The role of Phosphodiesterase 3, Phosphodiesterase 5, and the inhibitory γ subunit of the retinal cyclic GMP Phosphodiesterase, in Pulmonary Hypertension

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy

Fiona Murray

Institute of Biomedical and Life Sciences

University of Glasgow

2003

Abstract

Chronic treatment of rats (to induce pulmonary hypertension, PHT) for 14 days increased cGMP-inhibited, cAMP specific phosphodiesterase (PDE3), and cGMP binding, cGMP specific phosphodiesterase (PDE5) activities in selected branches of the pulmonary artery (MacLean *et al.*, 1997). The objective of this study was to establish the molecular basis for these changes in both animal and cell models of PHT, and also to investigate the effect the PDE3 inhibitor SKF94836, and the PDE5 inhibitor sildenafil, on isolated pulmonary arteries from normoxic and hypoxic rats.

It was shown that PDE3A/B gene transcription was increased in the main, first, intrapulmonary and resistance pulmonary arteries. Transcript and protein levels of PDE5A2 in the main and first branch pulmonary arteries (PAs) were also increased by chronic hypoxia. In addition, the expression of PDE3A was increased in cultured human pulmonary smooth muscle cells (hPASMC) maintained under chronic hypoxic conditions for 14 days, and this may be mediated via a protein kinase A-dependent mechanism. The treatment of cells with 8-Br-cAMP mimicked chronic hypoxia, inducing increased PDE3A expression, while treatment with the protein kinase A selective inhibitor, H8 peptide, abolished chronic hypoxia-induced expression of PDE3A. Finally, the treatment of cultured hPASMC, with the inhibitor of NF-κB degradation Tosyl-Leucyl-Chloro-Ketone (TLCK, 100μM), substantially reduced PDE5 transcript levels, suggesting a role for this transcription factor in the regulation of PDE5 gene expression. This is of interest because NF-κB is activated by hypoxia (Muraoka *et al.*, 2000; Aziz *et al.*, 1997). Taken together, our results show that phenotypic changes in the expression of PDE3 and PDE5 might provide an explanation for some of the changes in vascular reactivity of pulmonary vessels from rats with PHT.

Both SKF94836 (PDE3 inhibitor), and sildenafil (PDE5 inhibitor) were effective in producing a concentration-dependent relaxation in isolated PAs. The magnitude of the response to both SKF94836 and sildenafil were dependent on the preconstrictor used, (PE, 5-HT, or ET-1) the branch of the PA studied, and the presence of an intact endothelium. Hypoxia did not attenuate the maximum relaxation achieved by 3×10^{-5}M SKF94836 or 3×10^{-5}M sildenafil, providing evidence for the use of these inhibitors in the clinical treatment of PHT.

It has previously been reported that the inhibitory γ subunit of retinal cGMP PDE (PDEγ1/2) is expressed in non-retinal cells, and is involved in stimulating the p42/p44 mitogen-activated protein kinase (MAPK) by growth factors and G-protein coupled receptor agonists (unpublished data from the lab, Wan *et al.*, 2001, Tate *et al.*, 1998 Tate *et al.*, 2001). The possible role of PDEγ1/2 in modulating chronic hypoxic dependent mitogenic signalling pathways in both animal, and cell models of PHT was investigated.

The presence of PDEγ1/2 was demonstrated in rat PAs and in hPASMC, and the protein levels were shown to increase following hypoxic exposure. The increased expression of PDEγ1/2 with hypoxia was most marked in the resistance vessels that characteristically show the greatest remodelling with PHT. In both models of PHT the increase in PDEγ1/2 with hypoxia was correlated with an enhanced activation of p42/p44 MAPK. These studies identified a hypoxic-dependent change in the phenotypic expression of an intermediate protein regulating mitogenic signalling in pulmonary arteries in both the rat and the human. This may have a significant effect in the future investigations of arterial remodelling in PHT.

Table of Contents

Chapter 4 – Effect of PDE3 and PDE5 inhibitors on isolated pulmonary arterial rings

List of Tables

Chapter 1. Introduction

Chapter 2. Materials and Methods

List of Figures

Chapter 1. Introduction

Chapter 2 – Material and Methods

Chapter 3 – Evaluation of PDE3 and PDE5 in models of PHT

Chapter 4 – Effect of PDE3 and PDE5 inhibitors on isolated pulmonary arterial rings

Chapter 5: The role of PDEγ in chronic hypoxia

List of Publications

1. Fiona Murray, Margaret R MacLean and Nigel J. Pyne (2003), An assessment of the role of the inhibitory γ subunit of the retinal cyclic GMP phosphodiesterase and its effects on the p42/p44 mitogen-activated protein kinase pathway in animal and cellular models of pulmonary hypertension. *Brit. J. Pharmacol.*, 138, 1313-1319.

2. Fiona Murray, Margaret R MacLean and Nigel J. Pyne (2002), Increased expression of the cGMP-inhibited cAMP-specific (PDE3) and cGMP binding cGMP-specific (PDE5) phosphodiesterases in models of pulmonary hypertension. *Brit. J. Pharmacol.*, 137: 1187-1194.

3. Fiona Murray, Margaret R MacLean and Nigel J. Pyne (2001). Evaluation of phosphodiesterase-3 in normoxic and hypoxic human pulmonary artery smooth muscle cells. *Brit. J. Pharmacol.*, August 2001, P64.

4. Fiona Murray, Margaret R MacLean and Nigel J. Pyne (1999). Evaluation of transcript levels for phosphodiesterase-3 and phosphodiesterase-5 and associated inhibitory protein (γ) in pulmonary arteries from normoxic and hypoxic rats. Brit. J. Pharmacol., October 1999, P12

Acknowledgements

I wish to thank both Professor Nigel Pyne, and Professor Mandy MacLean for their guidance, supervision, and training throughout my PhD, all of which has allowed me to develop into an independent researcher. Together the University of Glasgow and the University of Strathclyde funded my studies, and offered facilities and resources that aided me in completing a very productive PhD.

I am indebted to my family, in particular to my parents who have supported me throughout, and given me the freedom to take all opportunities that have arisen in life. I would like to especially thank Rebecca, for being a true friend, for always being there to turn to, and being the only person that is as aloof as myself.

Throughout my PhD I have met many people whose friendship made my time in Glasgow a very happy and unforgettable one. I am grateful to all the help and encouragement that was given from fellow PhD students and postdocs in both the University of Glasgow and Strathclyde. I would like to specially mention a few of these; Peter, who stood by me throughout my PhD (working at the next bench), and who I always looked up to (only if I took my boots off); Dawn, for being my partner in crime, and giving me a roof over my head; Tom, the most corrupt president ever of the Pharmasoc, Steven, for teaching me dance moves I never dreamed possible; Graham for coaching me in pole dancing, and introducing me to Steve Wrights Sunday Love Songs; Lynne, for being super tech, and still enjoying a drink as much as myself; Ian for knowing everything about anything in the lab; Big Nigel, who over the past 3 years managed to destroy every piece of my self confidence, yet still left me smiling; Jenny, for just being so lovely; and finally Caroline, for being one of a kind, and a completely unforgettable flatmate. I enjoyed every minute of my PhD, so much so that I didn't really want to finish.

Author's Declaration

The composition of this thesis and the work described within it was carried out entirely by myself unless cited or acknowledged. Its contents have not previously been submitted for any other degrees. The research for this thesis was carried out between October 1998 and September 2002.

Signe[redacted]

DATE 13/05/03

Abbreviations

The main abbreviations used throughout this thesis are listed below. All abbreviations are also defined in the text when first cited.

AC .....Adenylyl cyclase

ACh .....Acetylcholine

ANP .....Atrial Natriuretic Peptide

BMPR-II .....Bone Morphogenetic protein receptor type II

8-Br-cAMP .....8-Bromoadenosine 3'5'-Cyclic monophosphate

cAMP .....Cyclic Adenosine Monophosphate

CCRC .....Cumulative Concentration Response Curve

COPD .....Chronic Obstructive Pulmonary Disease

CREB .....cAMP Response Element Binding Protein

cGMP .....Cyclic Guanosine Monophosphate

CH .....Chronic Hypoxic Rat

DAG .....Diacylgycerol

DMSO .....Dimethyl Sulfoxide

EGF .....Epidermal Growth Factor

ERK .....Extracellular Regulated Kinase

ET-1 .....Endothelin-1

GC .....Guanylyl cyclase

GPCR .....G-Protein Coupled Receptor

G3PDH .....Glyceraldehyde-3-Phosphate Dehydrogenase

H8 ..... N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide 2HCL]

HIF-1 ..... Hypoxic Inducible Transcription Factor 1

hPASMC ..... Human Pulmonary Smooth Muscle Cells

5-HT 5-Hydroxytriptamine

IP_3 ..... Inositol Trisphosphate

iNOS ..... Inducible Nitric Oxide

ISS ..... Isotonic Sucrose Solution

KCl ..... Potassium Chloride

LV ..... Left Ventricle

MAPK ..... Mitogen Activated Protein Kinase

MCT ..... Monocrotaline

MKP ..... MAPK Phosphatase

NF-κB ..... Nuclear Factor Kappa B

NO ..... Nitric Oxide

NOS ..... Nitric Oxide Synthase

ORF ..... Open Reading Frame

PA ..... Pulmonary Artery

PAP ..... Pulmonary Arterial Pressure

PBS ..... Phosphate Buffered Saline

PDE ..... Phosphodiesterase

PDEγ ..... Inhibitory γ subunit of the retinal cyclic GMP PDE

PDGF ..... Platelet Derived Growth Factor

PE .....Phenylepherine

PGI_2 .....Prostacyclin

PHT .....Pulmonary Hypertension

PK .....Protein Kinase

PLA_2 .....Phospholipase A_2

PLC .....Phospholipase C

PMSF .....Phenylmethylsulphonyl fluoride

PO_2 .....Partial Pressure Oxygen

PPHT .....Primary Pulmonary Hypertension

PVR .....Pulmonary Vascular Resistance

ROS .....Reactive Oxygen Species

RT .....Reverse Transcriptase

RT-PCR .....Reverse Transcriptase-Polymerase Chain Reaction

RTK .....Receptor Tyrosine Kinase

RV .....Right Ventricle

SMC .....Smooth Muscle Cells

SmGM .....Smooth Muscle Growth Medium

TLCK .....Nα-Tosyl-$_L$-Lysine Chloro-Methyl Ketone

TV .....Total Ventricle

URC .....Upstream Coding Region

CHAPTER 1

INTRODUCTION

Chapter 1. Introduction

1.1. Pulmonary Circulation

The pulmonary circulation is connected in series with the systemic circulation forming a continuous circuit. Blood is pumped from the right ventricle to the left side of the heart via the pulmonary circulation, while simultaneously the left ventricle pumps blood through the systemic circulation back to the right side of the heart. The main functions of the pulmonary circulation are: to allow the rapid uptake of oxygen for metabolic processes within the body and to remove carbon dioxide; to form a barrier between the external and internal environment (air/blood interface) to block foreign bodies, thrombi, air bubbles or fat particles; and also to act as a reservoir to store blood to allow the rapid readjustment of the circulation (for reviews of the pulmonary circulation see Barnes and Liu, 1995; Ganong, 1995; Levick, 1996).

Although the systemic and pulmonary vascular systems are superficially similar, important differences and adaptations are summarised in table 1.1, and are outlined throughout the text below. Essentially resistance is low throughout the pulmonary circulation, which is approximately one eighth of the resistance of the systemic circulation. Pulmonary arterial pressure (PAP) averages about 24/9mmHg with a mean arterial pressure of 15mmHg, much lower than the average arterial pressure in the systemic circulation, which is 120/80mmHg. A low-pressure gradient of around 7mmHg exists in the pulmonary circulation compared with a gradient of about 90mmHg in the systemic circulation. Unlike the systemic circulation, the normal pulmonary circulation, due to the low resistance and low pressure, is virtually maximally dilated at all times.

1.1.1. The pulmonary arterial tree

In the human pulmonary circulation there is approximately 17 or more orders of branching of the pulmonary artery (PA), which provides a route for the flow of deoxygenated blood into and around the entire lung (Singhal *et al.*, 1973). The PAs are shorter, thinner, and more distensible and have larger diameters than their systemic counterparts. These characteristics give the pulmonary arterial tree a high degree of compliance, which maintains a low-pressure environment and allows the pulmonary

vasculature to accommodate the entire stroke volume output of the right ventricle. Additionally, the thin walls of the PA gives them a capacitance function, allowing them to act as a variable blood reservoir, i.e. act as a transient source of blood from the left ventricle when output begins to increase, for example at the start of exercise.

The pulmonary arterial bed is more complex than the systemic, with regional differences in structure between branches (see figure 1.1). The branching of the pulmonary arterial system closely parallels that of the bronchial system. The mainstem bronchi give off lateral branches, which divide and subdivide to form bronchioles and in turn alveolar ducts. Gaseous interchange between air and blood may occur in all divisions beyond the bronchioles. Likewise, the main pulmonary artery divides and subdivides like the branches of a tree. The main and first branch PAs, known collectively as the conduit or elastic PAs, comprise of a number of elastic laminae. As branching continues, transition from an elastic PA (>1mm i.d.) to a muscular walled structure (100μm-1mm i.d.) occurs. Muscular PAs consist of increased smooth muscle, which reduces with further branching to form the partially muscular PAs. Additional branching results in the further loss of smooth muscle leading to the formation of the non-muscular PAs, then finally the pulmonary arterioles (<100μm i.d.). The difference in structure of each individual branch of the pulmonary arterial tree is thought to determine its function. For example, due to the many elastic laminae, the conduit PAs can accommodate stroke volume and recoil during diastole to maintain peripheral flow and the compliance of the circulation. However, the smaller PAs (resistance vessels 100μm-1mm i.d.), due to increased muscularisation, appear to be the main site in the pulmonary circulation of resistance to flow (Singhal *et al.*, 1973; Sasaki *et al.*, 1995).

1.1.2 The structure of pulmonary arteries

The varying presence of three layers of different cells; the intimae layer of endothelial cells, the medial layer of smooth muscle cells, and the outer adventitial layer of fibroblasts, achieve the structural and functional heterogeneity of PAs. Additionally, in each branch of the PA the medial layer consists of diverse populations of mature, intermediate, and immature smooth muscle cells, which are present in differing proportions in specific branches of the PA. Each smooth muscle cell has different vasoconstrictor/vasodilator, proliferative and matrix producing ability, due to expressing different cytoskeletal and contractile proteins, ion channels, and receptors

(Frid *et al.*, 1997; Sasaki *et al.*, 1995; Michelakis, 1997, MacLean *et al.*, 1994a/b). As the phenotype of pulmonary vascular smooth muscle is different throughout the pulmonary arterial tree, ligand-receptor interaction and response to stimuli may be regionally different (Meyrick and Reid, 1978; Frid *et al.*, 1997; Sasaki *et al.*, 1995). Due to the complexity of the pulmonary circulation it is always important to know which branch of the pulmonary arterial tree is being studied to allow the correct interpretation of experimental data.

The endothelium of the PA acts as a barrier preventing the passage of fluid, proteins and other blood components from the vessel lumen into the airspace. The endothelial cells, as in the systemic circulation, perform non-respiratory functions and have a role in controlling vascular tone. Endothelial cells have the ability to release both vasoconstrictor and vasodilator agents (Barnes and Liu, 1995). Changes in the endothelial layer may therefore have profound effects on the ability of the PA to respond to stimuli.

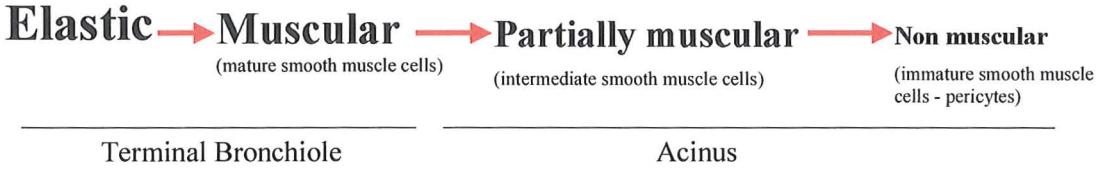


Figure 1.1 Muscularisation in the pulmonary arteries

With branching of the pulmonary artery, transition from an elastic PA (>1mm i.d.) to a muscular walled structure (100μm-1mm i.d.) occurs. Smooth muscle reduces to form the partially muscular PAs. Additional branching results in the further loss of smooth muscle leading to the formation of the non-muscular PAs, and pulmonary arterioles (<100μm i.d.).

1.1.3. Control of the pulmonary circulation

The pulmonary circulation is known to be a low resistance, low pressure circulation which is highly compliant. The control of the pulmonary circulation is predominately passive, influenced by lung volume or gravitational force that alter the distribution of pulmonary blood flow. In fact, there appears to be no baroreceptors comparable with those in the systemic circulation (regulation of the pulmonary vascualture is reviewed by Barnes and Liu, 1995). Despite the overall regulation of pulmonary blood flow being largely passive, the pulmonary circulation is also known to be under a degree of active regulation. Active factors such as autonomic nerves, humoral factors, and gasses all alter pulmonary vascular resistance and tone. The pulmonary vascular bed is supplied with sympathetic and parasympathetic nerve fibres. In fact, sympathetic nerve stimulation has been shown to increase pulmonary vascular resistance (PVR) by up to 70%, decrease compliance, and thereby increasing pulmonary arterial pressure (PAP, Kadowitz *et al.*, 1975; Piene 1976). Human pulmonary arteries are innervated and controlled by adrenergic, cholinergic, and nonadrenergic, noncholinergic nerves (NANC, Barnes and Liu, 1995). The increase in PVR as a result of sympathetic nerve stimulation appears to be mediated primarily by α_1-adrenoreceptors (Hyman, 1986). In contrast, β-adrenoreceptors have been shown to regulate vasodilation in response to sympathetic nerve stimulation (Hyman *et al.*, 1981). Cholinergic innervation seems to be less important in maintaining low pulmonary vascular tone, as blockade does not alter basal PAP or PVR (Murray *et al.*, 1986). However, human PAs have been shown to relax in response to acetlycholine, when an intact endothelium is present (Greenberg *et al.*, 1987). Additionally, in precontracted pulmonary arteries, electrical field stimulation initiates a frequency-dependent relaxation that is unaltered by adrenergic or/and cholinergic antagonists, indicating the presence of NANC nerves in the pulmonary circulation (Liu *et al.*, 1992a). Although NANC mediated vasodilation has been demonstrated *in vitro*, it has not been shown to occur *in vivo*, therefore its role in controlling pulmonary vascular tone requires further investigation. Active factors, although having a role in physiological conditions, are thought to be of particularly importance under pathological conditions where tone is altered (Fishman, 1985).

Many naturally occurring substances will selectively affect the vasomotor tone of PAs. Pulmonary vasoconstrictors include, thromboxane A_2, α-adrenergic agonists, angiotensin II, thrombin, angiotensin, prostaglandins, neuropeptides, and leukotrienes.

In contrast, β-adrenergic agonists (e.g. isoproterenol), prostacyclin, nitric oxide, acetylcholine, bradykinin and dopamine are pulmonary vasodilators. Some vasoactive agents such as 5-hydroxytryptamine (5-HT), endothelin (ET) and histamine have different vasoactive effects depending on the level of pre-existing pulmonary vascular tone, for example causing pulmonary vasoconstriction when tone is low or pulmonary vasodilation when tone is high (for reviews see Fishman, 1985; Barnes and Lui, 1995). In general the maintenance of the low pulmonary vascular tone in the pulmonary circulation appears to be due to a fine balance between vasoconstrictor/co-mitogens and vasodilator/antiproliferative mediators (Barnes and Liu, 1995). A number of the pulmonary vasoactive agents will be discussed below in more detail.

The pulmonary circulation is strongly influenced by respiratory gases. The systemic and pulmonary circulations differ dramatically in their response to changes in oxygen. Hypoxia is known to be one of the most potent pulmonary vasoconstrictors, whereas the systemic circulation dilates on exposure to low levels of oxygen (Fishman, 1976; Dumas *et al.*, 1999). Short-term exposure of the pulmonary circulation to low oxygen both *in vitro* and *in vivo* elicits a vasoconstrictor response that is totally reversible upon return to normal oxygen concentrations (Voelkel, 1986). A role of the pulmonary circulation is to match perfusion to ventilation in order to meet the varying demands of the living organism. Hypoxic pulmonary vasoconstriction is thought to be an inherent property of the lung, developed as a protective mechanism to divert blood from poorly ventilated alveoli to maintain or improve ventilation/perfusion relationships. In fact, the foetus relies on hypoxic pulmonary vasoconstriction to keep the pulmonary circulation closed, therefore allowing blood to be diverted through the ductus arteriosus. Hypoxic pulmonary vasoconstriction has been shown not to depend on substantial sympathetic innervation of the pulmonary vasculature as it occurs in isolated PA rings and isolated lung (Karamsetty *et al.*, 1996). Chronic hypoxia in the pulmonary circulation can cause right ventricular failure, and a decrease in pulmonary blood flow that may lead to the development of pulmonary hypertension (Fishman, 1985, Voelkel, 1986; Vender, 1994; Rabinovitch *et al.*, 1979). Pulmonary hypertension (PHT) is the disease state that is the basis for this study.

SYSTEMIC CIRCULATION	PULMONARY CIRCULATION
Serves many tissues	Serves only one tissue (lung)
Variable requirements	Single function
Many controls	Little control, no autoregulation
High pressure gradient	Low pressure gradient
High resistance	Low resistance (one eighth of systemic)
Long hydrostatic columns	Short hydrostatic columns
Vasodilator response to hypoxia	Vasoconstrictor response to hypoxia

Table 1.1: Differences between the systemic and pulmonary circulations

The systemic circulation and pulmonary circulation differ in a number of characteristics. These differences are summarised in the table above and may be related to the differences in the function and organisation of both the vascular systems.

1.2. Pulmonary Hypertension

Pulmonary hypertension (PHT) is known as abnormally high blood pressure in the arteries of the lung (reviewed by Rubin, 1997; Archer and Rich, 2000, Fishman, 1998; Vender *et al.*, 1994; Rabinovitch, 1997; Veyssier-Belot and Cacoub, 1999; Dumas *et al.*, 1999). When the mean pulmonary arterial pressure (PAP) is greater than 25mmHg. at rest or 30mmHg. during exercise, this is evidence of the presence of PHT (Fishman, 1998). Primary PHT (PPHT) is a relatively rare disorder, with an estimated incidence of 2-3 per million per year (Gaine and Rubin, 1998). The female/male ratio for PPHT is approximately 2:1, suggesting women may be more predisposed than men (Gaine and Rubin, 1998). All forms of PHT are difficult to both diagnose and manage, as early symptoms including fatigue and dyspnoea, dizzy spells and fainting are common for many respiratory disorders. Assessment of PHT is usually made using electrocardiograms, echocardiograms, pulmonary function tests, perfusion lung scans, or right heart catheterisation to evaluate the ability of right side of the heart to pump blood.

PHT is difficult to control and still presently incurable, usually leading to death. Current medical and surgical treatments for patients with PHT include anticoagulants, vasodilators and lung, or heart and lung transplantation (reviewed by Klings and Faber, 1999; Wanstall and Jeffery, 1998; Archer and Rich, 2000). Presently used vasodilators for the long-term therapy of PHT are calcium channel blockers, intravenous prostacyclin, and inhaled nitric oxide (NO), and more recently the dual endothelin receptor-blocking agent Bosentan (Tracleer®). The goal of vasodilators is to reduce the pressure and resistance in the PAs to increase the cardiac output, without reducing the systemic blood pressure. However, the limiting factor for therapeutic approaches to PHT appears to be the relative lack of specificity of drugs for the pulmonary circulation. Vasodilators shown to be effective in the pulmonary circulation tend to also be as effective in the systemic circulation, causing systemic hypotension. Present and possible novel drugs for the management of PHT will be outlined in more detail throughout, when discussing the drug target of interest. For the future successful treatment of PHT it is important to understand fully the physiology, pathophysiology and the triggers of the disease.

1.2.1 Classification of pulmonary hypertension

The World health Organisation recently classified PHT as either pulmonary arterial hypertension (PAH) or pulmonary venous hypertension (PVH). PHT is subdivided into primary pulmonary hypertension (PPH) or secondary pulmonary arterial hypertension (reviewed by Rubin, 1997; Fishman, 1998; Vender *et al.*, 1994; Rabinovitch, 1997; Veyssier-Belot and Cacoub, 1999; Dumas *et al.*, 1999; Jeffery and Wanstall, 2001; Archer and Rich, 2000). Secondary PAH occurs secondary as a serious complication of disorders such as HIV/AIDS infection, chronic obstructive pulmonary disease (COPD), pregnancy, Raynauds disease (vasospastic disorder), connective tissue diseases such as erythematosus and scleroderma, chronic pulmonary thromboembolism, cirrhosis of the liver, congenital heart disease or left ventricular failure. Additionally, a wide variety of stimuli can trigger the development of secondary PAH; including anorexic agents such as fenfluramine, dexfenfluramine and aminorex, long-term cocaine inhalation, and as mentioned above hypoxia. A genetic predisposition is thought to contribute to the susceptibility of individuals in developing PHT in response to some of the triggers mentioned above (reviewed by Archer and Rich, 2000, see below).

Abnormal vascular development or remodelling before birth can lead to persistent PHT of the newborn (PPHN), which has been shown to be responsible for a significant number of neonatal deaths occurring from 0-3 days old (Gersony, 1973; and reviewed by Weinberger *et al.*, 2001). Normally the transition from foetus to neonate results in an increase in pulmonary blood flow accompanied with a decrease in PAP and pulmonary vascular resistance (PVR) to allow the lung to function as the organ of gas exchange. PPHN develops when PVR and PAP does not decrease with the initiation of ventilation and oxygenation at birth. Neonates can develop PHT secondary to diaphragmatic hernia, or meconium distress, pulmonary hypoplasia or severe under development of the lung.

If the underlying cause of PHT cannot be identified it is called primary or idiopathic PHT (PPHT, Rubin, 1997; Archer and Rich, 2000). PPHT is thought to be a genetic disorder that may be the result of a sporadic or familial mutation. Genome wide research has shown there to be a link between PPHT and markers on chromosome 2q33 (Nichols *et al.*, 1997; Deng *et al.*, 2000). Furthermore, using microsatellite linkage marker analysis, a number of groups have identified that heterozygous mutations of the

gene encoding type II bone morphogenic protein receptor (BMPR2) appears to underlie familial PPHT, and has shown to occur in ≥26% cases of sporadic PPHT (Deng *et al.*, 2000; Machado *et al.*, 2001, Thomson *et al.*, 2000; Lane *et al.*, 2000). Currently 46 unique BMPR2 mutations have been identified, which may be useful for the screening and early detection of PPHT. These mutations include frameshifts, partial deletions, mis-sense, and splice-site mutations (Machado *et al.*, 2001). Bone morphogenic proteins are a group of cytokines within the transforming growth factor-β superfamily (TGF-β), which regulate growth, differentiation and apoptosis in various cell lines including epithelial cells (Sakou, 1998). It can be hypothesised that BMPR2 mutations may lead to PHT by disrupting the growth inhibitory effects of TGF-β members on vascular smooth muscle.

Furthermore, gene microarray technology has allowed the analysis of the gene expression profile of patients with PPHT to be compared to that from normal lung tissue (Geraci *et al.*, 2000; Golpon *et al.*, 2000). The expression of a total of 307 genes were found to be altered in the PPHT lung compared to the normal lung tissue. 133 of these genes were upregulated whereas 174 were down regulated in PPHT (Geraci *et al.*, 2000). Basically, an imbalance of genes involved in cell proliferation and apoptosis was observed in patients with PPHT. Genes encoding ribosomal, mitochondrial and cytoskeletal proteins, ion channels, enzymes, transcription factors and genes related to cyclin dependent kinases were differentially expressed with the development of PHT. Undoubtedly these gene mutations may have a role in pathogenesis of PHT.

1.2.2 Models of PHT

Due to the lack of lung tissue from patients with early stages of PHT, most data concerning the disease is drawn from animal models (reviewed by Jeffery and Wanstall, 2001). One of the most commonly utilised models of PHT is the chronic hypoxic rat (Hunter *et al.*, 1974; Rabinovitch *et al.*, 1979). In order to mimic the etiology of hypoxia induced PHT male Wistar rats are exposed to low oxygen by reducing the atmospheric pressure in a purposed built chamber (Hypoxic Hypobaric model). As the atmospheric pressure decreases, this leads to the decrease of the partial pressure of the gaseous components of air, and hence decreases the partial pressure of O_2 (pO_2) to a level that reduces inspired O_2 to ~10%. Low pO_2 has been shown to have a direct effect on the walls of the pulmonary artery (PA) of the rat lung. After 3 days of hypoxia, there

has seen to be elevated PAP, right ventricular hypertrophy and polycythemia. Furthermore, after 14 days of hypoxia, hypertrophy of the PA was noted (Wanstall *et al.*, 1992, Vender, 1994). Chronic hypoxia leads to PHT and right ventricular hypertrophy, which is associated with pulmonary vascular remodelling. The chronic hypoxic rat is the model for PHT used throughout this study.

PHT and right ventricular hypertrophy is also commonly induced by a single subcutaneous injection of monocrotaline (105mg/kg), a plant toxinpyrolizine alkaloid, to rats (MCT-treated rat model of PHT). MCT is converted to dehydromonocrotaline in the liver, which in turn is highly toxic to the pulmonary circulation (Meyrick *et al.*, 1980; Fishman, 1985; Dogrell *et al.*, 1998). A single injection of MCT in the rat can cause epithelial proliferation of the small PAs resulting in PHT and right ventricular hypertrophy (Dogrell *et al.*, 1998). Other widely used animal models for PHT include the fawn hooded rats, which have a hereditary tendency to develop idiopathic PHT (Stelzner *et al.*, 1992). In addition, the continuous infusion of perfused rabbit or lamb lungs with the stable thromboxane analogue A_2 mimetic, U46619, establishes stable PHT (Rimar and Gillis 1995; Schermuly *et al.*, 2000; Ichinose *et al.*, 1995a/b). PHT can also be surgically produced in fetal lambs (Hanson *et al.*, 1998b). Assessment of PHT in each model is characterised by hypertrophy and hyperplasia of the vascular smooth muscle (Hunter, *et al.*, 1974, Leach *et al.*, 1977). The remodelling is very similar among these models despite differences in the initiating mechanism.

In addition to animal models, cellular models are useful to study the mechanisms involved in hypoxia or ischemia. In cell culture conditions the extracellular environment can be easily manipulated to mimic conditions that are thought to occur *in vivo*. Through the use of cell cultures, more in-depth studies of the mechanisms involved in hypoxic injury can be more easily carried out. Hypoxia has been proposed to have a direct effect on smooth muscle cells (SMC). Studies have shown hypoxia can contract isolated pulmonary artery smooth muscle cells (PASMC) in culture (Murray *et al.*, 1990a). Prolonged hypoxia of 1-2 weeks can stimulate the proliferation of PASMC from human distal pulmonary arteries if cultered at low density (Yang *et al.*, 2002). However, many studies have also shown that serum-induced proliferation of PASMC can be inhibited under hypoxic conditions (Xiao, 1993; Yang *et al.*, 2002). Animal and cellular models are both helpful in understanding the disease and answering questions surrounding the disease. In this study the chronic hypoxic rat model will be utilised in

parallel with a hypoxic cell model for PHT, using human pulmonary artery smooth muscle cells (hPASMC).

