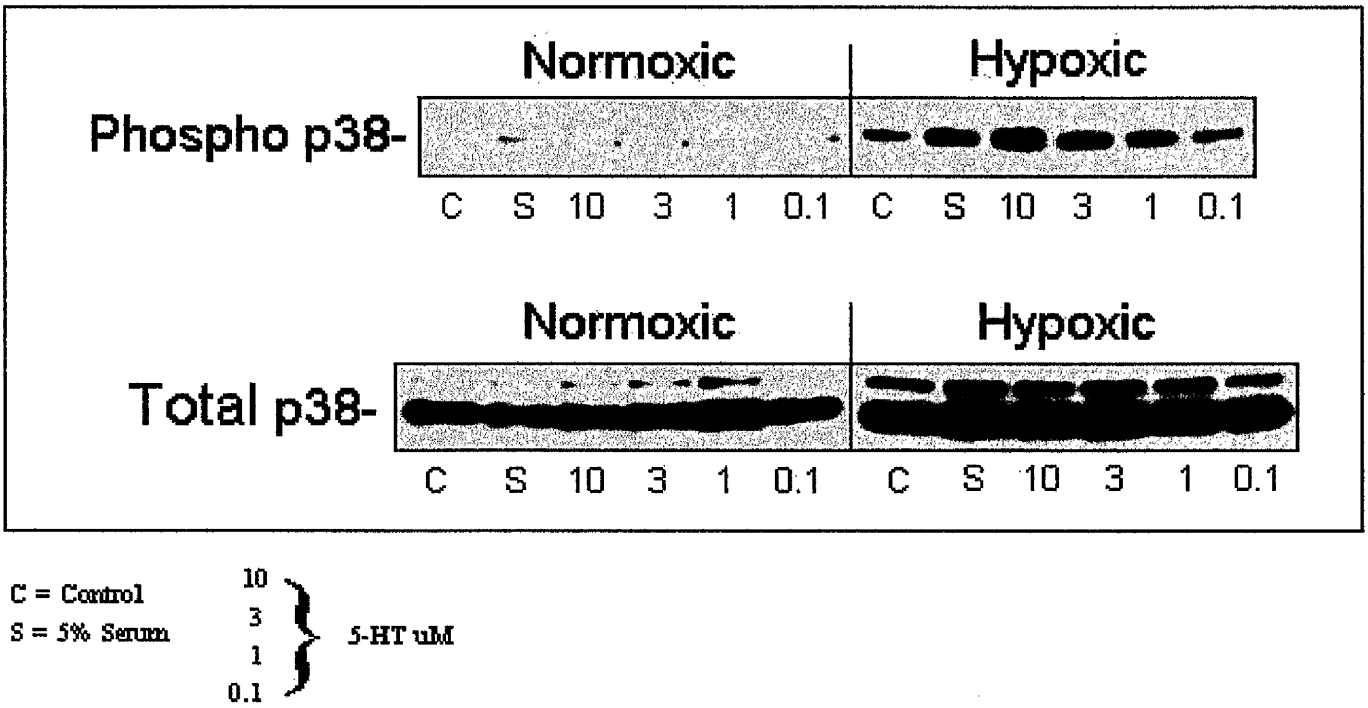


Figure 8.5 Effect of Increasing Concentration of 5-HT on p38 Activity in Pulmonary Artery Fibroblast Cells from Control and Chronically Hypoxic Rats

BPAF cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were then stimulated for 24h with a range of 5-HT concentrations as indicated. Assessment of phosphorylation was by SDS-PAGE gel electrophoresis and western blot analysis (Chapter 2.8). The experiment shown is one of 4 replicate experiments and is typical of those obtained. Band density is used as a measure of protein level.

Figure 8.6

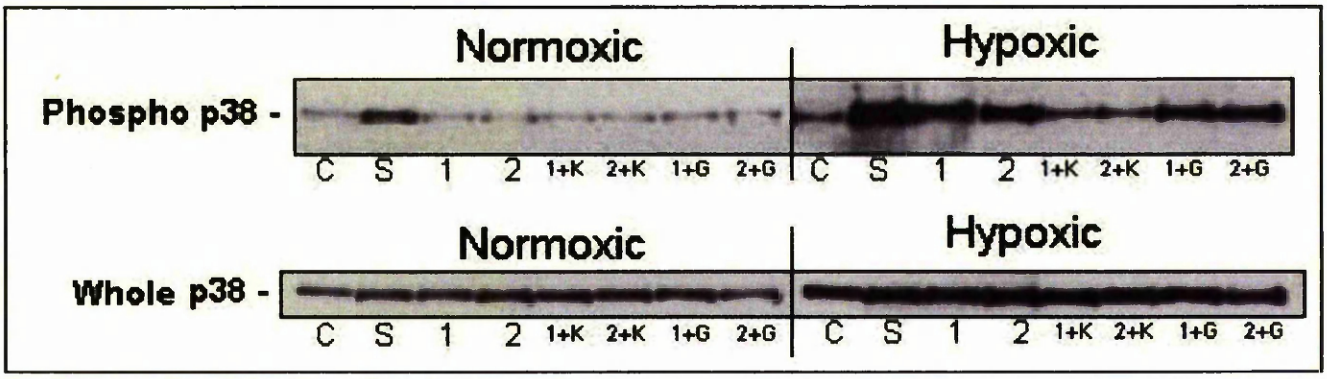


Figure 8.6 Effect of 5-HT on p38 Activity in Pulmonary Artery Fibroblast Cells from Control and Chronically Hypoxic Rats.

BPAF cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were then pre-incubated with 0.1μM ketanserin (K; a specific 5-HT_{2A} antagonist) or 1μM GR55562 (GR; a specific 5-$HT_{1B/1D}$ antagonist) for 2h prior to stimulated with 3 or 10μM 5-HT for 24h. The experiment shown is one of 4 replicate experiments and is typical of those obtained. Band density is used as a measure of protein level.

8.3.3 Effect of 5-HT and 5-HT Antagonists on p42/p44 MAP Kinase Phosphorylation

We wanted to see of 5-HT could further enhance p42/p44 MAP kinase phosphorylation already observed in pulmonary artery fibroblasts from hypoxic rats. Figure 8.7 shows the effect of increased doses of 5-HT on phosphorylated p42/44 MAP kinase activity in pulmonary artery fibroblast cells from control and chronically hypoxic animals. Again, in the unstimulated control cells from chronically hypoxic rats, a clear increase of p42/44 MAP kinase activity can be seen which is not apparent in the cells from normoxic animals. Neither cell type responded to 5-HT under any of the concentrations used with respect to increases in p42/p44 MAP kinase phosphorylation. The total amount of p42/p44 MAP kinase was examined (lower panel) to ensure equal loading of protein on the polyacrylamide gel.

Figure 8.8 shows the effects of 5-HT on phosphorylated p42/44 MAP kinase activity in pulmonary artery fibroblast cells from control and chronically hypoxic animals in the presence of 0.2% serum. In the unstimulated control cells from chronically hypoxic rats, a clear increase of p42/44 MAP kinase phosphorylation was observed in comparison to extracts from untreated normoxic rats. 3μM and 10μM 5-HT alone did not increase p42/p44 phosphorylation in either cell types as in Figure 8.7. In the presence of 0.2% serum, however, an increase in p42/p44 MAP kinase phosphorylation by 5-HT occurred in a dose-dependent fashion in the cells from chronically hypoxic animals. The total amount of p42/44 MAP kinase was examined to ensure equal loading of protein on the polyacrylamide gel.

Figure 8.9 shows the effect of 5-HT on phosphorylation of p42/44 MAP kinase in pulmonary artery fibroblast cells from control and chronically hypoxic animals. Unstimulated cells from chronically hypoxic animals displayed increased levels of p42/44

MAP kinase activity compared with normoxic cells. There was a further increase in p42/p44 MAP kinase phosphorylation with the addition of 5% serum, in RPAF from hypoxic animals. Neither 3μM nor 10μM 5-HT gave rise to increased p42/p44 MAP kinase phosphorylation in cells from chronically hypoxic animals or in the normoxic cells, over control levels. The addition of Ketanserin, a specific 5-HT_{2A} antagonist, and GR55562, a 5-HT_{1B-1D} antagonist had no effect on phosphorylation of p42/p44 MAP kinase. The total amount of p42/p44 MAP kinase was examined (lower panel) to ensure equal loading of protein on the polyacrylamide gel.

Figure 8.7

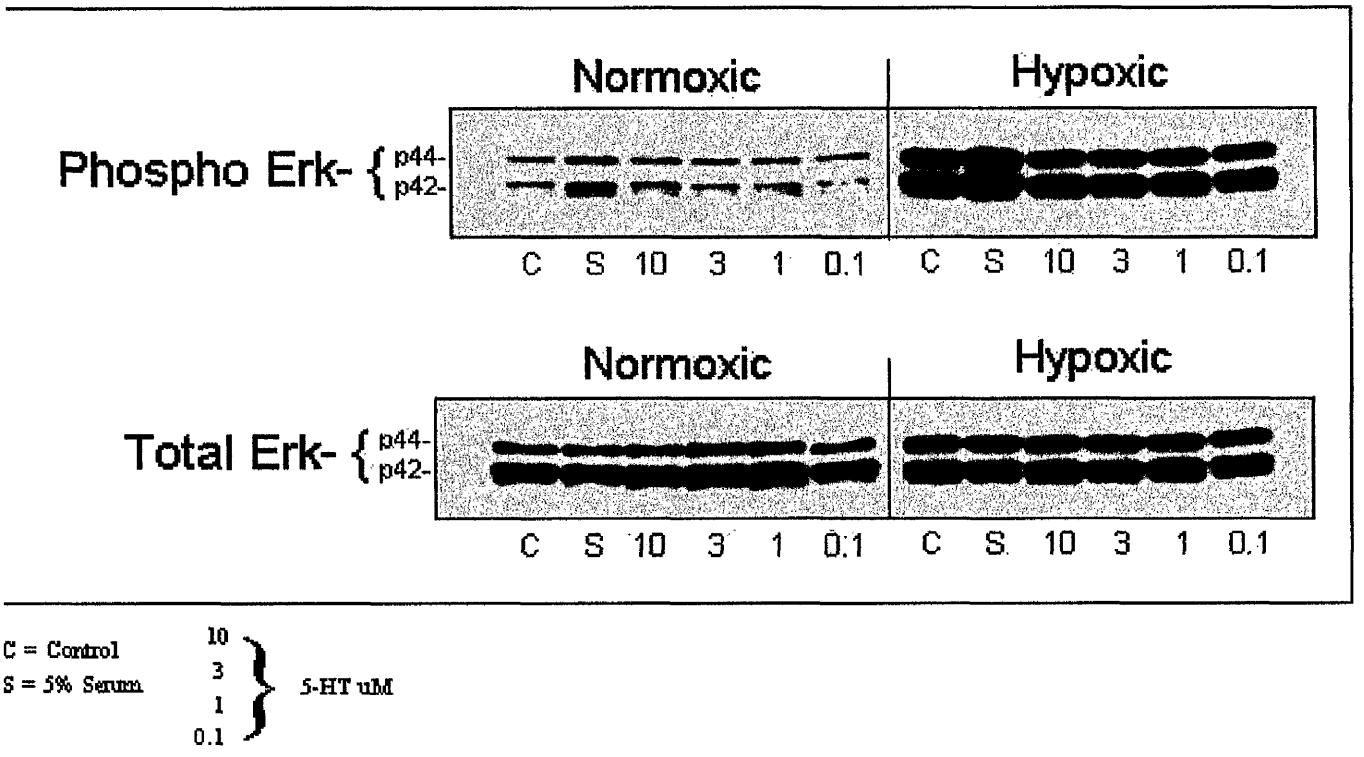
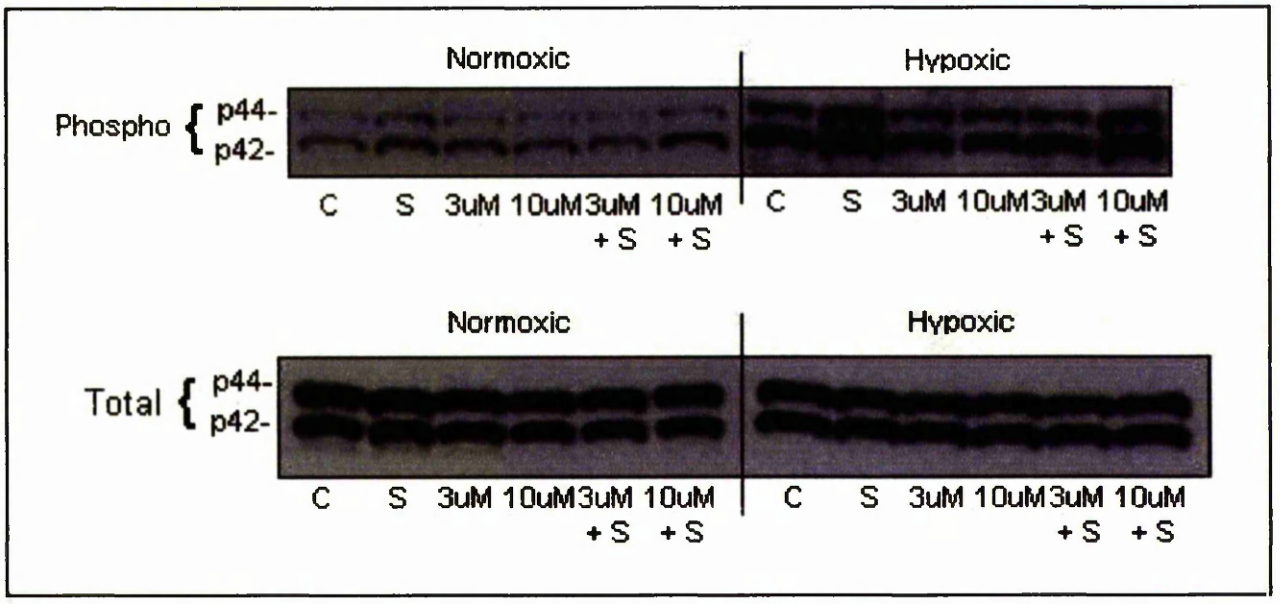