1.2.3 Pathology of PHT

Characteristics of PHT include abnormal vasoconstriction, pulmonary vascular remodelling and thrombosis *in situ* (reviewed by Jeffery and Wanstall, 2001; Rabinovitch, 1997; Meyrick and Reid, 1983). The pathology of PHT is characterised by the disturbance of the three layers of the PA. The classical remodelling seen with PHT includes, medial hypertrophy (increased wall thickness of the PA), significant muscularisation of the partially muscular and non-muscular peripheral PAs, advential hardening due to increased collagen and elastin, and right ventricular hypertrophy (Rabinovitch, 1997; Cowan *et al.*, 2000). Remodelling is frequently studied by removing lung tissue, fixing it in paraffin, staining it with haematoxylin-eosin and elastin (van Giesen), and viewing it directly by light microscopy. Remodelling can be quantified by counting the number of vessels (<50μm diameter) with a double elastic lamina and expressed as a % of the vessels examined.

A further feature of some forms of PHT, inparticular PPHT, is the formation of neointima and plexiform lesions that can be characterised using immune histology and *in situ* hybridisation technology (Voelkel *et al.*, 1997a). These appear usually on the resistance vessels, and comprise of a group of capillary-like channels separated by a matrix of proliferating cells and atypical endothelial cells. Plexiform lesions are widely used as a marker for PHT.

As outlined in section 1.1 there is marked heterogeneity in the endothelial, smooth muscle and fibroblast populations at specific locations within the pulmonary arterial tree. Each heterogeneous population of smooth muscle cells express different cytoskeletal and contractile proteins and channels. It is understood that in response to hypoxia there is a redistribution of smooth muscle cell phenotype, which can alter the proliferative and matrix producing abilities of each PA, thereby modify their response to vasoactive agents (Frid *et al.*, 1997). For example, increased matrix production reduces the compliance of the conduit PAs, which are thought to be the cause of increased right ventricular hypertrophy (Zuckerman *et al* 1991). In contrast, the increased muscularisation of the resistance vessels appears to be a major factor in the increased

resistance seen with PHT (Singhal *et al.*, 1973). Although resistance vessels are particularly sensitive to hypoxic stimuli, it is however likely that increased smooth muscle anywhere in the pulmonary circulation would increase the tone in response to hypoxia (Voelkel and Tuder, 2000). For example, in the CH large elastic PA there is an increase in endogenous tone (MacLean *et al.*, 1995; MacLean *et al.*, 1996; MacLean *et al.*, 1998a). The increased vascular tone of the pulmonary circulation in response to PHT appears to play a pivotal role in increasing its sensitivity to vasoactive mediators i.e. serotonin and ET-1 (MacLean, 1999a).

The endothelium is thought to play a crucial role in the pathogenesis of the PHT, influencing both vasoconstriction and structural remodelling (Dinh-Xuan *et al.*, 1991). In conduit PAs from CH, and MCT rat's, diminished endothelium-dependent relaxation and a decrease in acetylcholine-induced vasodilation have been established (Wanstall and O'Donnell, 1992; Oka *et al.*, 1993; Adnot *et al.*, 1991, MacLean *et al.*, 1995; MacLean *et al.*, 1996; MacLean *et al.*, 1998a). Furthermore, it has been demonstrated that endothelium-dependent vasodilators fail to relax pulmonary arterial strips isolated from MCT rats (Mathew *et al.*, 1995). Electron and light microscopy have both demonstrated alterations in the vascular endothelium in CH and MCT, showing increased density of microfilament bundles, increased density of rough endoplasmic reticulum and cell swelling (Rabinovitch, 1979). It is important to point out, that although endothelium-dependent relaxation is reduced in rat conduit PAs after hypoxia, it has however been reported to be increased in the resistance vessels from the CH (Adnot *et al.*, 1991; MacLean *et al.,* 1995; MacLean and McCulloch, 1998).

Decreased endothelium-dependent relaxation has been reported in patients with both PPHT and with PHT secondary to chronic obstructive lung disease (Dinh-Xuan *et al.*, 1991; 1993). It is possible that dysfunctional endothelium alters the release of endothelium-dependent factors such as prostacyclin, nitric oxide, endothelin, or 5-HT (Barnes and Lui, 1995). The endothelium dysfunction associated with PHT is also suspected to make patients sensitive to vasoactive agents. For example, endothelium removal was shown to potentiate the response to 5-HT and α-adrenoceptor agonists in human, rat, and bovine conduit PAs (MacLean *et al.*, 1994a; MacLean *et al.*, 1993a, Adnot *et al.*, 1991). As PHT is linked to a degree of endothelial dysfunction it would be beneficial if therapies for the disease still acted as effectively when endothelium is damaged.

1.2.4 Possible mediators of PHT

PHT appears to be the consequence of an elevation of pulmonary vascular tone and a thickening of the pulmonary vascular wall. The abnormal tone and vascular remodelling is thought to be due to an imbalance between the vasoconstrictor/vasodilator actions of endogenous mediators, and changes in their mitogenic/anti-mitogenic effects (reviewed by Fishman, 1998; MacLean, 1999a/b). Signal transduction plays a pivotal role in co-ordinating cellular functions, and reviews have suggested a variety of intercellular and intracellular messengers that appear to be possible mediators of PHT (reviewed by Jeffery and Wanstall, 2001; Archer and Rich 2000). It is possible that PHT is a result of synergy between a number of different signal transduction pathways, and the resulting 'cross-talk' augments the extent of vascular change. Therefore, manipulating various signal transduction pathways may allow more effective management of PHT. In the following section an outline of important mediators thought to be implicated in the physiology, and pathophysiology of PHT will be discussed.

1.2.4.1 The role of GPCRs in PHT.

Many circulating mediators and hormones act on the pulmonary circulation via multiple cell surface or intracellular receptors. Cell surface receptors include G-protein coupled receptor (7-transmembrane helical domain receptors), tyrosine-kinase receptors (RTKs), and ion channel linked receptors. GPCRs are heterotrimeric structures comprising of a guanine nucleotide-binding α subunit, and a complex of tightly associated β and γ subunits (for review see Marinissen and Gutkind, 2001; Hakonarson and Grunstein, 1998). On activation of the G-protein, guanosine diphosphate (GDP) is released in exchange for guanosine triphosphate (GTP) causing a conformational change resulting in dissociation of GTP-α from $\beta\gamma$. GTP-α is then free to diffuse in the membrane and associate with various enzymes and ion channels. Additionally, the GTPase activity of the α-subunit hydrolyses GTP to GDP terminating the process. Evidence also exists suggesting the $\beta\gamma$-complex may be involved in signal transmission to the effector proteins. For example, in the regulation of mitogen activated protein kinases (Luttrell *et al.*, 1997; Daub *et al.*, 1997).

G-proteins are classified according to the α-subunit. The main classes of G-protein including $G_{\alpha}s$, $G_{\alpha}i$, $G_{\alpha}q$, and $G_{\alpha}12$. Each class of G-protein leads to the activation/inactivation of various signalling pathways. Effector molecules include adenylyl and guanylyl cyclase, phospholipase A_2 (PLA_2), phospholipase C (PLC), ion channels and phosphoinositiole 3-kinase (P13Ks). GPCR agonist can control the production of second messengers, such, as the cyclic nucleotides, diacylglycerol (DAG), inositol (1, 4, 5)-triphosphate (IP_3), phosphatidyl inositol (3, 4, 5)-trisphosphate, and phosphatidic acid, and also stimulate an increase in intracellular calcium.

Activation of G proteins have been speculated to be critical in the early response to hypoxia, and the subsequent modulation of ion channel activity and cell depolarisation in various cell types (Mironov and Richter, 2000, Kobayashi *et al.*, 1998, Feldkamp *et al.*, 1999, Wenzlaff *et al.*, 1998). It has been demonstrated that $G_{\alpha}i/o$ is utilized for growth, and subpopulations of smooth muscle cells specifically with $G_{\alpha}i/o$ proliferate in response to hypoxia (Frid *et al.*, 1998). Additionally, vasoactive agents such as α_1-adrenoceptor agonists, endothelin-1 (ET-1), and 5-hydroxytryptamine (5-HT), which act via specific types of GPCRs, are reported to have a role in the development of PHT (reviewed by MacLean, 1999a/b/c; Jeffery and Wanstall, 2001; Archer and Rich 2000, see figure 1.1). Evidence for the role of some well characterised GPCRs and their ligands in PHT will be discussed below.

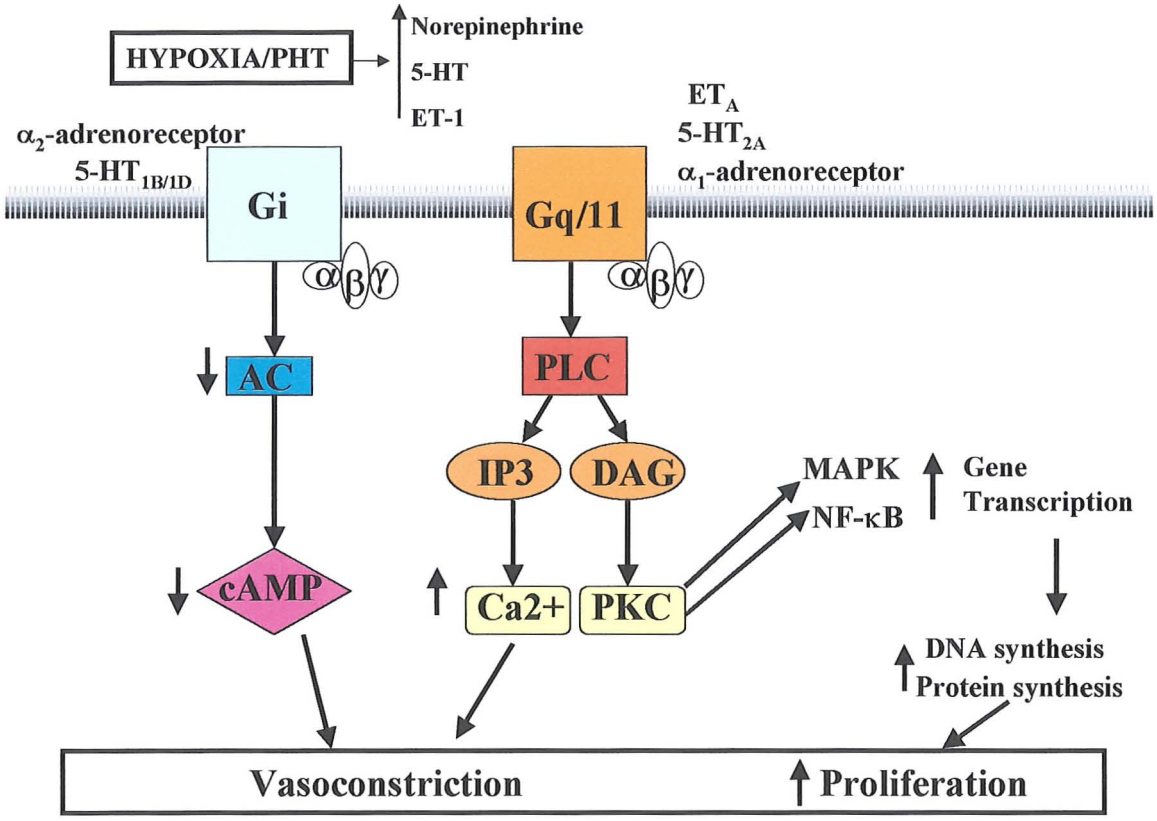


Figure 1.2 Signalling pathways of specific GPCRs thought to have a role in the development of PHT

In response to hypoxia/PHT vasoactive agents such as α_1-adrenoceptor agonists (norepinephrine), endothelin-1 (ET-1), and 5-hydroxytryptamine (5-HT), which act via specific G-protein coupled receptors (GPCRs), are increased. GPCRs coupled to $G_\alpha i$ induce vasoconstriction by negatively coupling to adenylyl cyclase (AC), thereby decreasing cAMP. In contrast, $G\alpha_{q/11}$ dependent GPCR agonists lead to the increased production of phospholipase C (PLC), which in turn generates diacylgycerol (DAG) and inositol triphosphate (IP_3). IP_3 releases calcium from internal stores, whereas DAG activates protein kinase C (PKC). PKC can phosphorylate mitogen activated protein kinase (MAPK) and nuclear factor κB (NF-κB) which are known to have a role in cellular proliferation (expanded in text). Figure is adapted from Sundeep, 1999; MacLean,1999.

1.2.4.1.1 The role of adrenoreceptors in PHT

Sympathetic stimulation causes changes in pulmonary vascular resistance mediated via GPCRs, namely the α- and β-adrenoreceptors (reviewed by Barnes and Liu, 1995; Bevan, 1989). The pulmonary vasculature expresses both α- and β-adrenoreceptors, which have been shown to regulate vasoconstriction and vasodilation respectively (Bevan, 1989). It is currently known that three subtypes of β-adrenoreceptor (β_1, β_2, and β_3), and two subtypes of α-adrenoreceptors (α_1 and α_2) exist. Using molecular techniques α-adrenoreceptors can be further classified into α_{1A}-, α_{1B}, α_{1C}-receptors and α_{2A}-, α_{2B}-, α_{2C}-, and α_{2D}- (Strasser *et al.*, 1992).

The α-adrenergic receptors regulate the increase in PVR and decrease in compliance that occur during sympathetic stimulation of the pulmonary circulation (Kadwitz *et al.*, 1975, 1976). In the intact and perfused lung, stimulation of the sympathetic nerves has been show to induce a frequency-dependent increase in perfusion pressure and PVR (Kadowitz and Hyman, 1973; Kadowitz *et al.*, 1976). This increase in perfusion pressure and PVR could be blocked by α-adrenoreceptor antagonists (Hyman and Katwitz, 1985). Furthermore, Hyman (1986), demonstrated that it appears to be the α_1-adrenoreceptors that primarily mediate the vasoconstrictive action of sympathetic nerve stimulation. In parallel, in both the pulmonary vascular bed and in isolated PAs, β-adrenoreceptors regulate vasodilation in response to sympathetic nerve stimulation and an increase in circulating catecholamines (Hyman *et al.*, 1981). This is thought to occur as inhibition of β-adrenoreceptors enhances the vasoconstrictor response to sympathetic nerve stimulation (Hyman *et al.*, 1981). Furthermore, by regulating DNA and protein synthesis, both α- and β-adrenoreceptors have been shown to have a role in smooth muscle proliferation (Nakaki *et al.*, 1990). These authors demonstrated that α_1-adrenoreceptor stimulation increased DNA synthesis, while stimulation of the β-adrenoreceptors inhibited this process.

Agonists for α-adrenoreceptors such as noradrenaline, and phenylephrine (PE) are thought to mediate pulmonary vasoconstriction primarily through increasing the levels of free calcium. By coupling to specific G proteins ($G_{\alpha}q$, $G_{\alpha}11$ or $G_{\alpha}13$), α_1-adrenoreceptors activate phospholipase C (PLC), which in turn generates diacylgycerol (DAG) and inositol triphosphate (IP_3). IP_3 binds to receptors on the endoplasmic

reticulum, which releases calcium from internal stores, whereas DAG activates protein kinase C (PKC). PKC can subsequently phosphorylate several protein kinases and activate transcription factors known to have a role in cellular proliferation, such as mitogen activated protein kinase (MAPK) and nuclear factor κB (Clemens, 1992, see figure 1.2). Furthermore, the blockage of potassium channels by stimulation of α_1-adrenoreceptors can also lead to an influx of calcium through voltage-dependent channels due to membrane depolarisation (Takizawa *et al.*, 1996).

The possible role of α_1-adrenoreceptor in PHT has been intensively studied (reviewed by Sundeep, 1999). Eckhart *et al.* (1996), showed hypoxia increased the *de-novo* synthesis of α_1-adrenoreceptors in smooth muscle cells both *in vivo* and *in vitro*. Upregulation of α_1-adrenoreceptors were also seen in the PAs of the CH (Jianming *et al.*, 1991). Furthermore, hypoxic exposure has been shown to increase the circulating levels of norepinephrine, a potent agonist for α_1-adrenorecepetors (Mardon *et al.*, 1998). The increase in α_1-adrenoreceptors with hypoxia may serve to induce vasoconstriction in the resistance vessels, redistributing blood flow to improve the ventilation/perfusion ratio.

Antagonists for α_1-adrenoreceptors were among the first drugs used as therapy for PHT (Sundeep, 1999). Studies by Brutsaert (1964), and Porcelli and Bergofsky (1973), demonstrated that α_1-adrenoreceptor blockers could either abolish or attenuate hypoxia-induced PHT. Furthermore, the selective α_1-blocker bunazosin can reduce the development of PHT and right ventricular hypertrophy in MCT (Inoue *et al.*, 1994). Unfortunately, due to their short half-life and intolerable systemic side effects of α_1-adrenoreceptor antagonists, they are no longer popular clinically (Cohen and Kronzon, 1981; Pickering *et al.*, 1982).

1.2.4.1.2 The role of endothelin-1 in PHT

Endothelin (ET) is a 21 amino-acid polypeptide, produced by endothelial cells (reviewed by Rubanyi and Polokoff, 1994; Inoue *et al.*, 1989, Yanigasawa *et al.*, 1988). Three structurally similar ETs have been characterised namely, ET-1, ET-2 and ET-3, which are encoded by different genes. Molecular cloning has identified endothelins act via two endothelin receptor subtypes ET_A (selective for ET-1 over ET-3), and ET_B (non selective, Miller *et al.*, 1993).

In the lung both ET-1 and ET-3 are abundantly expressed providing further evidence for a role in the pulmonary vasculature (Firth *et al.*, 1992). ET-1 is formed from preproendothelin-1, a 212-amino acid precursor, which is cleaved by an endopeptidase to form proendothelin-1 (big ET-1), a 38 amino acid peptide. This 38 amino acid peptide is then converted to the biologically active ET-1, catalysed by endothelin converting enzyme (reviewed by Inoue *et al.*, 1989, Yanigasawa *et al.*, 1988). ET-1 has a multifactorial action mediated by the two ET receptors (reviewed by MacLean *et al.*, 1998b). ET-1, via the ET_A receptor has been shown to cause a concentration-dependent contraction in PAs and veins *in vitro* and increase PVR *in vivo* (reviewed by Zamora *et al.*, 1993; MacLean *et al.*, 1998b). The ET_A selective antagonist BQ-123 has been shown to inhibit ET-1 induced contraction in human PAs, whereas the ET_B selective agonist, sarafotoxin, has been shown to have no effect (Buchan *et al.*, 1994). To initiate smooth muscle contraction ET-1 activates specific GPCRs in a similar manner to α_1-adrenoreceptor agonists (see figure 1.2). ET-1, through activation of PLC, leads to the production of IP_3 and DAG and stimulates the release of calcium from intracellular stores, and the activation of PKC. In parallel, binding of ET-1 to ET_B receptors has been shown to cause pulmonary vasodilation, possibly by mediating the release of NO, prostacyclin or activating adensine triphosphate (ATP)-gated potassium channels (Eddahibi *et al.*, 1991).

Evidence also exists for the contribution of ET_B receptors in ET-1 mediated vasoconstriction in both rat and human small PAs (MacLean *et al.*, 1994b; McCulloch *et al.*, 1998). MacLean and co-workers demonstrated that ET-1 mediated vasoconstriction in the large elastic PAs of the rat was blocked by the ET_A receptor antagonist FR 139317. In contrast, the response in the resistance vessels appeared to be due to "atypical" ET_B receptors. The ET-1 response in the resistance vessels could be mimicked by the ET_B receptor agonist sarafotoxin S6, and blocked only by a mixed ET_A/ET_B antagonist SB 209670 (Sato *et al.*, 1995; MacLean *et al.*, 1994b). In human PAs, a similar heterogeneity in the distribution of ET receptors is evident (McCulloch *et al.*, 1998). Additionally, both ET_A and ET_B have been demonstrated to be co-expressed and both have a role in vasoconstriction in the large elastic arteries and the small muscular arteries in the rabbit (Fukuroda *et al.*, 1994; Docherty and MacLean, 1998). Together, theses results demonstrate clear differences exist in ET receptor distribution between species and between branches of the pulmonary arterial tree, hence caution must be taken when interpreting experimental data.

In addition to being a potent vasoconstrictor, ET-1 has been shown to stimulate DNA synthesis and proliferation via ET_A receptors in PASMC and pulmonary fibroblasts. (Janakidevi *et al.*, 1992; Zamora *et al.*, 1993; reviewed MacLean *et al.*, 1998b). ET-1 has been shown to increase the incorporation of [^{3}H]-thymidine in bovine PASMC up to 140% over controls, providing evidence of its a co-mitogen action in PASMC (Hassoun *et al.*, 1992). In fact, increased growth of PASMC from fawn hooded rats has been attributed to their increased ET-1 levels (Zamora *et al.*, 1996). Due to the pulmonary vasoconstrictor and proliferative actions of ET-1, an increase in its circulating levels may contribute in part to the increased tone and remodelling observed with PHT (reviewed by MacLean, 1998b).

Lungs from the chronic hypoxic rat model of PHT exhibit both increased expression of ET-1 and the ET_A receptor (Li *et al.*, 1994). With hypoxia there has also been shown to be an increase in ET_A mediated vasoconstriction in the large and small PAs of the CH, the latter due to redistribution of ET_A receptors into these vessels (MacLean *et al.*, 1995; MacLean *et al.*, 1994b). Consistent with these results, ET-1 levels have been shown to be elevated in patients with both primary and secondary PHT (Stewart *et al.*, 1991; Cody *et al.*, 1992). These results would suggest that a common pathophysiological feature of PHT, regardless of the etiology, is an increase in the circulating levels of ET-1.

Researchers have also investigated the possible benefits of ET receptor antagonists in the treatment of PHT. For example, BQ-123 (ET_A selective antagonist), SB 217242 (ET_A/ET_B non-selective antagonist) and bosentan (ET_A/ET_B non-selective antagonist) have all been shown to reverse and/or prevent vascular remodelling and the increase in pulmonary arterial pressure elicited by hypoxia in rats (DiCarlo *et al.*, 1995; Chen *et al.*, 1995, Underwood *et al.*, 1998 and reviewed by MacLean *et al.*, 1998b). Importantly bosentan (Tracleer®), a dual endothelin-receptor antagonist (ET_A/ET_B), is presently used as a frontline treatment in PPHT. A double blind, placebo-controlled trial has shown bosentan can increase the exercise capacity and the haemodynamics of patients with PHT (Channick *et al.*, 2001). Thus a drug that reduces the vasoconstrictive/proliferative actions of ET-1 appears of advantage in controlling PHT.

1.2.4.1.3. The role of 5-Hydroxytryptamine in PHT

A further possible mediator of pulmonary vasoconstrictor is 5-hydroxytryptamine (5-HT, also known as serotonin, reviewed MacLean *et al.*, 2000). 5-HT is produced as a derivative of the amino acid tryptophan, and is released and acts locally. Recent evidence suggests there are at least 17 genetically different 5-HT receptors including 5-HT_{1A-F}, $5HT_{2A-C}$, 5-HT_3 and 5-HT_4 (reviewed by Hoyer *et al.*, 2001; MacLean, 1999b/c). In the pulmonary circulation the vasoconstrictive effect of 5-HT is mediated via 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors depending on the level of pre-existing tone and the species (reviewed by Zifa and Fillion, 1992, Morecroft and MacLean, 1998). In the absence of tone, the vasoconstrictive effect of 5-HT in bovine and rat PAs has been shown to be mediated via 5-HT_{2A}, whereas in the presence of tone, 5-HT elicits contraction via 5-$HT_{1B/1D}$ receptors (MacLean *et al*, 1994a; MacLean *et al.*, 1996). However, in human elastic and muscular PAs it is the 5-HT_{1B} receptor that mediates vasoconstriction, even in the absence of tone (MacLean *et al.*, 1996; MacLean *et al.*, 1999b/c). 5-HT acting on 5-$HT_{1B/1D}$ receptors induces contraction via a $G_{\alpha}i$ dependent mechanism, decreasing cAMP by negatively coupling to adenylyl cyclase. In parallel, contraction via 5-HT_{2A} receptors occurs through a $G_{\alpha}q$ dependent pathway, leading to the hydrolysis of IP_3 and DAG from PLC and the subsequent increase in intracellular calcium and activation of PKC (Summner and Humphrey, 1990, see figure 1.2). 5-HT has also been shown to have co-mitogenic effects in PASMC (Eddahibi *et al*, 1999; Lee *et al.*, 1994). These authors showed internalisation of 5-HT appears to be essential for its mitogenic effects.

The role of 5-HT in PHT was first proposed when patients taking anoregixens, such as fenfluramine and phentermine, were seen to develop PHT (Abenhaim *et al.*, 1996). Anorexigens have been shown to act by inhibiting 5-HT reuptake, stimulating the release of platelet 5-HT, and preventing 5-HT clearance by inhibiting monoamine oxidase (Seiler *et al.*, 1976; Buczko *et al.*, 1975). Administration of anoregixens increase the circulating level of 5-HT, and therefore prolong its vasoconstrictive effects. Plasma levels of 5-HT have been found to be elevated in patients using appetite suppressants, providing further evidence for a role for 5-HT in the development of PHT (Herve *et al.*, 1995). Additionally, circulating levels of 5-HT were shown to increase from 1-2nmol/L to about 30nmol/L with PPHT (Anderson *et al.*, 1987). In fact, in response to hypoxia, 5-HT is released from pulmonary neuroendocrine cells and

neuroepithelial bodies in the airway (Johnson and Georgieff, 1989). As with ET-1, evidence would suggest that increased 5-HT may have a role in the increased vasoconstriction and vascular remodelling associated with PHT (Reviewed by MacLean, 1999b/c).

5-HT sensitivity is also enhanced in the PAs from CH, MCT-treated rats, and from patients with PPHT (MacLean *et al.*, 1996; Eddahibi *et al.*, 1997; Brink *et al.*, 1988). The augmented response to 5-HT with PHT appears to be linked to an increase in 5-HT_{1B}-like receptor stimulation, which has been suggested to be related to a combination of increased tone and changes in cyclic nucleotide levels observed in these vessels. Sweeney *et al.*, 1995, demonstrated that a decrease in cGMP through NO inhibition enhanced the response to sumatriptan (5-HT_1-like agonist), while in parallel, an increase in cGMP inhibited the ability of 5-HT to constrict PAs. More importantly, increasing the tone in PAs from control rats resulted in the "uncovering" of $5HT_{1B/1D}$-mediated vasoconstriction to 5-HT (MacLean *et al.*, 1996). These results would suggest changes in cyclic nucleotide concentrations and tone in the PA are important in the pathophysiology of PHT, and alter the response of vasoactive mediators in the pulmonary circulation.

Unfortunately the 5-HT antagonist ketanserin has not been successful in the treatment of PHT, due to adverse effects on the systemic circulation (Herve *et al.*, 1995). MacLean *et al*, 1996, have shown that ketaserin (5-HT_{2A} specific antagonist) competitively antagonised 5-HT vasoconstriction in the PAs from both control and CH. However, ketanserin has also been shown to competitively antagonise 5-HT mediated vasoconstriction via 5-HT_{2A} receptors in the systemic circulation, which may explain its detrimental side-effects (Arneklo-Nobin *et al.*, 1988). Since a role of $5HT_{1D/1B}$ receptors in 5-HT mediated vasoconstriction appears to be uncovered in isolated human and rat PAs with PHT, this suggests a 5-$HT_{1B/1D}$-receptor antagonist may be more effective as a treatment.

1.2.4.2 The role of growth factors in PHT

Growth factors are a group of polypeptides and proteins than regulate cell function by interacting with receptors on the membrane of the cell. Well-characterised growth factors include nerve growth factor (NGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and insulin

like growth factor-1 (IGF-1). Growth factors commonly trigger intracellular pathways via tyrosine kinase receptors (RTKs). Characteristically, when these ligands bind to RTKs, the tyrosine kinase autophosphorylates the receptor, leading to the stimulation of several mitogen activated protein kinases (MAPK, Marshall 1995, Van Biesen *et al.*, 1995). The pathway initiated by the phosphorylation of the RTK and the activation of MAPK has a direct role in the activation of transcription factors in the nucleus that alter gene expression. Evidence for the involvement of growth factors, MAPKs and a wide number of transcription factors in pulmonary vasoconstriction and in PHT will be outlined below.

Growth factors are widely known to have a role in cell division and differentiation. Therefore, increased production of growth factors may have a role in the arterial remodelling observed in the PAs with PHT. For example, both PDGF and bFGF have been demonstrated to cause proliferation of rat PASMC (Rothman *et al.*, 1994). A number of growth factors and/or their mRNA have been shown to be elevated in PHT (reviewed Voelkel *et al.*, 1997). PDGF-A and PDGF-B (Arcot *et al.*, 1993; Katayose *et al.*, 1993), VEGF (Turder *et al.*, 1995, Christou *et al.*, 1998), TGF-β (Acrot *et al.*, 1993), bFGF (Arcot *et al.*, 1995), IGF-1 (Perkett *et al.*, 1992), and EGF (Gillespie *et al.*, 1989a) have all been shown to be increased with PHT. Upregulation of VEGF has in fact been associated with the development of plexiform lesions (Archer and Rich, 2000).

Xiao (1993), suggested that the proliferation of smooth muscle in PAs seen in response to hypoxia may be due to the increased secretion of growth factors from endothelial cells. This was concluded from their observations that hypoxic endothelial cells conditioned medium stimulated proliferation of PASMC, promoting them to enter the cell cycle and increase ^{3}H-thymidine incorporation into DNA. Increased expression of both PDGF-A and -B mRNA have been shown to occur in lungs as early as 3 days following hypoxic exposure, and remain elevated for the entire 3 weeks of the study (Katayose *et al.*, 1993; Li *et al.*, 1995). PDGF has also been shown to be a mediator in hypoxia-induced cell activation and cytokine release in human lung. For example, PDGF is involved in hypoxia-dependent expression of inflammatory cytokines, such as IL-6 and IL-8 in human pulmonary fibroblasts (Tamm *et al.*, 1998). Furthermore, the gene encoding VEGF is abundantly expressed in lung tissue induced by short- and long-term hypoxia (Voelkel *et al.*, 1996). The hypoxic upregulation of VEGF and PDGF

mRNA has been shown to correlate with the activation of p42/p44 MAPK and p38 MAPK (Jin *et al.*, 2000). These results suggest growth factors may have a role in the remodelling of the PA through the regulation of MAPK cascades.

1.2.4.3 The role of MAPK in PHT

MAPK cascades are activated not only by growth factors but also by a diverse array of stimuli including GPCR agonists, cytokines, neurotransmitters, hormones, and cellular stress (reviewed by Marshall, 1995; Van Biesen *et al.*, 1995; Seger and Krebs, 1995; Wildmann *et al.*, 1999). Agents such as ET, thromboxane A_2, prostaglandin H_2, prostaglandin F_2, thrombin, norepinephrine and acetylcholine have all been shown to activate these kinases. MAPK can be split into subfamilies based on their structure and function. The best characterised of the MAPK signalling molecules include the serine/theronine p42/p44 mitogen-activated protein kinase (p42/p44 MAPK or extracellular regulated kinases, ERK1/2), the c-Jun N-terminal kinases also known as stress activated protein kinases (JNKs/SAPKs), and the p38-MAPKs (Kolch, 2000; Widmann *et al.*, 1999; Seger and Krebs, 1995). The activation of p42/p44 MAPK has been associated with proliferation and differentiation, in contrast JNKs and p38 MAPK appear to be more involved in cellular response to stress such as cytotoxic insults and the pro inflammatory cytokines TNF-α and IL-1 (Orsini *et al.*, 1999). The general sequence of activation for MAPK is; MAPK kinase kinase (MAPKKK) phosphorylates and activates MAPK kinase (MAPKK), which in turn activates MAPK. Specific MAPKKK, and MAPKK and upstream mediators have been characterised to induce each MAPK subfamily (see figure 1.3).

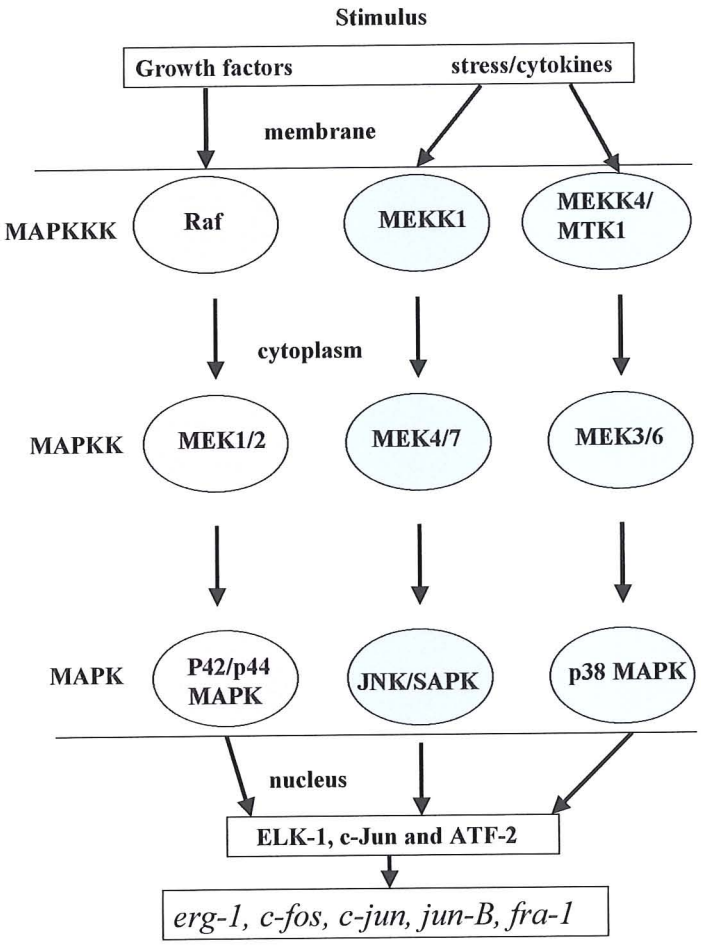


Figure 1.3 MAPK cascades

Above shows a schematic diagram of the mitogen activated protein kinase (MAPK) cascade. Three specific MAPK signalling cascades are represented above, which culminate in the activation of p42/p44 MAPK, JNK, and p38 MAPK. Active MAPK can translocate to the nucleus and regulate transcription through its action on transcription factors.

Due to association of the p42/p44 MAPK pathway with proliferation this section will focus on its regulation and possible role in the remodelling of the PA with PHT. The p42/p44 MAPK pathway can be initiated by the autophosphorylation of the RTK leading to the binding of the adapter protein Shc (SH2 domain-containing α_2-collagen related). This adapter protein is then phosphorylated itself allowing binding of a further adapter protein namely Grb2 (Growth factor receptor bound protein 2). The SH3 domain of Grb2 allows the constitutive binding of the guanine nucleotide exchange factor Sos (Son of Sevenless) leading to exchange of Ras-GDP for GTP at the plasma membrane. Activation of Ras, which is a regulatory GTPase, can then lead to the recruitment of Raf-1 to the plasma membrane through its interaction with its amino-terminal domain. Raf-1 (MAPKKK) is then phosphorylated, which in turn may phosphorylate MEK (MAPKK). MEK once activated phosphorylates p42/p44 MAPK. Downstream effectors of p42/p44 MAPK include transcription factors such as Elk-1 and c-Myc, cytoplasmic proteins such as cytosolic phospholipase A_2 ($cPLA_2$), and protein kinases such as p90 ribosomal S6 kinase (Seger and Krebs, 1995).

Recent studies have also suggested that GPCRs can mediate growth and differentiation through activation of p42/p44 MAPK (Daub *et al.*, 1996; Herrlich *et al.*, 1998). Classic GPCRs, such as ET-1, have been shown to increase smooth muscle proliferation due to MAPK activation, and the subsequent phosphorylation of transcription factors (Yamboliev *et al.*, 1998). It is understood that several different mechanisms may exist for the interaction of GPCRs and the p42/p44 MAPK pathway (reviewed by Gutkind, 1998; Murga *et al.*, 1999; and Gudermann *et al.*, 2000). Stimulation of GPCRs have been shown to induce tyrosine phosphorylation of the adapter protein Shc, formation of the Shc/Grb2 complex, and in turn activation of the MAPK pathway, once thought to be specific for RTKs (Bonfini *et al.*, 1996; Chen *et al.*, 1996; Van Biesen *et al.*, 1995; Daub *et al.*, 1997).

The mechanism by which G-proteins activate p42/p44 MAPK is thought to be attributed to $\beta\gamma$ subunit involvement in addition to α-subunit associated coupling (Lopez, 1998; Daub *et al.*, 1997). RTK transactivation has been demonstrated to occur in response to stimulation of GPCRs (such as ET receptors), through the release of $\beta\gamma$ and subsequent activation and tyrosine phosphorylation of RTKs (Luttrell *et al.*, 1997; Daub *et al.*, 1996; Daub *et al.*, 1997). Conway *et al.* (1999), demonstrated that PDGFRs utilise the G_αi-coupled receptors to regulate c-Src. This was suggested as addition of pertussis

toxin led to an approximate 40-50% decrease in the activation of c-Src and p42/p44 MAPK by PDGF. Das *et al.* (2001), presented further evidence demonstrating that pertussis toxin-sensitive G proteins are essential upstream signalling components of proliferation and activation of MAPK in response to hypoxia in PAs. These authors demonstrated that hypoxia-induced and serum-stimulated activation of p42/p44 MAPK and JNK, and increase in DNA synthesis were all markedly attenuated by pertussis toxin.

Activation of $G_{\alpha}i$ has also been suggested to modulate p42/p44 MAPK, through inhibition of adenylyl cyclase and subsequent decrease in cAMP and protein kinase A (PKA). PKA has been shown to phosphorylate Raf-1 kinase. Therefore increased PKA activity could prevent p42/p44 MAPK activation in certain cell types (Cook and McCormick, 1993). Additionally, $G_{\alpha}q/11$ have been shown to activate p42/p44 MAPK through the production of DAG and stimulation of calcium and the subsequent activation of PLCβ and protein kinase (PKC). For example, in the heart, p42/p44 MAPK is activated by GPCR agonists, such as α-adrenergic receptor agonists, AngII, and ET-1, leading to activation of the PLC cascade and ultimately the activation of PKC (Choukroun *et al*, 1998; Yamazaki *et al.*, 1999). PKC has been shown to phosphorylate and activate Raf-1 *in vitro* and in NIH3T3 cell clones (Kolch *et al.*, 1993, Widmann *et al.*, 1999). Furthermore, the activation of PKC has also been implicated in growth factor mediated proliferation of PASMC (Dempsey *et al.*, 1991).

Receptor internalisation is also thought to play a major role in the activation of MAPK via both RTKs and GPCRs. Cell receptor internalisation requires the fission of clathrin-coated vesicles from the plasma membrane into the cytosol, which is also known as endocytosis. Internalisation of receptors requires β-arrestins, which have been shown to serve as clathrin adaptors targeting agonist-occupied GPCRs to clathrin coated pits, and the GTPase activity of dynamin II (Daaka *et al.*, 1998). Binding of GTP, and the conversion of GTP to GDP leads to the assembly of dynamin II at the neck of clathrin coated pits, which is essential for vesicle budding (Damke *et al.*, 1994; Takei *et al.*, 1996). Dynamin II promotes endocytosis and pinching off of clathrin-coated endocytic vesicles, causing relocalisation of the receptor complex, bringing them closer to the machinery involved in activating the MAPK pathways. Activation of p42/p44 MAPK by GPCR agonists such as lysophosphatidic acid (LPA), and RTK agonists such as EGF, NGF, PDGF and IGF-1 is dependent on endocytosis and internalisation of the

receptor (Daaka *et al.*, 1998; Vieira *et al.*, 1996; Rakhit *et al.*, 2000, Alderton *et al.*, 2001a/b).

Substantial evidence exits for the activation of MAPKs in response to hypoxia. In endothelial cells it has been documented that hypoxia induces phosphorylation, nuclear translocation and activation p42/p44 MAPK (Minet *et al.*, 2000). In addition to the p42/p44 MAPK pathway mediating hypoxic responses, JNK and p38 pathways have been implicated in the cellular response to low oxygen (Seko *et a*l., 1997, Scott *et al.*, 1998, Jin *et al.*, 2000, Das *et al.*, 2001). Hypoxia has been shown to stimulate p42/p44 MAPK, JNK, and p38 MAPK in the PA from rat (Jin *et al.*, 2000). These authors showed an increase p42/p44 MAPK, JNK and p38 MAPK tyrosine phosphorylation and activities with hypoxia in the main and first branch PA from male Sprague-Dawley rats. JNK activation peaked at day 1, and p42/p44 MAPK and p38 MAPK activation peaked after 7 days of hypoxia. Furthermore, hypoxia stimulates both p38 MAPK and p42/p44 MAPK in fibroblasts derived from the PAs, but not from the aorta of CH (Welsh *et al.*, 2001). These authors suggested that the fibroblasts from the PA of CH appeared to have undergone a phenotypic switch, which causes them to exhibit enhanced proliferative responses compared to fibroblasts derived from the PA of normoxic rats.

Of particular interest, variable patterns of activation of ERK, JNK, and p38 MAPK in response to hypoxia have been documented depending on the cell type and the conditions under which the experiments are conducted. Studies such as those by Jin *et al.*, 2000, Das *et al.*, 2001, Scott *et al.*, 1998, all show the transient activation of p42/p44 MAPK, JNK, and p38 MAPK in response to hypoxia. For example, Scott *et al.* (1998), demonstrated that hypoxic stimulation of PA cells is mediated by activation of stress-activated protein kinases, with particular strong multi-phasic activation of the p38 MAPK pathway. These results may be due to cells responding and adapting to changes in oxygen concentrations.

1.2.4.4. The role of MAPK phosphatases in PHT

The duration and magnitude of MAPK can be regulated at different levels, in particular a major point of regulation occurs at the level of MAPK itself. MAPK phosphatases (MKPs) are known to dephosphorylate the threonine and tyrosine residues of MAPKs both *in vitro* and *in vivo*, thereby deactivating them (for review see Keyes, 1995, 2000; Haneda *et al.*, 1999). Currently 9 MKPs have been identified by PCR, or by searching

gene databases (Haneda *et al.*, 1999; Camps *et al.*, 1998). Multiple families exist to selectively dephosphorylate and inactivate particular MAPK isoforms. Some MKPs are tissue specific, however MKP-1 and MKP-2 are widely distributed and dephosphoylate p42/p44 MAPK, JNK, and p38 MAPK to various degrees (Chu *et al.*, 1996).

MKPs can be induced by environmental stimuli, such as mitogens, heat shock and oxidative stress (reviewed by Keyse, 1995, 2000; Haneda *et al.*, 1999; Fanklin and Kaft, 1997; Hirsch and Stork, 1997). Evidence also exists suggesting MKPs are induced in response to hypoxic stimuli. Northern and Western blot analyses verified that MKP-1 mRNA and protein levels were dramatically up-regulated by hypoxia in PC12 cells (Seta *et al.*, 2001). Furthermore, Laderoute *et al.*, 1999 demonstrated that the transient increase in MAPK activity induced by hypoxia, is correlated with both the transcriptional activation of MKP-1, and the enhanced protein expression of MKP-1. MKP-1 has been suggested to be a hypoxic response gene. The promoter region for the human MPK-1 gene contains cis-acting elements for AP-1 and CREB transcription factors, both of which are targets for JNKs and p38, and both shown to be increased with hypoxia (Kwak *et al.*, 1994; Kyriakis, 1994).

These results suggest that increased MKPs may explain the transient increase in MAPK activation induced by hypoxia even though the stimulus was not removed. Consistent with this theory, MKPs are induced in response to a robust stimulation of MAPK, suggesting they participate in the negative feedback control of MAPK activity (Alessi *et al.*, 1993; Sun *et al.*, 1993; Ward *et al.*, 1994). These authors suggested that the upregulation of MKP may have a protective role in hypoxic cells, preventing apoptosis that is known to occur in response to prolonged MAPK activation. MKP may in fact therefore contribute to the net increased growth and remodelling of PA in response to hypoxia.

1.2.4.5 The role of transcription factors in PHT

Transcription factors are the main targets for MAPK (reviewed by Faller, 1999; Angel and Karin, 1991). Sustained phosphorylation of specific Thr-X-Tyt motifs, activates MAPK allowing it translocate to the nucleus where they catalyses the phosphorylation of various transcription factors. In the nucleus the transcription factors bind to the regulatory domains of their target genes and activate/prevent transcription. Transcription factors activated by members of the MAPK family include; the early

growth response-1 transcription factor (Egr-1), the hypoxia-inducible transcription factor-1 (HIF-1), nuclear factor-κB (NF-κB), and the transcription factor Elk-1. These transcription factors in turn control the activation of genes encoding growth factors and other mediators (reviewed by, see Faller, 1999). For example, Elk-1 regulates members of the transcription activator protein-1 (AP-1) family. AP-1 is composed of the *Fos* family (*c-Fos, Fos-B, Fra-1* and *Fra-2*) and the *Jun* family (*c-Jun, JunB,* and *JunD*). MAPK has been shown to regulate AP-1 dependent transcription directly by both the *de-novo* synthesis *Jun* and *Fos* and/or by controlling their transactivation function (Karin *et al.*, 1997).

The cellular response to hypoxia appears to involve the activation of a number of transcription factors, perhaps as a molecular mechanism for adaptation (reviewed by Faller, 1999). Egr-1 is upregulated by hypoxia through the PKCα-mediated activation of the Ras/Raf/MAPK cascade in cultured monocytes and bovine aortic endothelial cells (Yan *et al.*, 1999; Lo *et al.*, 2001). In fact, *c-fos* gene expression is known to be "turned on" by hypoxia via the p42/p44 MAPK pathway (Muller *et al.*, 1997). Additionally, PDGF, via MAPK activation, induces the expression of a variety of immediate-early genes involved in increasing DNA synthesis, including *c-fos*, *c-jun*, and *jun-B* (Rothman *et al.*, 1994). Both PDGF-A and -B have been shown to be involved in hypoxic pulmonary remodelling (Katayose *et al.*, 1993). Therefore, the PDGF-dependent upregulation of transcription may be important in smooth muscle hypertrophy in the pulmonary circulation.

Central to the hypoxic induced response of the pulmonary circulation also appears to be the induction of the hypoxic response element, known as hypoxic-inducible factor-1 (HIF-1, Faller 1999, Richard *et al.*, 1999). HIF-1 is a heterodimeric transcription factor, which is stabilised post-translationally in response to hypoxia. The stabilised HIF-1 binds to the hypoxic response element (HRE) in the promoter of numerous genes upregulating their expression. Both HIF-1 mRNA and protein are rapidly induced by hypoxia in a variety of cell types, including pulmonary artery endothelial cells (Palmer *et al.*, 1998). These authors demonstrated that the induction of HIF-1 is required for the hypoxic induction of type II nitric oxide synthase (NOS) gene expression. Furthermore, it was shown mutation or deletion of HIF-1 binding sites abolished the hypoxic induction of type II NOS.

The p42/p44 MAPK pathway is known to phosphorylate the HIF-1α carboxyl-terminal domain and enhance the transcriptional activity of HIF-1 (Richard *et al.*, 1999). In turn HIF-1 induces the expression of various genes encoding VEGF, PDGF, 5-HT and ET-1, and erythropoietin (reviewed by Minet *et al.*, 2001; Semenza, 1996a; Lee *et al.*, 1997). Therefore, secretion of VEGF could be involved in an auto regulatory feedback loop through MAPK activation and HIF-1 phosphorylation (Minet *et al.*, 2001). It has been suggested that the upregulation of growth factors by HIF-1 occurs to stimulate the growth of new capillaries and red blood cell production, improving local oxygen delivery, and the oxygen carrying capacity of erythropoietin (Semenza, 1996b).

1.2.4.6 Role of NF-κB in PHT

Another transcription factor that is activated in response to inflammatory cytokines, mitogens, viral proteins and stress, is nuclear factor-kappaB (NF-κB). PKA, Caesin kinase II and MAPK have all been implicated in the phoshorylation of NF-κB (Makarvo, 2000; Shulze-Osthoff, 1997; Sibenlist *et al.*, 1994; Thanos and Maniatis, 1995). NF-κB is the collective name for members of the Rel family of ubiquitous, dimeric transcription factors (reviewed by Makarov, 2000, Baldwin, 1996; Ghosh *et al.*, 1998). Members of the Rel family include RelA (p65), RelB, c-Rel, NF-κB1 (p50/105) and NF-κB2 (p52/100), which can exist as homo- and heterodimers. Each Rel protein contains a Rel homology domain (RHD), which allows dimer formation, nuclear translocation, sequence-specific DNA recognition and interaction with IκB proteins. In mammals, the most commonly inducible form of NF-κB is p50/p65.

In unstimulated cells NF-κB is found inactive in the cytoplasm bound with the inhibitory proteins IκB. Exposure to stimuli results in phosphorylation of Ser 32 and Ser 36 of IκBα, ubiquitination, and the subsequent degradation of IκBα by the 26S proteasome. The enzymes controlling these processes are, IκB kinase (IKK), IκB ubiquitin ligase, and 26S proteasome respectively (reviewed by Karin, 2000; Li *et al.*, 1998; O'Connell *et al.*, 1998). The degradation of IκB results in the translocation of NF-κB to the nucleus where it can activate the transcription of a number of genes. In fact, more than 150 NF-κB responsive genes have been identified, including genes that encode for cytokines, mitogens, growth factors and adhesion molecules, (reviewed by Pahl, 1999; Sibenlist *et al.*, 1994; Thanos and Maniatis, 1995; Makarov, 2000, Baldwin,

1996; Ghosh *et al.*, 1998). Genes induced in response to stress or inflammation in the pulmonary circulation, such as TNFα, IL-1β, IL-6, IL-8, inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2), all contain NF-κB binding sites in their promoter region (Faller 1999; Pahl, 1999; Sibenlist *et al.*, 1994; Kim *et al.*, 1998).

Evidence exists for a possible role of NF-κB in PHT. Aziz *et al.* (1997), demonstrated that NF-κB activation could be induced by oxidative stress. Importantly, NF-κB has also been shown to modulate proliferation, branching, and morphogenesis in lung epithelium, therefore inhibition of NF-κB may reduce the remodelling seen with PHT (Muraoka *et al.*, 2000). In addition iNOS, which has a 5' flanking region containing a consensus sequence that binds to NF-κB, is unregulated by hypoxia (Xie *et al.*, 1993). Increased *de novo* expression of iNOS mRNA and protein expression has been noted in whole lung extracts and in large and small PA from rats and mice with chronic hypoxia induced PHT (Carville *et al.*, 1997; Le Cras 1996; Palmer *et al.*, 1998; Xue *et al.*, 1996; Kinnula *et al.*, 1995). The transcription of COX-2, which can also be regulated by NF-κB, has been reported to be increased more than 3-fold in isolated perfused lungs exposed to hypoxia (Childa and Voelkel, 1998). Together this data implicates NF-κB in the response of the pulmonary circulation to hypoxia, and possibly in the remodelling seen with PHT.

Anti-inflammatory drugs such as antioxidants, non-steriod anti-inflammatory drugs (NSAIDs), and immunosuppressants can all inhibit NF-κB (Epinat and Gilmore, 1999; Wissink *et al.*, 1998). The most commonly used NF-κB inhibitors are glucocorticoids, which are thought to act by either increasing the expression of IκB, or by inhibiting the transactivation of the NF-κB Rel A subunit (Karin, 1998; Markarov, 2000). These drugs however only inhibit NF-κB in a non-specific manner, acting on many other inflammatory mediators. More specific NF-κB inhibitors have been designed. For example, a peptide inhibitor capable of penetrating the nucleus and inhibiting NF-κB localisation has been described (Lin *et al.*, 1995). However, knockout animals have shown that altering NF-κB directly impaired or even prevented the development of normal immune function. Loss of NF-κB1, NF-κB2, c-Rel and RelB in knockout mice all caused defects in the activation of T and B cells, and decreased the immune response to pathogens (Gerondakis *et al.*, 1999). Due to the wide role of NF-κB in cell

regulation throughout the body, adverse side effects may limit its use as a therapeutic target in PHT.

1.2.4.7 The role of calcium and potassium channels in PHT

The ion channels known to play pivotal roles in determining pulmonary vascular tone are calcium (Ca^{2+}) and potassium (K^{+}), although a small amount of evidence does exist for a role of chloride, or sodium (reviewed by Reeve *et al.*, 1997). Importantly chronic hypoxia alters the membrane potential of rat PASMC from main and small PAs (Suzuki and Twarog *et al.*, 1982). Activation of Ca^{2+} gated channels can result in membrane depolarisation and the subsequent contraction of SMC. A number of intracellular pathways act be elevating intracellular calcium. For example, an increase in intracellular calcium can be mediated by GPCR agonists, such as ET-1 and 5-HT through stimulation of IP_3. In smooth muscle Ca^{2+} binds to calmodulin and activates calmodulin-dependent myosin light chain kinase, which catalyses the phosphorylation of myosin, allowing binding to actin, leading to contraction. Hypoxia has been shown to cause calcium influx through L-type voltage gated calcium channels (Premkumar *et al.*, 2000). The increase in intracellular calcium that occurs during hypoxia may suggest a number of calcium–dependent protein kinases and phosphatases are regulated including CAM-Kinase (Kilbourne *et al.*, 1992; Sheng *et al.*, 1990). An elevation in intracellular calcium may also have a possible role in cellular proliferation, as evidence suggests that Ca^{2+} controls the activation of PKC and MAPK. PKC activation has been shown to cause proliferation and mediate some of the effects of specific growth factors such as PDGF in PASMC (Dempsey *et al.*, 1991, 1990). Additionally, Conrad and co-workers, demonstrated that inhibitors of the calcium-calmodulin pathway inhibited p42/p44 MAPK activation in hypoxia-treated PC12 cells (Conrad *et al.*, 1999). Increased calcium may therefore contribute to both increased tone and remodelling throughout the pulmonary circulation of patients with PHT.

Ca^{2+} channel antagonists have been shown to prevent DNA synthesis and proliferation in response to growth factors in both rat and human VSMC (Kataoka *et al.*, 1997). The L- and T-type Ca^{2+} channel antagonist tetrandine (IC_{50}, 10-30μM) exerts an antiprolferative effect against a range of mitogenic stimuli such as PDGF and IL-1α in rat PASMC *in vitro* (Wang *et al.*, 2000). Hypoxia-induced pulmonary vasoconstriction is also attenuated by the calcium channel blocker verapamil, and enhanced by calcium

channel openers such as, BAY K 8644 (McMurtry *et al.*, 1980). Blocking of Ca^{2+} channels leads to hyperpolarisation and vasodilation. High doses of calcium antagonists, such as nifedipine (30-120mg per day) and diltiazem (120-720mg per day) are both successful treatments for PHT (Rich and Kaufmann, 1991). Inhibition of calcium channels by nifedipine has been shown to decrease PAP by more than 20% in two thirds of individuals with high PAP (Antezana *et al.*, 1998). Major adverse effects of calcium channel blockers are decreased cardiac output due to negative inotropic effects, reduced systemic blood pressure and salt and water retention (Rich and Kaufmann, 1991).

K^+ channels are present on airway smooth muscle and their activation results in hyperpolarisation and relaxation. Several types of K^+ channels have been classified in the pulmonary circulation according to their pharmacological, kinetic, and physiological characteristics; voltage-gated (Kv), inward rectifying (Kir), and calcium sensitive (Kca) channels (reviewed by Reeve *et al.*, 1997). Patch clamp studies suggest that K^+ channels are differently distributed throughout the pulmonary arterial tree. K^+ channels are tonically active, which allows a slow efflux of K^+ along their intracellular/extracellular concentration gradient. Inhibition of Kv channels result in accumulation of positively charged potassium ions, raising membrane potential, activating voltage gated calcium channels, increasing cytosolic calcium, and resulting in vasoconstriction.

Evidence suggests that the activity of voltage-sensitive K^+ channels may have a role in determining pulmonary vascular tone in PHT. In fact, the increase in tone seen with PHT has been suggested to be due to previously observed inactivation of K^+ channels (Ospenko *et al.*, 1998; McCulloch *et al.*, 1999). Studies have shown K^+ channels are inactivated in hypoxia, and dysfunctional in PASMC of patients with PPHT (Osipenko *et al.*, 1998; Weir *et al.*, 1998; Yuan *et al.*, 1998; Post *et al.*, 1992). Hypoxia induced pulmonary vasoconstriction has been shown to involve the inhibition of the voltage operated Kv channels in PASMC (Weir *et al.*, 1995; Post *et al.*, 1992). Exposure PASMC to hypoxia decreased K^+ through voltage gated K^+ channels (KV), decreased membrane potential, and increased intracellular Ca^{2+} and myosin light chain phosphorylation, all causing contraction. In patients with PPHT Kv (type 1.5) mRNA is reduced in PASMCs, which was associated with inhibition of potassium current, membrane depolarisation, and elevation in calcium channel activity (Yuan *et al.*, 1998).

Kca channels have also been shown to be associated with contraction, and play a role in regulating pulmonary vascular tone, as they are inhibited in response to hypoxia (Peng *et al.*, 1996; Post *et al*, 1992).

Potassium channel opening drugs are effective pulmonary vasodilators both *in vitro* and *in vivo* in a variety of animal species (reviewed by Weir *et al.*, 1998). The hypoxic decrease in outward potassium current and increase in inward calcium current, has been shown by patch clamping in cultured VSMC from PAs, to be blocked by the K+ channel opener cromakalin and enhanced by the potassium channel blocker glibenclamide (Yuan *et al.*, 1992). The pulmonary vasorelaxant effects of potassium channel opening drugs are dependent on the vasoconstrictor/spasmogen used to contract the preparations and are enhanced in preparations with PHT (Wanstall, 1996)

1.2.5 The role of cyclic nucleotide dependent pathways in PHT

1.2.5 1 The role of nitric oxide in PHT

Pulmonary blood flow is influenced by several local factors, which act via cyclic nucleotide dependent pathways (see figure 1.4), such as eicosanoids, and the endothelium-derived relaxation factor (nitric oxide). Nitric oxide (NO) is a well-characterised endogenous endothelium-dependent vasodilator, which is known to have a role in the regulation of pulmonary vascular tone (reviewed by see Hampl and Herget, 2000). NO is synthesised in endothelial cells during the conversion of the semi-essential amino acid L-arginine to L-citrulline, catalysed by nitric oxide synthase (NOS). There are at least three known isoforms of NOS; neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Endothelial NOS is expressed constitutively, although can be also modulated by factors such as shear stress and changes in intracellular Ca^{2+} (Barnes and Belvisi, 1993). The abundant expression of eNOS has been reported in the main PAs, whereas it is virtually absent from the small PAs (Xue *et al.*, 1994). Inducible NOS tends to be generated by proflammatory cytokines, for example, during airway inflammation and is regulated at the transcriptional level. Inducible NOS is expressed in many different cell types, and produces high levels of NO. Studies have shown that the basal formation of NO is due to the presence of the inducible Ca^{2+}/calmodulin-independent NOS (iNOS) isoform in VSMCs (Busse and Mulsch, 1990, Beasley *et al.*, 1991; Nunokawa *et al.*, 1993). Neuronal NOS is constitutively expressed associated with central and peripheral neurons. In addition, nNOS has also

been shown to be present in epithelial and VSMCs, including PASMC (Sherman *et al.*, 1999). Altered production of NO may be responsible for the development of a variety of diverse pathological events in mammalian organs.

Most of the biological effects of NO are mediated through its receptor protein, soluble guanylyl cyclase, which catalyses the conversion of guanosine triphosphate (GTP) into cyclic-guanosine monophosphate (cGMP) in smooth muscle cells. Increasing cGMP can, in turn, regulate the activation of protein kinase G (PKG). PKG reduces the intracellular Ca^{2+} concentration by inhibiting voltage and receptor operated calcium channels. Therefore NO prevents release of Ca^{2+} from the sarcoplasmic reticulum, resulting in the uncoupling of the contractile apparatus (Rembold, 1991). Additionally, PKG activation can also lead to PKG-dependent hyperpolarisation of the membrane by activating K^+ channels. In addition, cGMP reduces vascular tone and causes smooth muscle relaxation by activating myosin light chain phosphatase (Rembold, 1991). It has also been suggested that NO can regulate pulmonary vasodilation by direct activation of K^+ channels or by modulating expression and activity of angiotensin II receptors (reviewed by Weinberger *et al.*, 1999).

As the NO/cGMP pathway appears to play a key role in maintaining low vascular tone in the pulmonary circulation it is possible that altered NO production may occur with the development of PHT. However, this is still relatively controversial and it has not been resolved as to whether NO production or NOS expression is increased or decreased in the pulmonary circulation under hypoxic conditions. Discrepancies may be due to experimental models used, differences in NOS cofactor availability, natural history of PHT, hypoxic modulation of NOS enzyme activity, or responsiveness of the pulmonary vasculature to NO. Evidence for a role of both increased or decreased NO in PHT will be outlined in the sections below.

Inadequate local NO production has been implicated as part of the disease process of PHT. Endothelial NO production in rat main PA is attenuated by acute and chronic hypoxia (Shaul *et al.*, 1993). A subsequent decrease in [cGMP]i occurs in the main PA from CH, which is assumed to be the result of decreased endothelial NO production (Shaul *et al.*, 1993; MacLean *et al.*, 1996). These results in conjunction with the known loss of endothelial-dependent relaxation suggest a low availability of bioactive NO in CH rats. Likewise, reduced levels of NOS have also been shown in the pulmonary

vasculature of patients with PPHT (Giaid and Saleh, 1995). Additionally, PAs from patients with hypoxic lung disease have impaired release of endothelial NO on stimulation (Dinh-Xuan *et al.*, 1991). These studies suggest NO therapy might be beneficial in the treatment of PHT.

In contrast, a number of groups have demonstrated that lung NO production is enhanced with PHT (Archer *et al.*, 1998; Forrest *et al.*, 1999). In the PAs of CH, NOS mRNA and protein have both been demonstrated to be upregulated, suggesting an increase in endothelial NO release (Le Cras *et al.*, 1996, Resta *et al.*, 1997). *De novo* mRNA/protein expression of iNOS has also been demonstrated in PA CH (Carville *et al.*, 1997). For example, pulmonary NO production has been shown to be increased from the lungs of CH, and an increase in the *de-novo* expression and activity of NOS has been observed in the large PA from CH (Isaacson *et al.*, 1994, Xue *et al.*, 1994; Le Cras *et al.*, 1996, Resta *et al.*, 1997). Likewise, Shaul and co-workers reported NOS activity in whole lung homogenates, measured as [^{3}H]-arginine to [^{3}H]-citrulline conversion, doubled in rats with CH induced PHT compared with normoxic (Shaul *et al.*, 1995). Increased NOS appeared to correlate with the onset of increased muscularity in the small resistance PAs (Xue *et al.*, 1996).

It has been suggested that the increased levels of NO observed with prolonged hypoxia may be toxic to the pulmonary circulation. High concentrations of NO react with reactive oxygen species (ROS) i.e. superoxide, to form cytotoxic substances such as peroxynitrite. ROS are produced in response to injury in the lung hence high concentrations of NO and ROS may contribute to the remodelling associated with PHT (Kinnula *et al.*, 1995). Additionally, prolonged exposure to NO is known to downregulate PKG (Soff *et al.*, 1997). PKG is known to be involved in converting vascular smooth muscle from a dedifferentiated "synthetic" phenotype to a more contractile-like morphology (Boerth *et al.*, 1997). Therefore, NO-induced downregulation of PKG would promote the synthetic phenotype, which is a known feature of the vascular wall remodelling of pulmonary hypertension (Boerth *et al.*, 1997). The effect of NO in the pulmonary circulation may therefore be dependent on the levels of PKG. Taken together all these studies suggest the initial response of pulmonary circulation to hypoxia may be to increase NO, possibly in an attempt to restore normal tone. However prolonged hypoxic insult may increase NO production to a level at which the adverse effects of NO are dominant.