Figure 8.7 Effect of Increasing Concentration of 5-HT on p42/44 MAP Kinase Activity in Pulmonary Artery Fibroblast Cells fromControl and Chronically Hypoxic Animals

BPAF cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were then stimulated with a range of 5-HT concentrations as outlined in the figure for 24h. Assessment of kinase activity was by SDS PAGE gel electrophoresis and western blot analysis (See section 2.8). The experiment shown is one of 4 replicate experiments and is typical of those obtained. Band density is used as a measure of protein level.

Figure 8.8

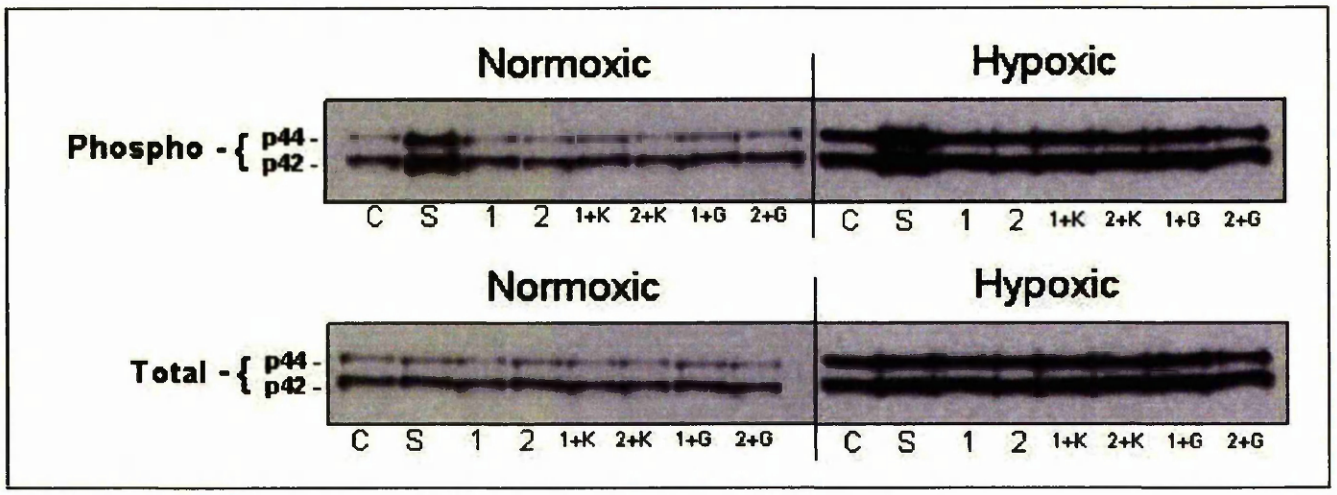


C = Control
S = 5% Serum
+S = 0.2% Serum

Figure 8.8 Effect of 5-HT and Serum on p42/44 MAP Kinase Activity in Pulmonary Artery Fibroblast Cells from Control and Chronically Hypoxic Rats.

BPAF cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were stimulated with 5% serum (S), 3 or 10μM 5-HT with or without 0.2% serum (+S) for 24h as indicated in the figure. Assessment of kinase activity was by SDS-PAGE gel electrophoresis and western blot analysis (See Chapter 2.8). The experiment shown is one of 4 replicate experiments and is typical of those obtained. Band density is used as a measure of protein level.

Figure 8.9



C = Control K = 0.1uM Ketanserin
S = 5% Serum GR = 1uM GR55562
1 = 3uM 5-HT
2 = 10uM 5-HT

Figure 8.9 Effect of 5-HT on p42/44 MAP Kinase activity in Pulmonary Artery Fibroblast cells from Control and Chronically Hypoxic Rats.

BPAF cells were grown to 90% confluency in 6-well plates and then quiesced for 24h in serum-free medium. The cells were then pre-incubated with 0.1μM ketanserin (a specific 5-HT_{2A} antagonist) or 1μM GR55562 (a specific 5-$HT_{1B/1D}$ antagonist) for 2h prior to stimulated with 3 or 10μM 5-HT for 24h. Assessment of kinase activity was by SDS PAGE gel electrophoresis and western blot analysis (See Chapter 2.8). The experiment shown is one of 4 replicate experiments and is typical of those obtained. Band density is used as a measure of protein level.

8.4 DISCUSSION

This chapter focused on the effects of 5-HT on the proliferation and cell signalling of RPAF cells from normoxic and chronic hypoxic rats. 5-HT alone did not produce a mitogenic response in pulmonary artery fibroblast cells from either control or chronically hypoxic animals. These results are in keeping with the work of others (Pitt *et al*, 1994; Seuwen and Pouyssegur, 1990) who studied 5-HT induced proliferation of bovine and rat pulmonary vascular smooth muscle cells in culture. In those studies, 5-HT had a co-mitogenic effect on the cells, requiring incubation with other growth factors such as PDGF and EGF. This is similar to the present work. The addition of a low concentration of serum (which in itself does not increase proliferation) in conjunction with 5-HT resulted in an increase of proliferation in both the normoxic and especially in the chronically hypoxic cells. This effect may require either binding of 5-HT to cell membrane receptors or active transport of 5-HT into the cell via the 5-HT transporter (5-HTT) (Eddahibi *et al,* 2000). One hypothesis could be that other co-mitogens change the structural conformity of the 5-HT receptor allowing 5-HT to bind, or that it opens up the 5-HTT allowing 5-HT to enter the cell. Previous work has demonstrated that the proliferative effect of 5-HT on [^{3}H]thymidine incorporation in bovine pulmonary artery smooth muscle cells was abolished after inhibition of the 5-HT transporter into the cell (Lee *et al*, 1991; Lee *et al*, 1994a).

Our previous work (Chapter 7) has demonstrated that cells from chronically hypoxic animals display increased basal levels of p38 and p42/p44 MAP kinase phosphorylation in comparison to those from normoxic cells, and further induced that phosphorylation which occurred in response to serum. In this present study, 5-HT increased pp38 above that of basal levels and in a dose-dependent manner to 5-HT. This occurred in the absence of any

co-mitogens. In contrast, 5-HT was unable to further increase p42/p44 MAP kinase phosphorylation above basal levels. However, in the presence of low levels of serum, there was a further enhancement of p42/p44 MAP kinase activation. Other work has demonstrated that 5-HT mediates its response by phosphorylating p42/44 MAP kinase (Florian and Watts, 1998; Watts, 1996). The mechanisms for this activation are unclear, however, signalling pathways for 5-HT have classically included activation of PLC and plasma membrane calcium channels that are sensitive to inhibition by dihydropyridines (Watts 1996). In our work, p42/p44 MAP kinase activation was activated by 5-HT only in the presence of co-mitogens. To date, this is the first study to show that pp38 is activated by 5-HT and also that the specific p38 MAP kinase inhibitor, SB203580, can block the enhanced hypoxia-associated proliferation with 5-HT.

Previous work has indicated that the 5-HT_1 receptor is involved in contraction and the 5-HT_2 receptor is involved in proliferation of cells (Frishman *et al* 1996). We were therefore particularly interested in the effects of 5-HT_1 and 5-HT_2 receptor antagonists on proliferation of RPAF cells and MAP kinase activation in these cells. Studies by other groups in rat aortic smooth muscle cells indicate that it is the 5-HT_{2A} receptor that mediates 5-HT stimulated phosphorylation and activation of the p42/p44 MAPKs (Kelleher *et al*, 1995). This may not be the case for RPAF cells since 5-HT alone does not increase p42/p44 phosphorylation. Other work has shown that the mitogenic effect of 5-HT on rat pulmonary vascular smooth muscle cells was attenuated by the 5-HT_2 receptor antagonist, ketanserin (Pitt *et al*, 1994). This would agree with our findings since the use of ketanserin blocked the increased proliferation in the cells from chronically hypoxic animals associated with hypoxia.

To be certain of which receptor was important in the proliferation and MAP kinase activation in chronically hypoxic animals with respect to 5-HT, further inhibitor studies in conjunction with specific agonist studies were undertaken. To date the 5-HT_2 receptor has 3 sub-types identified, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. Unfortunately, there are no specific 5-HT_{2A} agonists commercially available at this time, however, there are specific 5-HT_{2B} (BW72386) and 5-HT_{2C} (MK212) agonists and a specific 5-HT_2 agonist (α-methyl 5-HT). Use of these compounds allowed us to determine the specificity of the sub-types involved in proliferation of RPAF cells. There are also specific antagonists available naamely, 5-HT_{2A} (ketanserin), 5-HT_{2C} (RS102221) and a specific 5-$HT_{2B/C}$ antagonist (SDZ SER 082) which allowed us to determine further the action of the individual receptor sub-types.

The results demonstrated that, neither the actions of the 5-HT_{2B} nor 5-HT_{2C} agonists in the presence of low serum could produce a proliferative effect on either cell type. However, the selective 5-HT_2 agonist gave rise to a proliferative response in both cells types, which equalled the response in terms of magnitude of 5-HT itself. This suggests that the 5-HT_{2A} receptor may be responsible for proliferation of these cells. Inhibitor studies were then used to corroborate these results.

Again, the use of specific 5-$HT_{2B/C}$ inhibitors could not inhibit the replicative responses of these cells to 5-HT. The hypoxic-associated growth of these cells however, could be successfully abrogated by the use of ketanserin (5-HT_2 inhibitor). These findings agreed with the agonist studies and firmly pointed towards the 5-HT_{2A} receptor as being the receptor responsible for hypoxic-associated growth of pulmonary artery fibroblast cells from chronically hypoxic animals. The 5-HT_{2A} receptor also appears to signal via p38 MAP kinase because, by blocking the actions of this receptor we also blocked pp38 activation. pp38 has been shown previously in this thesis (Chapter 7) to be essential for hypoxic growth of pulmonary artery fibroblasts.

Chapter 9

General Discussion

9.1 Summary of Results

The data presented in this thesis shows that hypoxia has a marked effect on proliferation and cell signalling in pulmonary artery fibroblast cells. Cell models have been established to study the effects of acute and chronic hypoxia on fibroblast cells derived from the pulmonary and systemic circulations.

We have shown that in response to acute hypoxia:

- Acute hypoxia can stimulate pulmonary artery fibroblast cells to proliferate and can also increase the effect of mitogens. In contrast, acute exposure to hypoxia has no effect on proliferation of cells derived from the systemic circulation.
- This increase in proliferation also coincides with an increase in the second messenger IP_3 in pulmonary artery fibroblasts. Mitogens that caused increased proliferation to hypoxia in the pulmonary artery fibroblasts also gave rise to increases in IP_3 mass.
- Acute hypoxia also led to increased phosphorylation of members of the MAP kinase family of proteins that are known to be involved in cell growth and development.

We have also shown that in cells derived from chronically hypoxic rats but maintained in normoxia:

- Pulmonary artery fibroblast cells from chronically hypoxic animals have undergone a phenotypic switch whereby these cells proliferate to a level similar to those made acutely hypoxic.

- These cells also display increased MAP kinase phosphorylation when compared to those of control cells.
- Increased sensitivity to proliferation by 5-HT, a potent vasoconstrictor known to be released *in vivo* to hypoxia, was also apparent in the pulmonary artery fibroblasts from chronic hypoxic rats. This was again related to increased MAP kinase phosphorylation.
- The specific 5-HT receptor sub-type responsible for proliferation in the pulmonary artery fibroblasts from control and chronically hypoxic rats was also elucidated.