Despite the controversial role of NO it has still been possible to use it in the treatment of PHT. NO has been shown to attenuate ET-1 induced pulmonary arterial vasoconstriction (Lang and Lewis, 1991). Additionally, gene transfer studies have demonstrated that increased eNOS in the lung *in vivo* selectively reduces the increase in pulmonary vascular resistance in response to both ET-1, and hypoxia (Bivalacque *et al.*, 1999). Horstman and co-workers also demonstrated low doses of NO can attenuate the remodelling seen with PHT in both newborn and adult rats (Horstman *et al.*, 1998). Inhaled NO has in fact been shown to be successful as a therapy for some types of PHT, such as those that are short term and reversible i.e. heart/lung perioperative PHT (Frostell *et al.*, 1991; Clark *et al.*, 2000). Although inhaled NO improves oxygenation, long-term benefits are not seen due to its short action, and its potential to be a pulmonary irritant (Weinberger *et al.*, 2001; Troncy *et al*, 1997; Zapol *et al.*, 1994).

1.2.5.2 The role of prostacyclin in PHT

Arachodonic acid is metabolised through the cycloxygenase and lypoxygenase pathways to form prostaglandins and leukotrienes. Metabolites of arachodonic acid such as prostacyclin (PGI_2) and thromboxane (TxA_2) have been reported to exhibit vasoactive effects in the pulmonary circulation (reviewed by Christman, 1998; Terragno and Terragno, 1979). In endothelial cells PGI_2 synthase predominates and directs metabolism toward prostacyclin. Binding of PGI_2 in nanomolar concentrations to prostacyclin receptors causes elevation of intracellular cyclic adenosine monophosphate (cAMP) in smooth muscle by activating adenylyl cyclases (see figure 1.4). PGI_2 has been shown to be a very potent vasodilator in the pulmonary arterial bed (Shaul *et al.*, 1991). The vasodilatory properties of PGI_2 are well established and PGI_2 has been shown to decreases DNA synthesis in vascular smooth muscle cells (Shirotani *et al.*, 1991). Prostacylin is known to reduce PVR and attenuate vascular smooth muscle proliferation through signal transduction via ligand binding to its receptor. Thromboxane on the other hand is a potent vasoconstrictor, which has also be reported to act as a growth factor by activating PKC, suggesting a role regulating proliferation (Ko *et al.*, 1997). Abnormal eicosanoid production would therefore alter pulmonary vascular tone.

It has been suggested that endothelial dysfunction associated with PHT may alter eicosanoid synthesis. However, in common with NO, workers have reported PGI_2

levels to be both increased and decreased in response to hypoxia. Martin *et al.* (1992), measured eicosaniod production in cultured bovine pulmonary endothelial cells during constant flow and pressure perfusion at two oxygen tensions (Hypoxic 4% O_2, 5% CO_2, 91% N_2: normoxia 21% O_2, 5% CO_2, 74% N_2). These authors found production of the stable metabolite of prostacyclin, 6-keto PGF1α, was increased during hypoxia (normoxia 291 ±-27 vs. hypoxia 395 ± -35 ng/min/gm protein), whereas thromboxane and total leukotriene production did not change. Likewise, Peterson and co-workers found PGF1α was increased with the onset of PHT from 31 ± 3 to 842 ± 367 pg/ml (Peterson *et al.*, 1982). Additionally, PGI_2 has been shown to be increased 2.7 fold after 7 days of hypoxia (Shaul *et al.*, 1991). ET-1, of which levels are increased with PHT, can induce the production of prostacyclin in rat lung (Barnard *et al.*, 1991). Vasodilatory prostaglandins have been shown to promote rather than inhibit bovine PASMC proliferation (Pasricha *et al.*, 1992). These studies suggest in response to hypoxia the pulmonary circulation increases PGI_2, possibly in an attempt to restore normal tone.

In contrast, the synthesis of PGI_2 was significantly decreased in the distal PAs of neonatal calves exposed to hypoxia (Badesch *et al.*, 1989). Patients with severe PHT have reduced PGI_2 and PGI_2 receptor expression, which has been suggested to be involved in the remodelling of the PAs (Hoshikawa *et al.*, 2001; Tuder *et al.*, 1999). Transgenic mice over-expressing PGI_2 synthase were found to be protected from pulmonary smooth muscle hypertrophy and PHT following exposure to chronic hypoxia (Geraci *et al.*, 1999). PGI_2 and its analogues inhibited DNA synthesis and cell proliferation in distal human PASMCs, whereas proximal human PASMCs were comparatively unresponsive (Wharton *et al.*, 2000). These authors suggested regional heterogeneity and variation in receptor expression might contribute to the contrasting actions of PGI_2. Together the findings outlined in this section would suggest that PGI_2 would be a good candidate for therapeutic use in PHT .

Long term intravenous infusion of PGI_2 or its stable analogues, have been a major advance in the therapy of PHT, prolonging survival and delaying the need for lung transplantation. In rabbits with experimental PHT, evoked by the continuous infusion of the stable thromboxane mimetic U45519, nebulized prostacylin (56ng/kg.min) reduced the increase in PAP by approximately 30% (Schermuly *et al.*, 1999). Additionally PGI_2 and its stable analogues, such as iloprost, have been shown to inhibit

DNA synthesis and smooth muscle proliferation in the distal PAs through the stimulation of cAMP (Wharton *et al.*, 2000). Long-term continuous intravenous PGI_2 improves oxygenation and reduces the PAP in PHT patients (McLaughlin *et al.*, 1998; Barst *et al.*, 1996). Although producing both beneficial acute effects and long-term hemodynamic responses, the dosage of PGI_2 required to sustain these effects increases with time as tolerance develops (Barst *et al.*, 1996).

1.2.5.3 The role of cyclic nucleotides in PHT

Many of the vasoactive compounds outlined above exert all or some their biological actions by directly or indirectly regulating cyclic nucleotides (see figure 1.4). Cyclic nucleotides, adenosine 3', 5' cyclic monophosphate (cAMP) and guanosine 3', 5' cyclic monophosphate, are key second messengers involved in a multitude of cellular events including determining vascular tone and reactivity (reviewed by Schwede *et al.*, 2000; Schmidt *et al.*, 1993; Koyama *et al.*, 2001). Adenylyl cyclase (AC) and guanylyl cyclase (GC) catalyse the formation of cAMP and cGMP respectively, whereas specific families of phosphodiesterases (PDE) catalyse their hydrolysis. The main target for cAMP is protein kinase A (PKA). However, cAMP can also act directly on cyclic nucleotide gated channels, cAMP regulated guanine nucleotide exchange factors (cAMP-GEFs), phosphodiesterases (PDEs) or EPACs (Schwede *et al.*, 2000; Kwasaski *et al.*, 1998). In parallel, cGMP has been shown to regulate protein kinase G (PKG), cGMP-gated channels, and PDEs (De Rooij *et al.*, 1998; Kwasaki *et al.*, 1998). Stimulation of PKA and PKG each results in the phosphorylation of a number of downstream targets, regulating cellular processes and gene expression (Francis and Corbin 1994). The activation of both cyclic nucleotides also appears to be dependent on compartmentalisation of the signals, and feedback mechanisms that are present at many points of the pathways (Scott *et al.*, 2000: Conti *et al.*, 1995). In general, stimulation of cAMP and/or cGMP would lead to wide spread relaxation of smooth muscle, while conversely inhibition would lead to the contraction of smooth muscle. Indeed, both cAMP and cGMP have been shown to have key roles in the control of pulmonary vascular tone (Murray, 1990; Della Frazia *et al.*, 1997; Koyama *et al.*, 2001).

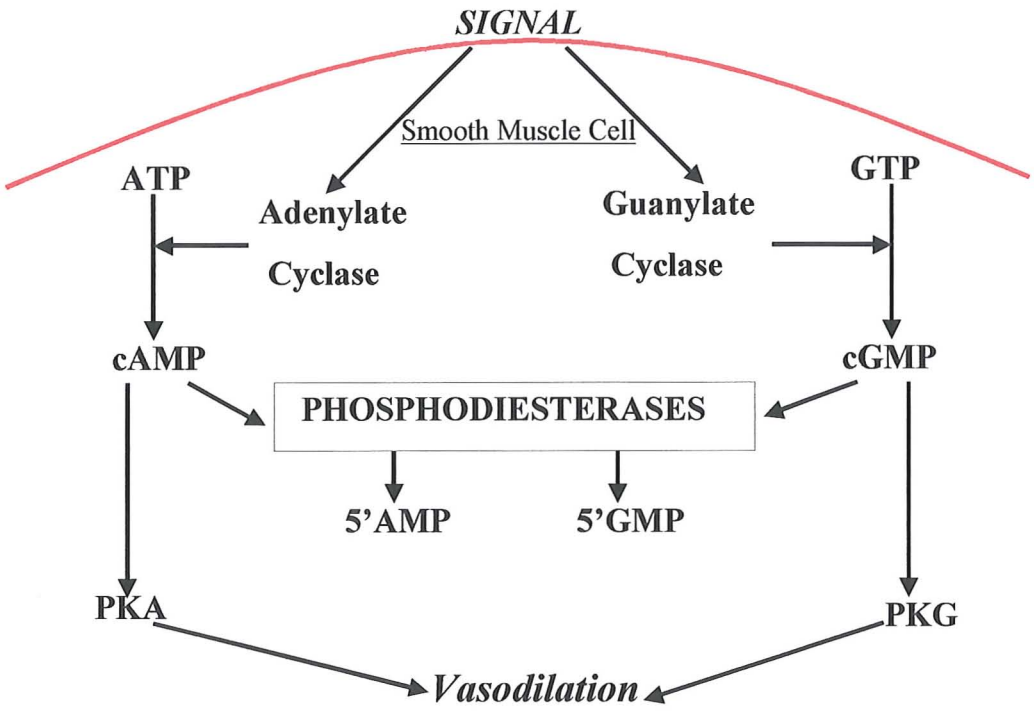


Figure 1.4 Schematic representation of cyclic nucleotide dependent pathways

Adenylyl cyclase (AC) and guanylyl cyclase (GC) catalyse the formation of cAMP and cGMP in response to stimuli such as prostacyclin and nitric oxide. In contrast, specific families of phosphodiesterases (PDE) catalyse their hydrolysis to their corresponding 5'nucleotide metabolite. The balance of the cyclases and the phosphodiesterases regulate cyclic nucleotide levels. The main targets for cAMP and cGMP are protein kinase A (PKA) and protein kinase G (PKG) respectively. Stimulation of the protein kinases results in the phosphorylation of a number of downstream targets, regulating cellular processes and gene expression, which can down regulate the contractile apparatus and cause vasodilation of the smooth muscle. Figure adapted from Manganiello *et al.* (1995).

An increase in either cGMP and/or cAMP can inhibit VSMC proliferation (reviewed by Southgate and Newby, 1990; Cornwell *et al.*, 1994; and Grosser *et al.*, 1995). The cAMP membrane permeable analogue, 8Br-cAMP, reduced the percentage of cells in the S phase of the cell cycle after serum stimulation, by preventing progression of the cell cycle from G0/G1 (Kronemann *et al.*, 1999; Hamad *et al.*, 1999b). It has been suggested that cAMP, via PKA activation, attenuates proliferation by antagonising mitogenic pathways in SMCs (Graves *et al.*, 1993; Bornfeldt and Krebs, 1999; Bonisch *et al.*, 1998; Zucker *et al.*, 1998). PKA is known to phosphorylate Raf-1 on serine 43 and serine 621 thereby inhibiting p42/p44 MAPK activation (Wu *et al.*, 1993; Hafner *et al.*, 1994). Additionally, the inhibition of the PI3K pathway by cAMP may be responsible for its antiproliferative action (Graves *et al.*, 1995).

It has also been shown that cGMP attenuates cellular proliferation. However, cGMP is apparently not as effective in controlling proliferation as cAMP (Kariya *et al.*, 1989; Southgate and Newby, 1990; Cornwell *et al.*, 1994; Yu *et al.*, 1997; Garg and Hassid, 1989). It has been suggested that cGMP, unlike cAMP, does not block but delays the G1/S transition in SMCs (Fukumoto *et al.*, 1999). cGMP via the activation of PKG and inhibition of Raf-1 has also been shown to reduce the activation of p42/p44 MAPK (Yu *et al.*, 1997). PKG has been reported to inhibit the proliferation of PASMC (Chiche *et al.*, 1998). Since PKG is known to be downregulated with the subculture of SMC, evidence exists that high concentrations of cGMP may activate PKA to inhibit cellular proliferation (Cornwell *et al.*, 1994; Cornwell and Lincoln, 1989). Natriuretic peptides, which regulate cGMP production, have been shown to be upregulated with PHT. Levels of natriuretic peptides, which bind to and activate the particulate transmembrane receptors that contain intracellular guanylyl cyclase domains, are increased in PHT (Zhao *et al.*, 1999). Furthermore, the atrial natriuretic peptide (ANP, a member of the natriuretic peptide family) is also increased in both human primary and secondary PHT (Morice *et al.*, 1990).

The abnormal tone and remodelling seen in all forms of PHT may therefore be due to defects in pulmonary vasodilatory pathways that regulate cyclic nucleotide concentrations. In the CH decreased intracellular concentrations of both cGMP and cAMP have been reported in the main, first branch and intrapulmonary arteries, however not in the resistance vessels (MacLean *et al.*, 1996). Likewise, Shaul *et al.* (1993), also demonstrated that chronic hypoxia in the rat resulted in a decrease in

[cGMP]i in the main PA. Variations in the phenotype of vascular smooth muscle cells in the pulmonary resistance vessels compared with those in the larger elastic PAs may explain the differences in cyclic nucleotide levels between these branches (Meyrick and Reid, 1978; Sasaki *et al.*, 1995). In contrast, Cohen and co-workers, found a nine fold increase in cGMP from isolated lung perfusate from CH compared to controls (Cohen *et al.*, 1996). Variations in results may, in part, be due to the net effect of the time the tissue was harvested and how the tissue was handled. The latter study suggests that the normal response to PHT may be an initial increase in the production of cyclic nucleotides, possibly in an attempt to restore normal tone. However, desensitisation of the cyclic nucleotide pathways may occur after prolonged exposure to hypoxia, which would explain the increased vasoconstriction seen with PHT. The response to the cyclic nucleotides appears to depend on the strength and duration of the signal. The increase tone in the pulmonary circulation in response to hypoxia may be due to decreased production of cyclic nucleotides, increased hydrolysis of cyclic nucleotides, or impaired signal transduction distal to cyclic nucleotide formation and hydrolysis.

Despite the controversy, many of the current treatments outlined for PHT act by increasing cGMP and cAMP levels. Major mediators of cGMP/cAMP production such as NO and PGI_2, as outlined in the sections above, are used to control PHT. Subcutaneous injections of 90mg/kg SCH 34826 twice a day, which elevates ANP and therefore cGMP, significantly prevented hypoxia induced pulmonary vascular remodelling and right ventricular hypertrophy (Stewart *et al.*, 1992). In addition, cAMP analogues have been shown to prevent the development of PHT and relax pulmonary vessels (Farrukh *et al.*, 1987). Stimulation with forskolin or a cAMP analogue inhibits proliferation induced by PDGF-BB and serum in distal human PASMC (Wharton *et al.*, 2000).

1.2.5.4. The role of the cAMP response element binding protein (CREB)

The cAMP pathway may provide the means of integrating a plethora of different signalling systems. In addition to increasing vasodilation and attenuating proliferation, cAMP can also potentially alter gene expression. cAMP modulates gene expression by the PKA mediated activation of the cAMP response element binding protein (CREB, reviewed by Shaywitz and Greenberg, 1999; Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989; Montminy, 1997; Roesler, 1999). CREB is a member of a large

family of transcription factors, which is phosphorylated on serine 133 by PKA to allow the transcriptional activation of genes with cis-regulatory cAMP-sensitive DNA elements (cAMP responsive elements, CREs). MAPK, Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), and PKC are also known to phosphorylate CREB at this particular residue. Moreover phosphorylation of CREB regulates expression of CRE containing genes such as *c-fos*, and somatostatin. CREB initiates gene expression that is known to persist long after the original stimulating cAMP has been degraded (Shywitz and Greenberg, 1999; Hai and Hartman, 2001, Mayr and Montminy, 2001). It has therefore been speculated that activation of CREB-mediated protein synthesis is a mechanism of transforming short-term effects of cAMP into long-term durable changes in the cell (Schwartz, 2001).

Hypoxia has been shown to initiate CREB serine phosphorylation, ubiquitination, and degradation (*in vitro* and *in vivo*). Mild hypoxia leads to the phosphorylation of CREB at the PKA phosphorylation site Ser133, resulting in the transcriptional activation of a number of genes (Beitner-Johnson and Millhorn, 1999). Taylor *et al.* (2000) demonstrated a time-dependent repression of protein phosphatase-1, a serine phosphatase important in CREB dephosphorylation and inactivation, from epithelial cells of rat exposed to hypoxia. Childa and Voelkel, 1996, proposed that the shear stress and the hypoxic induction of PGI_2 synthesis leads to the PKA activation of CREB via increased cAMP. These authors suggested the stimulation of CREB may be responsible for the increased COX-2 previously observed in the lung tissue from rats exposed to hypoxia. In addition the phosphorylation of CREB at Ser 133 has also been reported to be critical for growth factor induction of *c-fos* transcription (Ginty *et al*, 1994; Bonni *et al.*, 1995). Thus, these authors suggested that the growth factor mediated phosphorylation of CREB may supersede that mediated by PKA, thereby stimulating cell growth. CREB may therefore provide a possible further mechanism for hypoxic induced transcription. Further identification of CREB target genes would be important in understanding the possible molecular mechanisms involved in response to hypoxia of the pulmonary circulation.

1.2.5.5. The role of adenylyl cyclase and guanylyl cyclase in PHT

Intracellular levels of the cyclic nucleotides are determined by a balance of formation by the cyclases and degradation by the phosphodiesterases (PDEs). Adenylyl cyclase

(AC), and guanylyl cyclase (GC) catalyse the formation of cAMP and cGMP respectively. AC is activated by various extracellular stimuli mediated by receptors and their interaction with G-proteins. Adenylyl cyclases (AC), of which ten are currently identified, catalyse the formation of cAMP from adenosine triphosphate (ATP, reviewed by Hurley, 1998; Houslay and Milligan, 1997; Bently and Beavo, 1992). Each family of ACs have different tissue distributions and different glycosylation and regulatory properties (Deffer *et al.*, 2000). All isoforms are activated by the $G_{\alpha}s$ subunit of the heterotrimeric G protein, however specific isoforms are regulated by a variety of other protein regulators such as $G_{\alpha}i$, $G_{\beta\gamma}$, protein kinases (PKA, PKC and calmodulin kinase), phosphatases, calcium, and Ca^{2+}/CaM. The lung has been shown to express substantial levels of the two isoforms AC-6 and AC-8 (Hanoune *et al.*, 1997; Jourdan *et al.*, 2001). AC-6 is inhibited by low concentrations of calcium, PKA and by $G_{\alpha}i$, and $G_{\beta\gamma}$, allowing tight regulation of agonist-stimulated cAMP levels. In contrast, AC-8 activity is stimulated by calcium through calmodulin binding. Regulation of smooth muscle contraction, by AC-8 is not dependent on endogenous agonists but on intracellular calcium. These isoforms would be attractive targets in the treatment of PHT, as they may be limiting factors in cAMP production in the lung.

The formation of cGMP from guanosine triphosphate (GTP) is catalysed by guanylyl cyclases (GCs). Two families of GC exist, soluble (cytosolic) forms activated by NO donors, and particulate (membrane-bound) forms activated by natriuretic peptides (Hamad *et al.*, 1997; Lucas *et al.*, 2000; Bently and Beavo, 1992). Hormones, bacterial toxins, free radicals, calcium, and adenine nucleotides also regulate both families of GCs. Hamad *et al.*, 1999 demonstrated that both the soluble and particulate GCs are present in cultured hPASMC. Positive regulation of both GC and AC, would increase cAMP and cGMP, promoting smooth muscle relaxation.

The control of cyclic nucleotide levels in smooth muscle is critical in determining pulmonary vascular tone and reactivity, therefore changes in levels of either AC or GC may be responsible for the altered tone seen with PHT. In fact, soluble GC mRNA, protein, and enzyme activity have all been shown to be upregulated during the development of hypoxia-induced PHT in rats (Li *et al.*, 1999a). Additionally, Hamad *et al.* (1999b), demonstrated that the activation of soluble GC by NO donors, and the particulate GC by ANP both inhibit the proliferation of HASMCs in response to serum and thrombin. It has also suggested that desensitisation of guanylyl cyclase may occur

in response to an increase in PAP, due to the release of eNOS and the subsequent prolonged over production of NO (Wanstall *et al.*, 1992). However, Shaul *et al.* (1990), have shown that adenylyl cyclase activity is unaltered by hypoxia, even though there is an attenuated relaxation to forskolin. As adenylyl cyclase activity appears to be unaltered with PHT, the increased hydrolyses of cyclic nucleotide may explain the reduced relaxation to forskolin.

1.3 Phosphodiesterases

1.3.1 Introduction to phosphodiesterases

AC and GC catalyse the formation of cAMP and cGMP respectively, whereas phosphodiesterases (PDEs) catalyse the hydrolysis of these 3', 5'-cyclic nucleotides at their 3'-phosphoester bond, to form the corresponding 5'nucleotide metabolite (5'AMP and 5'GMP). There are presently eleven families of cyclic nucleotide phosphodiesterases with distinctive substrate specificities, regulatory characteristics, molecular sequences, tissue distribution and susceptibility to specific inhibitors (reviewed by Thompson, 1991; Beavo, 1995: Soderling *et al.*, 1998, 1999; Corbin and Francis, 1999; Fawcett *et al.*, 2000; Conti, 2000; Soderling and Beavo 2000; Yuasa *et al.*, 2000; Koyama *et al.*, 2001). In addition, most PDE families contain several distinct gene products with several different splice variants often expressed in a specific tissue, cellular or even subcellular manner. Some PDE families hydrolyse both cAMP and cGMP (PDE 1, 2, 3, 10, 11), others are highly specific for cAMP (PDE4, 7, 8), and some families are specific for the hydrolyse of cGMP (PDE5, 6, 9). Due to their central role in smooth muscle tone a variety of tissue specific PDE inhibitors are available. PDEs have already become an attractive target for drug development in a range of disorders such as asthma and thrombosis (reviewed by Thompson 1991).

The basic molecular structure of all PDEs is shown in figure 1.5. All mammalian PDEs are dimeric and each contains three functional domains, a conserved catalytic domain of ~270 amino acids, a regulatory N-terminus and a C-terminus. Protein-protein interaction domains, and phosphorylation domains exist at the N-terminus (i.e. for PKA, PKG, MAPK and CaMK), giving each family distinctive regulatory characteristics and allowing them to be subject to short-term allosteric action by endogenous activators or inhibitors. For example, a calmodulin binding domain is present in PDE1 allowing it to

be regulated by calcium concentrations, non-catayltic cGMP binding sites (GAF) are present in PDE2, 5, 6, 10, and 11, and phosphorylation site for various kinases are present in PDE1, 3, 4, 5, 6 and 7 (Conti *et al.*, 1995, see below for further details on specific PDEs). Within the catalytic domain there is ~30% amino acid identity between PDE families and ~80% identity between isoforms of the same family (Manganiello, 1995). The Zn^{2+} binding motifs located in the catalytic domain, which almost extends into the C-terminus of each enzyme, appears to be essential for the hydrolysis of the cyclic nucleotides (Corbin and Francis, 1999). The C-terminus has been suggested to contain domains to allow dimerisation.

The exact cellular and subcellular localisation of PDEs are thought to be important in the compartmentalisation of cAMP/cGMP signalling responses (Houslay and Milligan, 1997). It has been suggested that the targeting domains identified in most PDEs contribute to the association of isoforms to the membrane and subcellular compartments. For example, PDE3 contains a domain with six transmembrane helices that target the enzyme to the endoplasmic reticullum (Conti, 2000). The presence of two highly conserved regions, called upstream coding region 1 (UCR1) and UCR2, in the N-terminus of PDE4 are thought to be involved in targeting specific isoforms to the plasma membrane. The so called "short forms" of PDE4, such as PDE4D1 and 4D2, lack UCR1 and are therefore found solely in the cytosol. However, the "long forms" of PDE4, for example PDE4D3, PDE4D4, and PDE4D5, exhibit both UCR1 and UCR2 in the N-terminal domain, targeting them to both the cytosolic and particulate fractions (Bolger *et al.*, 1997). Compartmentalisation may provide a mechanism by which PDEs selectively alter specific cAMP/cGMP pools and regulate distinct signalling pathways. Chini *et al.*, 1997, showed PDE4 in mesangial cells regulates a cAMP pool that activates PKA involved in the inhibition of the production of reactive oxygen metabolites, while PDE3 regulates a cAMP pool that suppresses cell proliferation. Molecular cloning, biochemical and pharmacological analysis have allowed the understanding of PDEs to expand. Below summarises the major characteristics of the eleven currently known PDE families.

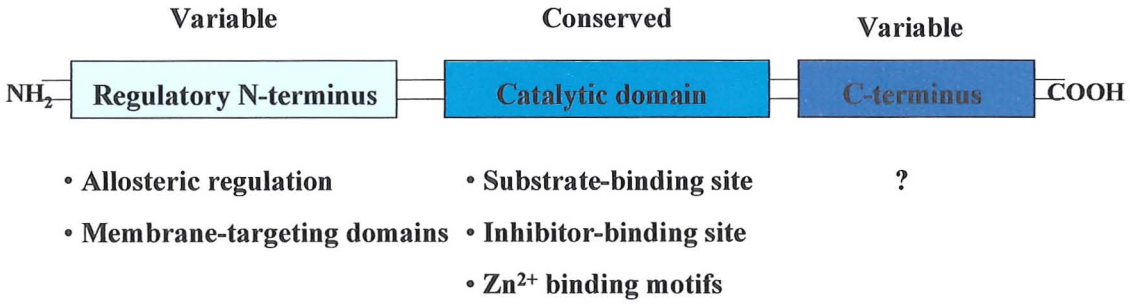


Figure 1.5 The basic molecular structure of PDEs

PDEs are known to possess a conserved catalytic domain proximal to the carboxyl terminus and regulatory domains or motifs near the amino terminus.

1.3.1.1 PDE1

Currently three genes encode PDE1 (*PDE1A, PDE1B*, and *PDE1C*), which is known to hydrolyse both cAMP and cGMP (Kakkar *et al.*, 1999). PDE1 has been found in high concentrations in the brain, lung, and heart. PDE1A and PDE1B hydrolyse cGMP more efficiently than cAMP, whereas PDE1C hydrolyses cAMP and cGMP with equal efficiency (Beavo, 1995). The *PDE1A* gene encodes the previously characterised 58kDa lung isoform (Sharma and Wang 1986). The kinetic constants for PDE1A (K_m) are for cAMP in the range of 34-40μM, and for cGMP in the range of 2-3μM (Beavo, 1995). The presence of calcium/calmodulin (Ca^{2+}/CaM) binding sites allows PDE1 to be activated and regulated by Ca^{2+} concentrations, hence it is often referred to as the Ca^{2+}/CaM-dependent PDE. Activation of PDE1 by calcium could therefore lead to an increase in cAMP/cGMP degradation, providing a mechanism for "cross-talk" between these second messengers. In addition, it has been demonstrated that CaM kinase II, PKC and PKA can also phosphorylate PDE1, thereby controlling its regulation by decreasing the affinity of the enzyme for CaM (Spence *et al.*, 1995; Sharma and Wang, 1986). Therefore, a cell can regulate the amplitude and duration of either cAMP or cGMP, depending on the affinity of the PDE1 isoform expressed for Ca^{2+}/CaM and on the phosphorylation state of the enzyme. Rybalkin *et al.*, 2002 have shown that PDE1C activity is required for maximal proliferation of human SMC. These authors showed that although PDE1C is absent in intact human aorta, it was readily detected in both cultured SMCs from the same donor, and in proliferating human SMCs in foetal aorta *in vivo*. It may be possible that the PDE1C is induced in the SMCs of the pulmonary

artery when they stimulated to proliferate in response to hypoxia. A commonly used potent PDE1 inhibitor is vinpocetine (Thompson, 1991, Kakkar *et al.*, 1999).

1.3.1.2. PDE2

PDE2 is known as the cGMP-stimulated PDE. The enzyme is expressed as at least three splice variants. PDE2 can be membrane bound or cytoplasmic, with both forms hydrolysing cAMP and cGMP with positive cooperative kinetics. cGMP is the preferred allosteric effector and substrate for PDE2 (Manganiello *et al.*, 1995). Upstream of the conserved catalytic domain in the N-terminal, PDE2, like PDE5, PDE6, PDE10, and PDE11 contains a further conserved region with two internal repeat sequences forming two homologous noncatalytic cGMP-binding sites. The cGMP-binding domain in PDE2, 5, and 6 share a conserved N(K/R)XnFX3DE motif (McAllister-Lucas *et al.*, 1994). The cGMP-binding domains in the N-terminal of all these PDEs, which are also conserved across a wide variety of proteins, are referred to as the GAF domain (cGMP binding and stimulated phosphodiesterases, *Anabaena* adenylyl cyclases and *Escherichia coli,* Aravind and Ponting, 1997). In the case of PDE2, cGMP binding to these non-catalytic binding domains increase the affinity of the catalytic site, increasing its activity under basal substrate conditions. For example, stimulation of PDE2 activity by increased cGMP would lead to a subsequent decrease in the normal levels of cAMP. Hence, PDE2, which is known to be abundant in the adrenal cortex and in areas of the brain, is a target for crosstalk between cAMP and cGMP. EHNA (erthro-9-[2-hydroxy-3-nonyl]adenine) has been reported to be a specific inhibitor for this cGMP-stimulated PDE (Michie *et al.,* 1996).

1.3.1.3. PDE3

PDE3 has a high affinity for both cAMP and cGMP, with K_m values of ~0.1-0.8μM (Manganiello *et al.*, 1995). However, binding of cGMP to its active sites is known to inhibit PDE3 activity, therefore preventing cAMP hydrolysis. For example, in human atrial SMC it has been demonstrated that nitrovasodilators through releasing NO and increasing cGMP concentrations, inhibit PDE3, and increase [cAMP]i (Kirstein *et al.*, 1995). Hence, PDE3 is known as the cGMP-inhibited, cAMP-specific PDE. Two genes, PDE3A and PDE3B, have been isolated from human tissue (Manganiello *et al.*, 1995). PDE3A and PDE3B have very similar kinetic and regulatory properties and

contain the same C-terminal domain (Soderling *et al.*, 1998; Doberman *et al.*, 1997). The hydrophobic N-terminus, made up of ~200 amino acids containing six transmembrane helices, are however different between the two isoforms and appear to be responsible for association with the membrane (Soderling *et al.*, 1998a; Kenan *et al.*, 2000). PDE3A has historically been thought of as the cardiovascular PDE3 (Meacci *et al.*, 1992), whereas PDE3B is often referred as adipocyte PDE3. Indeed PDE3B was originally cloned from rat adipocytes (Dergerman *et al.*, 1997, Taira *et al.*, 1993). Therefore, the differential tissue expression of PDE3A compared with PDE3B was initially thought to be one of the defining characteristics between the two sub-types (Reinhardt *et al.*, 1995). However, both PDE3A and PDE3B are expressed in rat aortic smooth muscle cells, and human aortic smooth muscle cells (Lui and Maurice, 1998, Palmer and Maurice, 2000).

PDE3 also contains a consensus sites for phosphorylation by PKA and PKB. Two phosphoylation sites on PDE3 have been identified using site directed mutagenesis, namely Ser302, and Ser273 (Degerman *et al.*, 1997). Adenylyl cyclases activators have been shown to stimulate PDE3 activity through an accumulation of cAMP and a subsequent increase in PKA (Gettys *et al.*, 1987). PKA has been shown to phosphoylate and activate PDE3 from platelets, heart, and aortic smooth muscle (Grant *et al.*, 1988; Rascon *et al.*, 1992). This suggests that PDE3 activation has a role in mediating negative feedback control of PKA systems, and thereby desensitisation of the cAMP signal. Desensitisation of PDE3 and also PDE4 has been shown to occur following chronic exposure to a cAMP elevating stimulus (Conti *et al.*, 1995). In addition, PDE3B is regulated by one or more insulin-dependent kinase (Lopez-Aparicio *et al.*, 1992). The overall effect of insulin appears to be to antagonise the actions of adenylyl cyclase activation and to inhibit cAMP-stimulated lipolysis. The antilipolytic effects of insulin are dependent on the rapid reduction of cAMP via phosphorylation of PDE3B (Manganiello *et al.*, 1992; Gettys *et al.*, 1988).

A number of highly selective inhibitors exist for PDE3, which include SKF94838, siguazodan, cilostamide, and milrinone (Thompson 1991). PDE3 inhibitors such as milrinone and cilostamide are positive inotropes, vasodilators and inhibitors of platelet aggregation (Beavo, 1995; Degerman *et al.*, 1997).

1.3.1.4. PDE4

PDE4, which is one of the most extensively studied PDE families. PDE4 is a cAMP-specific PDE with relatively high affinity for cAMP (Km < 2μM) and is sensitive to inhibition by rolipram. PDE4 has at least four genes *PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D* with over 18 isoforms as a result of alternative mRNA splicing (reviewed by Houslay *et al.*, 1998; Beavo, 1995). A characteristic feature of PDE4 is the presence of conserved domains known as UCR1 and UCR2 at the N-terminus. Alternative mRNA splicing produces long (containing both UCR1 and UCR2) and short (containing only UCR2) isoforms.

As with PDE3, PDE4 is also phosphorylated by PKA, therefore providing feedback regulation of cAMP (reviewed Manganiello *et al.*, 1995). Prolonged stimulation of cAMP has been shown to increase the mRNA and activity of PDE4, in particular PDE4D, suggesting regulation at the level of transcription (Swinnen *et al.*, 1989; Sette and Conti, 1996). Long-term upregulation of cAMP-PDE activity would therefore lead to the reduced potency of activators of adenylyl cyclase. In addition to regulation by PKA, the p42 MAPK has also recently been shown to regulate specific isoforms of PDE4 (Hoffmann *et al.*, 1999; MacKenzie *et al.*, 2000; Baillie *et al.*, 2000). p42 MAPK has the ability to either increase or decrease cAMP levels dependent upon the pattern of the cAMP-specific PDE (PDE4) isozyme expression. MAPK has been shown to increase cAMP levels in the cell by phosphorylating PDE4B, PDE4C and PDE4D3 (long forms of PDE4) at a single site (Ser579), and inhibiting their activity (Hoffmann *et al.*, 1999; Baillie *et al.*, 2000). In contrast, PDE4B2 and PDE4D1 (short forms of PDE4) are both activated by C-terminal phosphorylation by p42 MAPK (MacKenzie *et al.*, 2000; Baillie *et al.*, 2000).

Inhibitors of PDE4 include rolipram, imidazolidinone (Thompson, 1991). As PDE4 has been characterised in a number of inflammatory cells, inhibitors have been shown to have significant anti-inflammatory effects in both animal models of asthma and clinically to treat asthma (Tenor *et al.*, 1996; Banner and Page, 1995, Murray *et al.*, 1991; Doherty, 1999).

1.3.1.5. PDE5

PDE5 has been characterised as the cGMP-binding, cGMP-specific PDE, and has been identified as the main cGMP-binding protein in the lung (Francis *et al.*, 1990; Thomas *et al.*, 1990). Three variants of PDE5A have been isolated and characterised (Kotera *et al.*, 1999; Loughney *et al.*, 1998; Lin *et al.*, 2000). PDE5 isoforms possess unique N-terminal sequences and have different tissue expression. For example PDE5A3 has only been found in human penile cavernosum, whereas PDE5A1 and PDE5A2 are co-expressed in vascular smooth muscle (Kotera *et al.*, 1999; Lin *et al.*, 2000; Murthy, 2001).

PDE5 is composed of two allosteric cGMP-binding domains, one catalytic domain, and a phosphorylation site for PKA and PKG (Corbin and Francis, 1999; Thomas *et al.*, 1990; McAllister-Lucas *et al.*, 1993). Additionally, PDE5 contains two or more zinc atoms per monomer that bind with high affinity (Km = ~0.5μM), and appear necessary for catalysis (Francis *et al.*, 1994). When the allosteric cGMP binding sites of PDE5 are occupied by substrate, PKA and PKG can then phosphorylate a specific serine residue (Ser92) on PDE5 (Thomas *et al.*, 1990). In fact, it has been suggested binding of cGMP to the regulatory subunit changes the configuration of the PDE5 allowing it to be phosphorylated by cAMP and cGMP dependent kinases (Francis *et al.*, 1990). Burns *et al.*, 1992, demonstrated in guinea pig lung PDE5 activity could be stimulated in the presence of the catalytic subunit of PKA and ATP. These authors also reversed this activation of PDE5 by addition of phosphatases. In addition, the phosphorylation of the Ser92 site on PDE5 by PKA or PKG has been shown by Corbin *et al.*, 2000 to increase the activity of PDE5 by 50-70%. These results indicate PDE5 can be phosphorylated by PKA and PKG.

It is possible that the phosphorylation of PDE5 by PKG could be involved in the negative feedback regulation of cGMP levels. Increased cGMP has been suggested to occupy the catalytic sites of PDE5, which subsequently leads to the occupation of the GAF domain, inducing phosphorylation and activation of PDE5 by PKG. Activation of PDE5 subsequently lowers [cGMP]i (evidence by Corbin *et al.*, 2000; Turko *et al.*, 1998; Thomas *et al.*, 1990; Venkatesh *et al.*, 2001). It is difficult to study this proposed negative feedback regulation in intact cells, as few reliable PKG inhibitors, due to the possible involvement of PKA, and the fact cGMP analogues may bind to both the

catalytic, and GAF domains directly. Wyatt *et al.* (1998), have shown that treatment of rASMC with ANP results in an increase in cGMP levels, an increased incorporation of phosphate into immunoprecipitated PDE5, and is associated with an increase in PDE5 activity in the immunoprecipitate. Studies by Murthy (2001), also demonstrated that the generation of cAMP can lead to the PKA-dependent activation of PDE5 and attenuation of cGMP levels.

A large number of specific PDE5 inhibitors exist. The order of potency of common PDE inhibitors for PDE5 is sildenafil> zaprinast> dipyridamole> IBMX> cilostamide> theophylline> caffeine> rolipram (Thomas *et al.*, 1990; Ballard *et al.*, 1998).

1.3.1.6 PDE6

PDE6 is a key element in the proposed cyclic nucleotide cascade of vision, and is only known to be found in the photoreceptors of the eye, hence it is referred to as the photoreceptor PDE. PDE6 has high affinity for cGMP but low affinity cAMP, and is a cGMP-specific PDE. PDE6 shares common structural and functional properties with PDE5. Both PDE5 and PDE6 display a high degree of identity (45-48%) between the catalytic domain, posses both catalytic and non-catalytic cGMP binding sites (GAF), hydrolyse cGMP better than cAMP, and are both sensitive to a common set of competitive inhibitors. (McAllister-Lucas *et al.*, 1993; Gillespie and Beavo, 1989; Turko *et al.*, 1999; Gonzalez, 1999). PDE6 is a $\alpha\beta\gamma_2$ heterotetramer, where α, and β are the catalytic sites for cGMP hydrolysis and γ are the protein inhibitors of the enzyme. Rod and cone PDE6 exist, which despite physiological differences, are both activated in the same manner in the visual transduction pathway (Gillespie, 1990).

The phototransduction cascade involves rhodopsin (GPCR), G-protein receptor coupled kinase (GRK) and β-arrestin, which resembles signalling by growth factors and GPCRs in mammalian cell systems (Stryer, 1991). Photoexcitation of rhodopsin results in the GDP-GTP cyclical activation of the G-protein transducin ($T\alpha\beta\gamma$, see figure 1.6). Activated transducin ($T\alpha$-GTP), dissociates from $T\beta\gamma$ and binds to the inhibitory γ subunits of PDE6, thereby removing their inhibitory effect. Activation of PDE6$\alpha\beta$ leads to the hydrolysis of cGMP to 5'GMP resulting in the closure of cGMP-gated Na^+/Ca^{2+} channels on the plasma membrane. A decrease in Na^+ and Ca^{2+} into the cells results in hyperpolarisation and ultimately a decrease in neurotransmitter release

(review by Yafitz and Hurley, 1994). The occupancy state of the noncatalytic sites on PDE determines whether γ remains bound to activated PDE or dissociates from the holoenzyme, and may be relevant to light adaptation in photoreceptor cells (Norton *et al.*, 2000).

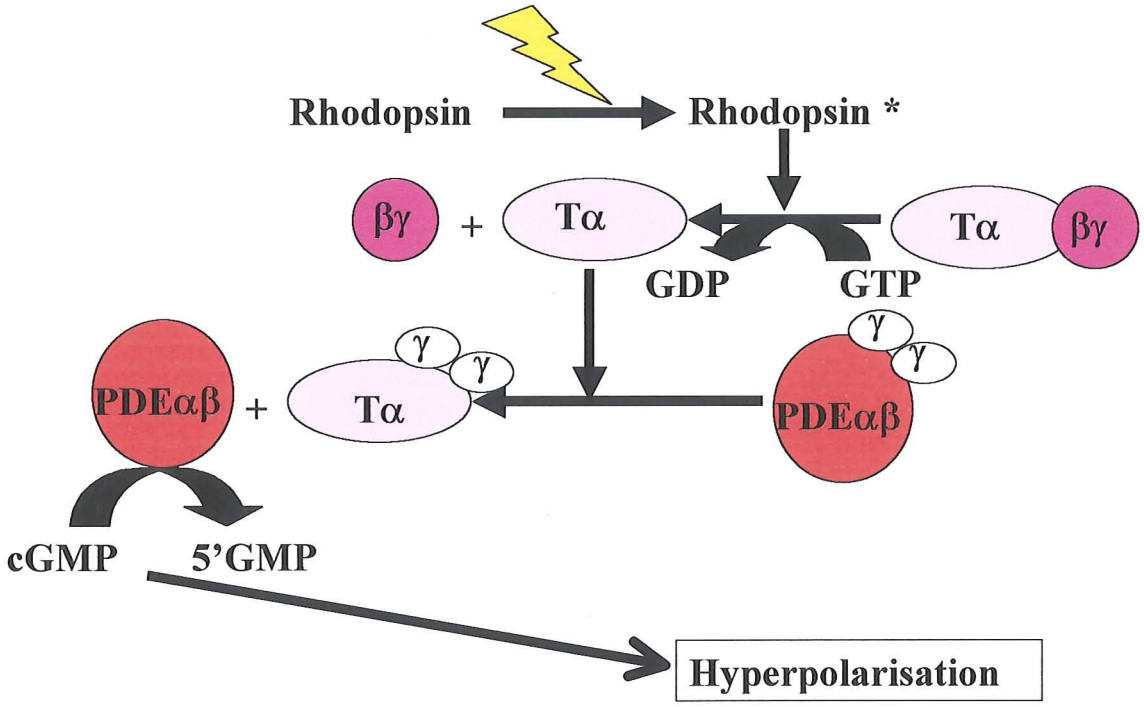


Figure 1.6 Schematic representation of the phototransduction cascade

The light activation of rhodopsin leads to the activation of the G-protein, transducin (T), in the photoreceptors. Activated transducin (Tα-GTP), dissociates from Tβγ and binds to the inhibitory γ subunits of PDE6. PDE6αβ leads to the hydrolysis of cGMP to 5'GMP resulting in hyperpolarisation of the cells (review by Yafitz and Hurley, 1994).

1.3.1.7. PDE7

PDE7 is a high affinity cAMP-specific PDE (low Km of ~0.2 μM), which is rolipram insensitive. High concentrations of PDE7 can be found in skeletal muscle, kidney and brain (Michaeli *et al.*, 1993). PDE7 is closely related to PDE4 in that it is unaffected by cGMP, and specifically hydrolyses cAMP. Two splice variants have been identified, PDE7A and PDE7B. In fact, Takashi and co-workers only recently identified PDE7B in human, with high levels in the striatum (Takashi *et al.*, 2000). PDE7 appears to have a role in T cell activation. CD3- and CD28-dependent induction of PDE7 is required for T cell activation (Li *et al.*, 1999b). These authors also showed that treating T-cells with antisense oligonucleotides specific to PDE7 reduced proliferation, and prevented interleukin-2 production by 80%. PDE7 may therefore be a useful target for the treatment T-cell-mediated pathologies, such as allergies and/or rheumatoid arthritis, however presently no specific inhibitors are readily available (Solderling and Beavo, 2000).

1.3.1.8. PDE8

PDE8 is a cAMP-specific (low Km of ~70nM), IBMX-insensitive PDE (Lava, 1985, Soderling *et al.*, 1998b; Solderling and Beavo 2000). Lava (1985), reported PDE8 to be the first example of a PDE that cannot be inhibited by the non-selective inhibitor IBMX. Two subfamilies exist namely PDE8A and PDE8B. Expression of PDE8A is high in testis, eye, and liver, whereas PDE8B expression appears to be high in the thyroid gland, brain, and kidney (Soderling *et al.*, 1998b; Hayashi *et al.*, 1998). PDE8 contains a single PAS domain (for Per, ARNT, and Sim proteins for which was originally identified) at the N-terminus (Soderling *et al.*, 1998b). PAS domains contain a small number of conserved amino acids within a sequence of about 90 residues, and are thought to act as an environmental sensor in many proteins. Fix L, haemoglobin that acts as an oxygen sensor, and the transcription factor HIF-1 both contain PAS domains (Perutz *et al.*, 1999; Gothie *et al.*, 2000). In these proteins the PAS domains are thought to be involved in the mechanism by which cells sense and respond to changes in oxygen. The PAS domain in PDE8 may serve to mediate protein-protein interactions that regulate subcellular distributions, or have a regulatory/sensory function similar to Fix L and HIF-1 (Soderling and Beavo, 2000). The role of PAS domains in PDE8

activation still requires to be elucidated. No highly specific inhibitors are available for PDE8, or any of the following more recently discovered PDEs.

1.3.1.9. PDE9

PDE9 is a cGMP-specific enzyme (low Km of ~0.07μM). It was originally cloned and characterised by Soderling *et al.*, 1998. In common with PDE8 the enzyme is insensitive to inhibition by IBMX. Alternative splice variants of PDE9 have been identified, although there functions are currently unknown (Guipponi *et al.*, 1998). PDE9 is expressed in small intestinal smooth muscle, kidney, lung, brain, testis and skeletal muscle (Soderling *et al.*, 1998). No highly specific PDE9 inhibitors are yet available.

1.3.1.10 PDE10

PDE10 hydrolyses both cAMP and cGMP (Km ~0.05μM and ~3μM respectively). The V_{max} for cAMP hydrolyses by PDE10 is five fold lower than for cGMP. These kinetics suggest cGMP hydrolyses by PDE10 can be inhibited by a low concentration of cAMP. Therefore, the enzyme has been designated as the cAMP-inhibited, cGMP-specific PDE (Fujishige *et al.*, 1999; Solderling *et al.*, 1999). PDE10, unlike PDE8 and PDE9, is sensitive to inhibition by IBMX. Two alternatively spliced transcripts of PDE10 have been characterised, namely PDE10A1 and PDE10A2 (Kotera *et al.*, 1999). Fusjishige *et al.*, 1999 cloned and characterised PDE10A using bioinformatics and found the primary structure to be analogous to other cGMP binding PDEs, such as PDE2, PDE5 and PDE6. Unlike the other cGMP-specific PDEs, the GAF domain in PDE10 is thought to have a function in addition to cGMP binding. This has been suggested as binding studies have shown the dissociation constant for cGMP binding is greater than 9μM, which is higher than *in vivo* concentrations of cGMP will ever reach in most cells (Soderling *et al.*, 1999).

1.3.1.11 PDE11

Fawcett *et al.*, 1999, cloned and characterised the most recently identified PDE, PDE11A. PDE11A can catalyses the hydrolysis of both cAMP and cGMP. PDE11A and its slice variants carry distinct GAF sequences in their N-terminal region. The enzyme is therefore related to other human genes for GAF-PDE such as PDE5A, and

PDE6A-C (Yuasa *et al.*, 2001). PDE11 appears to be abundant in the prostate and also found in the testes, thyroid gland, and liver (Yuasa *et al.*, 2000).

1.3.1.12 PDE inhibitors in the systemic circulation

Modulation of PDE function in cells is critical for maintaining cyclic nucleotide levels within a narrow rate limiting range of concentrations. The variation in distribution and physiological function of PDEs between tissues make them excellent pharmacological targets. In fact, PDE inhibitors have been shown to alter the contractile tone of various types of smooth muscle, including vascular smooth muscle, corpus cavernosal smooth muscle, and bronchial smooth muscle (reviewed by Polson and Strada, 1996; Corbin and Francis; Trophy, 1998). The functional consequence of selective PDE inhibition in a number of systemic arteries will be outlined below.

The effect of PDE inhibition has been widely studied in the systemic arteries. In vascular smooth muscle the main PDE isoforms present are PDE3, PDE4, and PDE5, and relatively selective inhibitors for these enzymes are available and have been extensively studied (Polson and Strada, 1996). PDE 3 inhibitors such as amrinone and cilostazol have both been shown to markedly relax precontracted rat aortic rings in a concentration-dependent manner. These responses were found to be partly dependent on the presence of an intact endothelium, as on removal of the endothelium the concentration-dependent relaxation curve was shifted to the right (Nakamura *et al.*, 2001; Van der Zypp *et al.*, 2000). Additionally, relaxation of rat aortic smooth muscle induced by isoprenaline has been shown to be potentiated by the PDE3 inhibitor cilostamide (100nM), which correlated with an increase in cAMP levels (Delpy *et al.*, 1996). The relaxation of the aorta and the increase in cAMP levels as a result of PDE3 inhibition was greater if the endothelium was intact (Delpy *et al.*, 1996). PDE3 inhibitors also posses antiplatelet, antiproliferative, and thrombolytic activities, suggesting they may be useful in treating cardiovascular disease and minimising restenosis seen after angioplasty (Indolfi *et al.*, 1997; Wang *et al.*, 2000). In fact, the first clinically used PDE inhibitor was amrinone for use in heart failure. Short-term use of PDE3 inhibitors such as amrinone, and milrinone were shown to increase cardiac index and stroke volume index, with a corresponding decrease in right atrial and pulmonary capillary wedge pressure, indicating an improvement in ventricular function (Benotti *et al.*, 1978; Baim *et al.*, 1983). However, in long-term clinical trials the

hemodynamic improvements seen early in therapy were not sustained, and an increase in mortality was observed (Uretsky *et al.*, 1990; Packer *et al.*, 1991)

In parallel, both PDE4 (rolipram) and PDE5 (zaprinast and DMPPO) inhibitors produce endothelium-dependent relaxations in precontracted aortic rings (Konas *et al.*, 1991; Yu *et al.*, 1995; Delpy *et al.*, 1996; Kukovetz *et al.*, 1979: Delpy and Le Monnier de Gouville, 1996). The endothelium-dependent relaxations produced by specific PDE4 and PDE5 inhibitors were inhibited by L-NMMA (inhibitor of the L-arginine-NO pathway) and by methylene blue (soluble guanylate cyclase inhibitor), suggesting vasorelaxations induced by these inhibitors are mediated by the endothelium-derived relaxing factor nitric oxide. Komas *et al.* (1991), also demonstrated that selective inhibition of PDE4 by denbufylline and rolipram relaxed rat aortic rings better in the presence than in the absence of a functional endothelium, and showed the biosynthesis and release of endothelium-derived relaxing factor was necessary for their effect. The PDE5 inhibitor E4021 dilates precontracted porcine large coronary artery in both the presence and absence of the endothelium. E4021 and was shown to be ~100 times more potent then zaprinast, and more effective if an intact endothelium was present. E4021 caused a significant increase in $[cGMP]_i$ levels in these endothelium-denuded porcine coronary artery, however had no effect on $[cAMP]_i$. These authors also demonstrated that E4021 induced a dose-dependent dilation of epicardial coronary artery in conscious pigs (Saeki *et al.*, 1995). Additionally, it has been shown that the PDE5 inhibitor MBCQ evokes a concentration-dependent relaxation in phenylephrine precontracted endothelium-intact resistance systemic arteries (Samson *et al.*, 2001). This concentration-dependent relaxation was potentiated significantly in the presence of the nitric oxide donor DEA NONOate (0.001nM-1μM), and attenuated significantly in the presence of the soluble guanylyl cyclase inhibitor, ODQ (3μM). The highly selective PDE5 inhibitor, sildenafil (10^{-7}-10^{-4}M), also significantly relaxed rat aorta and human coronary artery, which appeared to be due to an increase in cGMP levels and inhibition of the Ca^{2+}-dependent cascade for contraction (Medina *et al.*, 2000; Machida *et al.*, 2002). It is important to note, that for the maximal effect of any of the PDE inhibitors to be observed in systemic arteries, an intact endothelium is required.

In airway smooth muscle selective inhibitors of both PDE3 and PDE4 partially reverse spontaneous tone in human isolated bronchi (Rabe *et al.*, 1993; Cortijo *et al.*, 1993). These results are consistent with the presence of large amounts of both PDE3 and PDE4

in airway smooth muscle. Additionally, a combination of PDE3 and PDE4 or dual PDE3/PDE4 inhibitors such as zardaverine, have been shown to produce an even greater bronchorelaxant (Schudt *et al.*, 1991; Trophy *et al.*, 1993). *In vivo* PDE4 inhibitors have been shown to reverse bronchospasm induced by histamine, leukotiene D_4, or carbachol (Raeburn and Karlsson, 1997). It has been suggested by these authors and others, that an increase in basal cAMP might be required to see the full bronchorelaxant activity of both PDE3 and PDE4 inhibitors. As PDE 4 inhibitors are also known to reduce inflammatory and immunomodulatory responses, these data together suggest they may have a possible role in the treatment of asthma (Piaz and Giovannoni, 2000).

Widespread attention has recently focused on the relaxation of corpus cavernosum smooth muscle by PDE inhibitors (in particular PDE5 inhibitors) for the effective treatment of male impotence. In human corpus cavernosum *in vitro* the PDE5 inhibitor, sildenafil, was shown to enhance NO-dependent relaxation in a concentration-dependent manner, and was shown to be 240-fold more potent than the early PDE5 inhibitor zaprinast (Ballard *et al.*, 1998). More importantly, following sexual stimulation, sildenafil has been shown to enhance NO-stimulated cGMP-mediated smooth muscle relaxation, increasing blood flow to the penis, improving penile erection. Likewise, in rabbit, sildenafil and zaprinast both either alone or in combination with sodium nitroprusside, relax the corpus cavernosum and increase cGMP production, however do not alter cAMP levels (Jeremy *et al.*, 1997). In fact, sildenafil citrate (Viagra®) is currently used to treat male erectile dysfunction (Moreland *et al.*, 1999; Boolell *et al.*, 1996; Ballard *et al.*, 1998; Goldstein *et al.*, 1998; Corbin and Francis, 1999; Medina *et al.*, 2000). Due to the development of new highly selective PDE inhibitors, such as sildenafil, new and promising therapeutic applications have been suggested, for example in the treatment of pulmonary hypertension.

1.3.2. The role of PDEs in PHT

Modulation of PDE function in cells is critical for maintaining cyclic nucleotide levels within a narrow rate limiting range of concentrations. Increased PDE activity would reduce the level of cyclic nucleotides, and thereby decrease the ability of agents that act through raising cAMP/cGMP to relax SMC. The decrease cyclic nucleotide concentrations and the increased tone seen previously with PHT by MacLean *et al.*

(1996), may therefore be a result of increased PDE activity. The lung is known to express all PDEs, with the exception of PDE6 (Rabe *et al.*, 1994; Polson and Strada, 1996; Dent *et al.*, 1994; Soderling and Beavo 2000; Yuasa *et al.*, 2000; Koyama *et al.*, 2001). Therefore, the control of cyclic nucleotides in the PA may be dependent on the activities of several different PDE isoforms. The employment of selective (monoselective) PDE inhibitors in biochemical and functional studies has helped to further identify the possible roles of some of the PDE families in PHT. Using fast protein liquid chromatography Rabe *et al.* (1994) revealed the presence of high levels of a zaprinast sensitive PDE5, a Ca^{2+}/calmodulin stimulated PDE1, a cGMP-inhibited cAMP-specific PDE3, and a cAMP-specific rolipram sensitive PDE4. Additionally, these authors demonstrated that specific inhibitors for PDE3, PDE4, and PDE5 all relaxed isolated preconstricted human PAs (Rabe *et al.*, 1994).

MacLean *et al.*, 1997 reported an increase in both total cAMP and total cGMP PDE activity in the main, first branch, and intrapulmonary vessels from the CH, providing evidence for a role of PDEs in PHT. Furthermore, using specific inhibitors in the PDE assay, these authors determined how the activity of each PDE family studied was altered with chronic hypoxia. In the main, first branch and intrapulmonary arteries there was an increase in cilostamide-sensitive PDE3 activity. Additionally, in the majority of vessels studied, an increase in zaprinast-inhibited PDE5 activity was observed. No change was observed in PDE2 activity in any of the vessels studied, PDE1 was observed to only increase in the main PA, whereas PDE4 was found to decrease in the resistance vessels. These studies suggest that changes in cyclic nucleotide levels in CH appear to be associated, in most part, to increased PDE3 and PDE5 activity. The molecular mechanisms that may underlie the hypoxic-dependent increase in PDE3 and PDE5 activity in the PAs has not yet been established. Below will discuss further evidence for the role of PDE3 and PDE5 in PHT.

1.3.2.1. The role of PDE3 in PHT

As outlined above PDE3 is known as the cGMP-inhibited cAMP-specific PDE, of which two subfamilies, PDE3A and PDE3B, have been identified in rASMC and hASMCs (Lui and Maurice, 1997; Palmer and Maurice, 2000). Commonly used specific PDE3 inhibitors include milrinone, amrinone, and cilostamide. Furthermore, compounds such as SKF94120, SKF94836, and Org 9935 are 30-100 fold selective for

PDE3, and used at appropriate concentrations are tools to assess the functional importance of PDE3 (Reeves *et al.*, 1987; Murray *et al.*, 1991; Shahid *et al.*, 1991). A role of PDE3 is seen in response to cell proliferation, as exposure of SMC to hypoxia resulted in a time-dependent decrease in cAMP, which correlated with increased PDE3 activity (Pinsky *et al.*, 1993). In addition, PDE3 inhibitors have been shown to attenuate serum-stimulated proliferation in rASMC (Pan *et al.*, 1994). The PDE3 inhibitor SKF94836 has been shown to reduce serum stimulated DNA synthesis and proliferation by 30% in SMC, and enhance the antiproliferative effect of the cAMP elevating agent forskolin (Souness *et al.*, 1992). Additionally, Billington *et al.*, 1999, showed that siguazodan (PDE3 inhibitor) inhibited both [^{3}H]-thymidine incorporation and the increase in cell number induced by PDGF-BB (20ng/ml).

Functional studies have shown PDE3 inhibitors could be useful in the treatment of PHT. Both milrinone (0.01-156μM) and SCA40 (0.01-31μM) have been shown to be effective in relaxing preconstricted PAs. However, importantly both PDE3 inhibitors remained potent in the PAs from CH (Jeffery and Wanstall, 1998). In a similar study, SCA40 reversed the preconstriction induced by phenylephrine in the main and intrapulmonary PAs, and was 4.9-fold more potent in the hypoxic PAs than in the control PAs (Crilley *et al.*, 1998). Clarke *et al.* (1991), showed inhibition of PDE3 by amrinone reduces PVR in isolated perfused lung. Milrinone has been shown to significantly decrease the mean PAP and PVR in the hypoxic dog, and in early clinical trials to lower the pulmonary capillary pressure in patients with heart failure. (Kato *et al.*, 1998; Baim *et al.*, 1983, Jaski *et al.*, 1985). Furthermore, cilostamide has been shown to attenuate both acute and chronic hypoxia induced PHT (Phillips *et al.*, 2000). PDE3 inhibition has also been show to improve agonist-induced relaxation of PAs from CH. Wagner *et al.* (1997), demonstrated that a combination of the PDE3 inhibitor milrinone, and the PDE4 inhibitor rolipram, significantly reduced the magnitude of the contractile response to U46619 in PAs from CH.

PDE3 inhibitors are also known to have positive inotropic and vasodilatory actions in the systemic circulation, which would unfortunately lead to harmful side effects when treating PHT (Beavo, 1995). It may be possible that co-administration with other PDE inhibitors or pulmonary vasodilators would allow the use of subthreshold doses (which under clinical conditions do not have other cardiovascular side effects e.g. inotropy) of PDE3 inhibitors in the treatment of PHT.

1.3.2.2. The role of PDE5 in PHT

PDE5 has been characterised as the cGMP-binding, cGMP-specific PDE, and has been identified as the main cGMP-binding protein in the lung (Francis *et al.*, 1990; Thomas *et al.*, 1990). PDE5 was first purified from rat lung using sequential chromatography on DEAE-cellulose, blue sephrose CL-6B, zinc chelate adsorption and HPLC-TSK DEAE 5PW (Francis and Corbin, 1988). The predicted molecular weight of PDE5 from the open reading frame (ORF) of isolated cDNA was 99kDa (McAllister *et al.*, 1993). PDE5 has been cloned not only from rat (PDE5A1 and PDE5A2), but also from human (PDE5A1, and PDE5A2) lung tissue (Kotera *et al.*, 1999; Loughney *et al.*, 1998). The molecular weight of PDE5A1 and PDE5A2 from lung tissue, are 98kDa and 93kDa respectively.

PDE5 has been shown to have a role in modulating normal pulmonary vascular tone at birth. At birth there is known to be a dramatic increase in pulmonary blood flow and a decrease in PVR. Sanchez *et al.* (1998), demonstrated this decrease in PVR in both ovine and mice lungs could be correlated with a decrease in PDE5 activity, protein and mRNA. These results would suggest that a low level of PDE5 activity may play a role in controlling the low basal tone in the adult pulmonary circulation. With respect to PHT, increased PDE5 activity has been reported not only in the PAs of CH, but also in the lung from the ovine foetal model of PHT (MacLean *et al.*, 1997; Hanson *et al.*, 1998b). Hanson *et al.* (1998b), demonstrated that the increased PDE5 activity seen in pulmonary hypertensive animals compared with control animals may be due to an increase in the phosphorylation of PDE5. These results are consistent with findings by Black *et al.* (2001), showing PDE5 protein expression is increased in lambs with PHT, induced by aorta-pulmonary vascular graft replacement. Increase PDE5 would explain the impaired responsiveness to cGMP-dependent vasodilators such as SNP, and the decrease in cGMP in the conduit PAs from CH (Oka, 2001, MacLean *et al.*, 1998a).