9.2 Species Difference

Closer examination of the results indicates that the effects of hypoxia on pulmonary artery fibroblasts are not always consistent between different species. The most notable example is in the p42/p44 MAP kinase responses of pulmonary artery fibroblasts. Acute hypoxia caused only a small increase in p42/p44 MAP kinase activity in cells derived from the bovine model whereas the rat cells that had been exposed to chronic hypoxia showed high levels of p42/p44 MAP kinase phosphorylation. This was also true of other MAP kinase isoforms, for example, in the bovine model, JNK was activated in the face of hypoxia which was unlike the rat model. This was also the case when cells from different species were subjected to hypoxia in cell proliferation studies. In the bovine cells, acute hypoxia alone increased proliferation, whilst in rat cells hypoxia increased proliferation only in the presence of mitogens. This apparent conflict in results may be due to an underlying difference in the pharmacology or biochemistry of the pulmonary artery fibroblasts of the various

species. It must also be pointed out that bovine cells were exposed to low O_2 levels (~30mmHg) for a relatively short time (24h) whereas rat cells had been exposed (*in vivo*) to less severe hypoxic exposure for a longer time period (~330mmHg for 14 days). It is possible that the effect of hypoxia on cell proliferation is dependent on both the intensity and the duration of the hypoxic stimulus, but this remains to be addressed by further scientific investigation.

Alternatively, the difference may be due to the different order of pulmonary artery used from species to species. In both animals, we used the main branch of the pulmonary artery. However, in the bovine cells the piece of tissue was isolated from well down the main branch near to the tip of the lobe. In the rat, the tissue was isolated just as the artery entered into the lungs. As stated in chapter 1, the response of pulmonary arteries to hypoxia may differ between proximal and distal regions of the vessel. This may account for the experimental differences between the species. It would have been interesting to conduct a study comparing various regions of the pulmonary artery from each species. This would have been possible in the bovine lung where the vessels were large. However, the rat pulmonary artery rapidly decreases in size and explanting become increasingly difficult.

Whilst the effect of hypoxia differed between species, there was also differences in proliferation responses to various agonists. In pulmonary artery fibroblasts from the cow, endothelin-1 did not induce proliferation whereas in the rat, ET-1 was a good mitogen (Peacock and Aidulis, 1994). Also, in the bovine model, 5-HT acted as a mitogen on its own whilst in the rat model it only acted as a co-mitogen. It is possible that this may be due to changes in receptor abundance and conformity in fibroblasts from different species.

9.3 Role of Stress-Activated Protein Kinases

It is clear from the results that there are factors in both the pulmonary arteries from cow and rat that are important to the hypoxia-associated increases in fibroblast proliferation. The stress-activated protein p38 MAP kinase was activated in the face of acute hypoxia in the bovine model, and also in the cells from chronically hypoxic rats. The ability of a specific pharmacological inhibitor of this protein (SB203580) to attenuate hypoxic-stimulated DNA synthesis in growth-arrested cells implies that its biological function is to relay signals important for cells to re-enter the cell cycle. That hypoxia evokes the activation of multiple signalling pathways emphasises the intricate levels of control imposed on crucial cellular decisions such as those made prior to cell division. Ultimately, a complex process such as re-entry into the cell cycle will depend on the specific transcription factors that become activated by the terminal protein kinase of the pathways to which a receptor of a particular mitogen or stress is coupled. How these transcription factors combine in the nucleus to induce the co-ordinated expression of specific early genes is critical for cell cycle progression. Research efforts in the past few years have been successful in identifying some of the transcription factors regulated by these kinases. For example, $p62^{TCF}$/Elk-1 is a common nuclear target for both the p42/44 and p38 MAP kinases isoforms which were shown in the cells from chronically hypoxic animals to be in abundance when compared with control cells (Marais *et al*, 1993, Raingeaud *et al*, 1996). While ATF-2 is a substrate for p38 MAP kinase *in vitro* and in transfected cells (Raingeaud *et al*, 1996). In addition, specific nuclear targets for $p70^{s6k}$, MAPKAP kinase-2 and $p90^{rsk}$ are also coming to light (de Groot *et al*, 1994). While all of these factors would be predicted to become activated on exposure of pulmonary artery fibroblasts to hypoxia,

the full array of genes that they control, and the contribution that products of these genes make to the genetic program regulating the cell cycle still has to be investigated. A better understanding of the transcriptional control of genes will be necessary to provide information concerning how various signalling inputs impinging upon the nucleus are co-ordinated to initiate complex genetic responses. In the meantime, compounds such as Ketanserin and SB203580 used in this study will serve as valuable tools for identifying the specific nuclear apparatus by which transcription events are regulated by allowing researchers to dissect pathways involved in transcriptional control.

9.4 Hypoxia causing a Phenotypic Switch

The findings in this thesis that pulmonary artery fibroblasts from chronically hypoxic animals would appear to have undergone some form of phenotypic switch whereby signals associated with hypoxia are always switched on may help in the understanding of which genes are important in the response. It would be logical to assume that if the products of the gene were constantly available, then the genes that control their regulation would also be constantly in production. This would allow for comparisons of gene amount and presence to be easily ascertained.

Despite outlining some of the protein cascades involved in hypoxic-stimulated mitogenesis, a number of points remain unsolved regarding upstream events involved in their activation, the possible interplay between these pathways, and the nature of the hypoxic "receptor/s" that they are coupled to. While signal transduction by hypoxia is far from understood and it is unknown whether hypoxia signals via G-protein interactions or receptor tyrosine kinases, an observation in the BPA fibroblasts

was the resemblance of hypoxic mediated events to signalling characteristics of growth factors that activate receptor tyrosine kinases, such as PDGF. Thus, for example, activation of MAP kinase by hypoxia followed a slow and gradual time course rather than the rapid kinetics reported in other cell types that are often maximal within 2-5 minutes. The relative importance of G-protein-coupled receptor and tyrosine kinase receptor-mediated signalling events triggered by stresses and growth factors is currently a topic of considerable interest. Research within the past few years has provided novel paradigms to support the concept of physiologically relevant cross talk between G-protein-coupled receptors, and tyrosine kinase receptor-linked routes for communication within the cell (Daub *et al,* 1996).

9.5 Pulmonary vs. Systemic arteries

This thesis has demonstrated for the first time, to our knowledge, a fundamental difference in response to hypoxia between systemic and pulmonary artery cells. Unlike pulmonary artery fibroblasts, fibroblast cells from the systemic circulation in both the bovine and rat models do not proliferate to hypoxia, or indeed give rise to the phosphorylation of the proteins which we consider important. This finding is fundamental to this thesis as it mimics the known physiological response where hypoxia *in vivo* does not result in vasoconstriction or in remodelled vessels in the systemic arteries. It also supports the results from the pulmonary artery fibroblasts with respect to increased generation of signals resulting in proliferation, which are not seen in the systemic artery cells.

Despite questions that still need to be resolved, these studies have outlined a number of novel cellular consequences of hypoxia on cells, further defining the possible

mechanisms by which it is able to influence their behaviour. In particular, the studies highlight pathways that are likely to operate during vascular disease states like pulmonary hypertension and other hypoxic forms of lung disease where proliferation of these cell types contribute to blood vessel wall remodelling. A more conclusive role for these pathways will become apparent in future literature.

9.6 Future Work

The work of this thesis shows quite clearly that hypoxia is an important regulator of the pulmonary vasculature and the mechanisms by which hypoxia regulates remodelling during pulmonary hypertension may help us identify potential therapeutic targets.

While our picture of acute and chronic stimulation of cell signalling in our cell models of pulmonary hypertension is incomplete, it is clear that specific MAP kinase signalling pathways are being activated in cells from the pulmonary circulation. Furthermore, these pathways induce HIF-1 (Minet *et al,* 2000) which can lead to the upregulation of several genes such as the mitogen VEGF. This raises the possibility of exploiting differences between pathways as seen in this thesis in order to specifically inhibit hypoxia-induced signalling pathways in cardiopulmonary diseases. Achieving this goal requires a detailed biochemical and molecular understanding of the signalling pathways activated in pulmonary fibroblasts in response to hypoxia and a study of the regulatory events that these control.

Defining exactly how MAP kinase activation is involved in pulmonary hypertension is essential if we are to identify potential sites for therapeutic intervention. Although we have established that hypoxia causes an increase in proliferation of PAF cells

derived from the pulmonary artery of rats and cows, the effects of this on progression of these cells through the cell cycle is unknown. Recent work has shown that MAP kinase pathways converge to regulate cyclin D1 during hypoxic exposure of PC12 cells (Conrad *et al* 1999). Since hypoxia increases proliferation in our cell model, the role of cyclin D1 in hypoxia-induced proliferation of PAF cells and the regulation of its expression by p38 MAPK and ERK pathways is unclear and would be an attractive addition to this research.

We have shown that intracellular processes are specifically altered by decreased O_2 in pulmonary artery but not systemic fibroblasts. Now we should take the investigation one step further and look at which transcription factors are turned on by hypoxia and which genes are thus activated.

Along with the increase in proliferation of fibroblasts seen in pulmonary vascular remodelling, fibroblasts have an important role to play in laying down matrix proteins such as collagen, which render the vessels indistensible. Future work should include the examination of extracellular matrix protein production from normoxic and hypoxic pulmonary artery fibroblasts and compare such protein generation to those of systemic vascular cells. Studying how the intracellular signalling changes seen in fibroblast cells due to hypoxic exposure may effect extracellular matrix production could also ultimately hold a clue for future treatments of pulmonary vascular remodelling during pulmonary hypertension.

References

Abrams HD, Rohrschneider LR and Eisenman RN (1982) Nuclear location of the putative transforming protein of avian myelocytomatosis virus *Cell* **29:** 427-439

Aceto J. F. and Baker K. M. (1990) [Sar 1] angiotensin II receptor-mediated stimulation of protein synthesis in chick heart cells *Am. J. Physiol.* **158:** H806-H813.

Alexander A. F and Jenson R (1959) Gross cardiac changes in cattle with high mountain (Brisket) disease, and in experimental cattle maintained at high altitudes *Am. J. Vet. Res.*, **20:** 680.

Anderson NG, Maller JL, Tonks NK and Sturgill TW (1990) Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* **343:** 651-653.

Ankoma-Sey V, Wang Y and Dai Z (2000) Hypoxic stimulation of vascular endothelial growth factor expression in activated rat hepatic stellate cells. *Hepatology* **31(1):** 141-148.

Archer SL, Eaton LW and Nelson DP (1988) Oxygen radicals and antioxidant enzymes alter pulmonary vascular reactivity in the rat lung. *J. Appl. Physiol.*, **66**: 102-111

Asaoka Y, Nakamura S, Yoshida K and Nishizuka Y (1992a). Protein kinase C, calcium and phospholipid degradation. *TIBS.* **17:** 414-417.

Asaoka Y, Yoshida K, Oka M, Shinomura T, Mishima H, Matsushima S and Nishizuka Y (1992b). The signal-induced phospholipid degradation cascade and protein kinase C activation. *Ciba Foundation Symposium.* **164:** 50-54.

Auer KL, Contessa J, Brenz-Verca S, Pirola L, Rusconi S, Cooper G, Abo A, Wymann MP, Davis RJ, Birrer M and Dent P (1998) The Ras/Rac1/Cdc42/ SEK/JNK/c-jun cascade is a key pathway by which agonists stimulate DNA synthesis in primary cultures of rat hepatocytes. *Mol. Biol. Cell* **9:** 561-573.

Balboa MA, Firestein BL, Godson C, Bekk KS and Insel PA (1994) Protein kinase C mediates phospholipase D activation by nucleotides and phorbol ester in Madin-Darby canine kidney cells. Stimulation of phospholipase D is independent of activation of polyphosphoinositide-specific phospholipase C and phospholipase A_2. *J. Biol. Chem.* **269:** 10511-10516.

Ballou, L. M., Luther, H. and Thomas, G. (1991) MAP2 kinase and 70K S6 kinase lie on distinct signalling pathways. *Nature* **349:** 348-350

Beg, AA and Baldwin, AJ (1993). The IκB proteins: multifunctional regulators of Rel/NFKB transcription factors. *Genes and Development* **7:** 2064-2070.

Belham CM, Rithwelle TJ, Scott PH, Pemberton AD, Miller HRP, Wadsworth RM, Gould GW and Plevin R. (1996) Trypsin stimulates proteinase-activated receptor-2-

dependent and-independent activation of mitogen-activated protein kinases *Biochem. J.* **320:** 939-946.

Belknap JK, Orton EC, Ensley B and Stenmark KR (1997) Hypoxia increases bromodeoxyuridine labeling indicies in bovine neonatal pulmonary arteries. *Am. J. Respir Cell Mol Biol* **16:** 366-371.