It can be hypothesized that PDE5 inhibitors would increase cGMP levels in the lung and help to prevent the development of hypoxia-induced PHT. In fact, various PDE5 inhibitors have been shown to be effective pulmonary vasodilators. PDE inhibitors act by competing with cGMP to bind to the catalytic site, but not the allosteric sites, of PDE5 (Francis *et al.*, 1990; Corbin and Francis, 1999). In conscious rats previously exposed to chronic hypoxia, i.v. DMPPO (1μM) caused a dose dependent decrease in

PAP with no corresponding change in the systemic artery pressure or cardiac output (Eddahibi *et al.*, 1998). Chronic treatment with DMPPO during the 2 weeks of hypoxia reduced the muscularisation of the PA at the level of the alveolar wall and alveolar duct, preventing the development of pulmonary vascular remodelling (Eddahibi *et al.*, 1998). Additionally, Jeffery and Wanstall (1998), demonstrated, like milrinone, zaprinast (0.2μM-625μM) was also effective at relaxing main PA from CH in a concentration dependent manner. Importantly zaprinast remained potent in rats with established PHT (4 weeks). Inhaled zaprinast has also been shown to selectively dilate the pulmonary circulation in lambs with U46619-induced PHT, and to enhance to pulmonary vasodilatory effects of inhaled NO (McMahon *et al.*, 1993; Ichinose, 1995a; Ichinose *et al.*, 1995b; Steinhorn *et al.*, 2000).

Previous studies have shown that the PDE5 inhibitors such as zaprinast or dipyridamole lack specificity and potency. For example, zaprinast also inhibits at least one other isoform, PDE1, and requires prolonged periods of incubation to elevate cGMP levels (Murray *et al.*, 1991). Dypyridamole is also an adenosine reuptake inhibitor, which may result in misinterpretation of results (Zeigler *et al.*, 1995). E4021 and E4010 are new more selective PDE5 inhibitors with no detectable effects on PDE1, PDE3 or PDE4. E4010 has been shown to improve mortality in MCT-induced pulmonary hypertensive rats by 84.4% (Kodama and Adachi, 1999). These authors demonstrated that rats treated chronically with 0.1% E4010 in their diet showed reduced right ventricular hypertrophy and increased plasma cGMP levels, compared to rats treated with vehicle. Likewise, in the CH a single oral dose of 1.0mg/kg E4010 attenuated the development of hypoxia-induced PHT, reducing the increase in PAP and reducing the characteristically right ventricular hypertrophy and increased medial wall thickness, with no significant systemic side-effects (Hanasato *et al.*, 1999).

Furthermore, Cohen *et al.*, 1996, demonstrated that E4201, caused a dose-dependent inhibition of hypoxic vasoconstriction in isolated perfused lungs from chronically hypoxic treated rats. In PHT rat lungs, E4021 increased intracellular cGMP 3-fold and reduced hypoxic vasoconstriction by 58 ± 2% (Cohen *et al.*, 1996). E4021 also reduced PAP in conscious PHT rats by 12.6 ± 3.7%, with importantly no systemic effects (Cohen *et al.*, 1996). In MCT-treated rats, oral administration of the PDE5 inhibitor E4021 (100mg/kg/day) reduced the immunoreactivities of ET-1 and endothelial NOS, and reduced right ventricular overload and medial thickening (Takahashi *et al.*, 1998;

1996). These authors showed myofibril diameter, medial thickness and smooth muscle were significantly lower on treatment with E4021, demonstrating a role for increased PDE5 in smooth muscle proliferation in PHT. Together these studies suggest a positive role for PDE5 inhibitors in the treatment of PHT.

Another newly developed and potent PDE5 inhibitor is sildenafil (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1-H-pyrazolo[3,4-d]pyrimidin-5-yl)phenylsulfonyl]-4-methyl-piperazine). Sildenafil has been shown to have high affinity for PDE5 and PDE6 with the respective inhibition constants of K_i of ~3.5 and 33nM (Ballard *et al.*, 1998). The order of potency of sildenafil compared to some commonly known PDE5 inhibitors is sildenafil (most potent) > zaprinast > dipyridamole > IBMX > cilostamide > theophyylline > caffeine > rolipram (Thomas *et al*, 1990; Ballard *et al*, 1998). Sildenafil, like most PDE5 inhibitors, stimulates cGMP binding to the allosteric sites of PDE5 by interacting at the catalytic site of this enzyme, however does not compete with cGMP. Illarion and co-workers concluded that residues such as TYR602, HIS607, HIS643, and ASP754 appear important for the interaction of sildenafil with PDE5 (Illarion *et al.*, 1999). As outlined previously sildenafil citrate (Viagra®) is currently used to treat male erectile dysfunction (Moreland *et al.*, 1999; Boolell *et al.*, 1996; Ballard *et al.*, 1998; Goldstein *et al.*, 1998; Corbin and Francis, 1999; Medina *et al.*, 2000).

These studies opened up the possibility that this new PDE inhibitor may be effective in the treatment of PHT (Sanjay *et al.*, 2000). Osinski *et al.* (2001), demonstrated that sildenafil has an antimitogenic effect on SMC, which was significantly potentiated when administered in combination with organic nitrates. Zhao *et al.* (2001), examined the effect of sildenafil on hypoxia-induced PHT in mice and healthy human volunteers. PHT was induced in healthy male volunteers by breathing in low oxygen causing a 56% increase in blood pressure in the PA. Sildenafil (100mg) inhibited the hypoxic rise in PAP without significantly affecting the systemic circulation. The reduction in PAP was reproduced in isolated mouse lung. Additionally, sildenafil attenuated the increase in PAP, RV hypertrophy, and remodelling in mice chronically exposed to hypoxia (Zhao *et al.*, 2001). Both these results were consistent with increases in plasma cGMP levels, and show a role for PDE5 inhibition by sildenafil in the treatment of PHT. Furthermore, in a randomised controlled trial, sildenafil caused selective pulmonary

vasodilation and improved gas exchange in individuals with PHT secondary to lung fibrosis (Ghofrani *et al.*, 2002).

Interestingly from clinical trials with sildenafil only transient mild or moderate side effects were seen. A clinical trial by Goldstein *et al.*, (1998), found the main side effects to be headache, flushing, dyspepsia, rhinitis and visual disturbances. Although Sampson *et al.*, 1999, did show expression of PDE5 mRNA and protein in systemic resistance arteries, sildenafil is only a modest vasodilator in these vessels causing only a small decrease in systemic arterial pressure and a mild reduction in preload and afterload. Together, these results suggest sildenafil might be extremely beneficial in the treatment of PHT, as it appears to be pulmonary specific. In addition, researchers have now begun to develop PDE5 inhibitors that are even more selective than sildenafil, such as vardenafil hydrochloride, which may reduce PAP with even fewer unwanted side-effects (Bischoff *et al.*, 2001).

1.3.3. The role of PDEγ in PHT

As outlined in 1.3.1.6 the activity of PDE6 is regulated through its interaction with the inhibitory subunit PDEγ. In photoreceptors Pγ is known to inhibit PDE6 activation, thereby inhibiting cGMP hydrolysis. Each step of the GTP-hydrolytic cycle of transducin is closely related to molecular state of PDEγ (Morrison *et al.,* 1987). Two functionally similar PDEγ exist, PDEγ1 (rod) and PDEγ2 (cone), differing in their amino-terminal regions and their location in the retina (Hamilton and Hurley, 1990). The carboxyl-terminal domains, which are essential for the inhibitory action against PDE6 and for stimulating transducin GTPase, are almost identical (Brown, 1992; Lipkin *et al.*, 1990; Skiba *et al.*, 1995, Slepak *et al.,* 1995). Furthermore, as a result of a 41 base pair deletion, two isoforms of PDEγ2 exist, namely long PDEγ2 and short PDEγ2. It is thought the two forms of PDEγ2 may be important at different stages of development, or in different cell types. PDEγ is phosphorylated by several kinases including p42/p44 MAPK, PKC, PKA and PDEγ kinase (Hayashi *et al.*, 1991; Udovichenko *et al.*, 1994; Xu *et al.*, 1998).

It has recently been suggested that PDEγ may have a wider role in mammalian cells other than photoreceptors. Evidence for a role of PDEγ other than in the retina is the expression of PDEγ1 in lung, kidney, testes, liver, heart, airway smooth muscle and

HEK 293 cells, and its absence in all these tissues from PDEγ1 knockout mice (unpublished data from the lab, Wan *et al.*, 2001, Tate *et al.*, 1998; Tate *et al.*, 2001). The presence of PDEγ throughout the body cannot however be explained by its association with PDE6, as PDE6 is only found in the eye. Hence it was suggested that as PDE5 shares common structural and functional properties with PDE6, PDE5 may have its own PDEγ subunits controlling its regulation. Both PDE5 and PDE6 display a high degree of identity (45-48%) between the catalytic domain, posses cGMP binding sites, hydrolyse cGMP better than cAMP, and are both sensitive to a common set of competitive inhibitors. (McAllister-Lucas *et al.*, 1993; Gillespie and Beavo, 1989; Turko *et al.*, 1999; Gonzalez, 1999). These authors also demonstrated PDE5 contains a region that has some homology with the sites in the PDE6 catalytic domain subunits (residues 481-540) and 479-538 in PDE6αβ that interact with the polycationic region of Pγ. In fact, Lochhead *et al.*, 1997 identified two small molecular mass proteins termed p14 and p18 in guinea-pig airway smooth muscle cells and mouse lung (where PDE5 is the major cGMP binding protein, Burns *et al.*, 1992), which cross-reacted with antibodies raised to the polycationic mid-region and C-terminal region of PDEγ. p14/p18 was shown to form a complex with PDE5, as PDE activity was immunoprecipitated using antibodies against the PDEγ subunit. Furthermore, recombinant PDEγ and a peptide corresponding to amino acids 24-46 of PDEγ have been shown to modulate PDE5 activity by preventing its activation by PKA in a concentration-dependent manner (Tate *et al.*, 1998, and Lochhead *et al.*, 1997). It was suggested that PDEγ may, through its possible interaction with PDE5, govern the duration and kinetics of cGMP signalling in mammalian cells. It is possible that the binding of PDEγ to PDE-5 may be altered under hypoxic conditions, explaining the increase in PDE5 activity observed in PA of CH (MacLean *et al.*, 1997).

In addition, PDEγ has been shown to stimulate the proteolysis of PDE5 by caspase-3 and caspase-8 *in vitro* (Frame *et al.*, 2001). In both *in vitro* and in intact cells, Frame *et al.*, 2001 demonstrated that caspase1, 3, 6, 7, 8, and 11 all cleave PDE5A1, reducing its hydrolysing activity. In the presence of PDEγ1, caspase-3 induced an approximately 80% reduction in the activation of the partially purified preparation of PDE5A1. As caspases (cystinoaspartic acid specific proteases) are important mediators of apoptosis this study suggests that under conditions of cellular stress, PDEγ may promote cleavage of PDE5 by caspase-3, inducing apoptosis. PDE inactivation through a subsequent

increase in cGMP has been shown to regulate apoptosis via the nitric oxide pathway. Nitric oxide has been shown to induce apoptosis in cardiomyocytes and endothelial cells via a cGMP–dependent pathway (Shen *et al.*, 1998; Shimojo *et al.*, 1999). Therefore, it may be that caspases exert their apoptotic action by inhibiting PDE5A1, thereby increasing cGMP concentrations. It is possible that under stress PDEγ may promote cleavage of PDE5A1 by caspase-3, prolonging cGMP-mediated cell signalling.

Recently a wider role for Pγ in signal transduction has been suggested. As PDEγ is an important link between rhodopsin activated transducin and cGMP gated channels, it was proposed that PDEγ1 and PDEγ2 maybe expressed in other tissues where they regulate other receptor-G-protein-mediated pathways, such as p42/p44 MAPK. A role for PDEγ in regulating EGF- and thrombin mediated activation of p42/p44 MAPK has been recently identified in HEK293 cells (Wan *et al.*, 2001). These authors demonstrated that the EGF- and thrombin dependent activation of p42/p44 MAPK was prevented in human embryonic kidney (HEK) 293 cells transfected with anti-sense rod PDEγ, however augmented in HEK293 cells over expressing recombinant rod and cone PDEγ. Data also suggested that phosphorylation of the Thr-62 in rod PDEγ by GRK2 (G-protein-coupled receptor kinase 2) is required for the increase in p42/p44 MAPK activation following EGF and thrombin stimulation. This was based on evidence showing the over-expression of recombinant GRK2 and/or recombinant PDEγ increased the activation of p42/p44 MAPK by both EGF and thrombin. Furthermore, a GRK2 resistant rod Pγ mutant prevented the increase in the EGF- and thrombin-dependent activation of p42/p44 MAPK, acting as a dominant negative.

The formation of a complex between PDEγ and dynamin II induced by EGF and thrombin was also suggested. Thrombin was shown to stimulate the association of endogenous PDEγ1 with dynamin II, which may be required for the endocytosis of receptor signal complexes leading to the activation of p42/p44 MAPK and cell proliferation (Wan *et al.*, 2001). This interaction was increased in rod PDEγ and GRK-2 transfected cells. Dynamin II is known to interact with proteins containing SH3 binding sites. Rod PDEγ contains a SH3 binding site at 20PVTPRKGPP28, providing further evidence that it may interact with dynamin II via a SH3 domain containing protein. PDEγ was therefore speculated to be a GTPase activating protein, that interacts with dynamin II to promote the "pinching off" of endocytic vesicles, bringing activated

MEK closer to p42/p44 MAPK in the cytosol, leading to its phosphorylation and subsequent activation.

As PDEγ may have a role as an intermediate in p42/p44 MAPK signalling, it may be that changes in its expression have a profound effect on cellular proliferation in PA in response to hypoxia. Therefore it would be of interest to investigate the expression of PDEγ1/2 in rat PA and hPASMC, and to determine the effect of chronic hypoxia.

1.4. AIMS

The aim was to further investigate the possible roles of PDE5 and PDE3 in PHT. Initially the main objective was to establish the molecular mechanisms that may underlie the hypoxic-dependent increase in PDE3 and PDE5 activity in the PAs. As chronic hypoxia can also induce the activation of the NF-κB, a further aim was to investigate if PDE3 or PDE5 may be under control of this pathway.

Additionally, the vasorelaxant properties of the PDE3 inhibitor SKF94836, and the PDE5 inhibitor sildenafil, in the PA was evaluated. Furthermore, the aim was to determine if the vasorelaxant effects are dependent on the endothelium, the nature of the preconstrictor, and the size of the PA. It was also necessary to investigate if both inhibitors are still effective in the PAs from CH.

Also, as PDEγ is expressed in non-retinal tissue, and appears to have a role in the p42/p44 MAPK pathway, or modulating PDE5 activity, the final aim was to investigate whether PDEγ1/2 is expressed in rat PA and hPASMC, and to determine the effect of chronic hypoxia. These novel studies were intended to show a possible wider role of PDEγ in signal transduction and PHT. Completion of all these objectives are hoped to further elucidate pathways involved in the response to hypoxia in order to highlight possible novel targets for treatments of PHT.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2. Materials and Methods

2.1. Materials

Unless otherwise stated, all reagents were obtained from Sigma chemical company (U.K.), or BDH (U.K.).

Anachem (U.K.)

30% (w/v) Acrylamide/bis (29:1).

Amersham Pharmacia Biotech (U.K.)

DNA Polymerase Mix (dNTPS), GFX^{TM} PCR and Gel Purification Kit, Hybond^{TM}ECL^{TM} Nitrocellulose Membrane.

Amersham International p.l.c. (U.K.)

^{3}H-cAMP and ^{3}HcGMP (37mCi/mmol, 1850kBq/mmol)

BD Transduction Laboratories (U.K.)

Anti-phospho-p42/p44 MAPK, and Anti-total (p42) MAPK Antibodies

BIO-RAD (U.K.)

BIO-RAD Protein Assay Reagent, Mini-Protean II Electrophoresis cell, Trans Blot cell

BioWittaker (U.K.)

Human Pulmonary Artery Smooth Muscle Cells (hPASMC), Smooth Muscle Cell Growth Medium (SmGM-2 bulletkit system)

Calbiochem-Novabiochem (U.K.)

Anti-Phosphodiesterase 5 Antibody.

Clonetech laboratories Inc (USA)

Rat Glyeraldehyde-3-Phosphate Dehydrogenase (G3PDH) Control Amplimer Set.

Dr. R. Cote (University of New Hampshire, USA)

Anti-PDEγ Antibody raised to the C-Terminal Domain of Photoreceptor PDEγ.

Eastman Kodak Company (U.K.)

Kodak Digital Camera, Kodak Digital Science^{TM} ID Image Analysis Software.

GalaxoSmithkline (U.K.)

SKF94836 (PDE3 inhibitor, M.W. 270)

H.A. West (U.K.)

Kodak LX24 Developer and Kodak Industrex Fixer.

Helena Biosciences (U.K.)

Phoenix thermal cycler.

Life Technologies (U.K.)

DNase I Amplification Grade, Oligo dt(18), Primers (PDE3A, PDE3B, PDE5A, PDEγ), Superscript II Reverse Transcriptase, Taq Polymerase, 100bp DNA Ladder, All general cell culture materials.

PE-Applied Biosystems (U.K.)

BigDye Dye Terminator Cycle Sequencing Kit.

Pharmacia Biotech. (U.K.)

Genequant II, RNA/DNA Calculator.

Pfizer (U.K.)

Sildenafil (PDE5 inhibitor, M.W. 430)

Qiagen (U.K.)

QIA Shredder, RNeasy Total RNA Isolation Kit.

Royal Hallamshire Hospital, Sheffield (U.K.)

Hypobaric chamber.

RS Biotech. (U.K.)

Galaxy CO_2 incubator – Oxygen Control.

Scottish Antibody Production Unit (U.K.)

Horseradish peroxidase-linked Anti-rabbit IgG, and Anti-mouse IgG.

2.2. Methods

2.2.1. Models of Pulmonary Hypertension

2.2.1.1. Animal model - Hypoxic/Hypobaric Rat

In order to mimic the etiology of hypoxia induced pulmonary hypertension (PHT), male Wistar rats (specific pathogen free) were exposed to low oxygen by reducing the atmospheric pressure in a purposed built chamber (Hypoxic Hypobaric model). The hypoxic hypobaric chamber is designed and manufactured by the Royal Hallamshire Hospital, Sheffield, and can hold two standard rat cages each with a maximum of four rats. As the atmospheric pressure decreases, this leads to the decrease of the partial pressure of the gaseous components of air, and hence decreases the partial pressure of O_2 (pO2) inspired. The pressure within the chamber was decreased to 550mbar, this reduced the inspired pO2 to approximately 110mmHg (~10% equivalent). The chamber was ventilated with air at approximately $45 l min^{-1}$, and initially depressurised slowly

over a period of two days. Every three days, when the animals required fresh water and food, the chamber was gradually taken up to atmospheric pressure over two hours. Following cleaning of the cages, the pressure in the chamber was then returned to 550mbar again over two hours. Age matched controls were maintained under normoxic/normobaric room conditions (20% v/v oxygen) for two weeks.

In both the control and hypoxic animal studies, male Wistar rats were 28-30 days of age at the start of all experiments. All animals were maintained at 21-22^{0}C on a twelve hour light-dark cycle, and allowed free access to standard food and water. After the two weeks, the average weight of both the control and hypoxic rats was approximately 200g (see figure 3.3.1).

2.2.1.1.1. Sacrifice and dissection

All animals were killed via an overdose of sodium pentabarbitone (60mg/kg^{-1} i.p.). After weighing, the heart and lungs were carefully dissected free and placed in cold Krebs. Using a microscope the main pulmonary artery (4-5mm, i.d.), first branch pulmonary artery (2-3mm, i.d.), intrapulmonary (0.2-2mm, i.d.), and resistance vessels (100-300μm, i.d.) were removed and cleaned of adherent tissue. Pulmonary arteries were then either kept in cold gassed Krebs-Heinslet solution [118.4mM NaCl, 25mM $NaHCO_3$, 47mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 2.5mM $CaCl_2$, 11mM, pH 7.4] at 4^{0}C (for no more than 24hrs) for use in organ bath experiments (2.2.4.), or transferred directly into liquid nitrogen then stored at –80^{0}C to allow protein and RNA extraction at a later date (2.2.2, or 2.2.3.).

2.2.1.1.2. Assessment of pulmonary hypertension

Pulmonary hypertension is characterised by right ventricular hypertrophy (Hunter, *et al.*, 1974, Leach *et al.*, 1977). The ratio of right ventricular weight (RV) to total ventricular weight (TV) was used as an index of right ventricular hypertrophy in this study (Hunter, *et al.*, 1974). After the removal of the pulmonary arteries, the heart was dissected free from the remaining lung lobes, atria, and remaining vessels. The right ventricle (RV) was then cut from the septum and left ventricle, blotted and weighed. The left ventricle (LV) was incised to remove any blood clots, then together with the septum blotted, and both weighed with the right ventricle to give total ventricular weight (TV, See figure 3.3.2).

Measure of PHT = RV weight/TV weight

2.2.1.2. Cellular model – hPASMC

As cells are more amenable for biochemical analysis a cellular model was designed to try and mimic any changes that had been seen with hypoxia in the animal model. Human pulmonary artery smooth muscle cells (hPASMC) were obtained from Clonetics (Biowhittaker).

On receipt, the cryopreserved hPASMC [In SmGM-2 supplemented with 10% v/v fetal bovine serum, and 10% dimethyl sulfoxide (DMSO)], were immediately transferred into liquid nitrogen storage. Each vial of cryopreserved cells came with a certificate of analysis showing seeding efficiency, number of cells per ampule (guaranteed to contain $\geq$ 500,000 viable cells) and donor information. The growth medium was smooth muscle growth medium (SmGM), which had been optimised for the proliferation of smooth muscle cells (BioWhittaker). The SmGM was prepared using smooth muscle basal medium (SmBM, 500ml), and the addition of the following single-use aliquots; 0.5ml of 0.5μg/ml human recombinant epidermal growth factor, 1ml of 1μg/ml human recombinant fibroblast growth factor, 0.5ml of 5mg/ml insulin, 25ml foetal bovine serum, and 0.5ml each of 50mg/ml gentamicin, and 50μg/ml amphotericin-B (SmGM-2 bullet kit). On removal from liquid nitrogen, cells were seeded into T-25 flasks (growth area of 25cm^2) at the recommended seeding density of 3500cells/cm^2 using the following calculations:

Max. area that can be plated = No. of cells available/Recommended seeding density

Max. no. flasks set up = Max. Surface area that can be plated/Growth area of flask

The growth medium was always added to flasks prewarmed at 1ml SmGM-2 for every 5cm^2 surface area of the flask, this increased to 2ml per 5cm^2 surface area as confluency was reached. The day after seeding, in order to remove residual DMSO and unattached cells, the growth medium was changed, then every third day thereafter. All cells were initially kept in a RS Biotech–Galaxy CO_2 incubator set at 5%CO_2, 95%air, humidified incubator set 37^{0}C. The cultures were regularly examined microscopically for any signs of stress such as detachment, rounding-up or atypical morphology.

The cells were checked daily until 70-90% confluency had been reached, at which time each flask was split 1:3. The cells arrived at passage three (P3) and were used in each experiment before or at P8, as human cell systems have a finite lifespan *in vitro*.

For RNA or protein extraction, control/treated cells were washed with sterile phosphate buffered saline (PBS, 10mM KH_2PO_4, 10mM K_2HPO_4, 0.9% w/v NaCl), then two protocols were followed as in section 2.2.2.1, and 2.2.3.1 respectively.

2.2.1.2.1. Passage of hPASMC by Trypsination

Cells were passaged at 70%-90%, as over confluence resulted in irreversible contact inhibition (BioWhittaker). In order to subculture the hPASMC, filter sterilised trypsin [2.125g NaCl, 0.1g KCl, 0.288g Na_2HPO_4, 0.05g KH_2PO_4, 0.25g Trypsin (10%w/v), 0.45g Glucose, 0.0626g EDTA, pH7.2 in 250ml dH2O] was used to detach cells from flasks by proteolytic and collagenolytic enzyme degradation. After removing the medium, cells were washed with SmBM (serum free media), to allow quicker and more effective trypsination. On removal of the serum free medium, 1–2 ml (for T-75, all volumes were adjusted accordingly flask size) of trypsin was added to each flask, which were returned to the incubator for 4–5 minutes. Complete cell detachment was checked by examination under the microscope. The trypsin was then neutralised with at least an equal volume of SmGM-2, after which the cells were pipetted into centrifuge tubes and centrifuged at 750rpm for 2½ minutes. Once the supernatant had been aspirated off, the pellet was resuspended on addition of approximately 3ml SmGM-2. 1ml from each centrifuge tube was then transferred into a T-75 flask containing 9ml of prewarmed SmBM, then returned to the incubator. The medium was always changed the day after trypsination to remove residual trypsin and non-attached cells. 70-90% confluence was usually achieved after 7-10 days. During this study cells were used at or before passage 8 (P8).

2.2.1.2.2. Hypoxic hPASMC Model

In order to mimic the animal model as closely as possible, the cells, were maintained in 10% O_2. After changing the media following passage, flasks were split into two groups. Half the flasks were returned to the normoxic incubator (2.2.1.2.), and half were transferred to the hypoxic incubator (RS Biotech – Galaxy CO_2 incubator – oxygen control, 10% O_2, 5%CO_2, balanced N_2, humidified, set 37^0C). Initially cells were

grown in the hypoxic incubator for 24 hours to 2 weeks to determine optimal conditions. At all times the hypoxic cells were treated identically to the control cells i.e. fed every three days and passaged when 70 – 90% confluent.

2.2.1.2.3 Addition of Drugs to hPASMC.

In order to explain some of the hypoxic dependent changes, and to determine cellular signalling pathways involved, chosen inhibitors and stimulators were added to the cells in culture. Table 2.1 shows the drugs added, the concentrations that they were used at, how frequently they were added to cells, and whether the drugs were added to the cells in serum free medium. All drugs were dissolved in dH_2O. Before each experiment, cells were quiescent [this refers to growing the cells for 24 hours in serum free media (SmBM)].

DRUG	FINAL CONCENTRATION	NUMBER OF ADDITIONS AND PERIOD OF INCUBATION	MEDIUM DRUGS WERE ADDED TO
8-Br-cAMP	100μM	One addition for 24hours when fresh medium was added.	SmGM
TLCK	100μM	Replaced every three days for two weeks, on addition of new medium.	SmGM
H8	50μM	Replaced every three days for two weeks, on addition of fresh medium.	SmGM
EGF	50ng/ml	One addition for 5minutes	SmBM
PDGF	10ng/ml	One addition for 5minutes	SmBM

Table 2.1. Drugs used to treat hPASMC

Table of the drugs added to hPASMC cells, the final concentration in the flask, the period of incubation, and the medium the cells were growing in when exposed to each drug. The abbreviations used are: 8-Br-cAMP, 8-Bromoadenosine 3'5'-Cyclic Monophosphate; TLCK, Nα-p-Tosyl-$_L$-Lysine Chloro-Methyl Ketone; H8, N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide 2HCl]; EGF, Epidermal Growth Factor; PDGF, Platelet Derived Growth Factor; SmGM, smooth muscle growth medium; SmBM, serum free smooth muscle basal medium.

2.2.2. Molecular Analysis

2.2.2.1. RNA isolation from rat pulmonary artery and hPASMC

RNase free equipment was used at all time. For isolation of RNA from rat pulmonary arterial branches, the tissue was initially ground to a fine powder in liquid nitrogen with a mortar and pestle. 600µl buffer RNeasy lysis buffer (containing 14.5M β-mercaptoethanol) was then added according to manufacturers guidelines (Qiagen), and the tissue homogenised by passing the lysate through a 25 gauge (G) needle five times.

For isolation of RNA from hPASMC, initially the medium was aspirated and the cells washed with sterile PBS. The hPASMC were scraped in 600µl of RNeasy lysis buffer (containing 14.5M β-mercaptoethanol), then passed through a 25G syringe needle five times.

Both the tissue and cell lysates were then pipetted onto QIAshredder (Qiagen) columns sitting in 2ml collection tubes and centrifuged at maximum speed (12,000rpm). Total RNA was extracted according to the RNeasy protocol instruction (Qiagen). In order to prevent potential contamination from genomic DNA, an incubation step with 4 units of DNase at 37^0C for 15 minutes followed by a second RNA extraction (clean up protocol, Qiagen) were included. Total RNA was eluted in RNase free H_2O and stored at -20^0C.

To determine the concentration and purity of RNA the absorbency was measured at 260nm (A260) and 280nm (A280) in a spectrophotometer (genequant II). The RNA was only used if a yield of 25µg of RNA per ml or greater was achieved, with a ratio between 1.5 and 2 (ratio between the absorbency values at 260 and 280nm gives an estimate of RNA purity).

2.2.2.2. Reverse Transcription Reaction

First strand synthesis was carried out in each reaction using 1µg total RNA catalysed by the enzyme superscript II reverse transcriptase (200units). The reaction was primed using 500ng of oligo$(dt)_{18}$, in a final volume of 20µl. The reverse transcription mixture also contained 200µM dNTP, 1 x first-strand buffer [50mM Tris-HCl(pH 8.3), 75mM KCl, 3mM $MgCl_2$, life technologies], and 1mM DTT. First strand synthesis was carried

out for 90 minutes at 42^{0}C, then inactivated at 70^{0}C for 15 minutes. One fifth of the cDNA was used as a template in subsequent PCRs. Each time first strand synthesis was performed, a separate reaction was carried out minus reverse transcriptase (-RT), in order to establish a lack of genomic DNA.

2.2.2.3. Polymerase Chain Reaction – Semi Quantitative

The polymerase chain reaction (PCR) allows the amplification of specific DNA sequences *in vitro* by the simultaneous primer extension of complementary strands of DNA. PCR amplifications were performed using gene specific forward and reverse primers (see below for sequences). Primers were all designed to Genebank sequences for each product of interest, and all primers were checked in Genebank to exclude the possibility of sequence homology with other genes. The PDE3A and PDE3B primers were designed to amplify regions corresponding to 3011-3415 and 2902-3201 in human PDE3A and PDE3B respectively. The PDE5A primers were designed to amplify 2338-2637 in bovine PDE5A.

Primers:

PDE3A sense, 5'-CTG GCC AAC CTT CAG GAA TC-3'

PDE3A antisense, 5'-GCC TCT TGG TTT CCC TTT CTC-3'

PDE3B sense, 5'-AAT CTT GGT CTG CCC ATC AGT CC-3'

PDE3B antisense, 5'-TTC AGT GAG GTG GTG CAT TAG CTG-3'

PDE5A sense, 5'-CGA TGC TGA TGA CAG CTT GTG ATC-3'

PDE5A antisense, 5'-CAA GAG CTT GCC ATT TCT GCC-3'

PDEγ1 sense (Y00746 Forward), 5'-ATG AAC CTG GAG CCA CCC-3'

PDEγ1 antisense (Y00746 Reverse), 5'-GCT CAC ATA GCA GGG ATC AGA-3'

PDEγ1 antisense (C-terminal reverse), 5'-AAT GAT GCC ATA CTG GGC CAG-3'

PDEγ2 sense, 5'-CGG GAT CCC GCC ACC ATG AGC GAC AGC CCT TGC C-3'

PDEγ2 antisense, 5'-CCC AAG CTT GGG TCC TCA GAT GAT CCC GAA CTG-3'

G3PDH sense, 5'-TGA AGG TCG GTG TCA ACG GAT TTG GC-3'

G3PDH antisense, 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3'

Amplification was performed in a 50µl volume PCR mixture containing: PCR reaction buffer (10mM Tris-HCL, 50mM KCl), 1.5mM $MgCl_2$, 0.2mM dNTP mix, 1µM of each primer, 200ng of cDNA and 2.5 units of Taq DNA polymerase (heat stable enzyme that synthesises DNA from single-stranded templates in the presence of primers). Equal loading of cDNA was confirmed using rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) specific primers in the reaction mixture. Mineral oil was added over each sample to prevent evaporation during denaturation. The reaction was programmed in a Phoenix thermal cycler as follows: initial denaturation for 5 minutes at 95^0C, 15-35 cycles of amplification (each cycle consisted of denaturation for 30 seconds at 95^0C, annealing for 30 seconds at 50^0C , and extension for 1 minute 40 seconds at 72^0C), a final extension of 10 minutes at 65^0C, and storage at 4^0C. RT-PCR conditions that yielded linear amplification rates were used to obtain results for each gene transcript studied.