Benitz WE, Kelly RT, Anderson CM, Lorant DE and Bernfield M (1990) Endothelial heparin sulphate proteoglycan. Inhibitory effects on smooth muscle cell proliferation. *Am. J. Respir. Cell. Mol. Biol.* **2:** 13-24.

Berridge MJ (1987) Inositol lipids and cell proliferation. *Biochem. Biophys. Acta* **907:** 33-45

Berridge MJ (1993a) Inositol trisphosphate and calcium signalling. *Nature* **361:** 315-325.

Berridge MJ (1993b) Intracellular channels and calcium spiking. *Biomedical Research (Japan)* **14:** 21-27.

Berridge MJ and Galione A (1988) Cytosolic calcium oscillators. *FASEB J* **2**: 3074-3082

Berridge MJ and Irvine RF (1989) Inositol phosphates and cell signalling. *Nature* **341:** 197-203.

Berra E, Diaz-Meco MT, Dominguez I, Municio MM, Sanz L, Lozano J, Chapkin RS and Moscat J (1993) Protein kinase C isoform is critical for mitogenic signal transduction *Cell* **74:** 555-563.

Bishop JM (1985) Viral Oncogenes. *Cell* **42:** 23-38

Bitterman PB and Henke CA (1991) Fibroproliferative disorders. Chest **99:** 185-192.

Blancher C, Moore JW, Talks KL, Houlbrook S and Harris AL (2000) Relationship of hypoxia-inducible factor (HIF)-1 alpha and HIF-2 alpha expression to vascular endothelial growth factor induction and hypoxia survival in humans. *Cancer Res* **60(24):** 106-113.

Boarder M (1994) A role for phospholipase D in control of mitogenisis. *TIPS.* **15:** 57-62.

Bobrov VA, Fushtei IM and Polivoda SN (1985) Pulmonary hemodynamics and serotonin level in patients with chronic penumonia with arterial hypertension. *Vrach. Delo.* **12:** 33-5.

Bogoyevitch MA, Ketterman AJ. and Sudgen PH. (1995) Cellular stresses differentially activate c-jun N-terminal protein kinases and extracellular signal-regulated protein kinases in cultured ventricular myocytes. *J. Biol. Chem* **270:** 29710-29717.

Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ and Sugden PH (1996) Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. *Circ. Res.* **79:** 162-173.

Bogoyevitch MA, Glennon PE, Andersson MB, Clerk A, Lazou A, Marshall CJ, Parker PJ and Sugden PH (1994) Endothelin-1 and fibroblast growth factor stimulate the mitogen-activated protein kinase signalling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signalling pathways leading to myocyte hypertrophy. *J. Biol. Chem.* **269:** 1110-1119.

Bonfini L, Karlovich CA, Dasgupta C and Banerjee U. (1992) The son of sevenless gene product: A putative activator of Ras. *Science* **255:** 603-606.

Bowtell D, Fu P, Simon M and Senior P (1992) Identification of murine homologues of the Drosophila son of sevenless gene: Potential activators of ras. *Proc. Nat. Acad. Sci. USA.* **89:** 6511-6515.

Brenot F, Herve P, Petitpretz P, Parent F, Duroux P and Simmonneau G (1993) Primary Pulmonary hypertenstion and fenfluramine use. *Br Heart J* **70:** 537-41.

Brogden RN and Sorkin EM (1990) Ketanserin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in hypertension and peripheral vascular disease. *Drugs* **40:** 903-949.

Bunn HF and Poyton RO. (1996) Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76:** 839-885.

Bunn HF, Gu J, Huang LE, Park J-W and Zhu H (1998) Erythropoietin: a model system for studying oxygen-dependent gene regulation. *J. Exp. Biol.* **201:** 1197-1201.

Campbell JA (1927a) Further observations on oxygen acclilatisation. *J. Physiol.* **63:** 325-342.

Campbell JA (1927b) Note on some pathological changes in the tissues during attempted acclimatisation to alterations of O2-pressure in the air. *Br. J. Exp. Pathol.* **8:** 347-351.

Campbell JA (1927c) Prolonged alterations of oxygen pressure in the inspired air with special reference to tissue oxygen tension, tissue carbon dioxide and haemoglobin. J. Physiol. **62:** 211-231.

Campbell JA (1935) Further evidence that mammels cannot acclimatise to 10 p.c. oxygen or 20,000 feet altitude. *Br. J. Exp. Path.* **16:** 39-48.

Cardenn DL and Gill GN (1992) Receptor tyrosine kinases. *FASEB J.* **6:** 2332-2337.

Carney DH, Scott DL, Gordon EA and LaBelle EF (1985). Phosphoinositides in mitogenisis: neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. *Cell.* **42:** 479-488.

Carpenter CL, Auger KR, Chanudhuri M, Yoakim M, Schaffhaussen B, Shoelson S and Cantley LC (1993) Phosphoinositide 3- kinase is activated by phosphopeptides that bind to the SH2 domains of 85 kDa subunit. *J. Biol. Chem.* **268:** 9478-9483.

Carpenter G (1992) Receptor tyrosine kinase substrates: src homology domains and signal transduction. *FASEB J.,* **6:** 3283-3289.

Carsten ME and Miller JD (1990) In "Uterine function, molecular and cellular aspects" eds. Carsten ME and Miller JD p121-167. Plenum, New York.

Catling AD, Reuter CWM, Cox M, Parsons SJ and Weber MJ (1994) Partial purification of a mitogen-activated protein kinase from bovine brain. *J. Biol. Chem.* **269:** 30014-30021.

Chadwick CC, Saito A and Fleischer S (1990) Isolation and characterisation of the inositol trisphosphate receptor from smooth muscle. *Proc. Nat. Acad. Sci. USA* **87:** 2132-2136.

Chen RH, Sarnecki C and Blenis J (1992) Nuclear localisation and regulation of ERK- and RSK-encoded protein kinases. *MCB.* **12:** 915-927.

Chen YR, Wang X, Tempelton D, Davis RJ. and Tan TH. (1996) The role of c-Jun N-terminal (JNK) in apoptosis induced by ultraviolet C and gamma radiotion. Duration of JNK activation may determine cell death and proliferation. *J. Biol Chem* **271:** 31929-31936.

Clark JD, Schievella AR, Nalefski EA and Lin LL (1995) Cytosolic phospholipase A2 *Journal of Lipid Mediators and Cell Signalling.* **12:** 83-117

Clerk A, Michael A and Sugden P (1998) Stimulation of the p38 Mitogen-activated protein kinase pathway in neonatal rat ventricular myocytes by the G-protein-coupled receptor agonists, endothelin-1 and phenylephrine: A role in cardiac myocyte hypertrophy? *J. Cell Biol.* **142:** 523-535

Coburn RF and Baron CB (1990) Coupling mechanisms in airway smooth muscle. *Am. J. Physiol.* **258:** L119-L133.

Comroe JH, van Lingen B and Stroud RC (1953) Reflex and direct cardiopulmonary effects of 5 OH trypyamine (serotonin). Their possible role in pulmonary embolism and coronary thrombosis. *Am. J. Physiol.* **173:** 379-386.

Conrad PW, Rust RT Han J Millhorn DE and Beitner-Johnson D (1999) Selective Activation of p38α and p38γ by Hypoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells. *J. Biol. Chem.* **274:** 23570-23576

Cook SJ, Palmer S, Plevin R and Wakelam MJO (1990) Mass measurement of inositol 1,4,5-trisphosphate and sn-1, 2-diacylglycerol in bombesin-stimulated Swiss 3T3 mouse fibroblasts *Biochem. J.* **265:** 617-620

Cornfield DN, Stevens T, McMurtry IF, Abman SH and Rodman DM (1993) Acute hypoxia increases cytosolic calcium in foetal pulmonary artery smooth muscle cells. Am. J. Physiol. **265:** L53-L56

Coso OA, Chiariello M, Kalinec G, Kyriakis JM, Woodgett J and Gutkind S (1995) Transforming G protein-coupled receptors potently activate JNK (SAPK). J. Biol.Chem.**270:** 5620-5624

Cowley S, Paterson H, Kemp P and Marshall CJ (1994) Activation of MAP kinase kinase in necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77:** 841-852.

Crespo P, Simonds WF and Gutkind JS (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein betagamma subunits. *Nature* **369:** 418-420.

Crouch EC, Parks WC, Rosenbaum JL, Chang D, Whitehouse L and Wu LJ (1989) Regulation of collagen production by medial smooth muscle cells in hypoxic pulmonary hypertension. *Am Rev Respir Dis* **140(4):** 1045-51

Cryer A and Bartly WE (1974) The design and use of a hypoxic chamber for small animals. *Laboratory Practice* **23:** 713-715.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young P R and Lee JC (1995) SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364:** 229-233.

Dai T, Rubie E, Franklin CC, Kraft A, Gillespie DAF, Avruch J, Kyriakis JM and Woodget JR (1995). Stress-activated protein kinases bind directly to the δ domain of c-Jun in resting cells: implications for repression of c-Jun function. *Oncogene.* **10:** 849-855.

Dalton S and Treisman R (1992) Characterisation of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. *Cell* **68:** 597-612.

Das M, Dempsey EC, Bouchey D, Reyland ME and Stenmark KR (1999) Chronic hypoxia induces exaggerated growth responses in pulmonary artery adventitial fibroblasts. *Am. J. Resp. Cell Mol. Biol.* **22:** 15-25.

Datta SR, Brunet A and Greenberg ME (1999) Cellular Survival: a play in three Akts. *Gen. Dev.* **13:** 2905-2927.

Daub H, Weiss FU, Wallasch C and Ullich A (1996) Role for transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379:** 557-560.

Davies PF and Ross R (1978) Mediation of pinocytosis in cultured arterial smooth muscle and endothelial cells by platelet-derived growth factor. *J. Cel.l Biol.* **79,** 663-671.

Davis RJ (1993) The mitogen-activated protein kinase signal transduction pathway. *J.Biol.Chem.* **268:** 14553-14556

De Groot RP, Ballou LM and Sassone-Corsi P (1994) Positive regulation of the cAMP-responsive activator CREM by the p70 S6 kinase: an alternative route to mitogen induced gene expression. *Cell* **79:** 81-91.

de Vries-Smits AMM, Burgering BMT, Leevers SJ, Marshall CJ and Bos JL (1992) Involvement of p21ras in activation of extracellular signal-related kinase-1. *Nature* **357:** 602-604.

Dempsey EC, Frid MG, Aldashev AA, Das M and Stenmark KR. (1997) Heterogeneity in the proliferative response of bovine pulmonary artery smo.oth muscle cells to mitogens and hypoxia: Importance of protein kinase C. *Can J. Physiol. Pharmacol.* **75(7):** 936-944

Dempsey EC, McMurty and O'Brian RF (1991). Protein kinase C activation allows pulmonary artery smooth muscle cells to proliferate to hypoxia. *Am. J. Physiol.* **260:** L136-L145.

Dent P, Haser W, Haystead TAJ, Vincent LA, Roberts TM and Sturgill TW (1992) Activation of mitogen-activated protein kinase kinase by v-Raf in NIH3T3 cells and *in vitro Science* **257:** 1404-1406.

Derijard B, Hibi M, Wu IH, Barratt T, Su B, Deng T, Karin M and Davis RJ (1994) Jnk: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76:** 1025-1037.

Derijard B, Raingeaud J, Barrett T, Wu I-T, Han J, Ulevitch RJ and Davis RJ (1995) Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* **267:** 682-685.

Deuel TF, Tong BD and Huang JS. (1983) Platelet-derived growth factor: structure, function and roles in normal and transformed cells. *Curr. Top. Cell. Regul.* **26:** 51-64.

DeVivo M and Iyenrar R (1994) G protein pathways: Signal processing by effectors. *Mol. Cell. Endocrin.* **100:** 65-70.

Dosal DE, Baker KM and Peach MJ (1991) in Horizons in Endocrinology (Maggi, M. and Greenen V. eds) pp. 265-272, Raven Press, New York

Downes CP and Carter N. (1992) Phosphoinositide 3-kinase: a new effector in signal transduction. *Cellular Signalling.* **3:** 501-513.

Durmowicz AG, Orton EC and Stenmark KR (1993) Progressive loss of vasodilator responsive component of pulmonary hypertension in neonatal calves exposed to 4,570m. *Am J. Physiol.* **265:** H2175-2183.

Eddahibi S, Hanoun N, Lanfumey L, Lesch KP, Raffestin B, Hamon M and Adnot S (2000) Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. *Am J Respir Crit Care Med* **161 (3):** A143

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM and Weinburg RA (1993). Association of mSOS/Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363:** 45-51.

Elder H, Ben-Av P, Schmidt U-S, Livneh E and Liscovitch M (1993). Upregulation of phospholipase D activity induced by overexpression of protein kinase C-a. Studies in intact Swiss/3T3 cells and in detergent-solubilised membranes in vitro. J.Biol. Chem.. **268:** 12560-12564.

Escobedo JA, Navankasattusas S, Kavanaugh WM, Milfrey D, Fried VA and Williams LT (1991) cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI-3 kinase to the β-receptor. *Cell* **65:** 75-82.

Esterly JA, Glagov S and Ferguson DJ (1968). Morphogenesis of intimal oblitererative hyperplasia of small arteries in experimental pulmonary hypertension. An ultrastructural study of the role of smooth muscle cells. *Am. J. Pathol.*, **52:** 325-347

Evered D, Nudgent J and Whelan J. (1985) Growth factors in biology and medicine Ciba Found. Symp **116:** 1-283

Exton JH (1994) Phosphatidylcholine breakdown and signal transduction. *Biochem. Biophys. Acta.* **1212:** 26-42.

Exton JH (1997) Cell signalling through guanine-nucleotide-binding regulatory proteins (G proteins) and phospholipases. *Eur. J. Biochem.* **243:** 10-20.

Fandrey J, Huwiler A, Frede S, Pfeilschifter J and Jelkmann W (1994) Distinct signalling pathways mediate phorbol-ester induced and cytokine-induced inhibition of erythropoietin gene expression. *Eur. J. Biochem.* **226:** 335-340.

Fantl WJ, Johnson DE and Williams LT (1993) Signalling by receptor tyrosine kinases. *Ann. Rev. Biochem.* **62:** 543-481.

Faure M, Voyno-Yasenetskaya TA and Bourne HR (1994) cAMP and betagamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.* **269:** 7851-7854.

Feldkamp MM, Lau N, Rak J, Kerbel RS and Guha A (1999) Normoxic and hypoxic regulation of vascular endothelial growth factor is mediated by Ras. *Int. J. Cancer* **81(1):** 118-24.

Feng G, Goodridge H. S. Harnett M M, Wei X, Nikolaev A. V., Higson A. P. and Liew F. (1999) Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinase differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *J. Immunol.* **163:** 6403-6412.

Feng X and Cai Y (1996) Hypoxia and endothelin-1 stimulate DNA synthesis of pulmonary artery smooth muscle cells. *Chin. Med. Sci. J.* **11:1** 28-31

Ferris CD and Snyder SH (1992) Inositol 1,4,5-trisphosphate-activated calcium channels. *Annu. Rev. Physiol.* **54:** 469-488

Firth JD, Edbert BL Pugh CW and Ratcliffe PJ. (1994) Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: Similarities with the erythropoietin 3' enhancer. *Proc. Nat. Ac. Sc. Am.* **91**: No.14 6496-6500.

Florian JA and Watts SW (1998) Integration of mitogen activated protein kinase kinase activation in vascular 5-hydroxytryptamine 2_A receptor signal transduction. *J Pharmacol Exp Ther* **284:** 346-355.

Freshney IR (1994) from Culture of Animal Cells. A Manual of Basic Techniques 3rd Edition. Wiley-Liss & Sons Inc.

Freshney IR (1983) Culture of Animals Cells. A. R. Liss, New York. 99-118.

Frid MG, Aldashev AA, Dempsey EC and Stenmark KR (1997a) Smooth muscle cells isolated from discrete compartments of the mature vascular media exhibit unique phenotypes and distinct growth capabilities. *Circ. Res.* **81**: 940-952

Frid MG, Dempsey EC, Durmowicz AG and Stenmark KR. (1997b) Smooth muscle cell heterogeneity in pulmonary and systemic vessels. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1203-1209.

Frid MG, Moiseeva EP and Stenmark KR (1994) Multiple phenotypically distinct smooth muscle cell populations exist in the adult and developing bovine pulmonary arterial media in vivo. *Circ. Res.* **75:** 669-681.

Frishman WH, Huberfeld S, Okin S, Wang YH, Kumar A and Shareef B (1995) Serotonin and serotonin antagonism in cardiovascular and non-cardiovascular disease. *J. Clin. Pharmacol.* **35:** 541-72.

Fry MJ (1994) Structure, regulation and function of phosphoinositide 3-kinases. *Biochem. Biophys. Acta* **1226:** 237-268.

Gerrard J, Phillips D, Rad G, Plow E, Walz D, Ross R, Herker L and White J (1980) Biochemical studies of two patients with the gray platelet syndrome. Selective deficiency of platelet alpha granules *J. Clin. Invest.* **66:** 102-109

Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimuva S, Masaki T, Duguid WP and Stewart DJ (1993) *N. Engl. J. Med* **328:** 1732-1739

Gille, H, Kortenjann, M, Thomae, O, Moomaw, C, Slaughter, C, Cobb, M. and Shaw PE (1995) ERK phosphortylation potentiates ELK-1-mediated tertiary complex formation and transactivation *EMBO J.* **14:** 951-962

Gille H, Sharrocks AD and Shaw P (1992) Phosphorylation of trabscription factor p62TCF by MAP kinase stimulates ternary cemplex formation at c-fos promotor. *Nature* **358:** 414-421

Glover GH and Newsom IE (1915) Brisket disease (dropsy of high altitude). *Agric. Station of Col. Agric. Coll. Bull,* **204:** 3-24.

Goldberg HJ, Viegas MM, Margis BL, Schlessinger J and Skorecki KL (1990) The tyrosine kinase activity of the epidermal growth factor receptor is necessary for phoapholipase A2 activation. *Biochem. J.* **267:** 461-465.

Gonzales, GA (1991) Characterisation of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *MCB* **11:** 1306-1312.

Gosney J, Heath D, Smith P, Harris P and Yacoub M (1989) Pulmonary endocrine cells in pulmonary arterial disease. *Arch. Pathol. Lab. Med.* **113:** 337-41.

Granot Y, Erikson E, Fridman H, Van Putten V, Williams B, Schrier RW and Maller JL. (1993) Direct evidence for tyrosine and threonine phosphorlyation and activation of mitogen-activated protein kinase by vasopressin in cultured rat vascular smooth muscle cells. *J. Biol. Chem.* **268:** 9564-9569.

Grinstein S, Rotin D and Mason MJ (1989) Na^+/H^+ exchange and growth factor-induced cytosolic pH changes: role in cellular proliferation. *Biochem. Biophys. Acta* **988:** 73-97.

Grovner RF, Wagner WW, McMurty IF and Reeves JT (1983) Pulmonary circulation, in *Handbook of Physiology. Section 2: The cardiovascular system, vol.111: Peripheral Circulation and Organ Blood Flow. Am. Physiol. Soc.,* Bethesa MD, part 1, pp. 103-366

Ha KS and Exton JH (1993) Differential translocation of protein kinase C isozymes by thrombin and platelet-derived growth factor. *J. Biol. Chem.* **268:** 10534-10539

Han J, Lee J-D, Bibbs L and Ulevitch RJ (1994) A MAP kinase targeted by an endotoxin and hyperosmolarity in mammalian cells *Science* **265:** 808-811

Harris P and Heath D (1986) The human pulmonary circulation, Churchhill Livingstone, New York 279-280

Hart CM and Block ER (1989) Lung serotonin metabolism *Clin. Chest. Med.* **10:** 59-70.

Hasen NM, Parker PJ and Adams GE (1996) Induction and phosphorylation of protein kinase C-alpha and mitogen-activated protein kinase by hypoxia and by radiation in Chinese hamster V79 cells. *Rad. Res.* **145:** 128-133.

Hasleton PS, Heath D and Brewer DB (1968) Hypertensive pulmonary vascular disease in states of chronic hypoxia. *J. Pathol. Basceriol.* **95:** 431-440.

Hassoun PM, Thappa V, Landman MJ and Fanburg BL (1992) Endothelin-1: mitogenic activity on pulmonary artery smooth muscle cells and release from hypoxic endothelial cells. Proceedings of the Society of Experimental Biological Medicine **199:** 165-170.

Hata A, Akita Y, Suzuki K and Ohno S (1993) Functional divergence of protein kinase C (PKC) family members. *J. Biol. Chem.* **268:** 9122-9129.

Heath D, Smith P, Harris P and Yacoub M (1988) Plexiform lesion in bronchopulmonary anastomosis *Br. J. Dis. Chest* **82:** 294-299.

Heffner JE, Sahn SA and Repine JE. (1987) The role of platelets in the adult respiratory distress syndrome. Culprits or bystanders? *Am. Rev. Respir. Dis.* **135:** 482-92.

Heldin C-H and Westmark B (1984) Growth factors: mechanism of action and relation to oncogenes *Cell* **37:** 9-20.

Hesketh TR, Moore JP, Morris JD, Taylor MV, Rogers J, Smith G and Metcalfe JC (1985) A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. *Nature* **313:** 481-484

Hibi M, Lin A, Smeal T, Minden A and Karin M (1993) Identification of an oncoprotein- and UV-responsive protein kinase that bind and potentiate the c-jun activation domain. *Gen. Dev.* **7:** 2135-2148.

Hill CS and Treisman R (1995) Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80:** 199-211.

Hirano M, Hirai SI, Mizuno K, Osada SI, Hosaka M and Ohno S (1995) A protein kinase C isozyme, nPKCε, is involved in the activation of NF-κB by 12-o-tetradecanoylphorbol-13-acetate (TPA) in rat 3Y1 fibroblasts. *Biochem. Biophys. Res. Comm.* **206:** 429-436

Ho AK, Thomas TP, Chik CL, Anderson WB and Klein DC (1988) Protein kinase C: Subcellular redistribution of protein kinase C is involved in potentiation of beta-adrenergic stimulation of pineal cAMP and cGMP by K^+ and A23187. *J. Biol. Chem.* **263:** 9292-9297.

Holden CP and Storey KB (1997) Second messenger and cAMP-dependent protein kinase responses to dehydration and anoxia stresses in frogs. *J. Comp. Physiol.* **167:** 305-12

Horgan MJ, Fenton II JW and Malik AB (1987) Thrombin-induced pulmonary vasoconstriction *J. Appl. Physiol.* **63:** 1993-2000.

Housey GM, Johnson MD, Hsiao WLW, O'Brian CA, Murphy J, Kirschmeier P and Weinstein IB (1988) Overproduction of protein kinase C causes disordered growth control in rat fibroblasts *Cell* **52:** 343-354

Howe LR, Leevers SJ, Gomez N, Nakielny S, Cohen P and Marshall CJ (1992) Activation of the MAP kinase pathway by the protein kinase raf. *Cell* **71:** 335-342.

Hoyer D, Clarke DE, Fozard JR, Hertig PR, Martin GR, Mylecharane EJ, Sexena PR and Humphrey PPA (1994) VII. International Union of Pharmacology classicication of receptors for 5-hydroxytryptamin (serotonin). *Pharmacol. Rev.* **46:** 157-203.

Huang CL and Ives HE (1987) Growth inhibition by protein kinase C late in mitogenisis. *Nature (Lond.)* **329:** 849-850.

Huang W, Alessandrini A, Crew CM and Erikson RL (1993) Raf-1 forms a stable complex with Mek 1 and activates Mek 1 by serine phosphorylation. *Proc. Natl. Acad. Sci. USA* **90:** 10947-10951

Hug R and Sarre TF (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* **291:** 329-343

Hunter C, Barer GR, Shaw JW & Clegg EJ (1974) Growth of the heart and lungs in hypoxic rodents : A model of human hypoxic disease. *Clin. Sci. Mol. Med.* **46**: 375-391.

Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M and Matsumoto K, Miyazono K and Gotoh Y (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **275,** 90-94.

Iismaa TP & Shine JG (1992) G protein-coupled receptors. *Curr. Opinion Cell. Biol.* **4:** 195-202.

Janssens SP, Thompson BT, Spence CR and Hales CA (1991) Polycythemia and vascular remodelling in chronic hypoxic pulmonary hypertension in guinea pigs *J. Clin. Invest.* **96:** 1823-1833.

Johnson DA and Georgieff MK (1989) Pulmonary neuroendocrine cells. Their secretory products and their potential roles in health and chronic lung disease in infancy. *Am. Rev. Respir. Dis.* **140:** 1807-12.

Jones R and Reid L (1995) Vascular remodelling in clinical and experimental pulmonary hypertensions. In: Pulmonary vascular remodelling. Edited by Bishop JE. Reeves JT Luarent GJ eds: London: Portland Press; 47-115.

Kazlauskas A and Cooper JA (1989) Autophosphorylation of the PDGF receptor in the kinase insert region regulates the interactions with cellular proteins. *Cell* **58:** 1121-1133.