The amplification products were analysed by agarose gel electrophoresis. An agarose gel containing 1% agarose, and 0.5µg/ml ethidium bromide in Tris-Borate-EDTA [(TBE) working solution 0.045M Tris-Borate 0.001M EDTA] was initially prepared in a Mini Q apparatus (Bioscience services), allowed to set, then covered with TBE buffer on removal of the combs. 15µl of each PCR product were mixed with 3µl of gel loading buffer (50% v/v glycerol, 0.05% w/v bromophenol blue, 0.05% w/v xylene cyanol). All 18µl of the samples were then loaded into the wells, along side 10µl of 100 bp DNA ladder [consisting of 15 blunt ended fragments between 100 and 1500bp in multiples of 100bp and an additional fragment at 2072 bp, (life technologies)]. Electrophoresis was at 70 volts for ~1 hour, then the amplified DNA bands were visualized with a U.V. transilluminator and photographed using a Kodak Digital camera and Kodak Digital Science ID image analysis software (Eastman Kodak Company).

2.2.2.4. Sequence Analysis

In order to verify the sequence of each product, the amplified DNA bands were excised from the agarose gel and purified using the GFX™ PCR gel band purification kit (Amersham Pharmacia Biotech). The purified amplicons were sequenced, in both

directions using the specific forward and reverse primers for the expected product, on a PE-Applied Biosystems Division Model 373A automated DNA sequencer and a BigDye Dye terminator cycle sequencing kit. Sequencing was carried out by the molecular biology facility at the University of Strathclyde.

2.2.3. Biochemical Analysis

2.2.3.1. Protein isolation from rat pulmonary artery and human pulmonary smooth muscle cells

To isolate protein from both tissue and cells, the homogenisation buffer isotonic sucrose solution (I.S.S.) was used with composition: 0.25M sucrose, 10mM Tris HCl, 1mM EDTA, 0.1mM phenylmethylsulphonyl fluoride (PMSF) and 2mM benzamidine, pH 7.4. The PA branches were initially ground to a fine powder in liquid nitrogen using a mortar and pestle, then homogenised by adding 500µl I.S.S. and passing through a 25G syringe needle five times.

After the removal of the media, cells were washed with sterile PBS, then scraped in 600µl I.S.S. per T-75 flask. To shear the cells, the lysate was passed five times through a 25G syringe needle. Both the homogenised tissue and cells were centrifuged for 2 minutes at 12,000 rpm, and only the supernatant used in subsequent experiments.

2.2.3.2. Protein Assay

Protein content of each sample was determined using the BIO-RAD micro protein assay system (Bradford, 1976). 10µl of sample was added to 200µl BIO-RAD reagent and 800µl dH2O. Protein content was estimated by measuring the A595 of each sample using a spectrophotometer (Jenway, 6105 U.V./VIS.), then comparing the values against a standard curve previously constructed using a range of known bovine serum albumin (BSA) concentrations. In order to add equal amounts of each protein sample, I.S.S. was added to dilute samples with higher protein concentrations.

2.2.3.3 SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a rapid method for quantifying, comparing, and characterising proteins. Electrophoresis was

carried out following the method described by Laemmli, (1970). Samples were prepared by adding equal volumes of sample buffer [62.5mM, Tris/HCL (pH 6.7), 0.6M mercaptoethanol, 12.5% glycerol (v/v), 1.25% SDS (w/v) and 0.02% (w/v) bromophenol blue]. Before loading, each sample was boiled for ~5minutes to ensure disruption of the disulphide bonds and denaturing of the proteins.

Acrylamide gels were cast in BIO-RAD mini-protean units (10 x 7cm). Initially separating gels were prepared, containing a final concentration of 12 or 14% acrylamide [components of separating gel: 12/14% acrylamide, 0.375M Tris base (pH8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.05% (v/v) TEMED, and dH2O] depending on the size of the protein of interest. After setting, a 6% acrylamide stacking gel [components of stacking gel: 6% acylamide, 0.125M Tris base (pH6.7), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.1% (v/v) TEMED, and dH2O] was poured on top and a ten welled comb inserted. The gels were then added into the electrophoresis equipment, afterwhich the combs were removed, and the upper and lower chambers were filled with electrophoresis running buffer (0.21M glycine, 3.5M SDS, and 25mM Tris base). The samples and molecular weight standards were loaded using a hamilton syringe. The gels were then run at a constant voltage of 200 volts/1mA until the dye front was at the bottom.

The molecular weight standards (10µl added to each gel) were prestained SDS-PAGE molecular weight markers (α_2-macroglobulin: 230kDa, β-glactosidase: 135kDa, fructose-6-phosphate: 97kDa, pyruvate kinase: 78kDa, fumarose: 57.5kDa, lactic dehydrogenase: 38.5kDa, and triosephosphate isomerase: 33.5kDa). These molecular weight markers were used to estimate the molecular weights of the unknown protein. The above molecular weights given for the molecular weight markers are the apparent molecular weights for each protein when run on SDS-PAGE, and not the native molecular weights.

2.2.3.4. Transfer of proteins to a nitrocellulose membrane

Proteins from the gel were then transferred to nitrocellulose using BIO-RAD trans blot apparatus following the procedure described by Towbin *et al.*, (1979). The transblotting sandwich was assembled (sponge, blotting paper, gel, nitrocellulose membrane, blotting paper, sponge), all of which had been previously soaked in transfer buffer (0.21M

glycine and 25mM Tris Base in 20% (v/v) methanol). The side with the gel was positioned nearest the negatively charged terminal, allowing the proteins (negatively charged) to transfer onto the nitrocellulose membrane. The transfer unit was immersed in transfer buffer then ran for at least 60 minutes at 100 volts/0.6mA.

2.2.3.5. Western blotting

Following the transfer of proteins the nitrocellulose was incubated in blocking buffer for 2-3 hours to prevent non-specific binding. On removal of the blocker the primary antibody was added overnight, after which the membrane was washed 3 x 10 minutes in washing buffer. A horseradish peroxidase (HRP)-linked secondary antibody was added for 1 hour, which was followed by 3 x 10minute washes in washing buffer. All stages of the protocol are carried out on an orbital shaker to ensure equal coverage and washing of the membrane. Table 2.2 shows specific conditions for all the antibodies used.

Immunoreactive bands were detected using an enhanced chemiluminesence detection kit (ECL). ECL allows the detection of specific antigens that are attached to HRP-antibodies (directly or indirectly) by emitting light which can be detected by exposure to x-ray film. After removing excess washing buffer, equal volumes of ECL detection reagents 1 (2.5mM luminol, 1.1mM p-coumaric acid, 0.1M Tris base, pH8.5) and 2 (0.02% hydrogen peroxide, 0.1M Tris base, pH8.5) were mixed to give a final volume of 125ml/cm2 on the membrane. The mixed ECL reagents were applied for 1 minute onto the side of the membrane onto which the protein was electro-blotted. After removing surplus ECL reagents the membrane was placed in an autoradiographic cassette then covered with sparkle film. In a dark room under safety lights, the membrane was exposed to X-ray film for 1-10 minutes depending on the intensity of the signal. The film was then developed and fixed, using Kodak GBX developer and Kodak GBX fixer (made to manufactures instructions) to visualise immunoreactive bands.

2.2.3.6. Reprobing of nitrocellulose blots

Nitrocellulose membranes were regularly reprobed with new antibody. This involved incubating the blot with stripping buffer (100mM β-mercaptoethanol, 2%SDS, 62.5mM Tris HCl, pH 6.7) at 70^0C for 1 hour with gentle agitation. The membranes were then washed 3 x 10 minutes in the wash buffer for the new antibody (table 2.2), which was subsequently applied as in 2.2.3.5.

1^0 Antibody	Wash Buffer	Blocker	2^0 Antibody
Anti-PDE5 (1:2000) in 5% milk (w/v) in TPBS, 4^0c	PBS/0.1%Tween (v/v)/0.001%Thiomerosol (w/v) (TPBS)	5% milk (w/v) in TPBS, room temperature	Anti rabbit IgG (1:2000) in 5% milk (w/v) in TPBS, room temperature
Anti-PDEγ (1:10 000) in 1% gelatin (w/v) in IPBS, 37^0C	PBS/0.05% Igepal CA-630 (v/v)/0.001%Thiomerisol (w/v) (IPBS)	3% gelatin in PBS, 37^0C	Anti-rabbit IgG (1:2000) in 1% gelatin (w/v) in IPBS, 37^0C
Anti-phospho p42/p44 MAPK, (1:1000) in 3% BSA (w/v) in TTBS, 4^0c	TBS/0.1%Tween (v/v)/0.001%Thiomerosol (w/v) (TTBS)	5% milk in TTBS, room temperature	Anti-rabbit IgG (1:2000) in 5% milk (w/v) in TTBS, room temperature
Anti-P42 MAPK (1:1000) in 3% BSA (w/v) in TTBS, 4^0c	TBS/0.1%Tween (v/v)/0.001%Thiomerosol (w/v) (TTBS)	5% milk in TTBS, room temperature	Anti-mouse IgG (1:2000) in 5% milk (w/v) in TTBS, room temperature

Table 2.2. Specific conditions for all the antibodies used, following 2.2.3.5

The abbreviations used are: PDE, Phosphodiesterase; MAPK, Mitogen Activated Protein Kinase; PBS, Phosphate Buffered Saline (10mM KH_2PO_4, 10mM K_2HPO_4, 0.9% w/v NaCl); TPBS, PBS/0.1%Tween (v/v)/0.001%Thiomerosol (w./v); NPBS, PBS/0.05% Igepal CA-630 (v/v)/0.001%Thiomerisol (w/v); TBS, Tris Buffered Saline (10Mm Tris base, 100Mm NaCl, pH7.6); TTBS, TBS/0.1%Tween (v/v)/0.001%Thiomerosol (w./v)

2.2.3.7. Phosphodiesterase Assay

The assay of PDE activity was by the two-step radiotracer method using 0.5μM [^{3}H]cAMP or [^{3}H]cGMP according to Thompson W.J., and Appleman M.M., (1971). This assay monitors the conversion of [^{3}H]cGMP/[^{3}H]cAMP into ^{3}H-guanosine/^{3}H-adenosine. Samples were prepared as in 2.2.3.1, in I.S.S. for both the tissue and cells, and equalised for protein. On ice, 25μl of sample was added to 25μl I.S.S. and 50μl ^{3}H-cAMP/cGMP working stock (50μl 1850kBq ^{3}H-cAMP/^{3}H-cGMP in 10mls of 1μM cGMP (unlabelled cAMP/cGMP, 10mM Tris Base, 5mM $MgCl_2$, pH 7.4). Blanks only contained 50μl I.S.S, and 50μl and 50μl ^{3}H-cAMP/cGMP working stock. Each tube contained 9.25kBq of labelled cyclic nucleotide. All tubes were incubated at 30^{0}C for 10 minutes over which activity is linear, then boiled for 2 minutes and allowed to cool on ice again to terminate the reaction. 25μl of a 1mg/ml snake venom (*Hannah ophiophagus*) was then added to each sample to convert ^{3}H-AMP/^{3}H-GMP to ^{3}H-adenosine/guanosine respectively. After an incubation of 10 minutes, 400μl Dowex was added. Samples were then vortexed every five minutes for a period of 15minutes, then centrifuged for 2minutes at 12,000 rpm. 150μl supernatant was added to 2ml scintillant, which was then counted (Wallac, 1209 Rackbeta, Liquid Scintillation Counter, programme 1 - ^{3}H, 60 seconds). In every experiment each assay was done in triplicate. Specific activity was expressed as pmol/min/mg.

In order to measure the relative contribution of PDE3 activity to total PDE activity, 10μM SKF94836 in DMSO was added into the assay. Experiments were controlled by adding 1μl of the vehicle (DMSO) into parallel samples that had not been treated with inhibitor. These controls were necessary to show a true effect of the inhibitors.

2.2.4. Pharmacological Analysis

2.2.4.1. 10ml Organ Bath Set-up for isolated main and first branch pulmonary artery

The main and first branch pulmonary arteries (PA) were dissected as described in section 2.2.1, afterwhich each branch was cut into two equal size rings (~2-5mm). Standard organ bath procedures were used. Figure 2.2 shows a schematic diagram of the organ bath set up. Each PA was suspended by two wire supports. The top wire support (hook shaped) was then connected by thread to a force displacement transducer, while the bottom wire support, which was attached to a glass rod, was clamped in place. The isometric force transducer was connected via an amplifier to Mac lab (Chart V3.5, MacLab Data Acquisition System, Version 8E, AD Instruments Pty Ltd, Australia), a computer based data handling system which recorded vessel contraction/relaxation as in a pen chart recorder. The rings were mounted 10ml organ baths containing modified Krebs-Heinslet solution (Krebs) as described in 2.2.1. continuously oxygenated with 16% O_2, 5% CO_2, and 79% N_2, and maintained at 37°C. These conditions were used to mimic the internal environment of the pulmonary artery as closely as possible.

Rings were placed under a resting tension of 1.5g, which was maintained throughout all experiments. A tension of 1.5g is set, as it is known to be the optimal tension to produce a maximal contraction to 50mM potassium chloride (KCl) in control PA, and can be said to mimic the *in vivo* tension. Initially all PA were equilibrated for 45 minutes after which each vessel was contracted with 50mM potassium chloride (KCl). 50mM KCl has previously been demonstrated to produce a maximum contractile response (MacLean *et al.*, 1994a). These authors demonstrated that higher concentrations of KCl resulted in a decrease in contractile response. After washing with Krebs, an additional 50mM KCl was always added to each bath to ensure maximal contraction had been achieved by the first addition of KCl. After a further equilibration period of approximately 45 minutes following washing, endothelium function was always checked. Functional endothelium was assessed by the ability of 10^{-6}M acetylcholine (ACh) to significantly relax PA rings pre-contracted with 10^{-6}M phenylepherine (PE). In selected experiments the endothelium was removed by gently rubbing the luminal surface of the rings with ridged forceps. When no response was

achieved with 10^{-6}M ACh, the vessels were considered to be denude of functional endothelium.

2.2.4.2. Preconstrictors

Before commencing protocols, each vessel was washed and allowed to return to resting tension. Cumulative concentration response curves (CCRCs) were constructed for PE (10^{-9}-10^{-5}M), 5-HT (10^{-9}-10^{-5}M), ET-1 (10^{-11}-10^{-7}M) in half log steps for both the main and first branch pulmonary artery. The periods between additions were dictated by the time taken for the responses to stabilise, which was usually 5 minutes. In further experiments a concentration of each vasoconstrictor was used that resulted in a sustainable contraction that was approximately 90-100% of the maximum KCl response.

2.2.4.3. Effect of PDE3 and PDE5 inhibitors on preconstrictor responses

Both the PDE5 inhibitor sildenafil, and the PDE3 inhibitor SKF94836 were stored at a stock concentration of 10^{-2}M in 1% DMSO. CCRCs were constructed using the PDE5 inhibitor sildenafil (10^{-9}-10^{-5}M in 1% DMSO), and the PDE3 inhibitor SKF94836 (10^{-9}-10^{-5}M in 1% DMSO) in half log steps for each of the three preconstrictor agents, in the main and first branch pulmonary artery from both control (+/- endothelium) and hypoxic animals. Additions of both inhibitors only commenced once a stable plateau had been reached for the preconstrictor used. In all experiments, in order to show the true effect of the inhibitors, one half of the branch was always used as a time control where only 1%DMSO was added in to the bath (final concentration 0.01% DMSO).

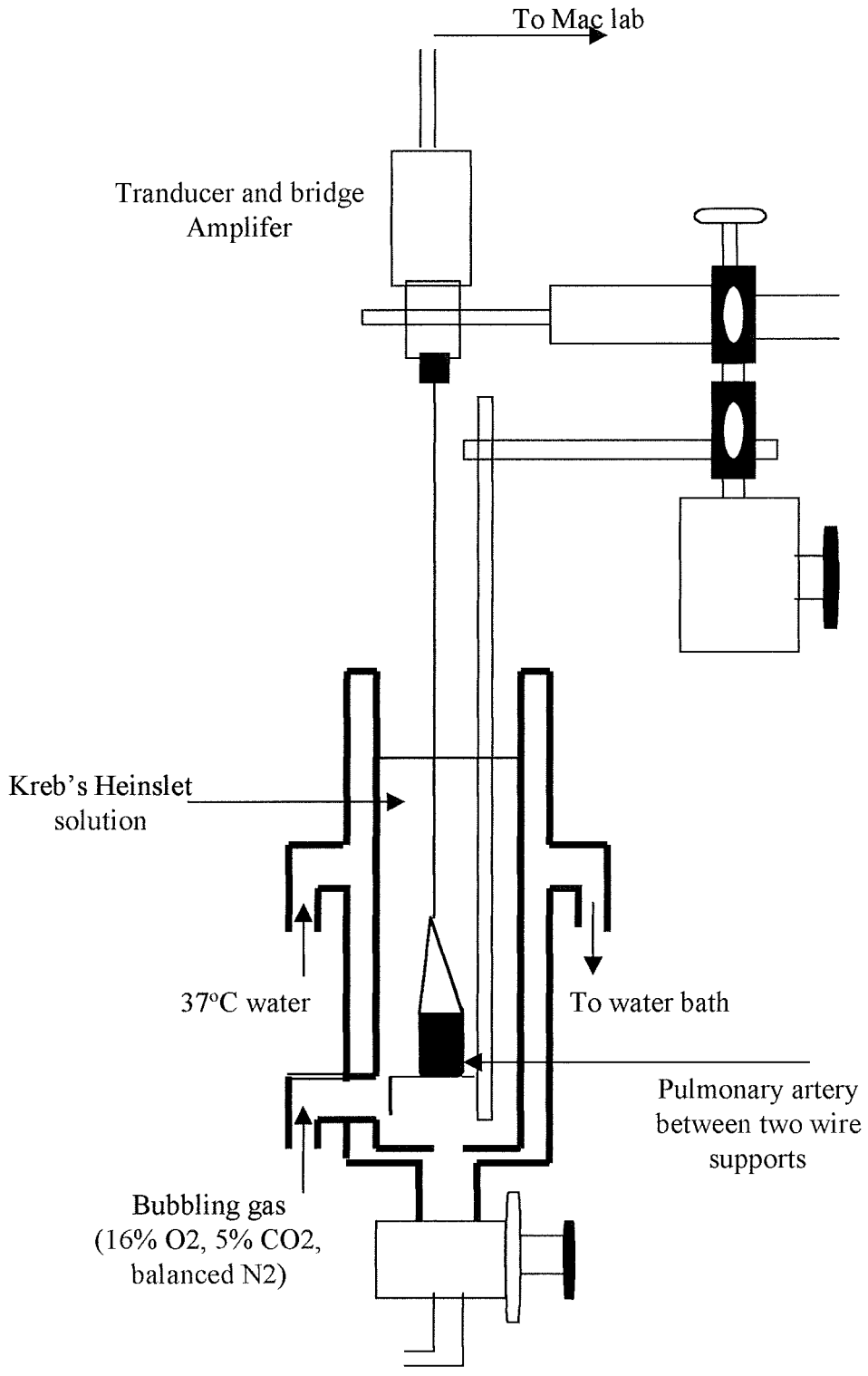


Figure 2.2. 10ml organ bath experimental apparatus

Diagrammatic representation of the 10ml organ bath experimental apparatus for the pharmacological analysis of isolated main and first branch pulmonary arteries (not to scale).

2.2.5. Data Analysis

In all analyses comparisons between two groups of data were made using Student's *t*-test for paired or unpaired data where appropriate. The statistic software package prism (Graphpad Prism, San Diego, CA, USA) was used to handle raw data, where *P<0.05 was considered to be statistically significant. RT-PCR and Western blotting results were quantified by densitometry using a Bio RAD imaging densitometer (Model G.S.-690) in conjunction with Molecular Analyst Software, Version 2.1 (Bio Rad laboratories (U.S.). Optical densities were expressed as arbitrary units. In all experiments "n" either indicates the number of different animals used or number of different populations of cultured cells.

In the organ bath experiments all data were expressed as percentage of the reference response to 50mM KCl in each vessel. Each point is the mean ± s.e.m. Statistical comparisons of the means of groups of data were made by use of Student's *t*-test for paired or unpaired data where appropriate. A level of probability of P<0.05 was taken to indicate statistical significance. As above, n equals the number of different animals used.

To take into account a possible effect of the vehicle (DMSO), the response of the PA to DMSO alone was subsequently subtracted from the parallel response to the PDE inhibitor. These calculations allowed results to be obtained that could only be attributable to the PDE inhibitors.

CHAPTER 3

EVALUATION OF PDE3 AND PDE5 IN MODELS OF PHT

Chapter 3 – Evaluation of PDE3 and PDE5 in models of pulmonary hypertension

3.1. Introduction

Pulmonary hypertension (PHT) is a disease associated with increased vascular resistance and pulmonary vascular remodelling, and is characterised as abnormally high blood pressure in the arteries of the lung (reviewed by Rubin, 1997; Archer and Rich, 2000, Fishman, 1998; Vender *et al.*, 1994; Rabinovitch, 1996; Veyssier-Belot and Cacoub, 1999; Dumas *et a*l., 1999). PHT can occur as a primary illness (PPHT), thought to be the result of a sporadic or familial mutation, or more frequently secondary as a serious complication of disorders such as HIV/AIDS infection, chronic obstructive pulmonary disease (COPD), pregnancy, Raynauds disease (vasospastic disorder), connective tissue diseases such as erythematosus and scleroderma, chronic pulmonary thromboembolism, cirrhosis of the liver, congenital heart disease or left ventricular failure. Exposure to low oxygen results in pulmonary vascular remodelling and increased pulmonary arterial vasoreactivity associated with PHT; therefore the chronic hypoxic rat is used as a reliable model (Hunter *et al.*, 1974; Rabinovitch *et al.*, 1979, Voelkel and Tuder, 2000). The mechanisms involved in the development and maintenance of PHT are still not fully understood. It is however thought that defects in pulmonary vasodilatory, vasocontractile and/or proliferative pathways may account for the abnormal vascular tone and increased proliferation seen in all forms of PHT.

The second messengers cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP) have been shown to play key roles in the control of pulmonary vascular tone (Murray *et al.*, 1990b). cAMP and cGMP relax smooth muscle through the activation of protein kinase A (PKA) and protein kinase G (PKG) respectively, which in turn phosphorylate key contractile proteins and specific types of K^+, Cl^-, and Ca^{2+} channels (Hakonarson and Gruinstein, 1998). Furthermore, cGMP and/or cAMP both control smooth muscle proliferation (reviewed by Southgate and Newby, 1990; Cornwell *et al.*, 1994; and Grosser *et al.*, 1995). It has been suggested that cAMP and cGMP, via the activation of PKA and PKG respectively, attenuates proliferation by antagonising mitogenic pathways through inhibition of Raf-1 (Graves *et al.*, 1993; Bornfeldt and Krebs, 1999; Bonisch *et al.*, 1998; Zucker *et al.*, 1998; Yu *et al.*, 1997).

Cyclic nucleotide levels are reduced in vessels from rats chronically (14 days) exposed to hypoxia (MacLean *et al.*, 1996). This may explain the reduced sensitivity to agents such as nitric oxide in promoting relaxation of pre-contracted pulmonary vessels in CH and in patients with PHT (MacLean *et al.*, 1996).

Intracellular cAMP/cGMP are regulated by adenylyl/guanylyl cyclase and the phosphodiesterases. Phosphodiesterases (PDEs), of which eleven are currently identified, catalyse the hydrolysis of both cAMP and cGMP. PDEs reduce the intracellular concentrations of the cyclic nucleotides, hence determine the size and duration of vasodilatory and proliferative stimuli (Kauffma *et al*, 1987). Each of the 11 PDE families have differing tissue distribution, regulatory properties, amino acid sequences, and kinetic characteristics (reviewed by Thompson, 1991; Beavo *et al.*, 1994; Beavo, 1995: Soderling *et al.*, 1998, 1999; Corbin and Francis, 1999; Fawcett *et al.*, 2000; Conti, 2000; Soderling and Beavo 2000; Yuasa *et al.*, 2000; Koyama *et al.*, 2001). It has been reported that cAMP and cGMP PDE activity is elevated in pulmonary arteries (PA) from chronic hypoxic rats (CH), most significantly PDE3 and PDE5 (MacLean *et al.*, 1997).

PDE3 is a cGMP inhibited cAMP, specific PDE, which is expressed as two isoforms, PDE3A and PDE3B. An increase in PDE3 leads to a decrease in cAMP, preventing the threshold activation needed to activate PKA, thereby reducing the ability of smooth muscle to vasodilate. PDE3 has been reported to have a role in the pulmonary circulation, as Wagner *et al.* (1997,) reported that the inhibition of PDE3 potentiated β-adrenergic agonist receptor-mediated and forskolin-mediated relaxation in pulmonary arterial rings. The activity of PDE3 is increased in main, first branch, and intrapulmonary arteries from rats maintained under chronic hypoxic conditions (MacLean *et al.*, 1997).

PDE5 is important in the pulmonary circulation due to being the major cGMP specific binding protein in the lung (Francis *et al.*, 1980; Thomas *et al.*, 1990). Three sub-types exist termed PDE5A1 PDE5A2 and PDE5A3. Functional studies have shown the potential of PDE5 to modulate the pulmonary circulation. Zeigler *at al.* (1995), showed that zaprinast, a potent PDE5 inhibitor, can vasodilate the pulmonary circulation. Furthermore, Cohen *et al.* (1996), demonstrated that the PDE5 inhibitor E-4201, inhibited hypoxic vasoconstriction in isolated perfused lungs from chronically hypoxic

treated rats in a dose-dependant manner. Inhibition of PDE5 has been reported to enhance nitric oxide-stimulated, cGMP-mediated, smooth muscle relaxation, increasing blood flow in the pulmonary circulation (McMahon *et al.*, 1993; Ichinose *et al*, 1995a; Ichinose *et al.*, 1995b; Black *et al.*, 2001). PDE5 activity is increased in the first branch and intrapulmonary artery from rats maintained under chronic hypoxic conditions (MacLean *et al.*, 1997).

The molecular mechanisms regulating these two PDEs in hypoxic conditions have however not yet been detailed. Hypoxic-dependant changes in PDE activity may be due to the *de-novo* synthesis of the enzyme or via post-translational modifications e.g. phosphorylation, or association with regulatory proteins. The limiting factor in developing new treatments for PHT may be that the molecular and cellular pathogenesis of the condition is poorly understood. Therefore, the aim of these experiments was to establish the molecular mechanism that underlie the hypoxic changes in PDE3 and PDE5 activity by applying semi-quantitative RT-PCR and quantitative Western blotting analysis to rat pulmonary arterial branch homogenates. It was also intended to develop a cellular model of PHT, using human pulmonary smooth muscle cells (hPASMC), and to further elucidate the pathways involved in any hypoxic induced changes that were observed.

3.2 Materials and Methods

3.2.1 Materials

All reagents, unless otherwise stated, were obtained from Sigma chemical company (U.K.), or BDH (U.K.). Cell culture supplies were from life Technologies (U.K.). RNeasy total RNA isolation kit and QIA shredder were from Qiagen (U.K.). Superscript II reverse transcriptase, DNase I Amplification Grade, Oligo dt (18), Taq Polymerase, and primers were from Life Technologies (U.K.). DNA Polymerase Mix (dNTPS), GFX^{TM} PCR and Gel Purification Kit, Hybond^{TM}ECL^{TM} nitrocellulose membranes were from Amersham Pharmacia Biotech (U.K.). Rat glyeraldehyde-3-phosphate dehydrogenase (G3PDH) control amplimer set was from Clonetech laboratories Inc (USA). BigDye Dye terminator cycle sequencing kit was from PE-Applied Biosystems (U.K.). Anti-phosphodiesterase 5 antibody from Calbiochem-Novabiochem (U.K.). PDE3 inhibitor SKF94836 (M.W. 270) was from

GalaxoSmithkline (U.K.). ^{3}H-cAMP and ^{3}HcGMP (37mCi/mmol, 1850kBq/mmol) both from Amersham International p.l.c. (U.K.).

3.2.2 Animal Studies – Chronic Hypoxic Rat

Male Wistar rats of 28-30 days old (at start of experiment) were housed in a specially designed perspex hypobaric chamber (Royal Hallamshire Hospital, Sheffield). The pressure within the chamber was decreased to 550mbar; this reduced the inspired pO_2 to approximately 110mmHg (~10% equivalent). The temperature of the chamber was maintained at 21-22°C and the chamber was ventilated with air at ~451 min^{-1}. Animals were maintained in these hypoxic/hypobaric conditions for 14 days. Age-matched controls were housed under normoxic/normobaric room conditions (20% v/v oxygen). Following sacrifice the right ventricle of the heart was dissected free of the septum and left ventricle and these were blotted and weighed. PHT was assessed by measuring the ratio of right ventricle (RV)/total ventricular (TV) weight. This is a well-established index of the degree of PHT in the rats (Hunter, *et al.*, 1974). Pulmonary arteries were then dissected and taken for biochemical analysis.

3.2.3 Cell Culture

Human pulmonary artery smooth muscle cells (BioWittaker, U.K., from main and first branch PA) were maintained in smooth muscle cell growth medium (SmGM-2 bulletkit system, BioWittaker, U.K.). Following passage, flasks were split into two groups. Half the flasks were returned to the normoxic incubator (RS Biotech – Galaxy CO_2 incubator set at 5%CO_2, 95% air, humidified, set 37^{0}C), and half were transferred to the hypoxic incubator (RS Biotech – Galaxy CO_2 – oxygen control incubator 10% O_2, 5% CO_2, balanced N_2, humidified, set 37^{0}C). Cells were grown in the hypoxic incubator for 24 hours to 2 weeks to determine optimal conditions.

3.2.4 Homogenate preparation

To isolate protein from both tissue and cells, the homogenisation buffer isotonic sucrose solution (I.S.S.) was used with composition: 0.25M sucrose, 10mM Tris HCl, 1mM EDTA, 0.1mM phenylmethylsulphonyl fluoride (PMSF) and 2mM benzamidine, pH 7.4. The PA branches were initially ground to a fine powder in liquid nitrogen using a mortar and pestle, then homogenised by adding 500µl I.S.S. and passing through a 25G

syringe needle five times. After the removal of the media, cells were washed with sterile PBS, and then scraped in 600µl I.S.S. per T-75 flask. To shear the cells, the lysate was passed five times through a 25G syringe needle. Both the homogenised tissue and cells were centrifuged for 2 minutes at 12,000 rpm, and only the supernatant used in subsequent experiments.

3.2.5 Total RNA extraction

For isolation of RNA, rat pulmonary arterial branches were ground to a fine powder in liquid nitrogen with a mortar and pestle. 600µl buffer RNeasy lysis buffer was added according to manufacturers guidelines (Qiagen), and the tissue was then homogenised by passing the lysate through a 25G syringe needle five times. For isolation of RNA from hPASMC, the medium was aspirated and the cells washed with sterile PBS. The hPASMC were scraped in 600µl of RNeasy lysis buffer, then passed through a 25G syringe five times. Both the tissue and cell lysates were then pipetted onto a QIAshredder (Qiagen). Total RNA was extracted according to the RNeasy protocol instruction (Qiagen). To prevent potential contamination from genomic DNA, an incubation step with 4 units of DNase at 37^0C for 15 minutes followed by a second RNA extraction (clean up protocol, Qiagen) were included. Total RNA was eluted in RNase free H_2O and stored at -20^0C.