Kelleher MD, Abe MK, Chao TSO, Jain M, Green JM, Solway J, Rosner MR and Hershenson MB (1995) Role of MAP kinase activation in bovine tracheal smooth muscle mitogenisis. *Am J Physiol* **268:** L894-L901.

Kelly K, Cochran BH, Stiles CD and Leder P (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor *Cell* **35**, 603-610

Keyse SM (1999) The role of protein phosphatases in the regulation of mitogen and stress activated protein kinases. *Free Radical Research* **31:** 341-349.

Khan WA, Blobe G, Halpern A, Taylor W, Wetsel WC, Burns D, Loomis C and Hannun YA (1993) Selective regulation of protein kinase C isoenzymes by oleic acid in human platelets. *J. Biol.Chem.* **264:** 1483-1487.

Kivinen L and Laiho M (1999) Ras- and mitogen-activated protein kinase kinase-dependent and-independent pathways in p21Cip1/Waf1 induction by fibroblast growth factor-2, platelet-derived growth factor, and transforming growth factor-beta 1. *Cell Growth Differ.* **10:** 621-8.

Kohno M, Chatani Y, Tanaka E, Hattori A and Nishizawa N. (1992) Mitogen-induced tyrosine phosphorylation of 41 kDa and 43 kDa proteins: Potential role in integrating multiple mitogenic signalling pathways. *Biochem. J.* **287:** 917-924.

Kolch W, Heidecker G, Kocks G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D and Rapp UR (1993) Protein kinase Cα activates Raf-1 by direct phosphorylation *Nature* **364:** 249-252

Kolesnick R and Goldie DW (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signalling *Cell* **77:** 325-328

Kyriakis JM, Force TL, Rapp UR, Bonventre JV and Avruch J (1993) Mitogen regulation of c-Raf-1 protein kinase activity toward mitogen-activated protein kinase-kinase. *J. Biol. Chem.* **268:** 16009-16019.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Avruch J. and Woodgett JR (1994) The stress-activated protein kinase subfamily of c-jun kinases *Nature* **369:** 156-160

L'Allemain G, Pouyssegur J and Beber MJ (1991) P42-MAP kinase (mitogen activated protein kinase) as a converging target for different growth factor signalling pathways: use of pertussis toxin as a discrimination factor. *Cell Regulation* **2:** 674-684.

Laderoute KR, Mendonca HL, Calaoagan JM, Knapp AM, Giaccia AJ and Stork PJS (1999) Mitogen-activated protein kinase phosphatase-1 (MKP-1) expression is induced by low oxygen conditions found in solid tumour microenvironments: A candidate MKP for the inactivation of hypoxia-inducible stress-activated protein kinase/c-jun N-terminal protein kinase activity. *J. Biol. Chem.* **274:** 12890-12897.

Lafont A, Guzman LA, Whitlow PL, Goormastic M, Cornhill JF and Chisholm GM (1995) Restenosis after experimental angioplasty: intimal, medial and adventitial changes associated with constrictive remodelling *Circ. Res.* **76:** 996-1002.

Lane HA, Fernandez A, Lamb NJC and Thomas G (1993). $p70^{s6k}$ function is essential for G1 progression. *Nature* **363:** 170-172.

Lange-Carter CA, Pleimen CM, Gardener AM, Blumer KJ and Johnson GL (1993) A divergence in the MAP kinase regulatory network defined by MAK kinase and Raf. *Science* **260:** 315-319.

Lark JD, Schievella AR, Nalefski EA and Lin LL (1995) Cytosolic phospholipase A2. *Journal of Lipid Mediators and Cell Signalling.* **12:** 83-117.

Lauweryns JM, de Bock V, Guelinckx P and Decramer M (1983) Effects of unilateral hypoxia on neuroepithelial bodies in rabbit lungs. *J Appl Physiol* **55:** 1665-8.

Lavoie J, L'Allemain GL, Brunet A, Muller R and Poussegur J (1996) Cyclin D1 expression is regulated positively by the p42/p44^{MAPK} and negatively by the p38/HOG^{MAPK} pathway. *J. Biol. Chem.* **271:** 20608-20616.

Leach KL, Ruff VA, Wright TM, Pessin MS and Rabin DM (1991) Dissociation of protein kinase C activation and *sn*-1, 2-diacylglycerol formation. *J. Biol. Chem.* **266:** 3215-3221.

Lechleiter J, Girard S, Peralta E and Clapham D (1991) Spiral calcium wave propagation and annihilation in Xenopus Laevis oocytes. *Science* **252:** 123-126

Lee SB and Rhee SG (1995) Significance of PIP_2 hydrolysis and regulation of phospholipase C isozmes. Current Opinions in *Cell Biol.* **7:** 183-189.

Lee SL, Wang WW, Finlay GA and Fanburg BL (1999) Serotonin stimulates mitogen-activated protein kinase activity through the formation of superoxide anion. *Am J Physiol.* **277:** L282-91

Lee SL, Wang WW, Lanzillo JJ and Fanburg BL (1994a) Regulation of serotonin-induced DNA synthesis of bovine pulmonary artery smooth muscle cells *Am. J. Physiol.* **266:** L53-L60.

Lee JC, Laydon JT, McConnell PC, Gallagher TF, Kumar S, Green D, McNulty D, et.al (1994b) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis *Nature* **372:** 739-746.

Lee SL, Wang WW, Moore BJ and Fanburg BL (1991) Dual effect of serotonin on growth of bovine pulmonary smooth muscle cell in culture. *Circ. Res.* **68:** 1362-1368.

Lew BD, Dempsey KB, Zhao Y, Muthalif M, Fatima S and Malik KU (1999) β-Hexosamimidase-induced activation of p44/42 mitogen-activated protein kinase is dependent on p21 Ras and protein kinase C and mediates bovine airway smooth-muscle proliferation. *Am. J. Respir. Cell. Mol. Biol.* **21:** 111-118.

Lin LL, Wartmann M, Lin AY, Knopf JL Seth A and Davis RJ (1993) $cPLA_2$ is phosphorylated and activated by MAP kinase *Cell* **72:** 286-290.

Lindpainter K. and Ganten D. (eds) (1994) in The Cardiac Renin-Angiotensin System, Fatura Publishing Co., Armonk, NY

Lippton HL, Hauth TA, Summer WR and Hyman AL (1989a) Endothelin produces pulmonary vasoconstriction and systemic vasodilation. *J. Appl. Physiol.* **66(2):** 1008-12

Lippton HL, Pellett A, Cairo J, Summer WR, Lowe RF, Sander GE, Giles TD, Cohen G and Levitzky MG (1989b) Endothelin produces systemic vasodilation independent of the state of consciousness. *Peptides* **10(5):** 939-43

Liu JP (1996) Protein kinase C and its substrates. *Mol. Cell. Endocrin.* **116:** 1-29.

Lou Y, Oberpriller JC and Carlson EC (1997) Effect of hypoxia on the proliferation of retinal microvessel endothelial cells in culture. *Anat. Rec.* **248:3** 366-73

Lowrey OH, Rosebrough NJ, Farr AL and Randell RJ (1951) Protein measurements with the folin phenol reagent. *J.Biol. Chem.* **193:** 265-275.

MacDonald SG, Crews CM, Wu L, Driller J, Clark R, Erikson RL and McCormick F. (1993) Reconstitution of the raf-1-MEK-ERK signal transduction pathway *in vitro* *MCB* **13:** 6615-6620.

MacLean MR, McCullock KM and Baird M (1995) Pulmonary arterial ET_A-and ET_B-receptor-mediated vasoconstriction, tone and nitric oxide mediation in control and pulmonary hypertensive rats. *J. Cardiovasc. Pharmacol,* **26:** 822-830.

McCormick F (1993) Signal transduction. How receptors turn Ras on. *Nature* **363:** 15-16.

Madden BP, Gosney J, Coghlan JG, Kamalvand K, Caslin AW, Smith P, Yacoub M and Heath D (1994) Pretransplant clinicopathological correlation in end-stage primary pulmonary hypertension. *Eur. Respir. J.* **7:** 672-8.

Malarkey K, Belham C, Paul A, Graham A, McLees A, Scott PH and Plevin R (1995a) The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors *Biochem. J.* **309:** 361-375

Malarkey K, Chilvers ER, Lawson MF and Plevin R (1995b) Stimulation by endothelin-1 of mitogen activated protein kinases and DNA synthesis in bovine tracheal smooth muscle cells. *Br. J. Pharm.* **116:** 2267-2273.

Malek A and Izumo S (1992) Physiological fluid shear stress causes downregulation of endothelin-1 mRNA from bovine aortic endothelium *Am. J. Physiol* **263:** C389-C396.

Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF and Ahn NG (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265:** 966-969.

Marais R, Wynne J and Treisman R (1993) The SRF accessory protein ELK-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73:** 381-393

Marshall RP, McAnulty RJ and Laurent GJ (2000) Angiotensin II is mitogenic for human lung fibroblasts via activation of the autocrine action of TGFβ. *Am J Respir Crit Care Med* **161(6):** 1999-2004.

McGoon MD, Vliestra RE (1984) Vasodilator therapy for primary pulmonary hypertension. *Mayo. Clin. Proc.* **59:** 672-677.

McNamara C A, Sarembock I J, Gimple LW, Fenton II JW, Coughlin SR and Owens GK (1993) Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. *J. Clin. Invest.* **91:** 94-98.

Mecham RP Whitehouse LA and Wrenn DS (1987) Smooth muscle mediated connective tissue remodelling in pulmonary hypertension. *Science* **237:** 423-426

Mehmet H, Nanberg E, Lehmann W, Murrey MJ and Rozenqurt E (1990) Early signals in the mitogenic response of Swiss 3T3 cells: a comparitive study of purified PDGF homodimers *Growth Factors* **3:** 83-95.

Meloche S, Seuwen K, Pages G and Pouyssegur J (1992) Biphasic and synergistic activation of p44 MAPK (ERK1) by growth factors: correlation between late phase activation and mitogenicity. *Mol. Endocrin.* **6:** 845-854.

Meyer T and Stryer L (1991) Calcium spiking. *Annu. Rev. Biophys. Biophys.Chem.* **20:** 153-174

Meyrick B and Reid L (1980) Hypoxia-induced structural changes in the media and adventitia of the rat hilar pulmonary artery and their regression. *Am. J. Pathol.* **100:** 151-78

Meyrick B and Reid L (1979) Hypoxia and incorporation of [^{3}H]thymidine by cells of the rat pulmonary arteries and alveolar walls. *Am. J. Pathol.* **96:** 51-70.

Milanini J, Vinals F, Pouyssegur J and Pages G (1998) p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. *J. Biol. Chem.* **273:** 18165-18172.

Minami M, Kanayama Y, Inariba H, Negoro N, Inoue T and Takeda T (1991) Angiotensin II induced biphasic inositol 1,4,5-bisphosphate response in rat vascular smooth muscle cells. *Eur. J. Pharmacol.* **208(1):** 93-95

Minden A, Lin A, McMahon M, Lange-Carter C, Derijard B, Davis RJ, Johnson GL and Karin M (1994) Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK *Science* **266:** 1719-1723.

Minet E, Arnould T, Michel G, Roland I, Mottet D, Raes M, Remacle J and Michiels C (2000) ERK activation upon hypoxia: involvement in HIF-1 activation. *FEBS Lett* **468**:53-58.

Morin FC and Stenmark KR (1995) Persistent pulmonary hypertension of the newborn. *Am. J. Resp. Crit. Care Med.* **151:** 2010-2032

Morooka H, Bonventre JV, Pombo CM, Kyriakis JM and Force RJ (1995) Ischemia and reperfusion enhance ATF-2 and c-jun binding to the cAMP response elements and to an AP-1 binding site from the c-jun promotor. *J. Biol. Chem.* **270:** 30084-30092.

Morrison DK, Kaplan DR, Rapp UR and Roberts TM (1988) Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. *Proc. Nat. Aca. Sci. USA* **85:** 8855-8859.

Mulchahy L, Smith M and Stacy D (1985) Requirement for ras proto-oncogene function during serum-stimulated growth of NIH3T3 cells. *Nature* **313:** 241-243

Murphy JD, Rabinovitch M, Goldstein JD and Reid LM (1981)The structural basis of persistant pulmonary hypertension of the newborn infant. *J. Pediatr.* **98:** 962-967

Musashi M, Ota S and Shiroshita N (2000) The role of protein kinase C isoforms in cell proliferation and apoptosis. *Int. J. Hem.* **72(1):** 12-19.

Naeije R, Maggiorini M, Delcroix M, Leeman M and Melot C (1996) Effects of chronic dexfenfluramine treatment on pulmonary hemodynamics in dogs. *Am. J. Respir. Crit. Care. Med.* **154:** 1347-50.

Naeije, RL (1961) Kyphoscoliosis and cor pulmonale. A study of the pulmonary vascular bed. *Am. J. Pathol.* **38:** 561-73

Naftilan AJ, Gilliland GK, Eldridge CS and Kraft AS (1990) Induction of the proto-oncogene c-jun by angiotensin II. *Mol. Cell. Biol.* **10:** 5536-5540

Nakanishi H, Brewer KA and Exton JH (1993) Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **268:** 13-16.

Naor Z, Shearman MS, KishimotoA and Nishizuka Y (1988) Calcium-independent activation of hypothalamic type 1 protein kinase C by unsaturated fatty acids. *Mol. Endocrin.* **2:** 1043-1048.

Nishizuka Y (1992) Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**: 607-614.

Northwood IC, Gonzalez FA, Wartmann M, Raden DL and Davis RJ (1991) Isolation and characterisation of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. *J. Biol. Chem.* **266:** 15266-15276.

Novotna J and Herget J (1998) Exposure to chronic hypoxia induces quantitative changes of collagen in the walls of peripheral pulmonary arteries. *Life Sci.* **62(1):** 1-12

Ono S and Voelkel NF (1991)PAF antagonists inhibit monocrotaline-induced lung injury and pulmonary hypertension. *J. Appl. Physiol.* **71:** 2483-2492.

Orsini MJ, Krymskaya VP, Eszterhas AJ, Benovic JL, Panettieri RA and Penn RB. (1999) MAPK superfamily activation in human airway smooth muscle: mitogenisis requires prolonged p42/p44 activation. *Am. J. Physiol.* **277:** L479-L488.

Orton EC, Raffestin B, and McMurty IF (1990) Protein kinase C influences in rat pulmonary vascular reactivity. *Am. Rev. Res. Dis.* **141:** 654-658.

Owens GK and Schwartz S (1983) Vascular smooth muscle cell hypertrophy and hyperploidy in the Goldblatt hypertensive rat *Circ. Res.* **53:** 491-501

Packer L and Fuehr K (1977) Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature (London)* **267:** 423-425.

Pages G, Lenormand P, L'Allemain G, Chambard J-C, Meloche S and Pouyssegur J. (1993) Mitogen-activated protein kinases $p42^{mapk}$ and $p44^{mapk}$ are required for fibroblast proliferation. *Proc. Natl. Acad. Sci USA* **90:** 8319-8323

Pai J-K, Patcher JA, Weinstein BI and Bishop RW (1991) Overexpression of protein kinase C β1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. *Proc. Natl. Acad. Sci. USA* **88:** 598-602.

Pakala R, Willerson JT, Benedict CR. (1994) Mitogenic effect of serotonin on vascular endothelial cells. *Circulation* **90:** 1919-26.

Panettieri RA (1996) Airway smooth muscle cell growth and proliferation. In, Airway smooth muscle: Development and regulation of contractility. Ed. Raeburn D and Giembycz. Birkhauser Verlag, Basel, Switzerland.

Panettieri RA, DePalo LR, Murray PA, Yadvish PA and Kotlikoff MI (1989) A human airway smooth muscle cell line that retains physiological responsiveness. *Am. J. Physiol.* **256:** C329-335.

Pardee AB (1989) G1 events and regulation of cell proliferation. *Science* **24:** 603-608

Pazin MJ & Williams LT (1992) Triggering signalling cascades by receptor tyrosine kinases. *TIBS* **17:** 374-378.

Peacock AJ and Aidulis D (1994) Species variation in replicative response of pulmonary artery fibroblasts to endothelin-1. *Am. J. Resp. Crit. Care Med.* **149**: A825.

Peacock AJ, Dawes KE, Shock A, Gray AJ, Reeves JT and Laurent GJ. (1992) Endothelin-1 and endothelin-3 induce chemotaxis and replication of pulmonary artery fibroblasts. *Am. J. Respir. Cell. Mol. Biol.* **7:** 492-499.

Peroutka SJ (1995) 5-HT receptors: past, present and future. Trends Neurosci. **18:** 68-69

Perry RR and Vinik AI (1996) Endocrine tumors of the GI tract. *Annu. Rev. Med.* **47:** 59-62.

Pitt BR, Weng W and Steve AR (1994) Serotonin increases DNA synthesis in rat proximal and distal pulmonary vascular smooth muscle cells in culture. *Am. J. Physiol.* **266:** L178-186.

Poiani GJ, Tozzi CA, Yohn SA, Pierce RA, Belsky SA, Berg RA, Yu SY, Deak SB and Riley DJ (1990) Collagen and elastin metabolism in hypertensive pulmonary arteries of rats. *Circ. Res.* **66:** 968-978

Pribilova NN (1976) Serotonin metabolism and pulmonary hypertension in chronic lung disease. *Sov. Med.* **12:** 107-111.

Rabinovitch M, Gamble W, Nadas AS, Miettinen OS and Reid L (1979) Rat pulmonary circulation after chronic hypoxia: haemodynamic and structural features. *Am. J. Physiol.* **236:** H818-27.

Rabinovitch M (1987) Prostaglandins and structural changes in pulmonary arteries. *Am. Rev. Respir. Dis.* **136:** 777-779

Rahda TG, Venkitasubramanian TA, Viswanathan R. (1976) Effect of acute hypoxia on blood serotonin in human beings and rats. *Respiration* **33:** 64-69.

Raingeaud J, Whitmarch AJ, Barrett T, Derijard B and Davis RJ (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* **16:** 1247-1255.

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ and Davis RJ (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine *J. Biol. Chem.* **270:** 7420-7426

Raizada MK, Philips MI and Summers C (eds) (1993) in Cellular and Molecular Biology of Renin-Angiotensin Systems, CRC Press, Inc., Boca Raton, FL

Rao GN, Baas AS, Glasgow WC, Eling TE, Runge MS and Alexander RW (1994) Activation of mitogen-activated protein kinases by arachidonic acid and its metabolites in vascular smooth muscle cells. *J. Biol. Chem.* **269:** 32586-32591.

Rao GN, Lassegue B, Griendling KK and Alexander RW (1993) Hydrogen peroxide stimulates transcription of c-jun in vascular smooth muscle cells: role of arachidonic acid. Oncogene: 2759-2764.

Rebello A and Martinez CA (1999) Ras proteins: recent advances and new functions. *Blood* **94:** 2971-2980

Reid LM and Davies P (1989) in Lung Biology in Health and Disease (Lenfant, C, exec.ed). Pulmonary Vascular Physiology and Pathophysiology. (Weir, E.K. and Reeves, J. T., eds), **38:** 541-611, Marcel Decker, NY.

Reid L and Meyrick B (1982) Microcirculation: definition and organisation at tissue level. *Ann. N.Y. Acad. Sci.*, **384:** 3-20

Reuter CWM, Morgan MA and Bergmann L (2000) Targeting the Ras signalling pathwaay: a rational, mechanism-based treatment for hematologic malignancies? *Blood* **96:** 1655-1669.

Richard DE, Berra E and Pouyssegur J (2000) Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1 alpha in vascular smooth muscle cells. **275(35):** 26765-71.

Richard DE, Berra E, Gothie E, Roux D and Pouyssegur J (1999) p42/p44 Mitogen-activated Protein Kinases Phosphorylate Hypoxia-inducible Factor 1α (HIF-1α) and Enhance the Transcriptional Activity of HIF-1. *J. Biol. Chem.* **274:** 32631-32637.

Riley DJ, Poiani GJ, Tozzi CA, Rosenbloom J, Pierce RA and Deak SB. (1986) Collagen and elastin gene expression in the hypertensive pulmonary artery of the rat. *Trans. Assoc. Am. Phys.* **99:** 180-188.

Rivera VM, Miranti CK, Misra RP, Ginty DD, Chen RH, Blenis J and Greenberg ME (1993) A growth factor-induced kinase phosphorylates the serum response factor at a site that regulates its DNA-binding activity. *MCB* **13:** 6260-6273.

Robinson MJ and Cobb MH (1997) Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol* **9:** 180-186.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD and Downward J (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370:** 527-532.

Roony TA, Renard DC, Sass EJ and Thomas AP (1991) Beta-adrenergic receptor-mediated phospholipase C activation independent of cAMP formation in turkey erythrocyte membranes. *J. Biol. Chem.* **226:** 12272-12282

Rorie DK, Tyce GM, Edwards W, Sittipong R and Kaye MP (1988) Chronic hypoxia alters structure and transmitter dynamics in dog pulmonary artery. *Respir. Physiol.* **74:** 211-127.

Ross R, Raines EW and Bowden-Pope DF (1986) The biology of the platelet-derived growth factor. *Cell* **46:** 155-169.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-llamazares A, Zamanillo D, Hunt T and Nebrada AR (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins *Cell* **78:** 1027-1037.

Ryland D and Reid L (1975) The pulmonary circulation in cystic fibrosis. *Thorax* **30:** 285-291

Sachinidis A, Locher R, Vetter W, Tatje D and Hoppe J (1990) Different effects of platelet-derived growth factor isoforms on rat vascular smooth muscle cells *J. Biol. Chem.* **265:** 10238-10243.

Sadoshima J and Izumo S (1993) Signal transduction pathways of angiotensin II-induced c-fos gene expression in cardiac myocyctes *in vitro.* Roles of phospholipid-derived second messengers. *Cir. Res.* **73:** 424-438

Saed GM, Zhang W, Chegini N, Holmdahl L and Diamond MP (1999) Alteration of type I and III collagen expression in human pertonea mesothelial cells in response to hypoxia and transforming growth factor-beta 1. *Wound repair Regen.* **7(6):** 504-10.

Sanghera JS, Peter M, Nigg EA and Pelech SL (1992) Immunological characterisation of avian MAP kinases: Evidence for nuclear localisation. *Mol. Biol. Cell* **3:** 775-787.

Santilli SM, Fiegel VD, Aldridge DE and Knighton DR (1991) Rabbit aortic endothelial cell hypoxia induces secretion of transforming growth factor beta and augments macrophage adhesion in vitro. *Ann. Vasc. Surg.* **5(5):** 429-438.

Sarembock IJ, Gertz SD, Gimple LW, Owen RM, Powers ER and Roberts WC (1991) Effectiveness of recombinant desulphatohirudin in reducing restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. *Circulation.* **84:** 232-243.

Satoh T and Kaziro Y (1992) Ras in signal transduction. *Sem.Cancer Biol.* **3:** 169-177.

Schwartz SM and Reidy MA (1987) Common mechanisms of proliferation of smooth muscle in atherosclerosis and hypertension. *Hum. Pathol.* **18:** 240-247.

Scott PH, Paul A, Belham CM, Peacock AJ, Wadsworth RM, Gould GW, Welsh D and Plevin R (1998) Hypoxic stimulation of the stress-activated protein kinases in pulmonary artery fibroblasts. *Am. J. Respir. Crit. Care Med.* **158:** 958-962.

Scott PH and Peacock AJ (1995) Cell signalling in pulmonary vascular cells: Do not shoot the messenger! *Thorax* **51:** 864-866.

Scott PH, Belham CM Peacock AJ and Plevin R (1997) Intracellular signalling pathways that regulate vascular cell proliferation: Effect of hypoxia *Advances Exp. Physiol.* **82:** 317-326

Segal RA and Greenberg ME (1996) Intracellular signalling pathways activated by neurotrophic factors. *Ann Rev. Neurosci.* **19:** 463-489

Seger R, Seger D, Lozeman FL, Ahn NG, Graves LM, Campbell JS, Ericsson L. Harrylock M, Jensen AM and Krebs EG (1992) Human T-cell mitogen-activated protein kinase kinase are related to yeast signal transduction kinases. *J. Biol. Chem.* **267:** 25628-25631

Seko Y, Tobe K, Ueki K, Kadowaki T and Yazaki Y (1996) Hypoxia and hypoxia/reoxygenation activate Src family tyrosine kinases and p21(ras) in cultured rat cardiac myocytes *Cir.Res.* **78:** 82-90

Semmens M and Reid L (1974) Pulmonary artery muscularity and right ventricular hypertrophy in chronic bronchitis and emphysema. *Br. J. Dis. Chest.* **68:** 253-7

Seuwen K and Pouyssegur J (1990) Serotonin as a growth factor. *Biochem. Pharmacol.* **39:** 985-990.

Shapiro PS, Evans JN, Davis RJ and Posada JA (1996) The seven-transmembrane-spanning-receptors for endothelin and thrombin cause proliferation of airway smooth muscle cells and activation of the extracellular regulated kinase and c-Jun NH2-terminal kinase groups of mitogen-activated protein kinases. *J. Biol. Chem.* **271:** 5750-5754.

Sharkey AM, Day K, McFerson A, Malik S, Licence D, Smith SK and Charnock-Jones DS (2000) Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia. *J. Clin. Endocrinol. Metab.* **85(1):** 402-9

Short AD, Bian J, Ghosh TK, Waldron RT, Rybak SL and Gill DL (1993) Intracellular Ca^{2+} pool content is linked to control of cell growth. *Proc. Natl. Acad. Sci. USA* **90:** 4986-4990.

Simonson MS, Wann S, Mene P, Dubyak GR, Kester M, Nakazato Y, Sedor JR, and Dunn MJ. (1989) Endothelin stimulates phospholipase C, Na+/H+ exchange, c-fos expression and mitogenisis in rat mesangial cells. *J.Clin. Invest.* **83:** 708-712

Singhal, Henderson R, Horsfield K, Harding K and Cummings G (1973) Morphometry of the human pulmonary arterial tree. *Circ. Res.* **33:** 190-197.

Sobin SS, Tremer HM, Hardy JD and Chiodi HP (1983) Changes in arteriole in acute and chronic hypoxic pulmonary hypertension and recovery in rat. *J. Appl Physiol: Respir. Environ. Excercise Physiol.* **55:** 1445-1455

Sodhi A, Montaner S, Patel V, Zohar M, Bais C, Mesri EA and Gutkind JS (2000) The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia –inducible factor alpha. **60(17):** 4873-80

Somlyo AP and Somlyo AV (1992) In "The Heart and Cardiovascular System" eds. Fozzard HA, Jennings RB, Haber E, Katz AM and Morgan HE 2nd ed. P1294-1324.

Souhami RL and Moxam J (Eds) (1998) Textbook of Medicine 3rd Edition pp 507. Churchill Livingstone.

Sozeri O, Vollmer M, Liyanaage D, Firth G, Kour GE, Mark GE and Stabel S. (1992) Activation of the c-raf protein kinase by protein kinase C phosphoryltion. *Oncogene* **7:** 2259-2262

Starksen NF, Simpson PC and Bishopric N (1986) Cardiac myocyte hypertrophy is associated with c-myc protooncogene expression. *Proc. Natl. Acad. Sci. USA* **83:** 8348-8350.

Stenmark KR, Badesch DB, Durmowicz AG and Voelkel NF (1991) Control of vascular tone and cell proliferation in the pulmonary circulation. In Basic Mechanisms of Paediatric Respiratory Disease Cellular and Integrative, ed. V Chernick, RB Mellins, pp 169-87. Philadelphia: Decker.

Stenmark KR, Frid M, Nemenoff R, Dempsey and Das M (1999) Hypoxia induces cell-specific changes in gene expression in vascular wall cells: implications for pulmonary hypertension. *Adv. Exp. Med. Biol.* **474:** 231-258

Stenmark KR and Mecham RP (1997) Cellular and molecular mechanisms of pulmonary vascular remodelling. *Annu. Rev. Physiol.* **59:** 89-144

Stenmark KR, Orton EC, Crouch EC, Parks WC, Voelkel NF, Crouch EC, Parks WC and Mecham RP (1988) Vascular remodelling in neonatal pulmonary hypertension: role of the smooth muscle cell. *Chest* **92:** S127-32.

Storch TG and Talley GD (1988) Oxygen concentrations regulates the proliferative response of human fibroblasts to serum and growth factors. *Exp. Cell Res.* **175:** 317-25.

Sturgill TW, Ray LB, Erikson E and Maller JL (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein kinase II. *Nature* **334:** 715-718.

Sudhir K, Wilson E, Chatterlee K and Ives HE (1993) *J. Clin. Invest.* **92:** 3003-3007.

Sumpio BE, Banes AJ, Link WG and Johnson G (1988) Enhanced collagen production by smooth muscle cells during repetitive mechanical streching. *Arch. Surg.* **123:** 1233-1236

Susa M, Vulevic D, Lane HA and Thomas G (1992) Inhibition of downregulation of protein kinase C attenuates late phase p70s6k activation induced by epidermal growth factor but not by platelet-derived growth factor or insulin. *J. Biol. Chem.* **267:** 6905-6909.

Suzuki E, Nagata D, Kakoki M, Hayakawa H, Goto A, Omata M and Hirata Y (1999) Molecular mechanisms of endothelin-1 induced cell-cycle progression involvement of extracellular signal-regulated kinase, protein kinase C and phosphatidylinositol 3-kinase at distinct points. *Circ. Res.* **84(5):** 611-619.

Takahashi E and Berk BC (1998) MAP kinases and vascular smooth muscle function. *Acta. Physiol. Scand.* **164:** 611-621.

Tamemoto H, Kadowaki T, Tobe K, Ueki K, Izumi T, Chatani Y, Kohno M, Kasuga M, Yazaki Y and Akanuma Y (1992) Biphasic activation of two mitogen-activated protein kinases during the cell cycle in mammalian cells *J.Biol.Chem.* **267:** 20293-20297.

Taubman MB, Berk BC, Izumo S, Tsuda T, Alexander RW and Nadal-Ginard B. (1989) Angiotensin II induces c-fos mRNA in aortic smooth muscle. Role of $Ca2^{+}$ mobilisation and protein kinase C activation. *J. Biol. Chem.* **264:** 526-530.

Taylor W, Camalier R and Sanford K (1978) Density-dependent effects of oxygen on the growth of mammalian fibroblasts in culture. *J. Cell. Physiol.* **95:** 33-40.

Taylor CW and Richardson A (1991) Structure and function of inositol trisphosphate receptors. *Pharmacol. Ther* **51:** 97-137.

Tecott L, Shtrom S and Julius D (1995) Expression of a serotonin-gated ion channel in embronic neural and nonneural tissues. *Mol. Cell. Neurosci.* **6:** 43-55.

Theodosiou A, Smith A, Gillieron C, Arkinstall S and Ashworth A (1999) MKP5, a new member of the MAP kinase phosphatase family which selectively dephosphorylates stress-activated kinases. *Oncogene* **18:** 6981-6988

Timiras PS, Krum AA and Pace N (1957) Body and organ weights of rats during acclimatisation to an altitude of 12,470. *Am. J. Physiol.* **191:** 598-604.

Traver G, Cline MG and Burrows S (1979) Predictors of mortality in chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.* **119:** 895-902.

Traynor-Kaplan AE, Harris A, Thompson B, Taylor P and Sklar LA (1988) An inositol tetrakisphosphate-containing phospholipid in activated neurophils. *Nature* **334:** 353-334.

Trejo J, Connolly J and Coughlin SR (1996) The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from knockout mice. *J. Biol. Chem.* **271:** 21536-21541

Tsien BW and Tsein RY (1990) Calcium channels, stores and oscillations *Annu. Rev. Cell Biol.* **6:** 715-760

Vender RL (1994) Chronic hypoxic pulmonary hypertension. *Chest* **106:** 236-243.

Vender RL, Clemmons DR, Kwock L, Friedman M (1987) Reduced oxygen tension induces pulmonary endothelium to release a pulmonary smooth muscle cell mitogen(s). *Am. Rev. Respir. Dis.* **135:** 622-627.

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Kuks Z and Kolesnick RN (1996) Requirement

for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature* **380:** 75-79.

Voelkel NF (1986) State of the art. Mechanisms of hypoxic pulmonary vasoconstriction. Am Rev Respir Dis **133:** 1186-95.

von Euler, U S. and Liljestrand G. 1946. Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiol. Scand.* **12:** 301-320

Vouret-Craviari V, Van Obberghen-Schilling E, Rasmussen UB, Pavirani A, Lecocq JP and Pouyssegur J (1992) Synthetic α-thrombin receptor peptides activate G protein-coupled signalling pathways but are unable to induce mitogenisis. *Mol. Cell. Biol.* **3:** 95-102.

Wagner EM and Mitzner WA (1988) Effects of hypoxia on bronchial circulation *J. Appl. Physiol.* **65 (4):** 1627-33.

Wang P, Anthes JC, Siegal MI, Egan RW and Billah MM (1992) Existence of cytosolic phospholipase D. *J. Biol. Chem.* **266:** 14877-14880.

Watts SW (1996) Serotonin activates the mitogen-activated protein kinase pathway in vascular smooth muscle: Use of the mitogen-activated protein kinase kinase inhibitor PD098059. *J. Pharmacol. Exp. Ther.* **279:** 1541-1550.

Watts SW (1998) Activation of the mitogen activated protein kinase pathway via the 5-HT_{2A} receptor. *Ann. NY Acad. Sci.* **15;861:** 162-8.

Weibke JL, Montrose-Rafizadeh C, Zeitlin PL and Guggino WB (1992) Effect of hypoxia on endothelin-1 production by pulmonary vascular endothelial cells. *Biochem Biophs Acta* **1134:** 105-111

Weinberg RA (1985) The action of oncogenes in the cytoplasm and nucleus *Science* **230:** 770

Weir EK and Archer SL (1995) The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *FASEB J.* **9:** 183-189.

Welsh D, Scott P, Plevin R, Wadsworth R and Peacock AJ (1996) Effects of hypoxia on IP3 generation and DNA synthesis in bovine pulmonary artery fibroblasts (abstract). *Am. J. Respir. Crit. Care Med.* **153**: A576.

Welsh DJ, Scott P, Plevin R, Wadsworth R and Peacock AJ (1998). Hypoxia enhances cellular proliferation and inositol 1,4,5-triphosphate generation in fibroblasts from bovine pulmonary artery but not from mesenteric artery. *Am. J. Resp. Crit. Care. Med.* **158:** 1757-1762.

Widmann C, Gibson S, Jarpe MB and Johnson GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* **79:** 143-180

Wilkinson LO and Dourish CT (1991) in Serotonin Receptor Subtypes: Basic and Clinical Aspects (Peroutka S. J. eds) **Vol 15,** pp. 147-210 Wiley-Liss, New York

Wilson DJ, Fortner KA, Lynch DH. et al (1996) JNK, but not MAPK activation is assiciated with Fas-mediated apoptosis in human T cells. *Eur J Immunol.* **26:** 989-994.

Wu X, Hoh SJ, Zhou G, Dixon JE and Guan KL (1996) Selective activation of MEK1 and MEK2 by A-Raf from epidermal growth factor-stimulated Hela cells. *J. Biol. Chem.* **271:** 3265-3271.

Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenburg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases in apoptosis. *Science* **270:** 1326-1331.

Xu Z, Wu K, Yan Y, Liu J and Liu B (1995) Changes of collagen distribution in extrapulmonary arteries of hypoxia-induced pulmonary hypertensive rats. *Chung Kuo I Hsueh Ko Hsueh Yuan Hsueh Pao* **17(3):** 167-71.

Yamaguchi K, Shirakabe K, Shibuta H, Irie K, Oishi I, Ueno N, Taniguchi T, Nishida E and Matsumoto K (1995) Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. *Science* **270:** 2008-2011.

Yamakawa H, Banno Y, Nakashima S, Sawada M, Yamada J, Yoshimura S, Nishimura Y, Nozawa Y and Sakai N (2000) Increased phospholipase D2 activity

during hypoxia-induced death of PC12 cells: its possible anti-apoptotic role. *Neuroreport* **9:** 3647-3650.

Yan M and Templeton DJ (1994) Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by Raf and MEK kinase. *J. Biol. Chem.* **269:** 19067-19073.

Yan M, Dai T, Deak JC, Kyriakis JM, Zon LI, Woodgett JR and Templeton DJ (1994) Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* **372:** 798-800.

Yan SH, Zoa YS, Gao Y, Zhai C, Mackman N, Lee SL, Milbrandt J, Pinsky D, Kisiel W and Stern D (1998) Tissue factor transcription driven by Erg-1 is a critical mechanism of murine pulmonary fibrin deposition in hypoxia. *Proc Natl. Acad. Sci* **95**: 8298-8303.

Yokokawa K, Kohno M, Minami M, Ikeda M, Horio T, Kano H, Hanehira T, Yasunari K and Tekeda T (1995) Enhanced phosphoinositide turnover signalling stimulated by endothelin B-type receptor in endothelial cells from spontaneously hypertensive rats. *Clin. Exp. Pharm. Physiol.* **22:** S195-196.

Zechner D, Thuerauf DJ, Hanford DS, McConough M and Glembotski C (1997) A role for the p38 mitogen-activated protein kinase pathway in myocardial cell growth, sarcomeric organisation and cardiac-specific gene expression. *J. Cell Biol.* **139:** 115-127.

Zhang J, Salojin KV, Gao JX, Cameron MJ,Bergerot I and Delovitch TL (1999) p38 mitogen-activated protein kinase mediates signal integration of TCR/CD28 co-stimulation in primary murine T cells. *J. Immunol.* **162:** 3819-3829.

Zheng CF and Guan KL (1993) Properties of MEKs, the kinase that phosphorylate and activate the extracellular signal-related kinases. *J.Biol. Chem.* **268:** 23933-23939.

Zheng CF and Guan KL (1994) Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. *EMBO J.* **13:** 1123-1131.