3.2.6 RT-PCR

First strand synthesis was carried out using 1µg total RNA catalysed by the enzyme superscript II reverse transcriptase. The reaction was primed using 500ng of oligo $(dt)_{18}$. This mixture was heated to 70°C for 10 minutes and quick chilled on ice. The reverse transcriptase reaction was incubated at 42°C for 90 minutes and terminated at 70°C for 15 minutes.

The PCR was carried out using the following protocol: initial denaturation for 5 minutes at 95^0C, 15-35 cycles of amplification (each cycle consisted of denaturation for 30 seconds at 95^0C, annealing for 30 seconds at 50^0C, and extension for 1 minute 40 seconds at 72^0C), a final extension of 10 minutes at 65^0C, and storage at 4^0C.

RT-PCR with specific with specific forward and reverse oligonucleotide primers were used to amplify PDE transcripts. The PDE3A forward primer was 5'-CTG GCC AAC CTT CAG GAA TC-3' and the reverse primer was 5'-GCC TCT TGG TTT CCC TTT CTC-3'. The PDE3B forward primer was 5'-AAT CTT GGT CTG CCC ATC AGT CC-3' and the reverse primer was 5'-TTC AGT GAG GTG GTG CAT TAG CTG-3'. The PDE5A forward primer was 5'-CGA TGC TGA TGA CAG CTT GTG ATC-3' and the reverse primer was 5'-CAA GAG CTT GCC ATT TCT GCC-3'. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) forward primer was, 5'-TGA AGG TCG GTG TCA ACG GAT TTG GC-3' and the reverse primer was, 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3'. The PDE3A and PDE3B primers were designed to amplify regions corresponding to 3011-3415 and 2902-3201 in human PDE3A and PDE3B respectively. The PDE5A primers were designed to amplify 2338-2637 in bovine PDE5A.

3.2.8. Sequence analysis

The purified amplicons were sequenced, in both directions, on a PE-Applied Biosystems Division Model 373A automated DNA sequencer using the PCR primers and a BigDye terminator cycle sequencing kit.

3.2.9 PDE Assay

The assay of PDE activity was by the two-step radiotracer method using 0.5 μM [^{3}H] cAMP or [^{3}H] cGMP according to Thompson and Appleman, 1971.

3.2.10 Western Blotting

Nitrocellulose sheets were blocked in 5% non-dried milk (w/v) in PBS plus 0.1% Tween-20 (v/v) and 0.001% thimerisol (w/v) at 4°C for 1 hour. The nitrocellulose sheets were incubated overnight at 4°C in blocking solution containing anti-PDE5 antibodies. After this time, the nitrocellulose sheets were washed in PBS plus 0.1% Tween-20 (v/v). Detection of immunoreactivity was by incubating nitrocellulose sheets for 2 hours at 37°C with a reporter HRP-linked anti-rabbit antibody in blocker. After washing the blots again as described above, to remove excess reporter antibody, immunoreactive bands were detected using an enhanced chemiluminesence detection kit.

3.2.11 Quantification

RT-PCR and Western blotting results were quantified by densitometry (linear range of optical density between 0-1 arbitrary unit).

3.2.12 Statistics

In all analysis comparisons between two groups of data were made using Student's *t*-test for paired or unpaired data where appropriate. The statistical software package Prism (Graphpad Prism, San Diego, CA, USA) was used to handle raw data, where *P<0.05 was considered to be statistically significant. In all experiments "n" either indicates the number of different animals used, or number of different populations of cultured cells.

3.3 Results

3.3.1 Chronic Hypoxic rat (CH)

The exposure of male Wistar rats to 10% O_2 for 2 weeks resulted in a significant decrease (P<0.05) in body weight from 221.3g ± 2.6 to 199.7g ± 2.4 (n=80, P<0.05, Student's *t*-test) as seen in figure 3.3.1. This is not unexpected due to a loss of appetite that occurs in the Wistar rats when they are initially exposed to hypoxia. Although the CH were slightly lighter, they always appeared healthy throughout the 2 weeks in reduced oxygen, and showed no apparent signs of anxiety, excess aggressive behaviour or change in social interaction.

The development of PHT was characterised by right ventricular hypertrophy. The ratio of right ventricular weight (RV) to total ventricular weight (TV) was used as an index of right ventricular hypertrophy. RV/TV ratios were 0.202 ± 0.001 and 0.336 ± 0.006 for normoxic and hypoxic rats respectively (figure 3.3.2, n=80, P<0.05, Student's *t*-test). As the RV/TV ratio is significantly increased with hypoxia this confirmed that right ventricular hypertrophy had occurred, and hence pulmonary hypertension had developed in the rats exposed to hypoxia for 2 weeks. Throughout this study "hypoxic" refers to rats subjected to 14 days of chronic hypoxia.

3.3.2 Linear amplification of PDE3A, PDE3B, PDE5A, and G3PDH transcripts by RT-PCR

In order to perform semi-quantitative RT-PCR, each gene transcript of interest had to be amplified at various cycle lengths (conditions as in 2.2.2.4). A cycle length at which all the transcripts did not show maximal amplification, was used in all subsequent reactions (e.g. linear amplification conditions was used). Non-maximal amplification allows any changes in transcript to be detected. At 25 cycles none of the transcripts showed maximal amplification (figure 3.3.3). Hence, 25 cycles were used in all subsequent RT-PCR reactions. First branch pulmonary arteries were used in these experiments, as more were readily available.

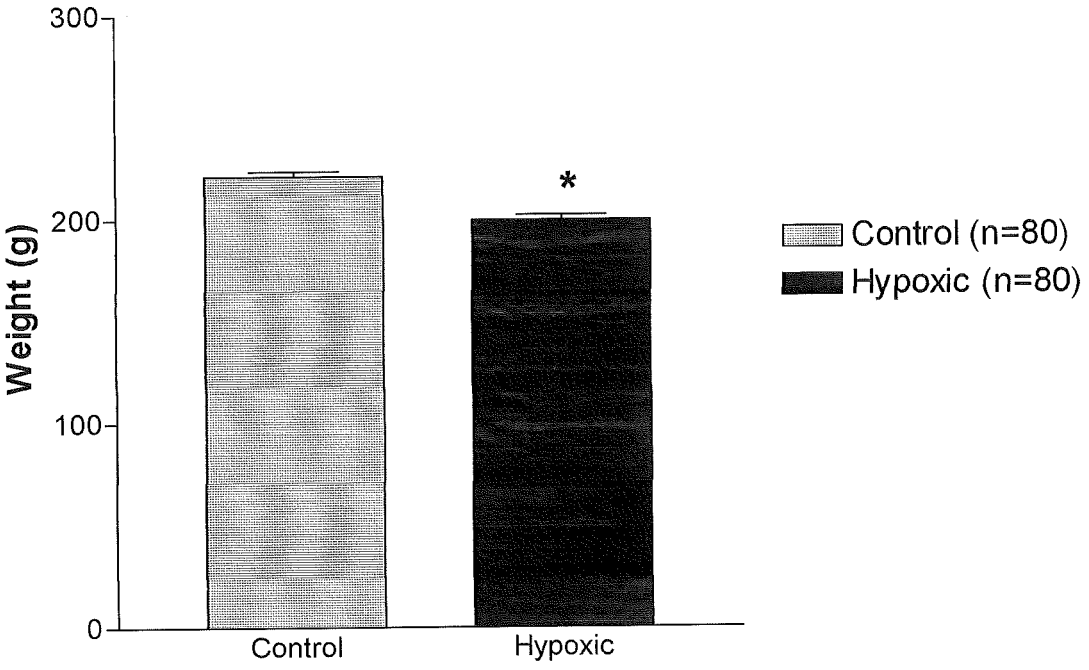


Figure 3.3.1. The effect of chronic hypoxia on mean Wistar rat weight

As in 2.2.1.1, Wistar rats were maintained under hypoxic conditions (pO_2 of ~110mmHg [~10% equivalent]) for 2 weeks (hypoxic). Age match controls were housed in normoxic/normobaric room conditions also for 2 weeks (control). All animals were weighed before dissection. Data are expressed in the above histogram as mean weight in grams ± s.e.m (n=80). * Denotes the data are significantly different ($P<0.05$, by Student's *t*-test)

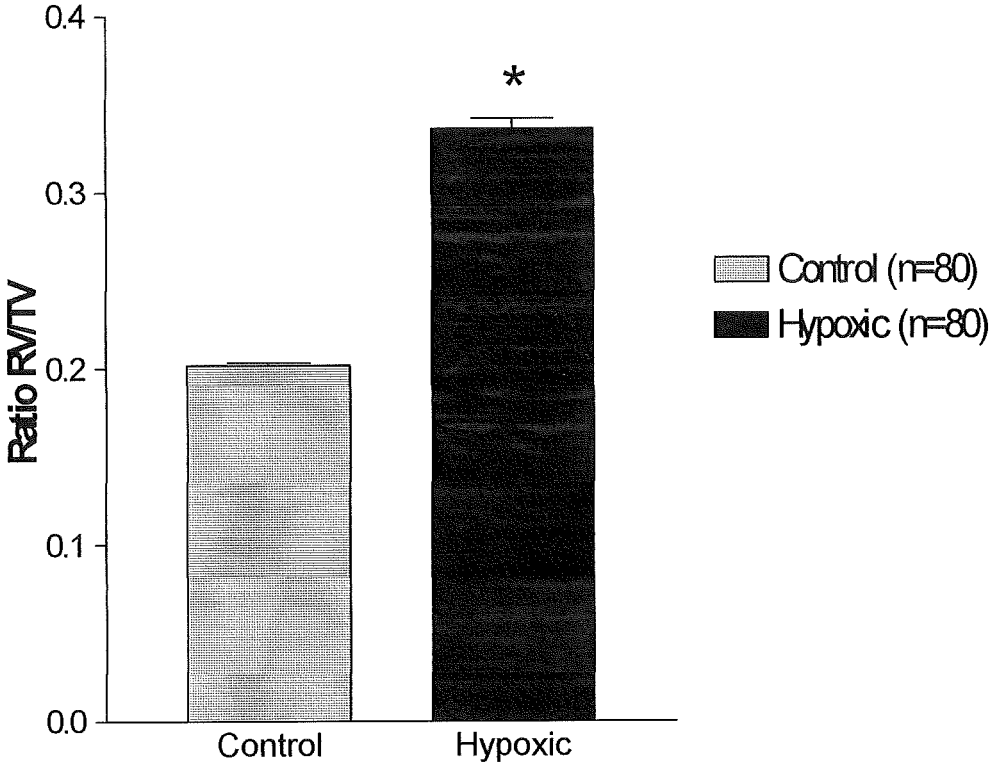


Figure 3.3.2. The effect of chronic hypoxia on right ventricular hypertrophy

Pulmonary hypertension is characterised by the ratio of right ventricular weight (RV) to total ventricular weight (TV), which is an index of right ventricular hypertrophy (see 2.2.1.1.2.). The data in the histogram above are expressed as the ratio of RV/TV ± s.e.m (n=80) from Wistar rats maintained under normoxic (control) and chronic hypoxic conditions (hypoxic). * Denotes the data are significantly different ($P<0.05$, by Student's *t*-test)

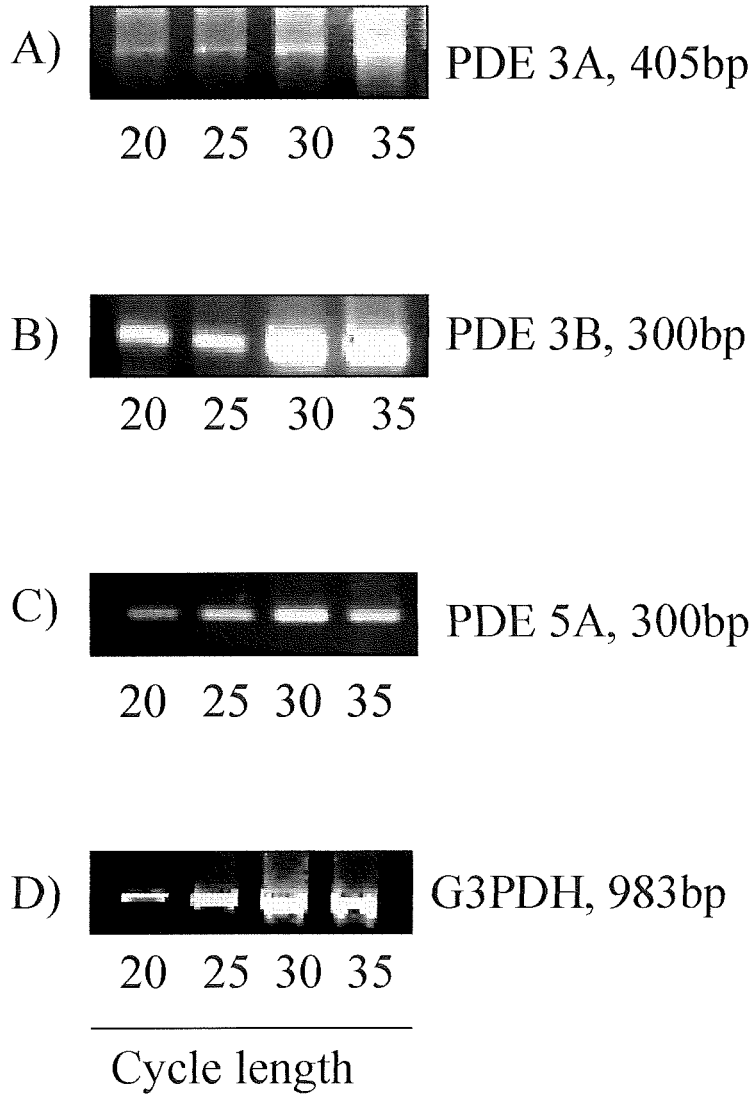


Figure 3.3.3. RT-PCR of the linear amplification of PDE3A, PDE3B, PDE5A, and G3PDH transcripts in rat control first branch pulmonary arteries

RT-PCR amplification using specific primers, as described in 2.2.2.4, of: (A) PDE3A, 405bp; (B) PDE3B, 300bp; (C) PDE5A, 300bp; and (D) G3PDH, 983bp from control rat first branch pulmonary arteries. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Each reaction was carried out using 20, 25, 30, and 35 cycles of amplification. Above is a representative result of 3 individual experiments, quantified by densitometry.

3.3.3 Effect of hypoxia on PDE3A, 3B transcript levels in rat PA

Figures 3.3.3 and 3.3.4 show the RT-PCR amplification (25 cycles) of PDE3A (405bp product) and PDE3B (300bp product) from the main PA, first branch PA, intrapulmonary PA and resistance vessels of both normoxic and hypoxic rats. With chronic hypoxia it can clearly be seen from figure 3.3.4 that both PDE3A and PDE3B transcript increased in all of the pulmonary arterial branches studied. The % increase in PDE3A and PDE3B transcripts in hypoxic *versus* normoxic rats were: main branch, PDE3A, 32. ± 10%; PDE3B, 44 ± 11%; first branch, PDE3A, 75 ± 18%; PDE3B, 78 ± 16%; intrapulmonary, PDE3A, 86% ± 4%; PDE3B, 37 ± 12%; resistance vessels, PDE3A, 90 ± 21%; PDE3B, 48 ± 4%, (n=4, $P<0.05$ *versus* normoxic animals, Student's *t*-test).

Data can also be expressed as a ratio of G3PDH. The PD3A/G3PDH transcript ratio in normoxic and hypoxic animals respectively were: main branch, 1.03 ± 0.02, 1.47 ± 0.08; first branch, 1.01 ± 0.04, 1.92 ± 0.11; intrapulmonary, 1.03 ± 0.05, 2.07 ± 0.2; resistance vessels, 0.91 ± 0.03; 1.92 ± 0.2 (n=4, $P<0.05$ *versus* normoxic animals, Student's *t*-test). The PD3B/G3PDH transcript ratio in normoxic and hypoxic animals respectively were: main branch, 1.02 ± 0.04, 1.6 ± 0.1; first branch, 1 ± 0.08, 1.95 ± 0.09; intrapulmonary, 1.01 ± 0.06, 1.52 ± 0.11; resistance vessels, 0.99 ± 0.05; 1.2 ± 0.02 (n=4, $P<0.05$ *versus* normoxic animals, Student's *t*-test). Alignment of the PDE3A (405bp) and PDE3B (300bp) with the corresponding human PDE3A and PDE3B (either from the published sequence or the sequence obtained experimentally using the hPASMC) can be seen in figures 3.3.8 and 3.3.9, revealed 90% and 92% similarity in their nucleotide sequences respectively. The enhanced transcription of both PDE3A/B seen with chronic hypoxia in all the PA branches may explain the increased PDE3 activity that had previously been shown to occur under hypoxic conditions in these vessels (MacLean *et al.*, 1997).

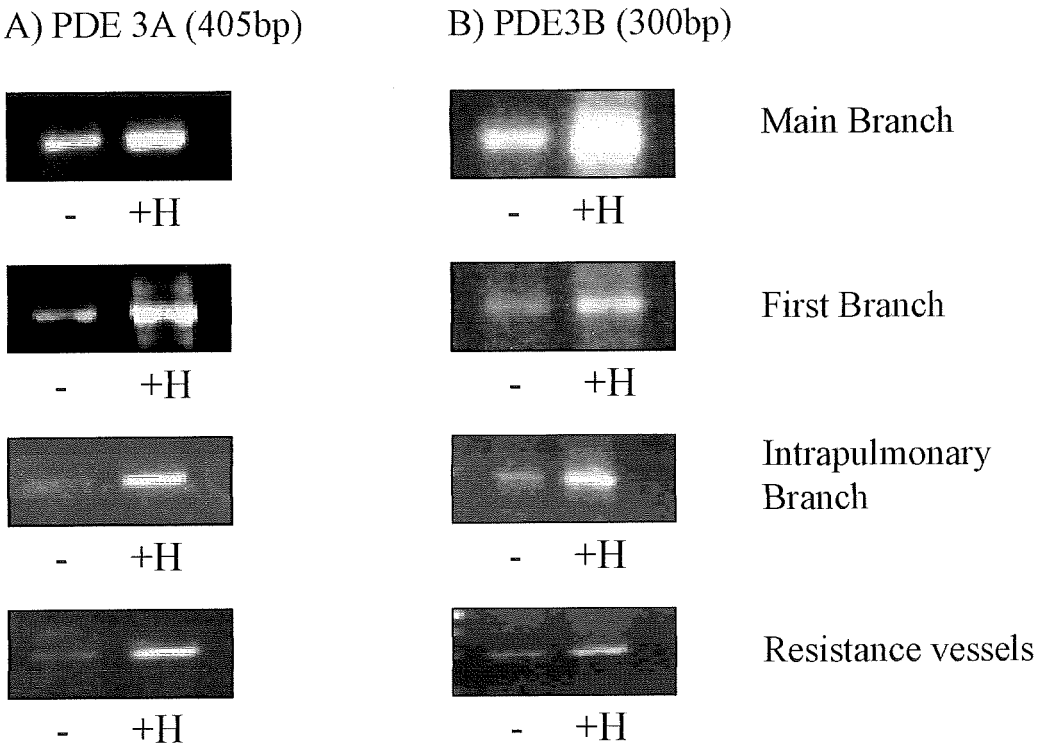


Figure 3.3.4. RT-PCR of PDE3A and PDE3B transcripts from control and hypoxic rat pulmonary arterial branches

RT-PCR with specific primers, as described in 2.2.2.4, for (A) PDE3A and (B) PDE3B from main, first branch, intrapulmonary, and resistance vessels from Wistar rats maintained under normoxic (-) and chronic hypoxic (+H) conditions. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 4 individual experiments, quantified by densitometry.

3.3.4 Effect of hypoxia on PDE5A transcript and protein levels in rat PA

A product of 300bp was amplified (25 cycles) from the main, first branch, intrapulmonary and resistance vessels by RT-PCR using PDE5A specific primers, therefore confirming its expression (figures 3.3.3 and 3.3.5A). The PDE5A primers used are common for all PDE5A subtypes. Chronic hypoxia only enhanced the PDE5A transcript in the main and first branch PAs, increasing 135 ± 24% and 150 ± 11% respectively (n=4, $P<0.05$ *versus* normoxic animals, Student's *t*-test). In contrast, no change in PDE5A transcript was seen in the intrapulmonary or resistance vessels with chronic hypoxia. The % change in the PDE5A transcript in hypoxic versus normoxic rats in the intrapulmonary and resistance vessels were non significant: -1 ± 5%, and –3 ± 5% respectively (n=4, $P<0.05$ *versus* normoxic animals, Student's *t*-test). The PD5A/G3PDH transcript ratio in normoxic and hypoxic animals respectively were: main branch, 1 ± 0.02, 1.4 ± 0.04; first branch, 1.01 ± 0.05, 1.65 ± 0.05 (n=4, $P<0.05$ *versus* normoxic animals, Student's *t*-test). The PD5A/G3PDH transcript ratio in normoxic and hypoxic animals respectively were: intrapulmonary, 1.02 ± 0.04, 1.01 ± 0.05; resistance vessels, 0.97 ± 0.04; 0.98 ± 0.11 (n=4, NS *versus* normoxic animals, Student's *t*-test). Alignment of the PDE5A (300bp) with the corresponding bovine and human PDE5A (either from the published bovine sequence or the sequence obtained experimentally using the hPASMC) can be seen in figure 3.3.10, all revealed 92% similarity in their nucleotide sequences.

As protein levels may not correspond with changes in mRNA levels, and antibodies for PDE5A are commercially available (Calbiochem-Novabiochem), Western blotting was carried out as using homogenates from the main, first branch, intrapulmonary and resistance vessels. PDE5A2 (M_r=93kDa) was expressed in both the main pulmonary artery and the first branch pulmonary artery (figure 3.3.6A). The levels of PDE5A2 in the first branch pulmonary artery were consistently low, and barely detectable unless Western blots were heavily over-expressed. The percentage increase in PDE5A2 protein expression was 94 ± 22% in the main PA, and 176 ± 17% in the first branch PA (n=3, $P<0.05$ *versus* normoxic animals, Student's *t*-test). These results suggest that the increased PDE5 activity that was observed in the first branch PA by MacLean *et al.* (1997), maybe due to increased protein expression of PDE5A2 as a result of increased mRNA transcription. MacLean *et al.* (1997), did not report a significant increase in

PDE5 activity with hypoxia in the main PA, which does not correlate with the increased PDE5A transcript and protein shown in these experiments.

PDE5A2 and the slightly heavier isoform PDE5A1 (Mr=98kDa) were both detected by Western blot in the intrapulmonary and resistance vessel homogenates (figure 3.3.6B-C). The % change in both the PDE5A1 and PDE5A2 protein expression in the intrapulmonary (-4 ± 3, -2 ± 3) and resistance vessels were (4 ± 4, 1 ± 8) respectively (n=3, NS, *versus* normoxic animals, Student's *t*-test). Chronic hypoxia did not modulated PDE5A1/PDE5A2 protein vessels in the intrapulmonary and resistance vessels. Therefore, the hypoxic dependent increase in PDE activity in the intrapulmonary arteries seen by MacLean *et al.* (1997), cannot be explained by increases in either PDE5A protein or mRNA.

3.3.5 Controls in all RT-PCR reactions

3.3.5.1 Effect of hypoxia on G3PDH transcript levels in rat PA

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is a glycolytic enzyme, which was used as an internal control for RT-PCR to verify the loading of equal total RNA. A product of 983bp was amplified (25 cycles) from the main, first branch, intrapulmonary and resistance vessels by RT-PCR using G3PDH specific primers, confirming its expression. No change in G3PDH transcript level was observed under normoxic and hypoxic conditions from each vessel studied (figure 3.3.5B). The percentage change in G3PDH transcripts from hypoxic versus normoxic rats were: main branch, -1 ± 2%; first branch, -1 ± 1%; intrapulmonary arteries, -1 ± 1%; resistance vessels; 3 ± 6% (n=4, NS *versus* normoxic animals, Student's *t*-test). As no significant differences could be seen in the levels of G3PDH this confirms that equal amounts of total RNA had been used for the amplification of the PDE3A/3B and PDE5 transcripts.

3.3.5.2 Verify removal of genomic DNA

In order to verify that the results obtained using RT-PCR are not due contamination/cross-over of genomic DNA, a negative control was included during DNA synthesis. This involved a separate cDNA reaction containing RNA and all other reagents except the reverse transcriptase. PCR reactions using this minus reverse transcriptase control as a template were always run in parallel with RT-PCR reactions

using the experimental cDNA samples. The generation of a PCR product from this negative control indicated the presence of genomic DNA contamination. If genomic DNA was found in the "minus reverse transcriptase" PCR, the parallel RT-PCR results were discarded.

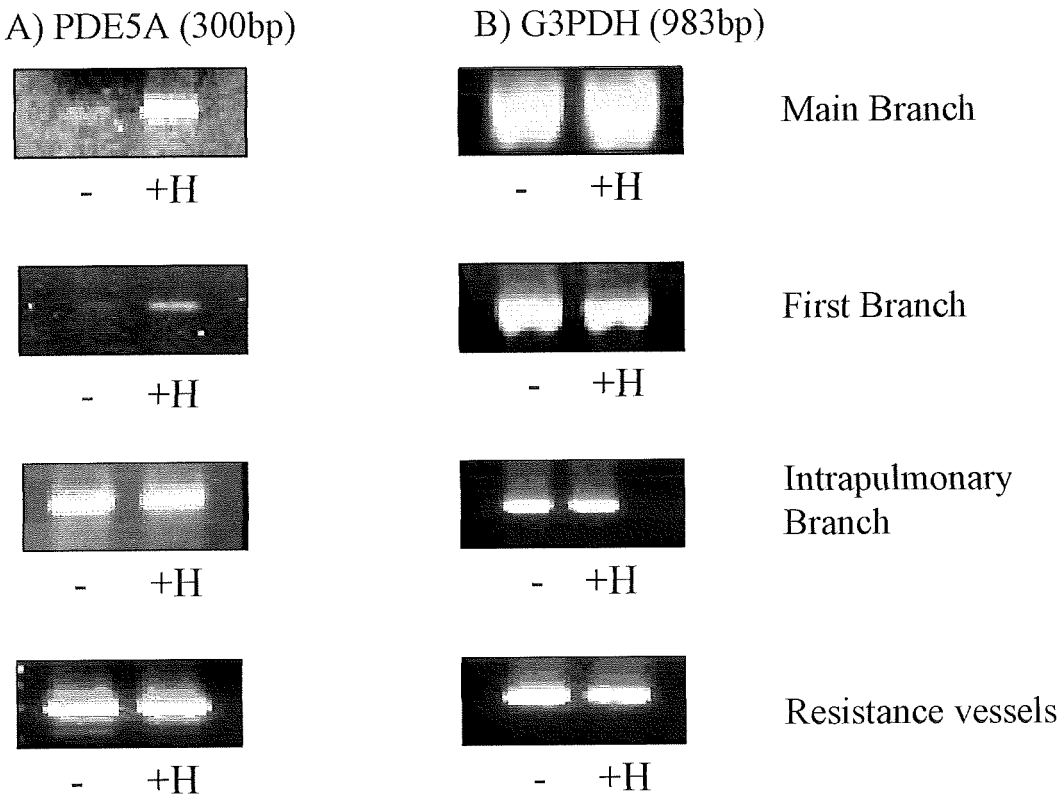


Figure 3.3.5 RT-PCR of PDE5A and G3PDH transcripts from control and hypoxic rat pulmonary arterial branches

RT-PCR with specific primers as described in 2.2.2.4, for (A) PDE5A and (B) G3PDH from main, first branch, intrapulmonary, and resistance vessels from Wistar rats maintained under normoxic (-) and chronic hypoxic (+H) conditions. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 4 individual experiments, quantified by densitometry

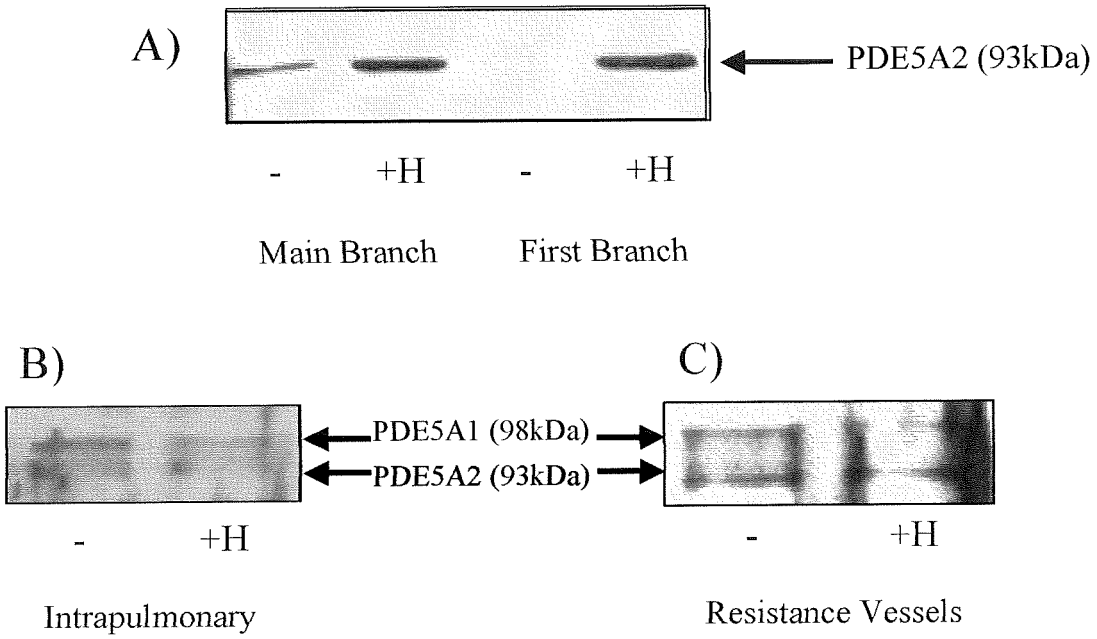


Figure 3.3.6. Western blot analysis of PDE5A from control and hypoxic rat pulmonary arterial branches

Western blot analysis using an anti-PDE5A antibody, as described in 2.2.3.3-2.2.3.5, showing the expression level of PDE5A1 and PDE5A2 in homogenates from main, first branch, intrapulmonary and resistance vessels from rat maintained under normoxic (-) and chronic hypoxic (+H) conditions. 10μg protein/sample were loaded onto SDS-PAGE. Above is a representative result of 3 individual experiments, quantified by densitometry.

3.3.6 Development of a model of PHT using cultured human pulmonary smooth muscle cells (hPASMCs)

Development of a cellular model allows easier biochemical manipulation and analysis. Initially, gene transcripts of interest were subjected to various cycle lengths (conditions as in 2.2.2.4) to show linear amplification. Cycle lengths showing linear amplification, were used in all subsequent reactions. From figure 3.3.7 it can be seen that none of the transcripts show maximal amplification at 25 cycles, hence this cycle length was used in all subsequent RT-PCR.

As with the all RT-PCRs performed, parallel reactions for the housekeeping gene G3PDH verified equal loading of total mRNA. Negative control reactions in which reverse transcriptase was omitted were also performed to ensure that the RT-PCR product was the result of the amplification of the cDNA template. If contamination of genomic DNA was found in the PCR reaction of the negative control, the corresponding RT-PCR results were not used.

Figure 3.3.7 shows that hPASMC express PDE3A, PDE3B, PDE5A and G3PDH. hPASMC were subjected to 10% O_2 for 6hours, 24 hours, 3 days, 7 days, and 14 days. As seen from figure 3.3.11 no significant differences were observed in any of the transcripts, after 6 hours, 24 hours, 3 days, and 7 days (densitometry not shown). However, after 14 days of sustained hypoxia PDE3A, but not PDE3B, PDE5A or G3PDH transcript level increased (figure 3.3.12). The percentage increase in PDE3A transcript in chronic hypoxic versus normoxic hPASMC was 90 ± 12% (n=3, $P<0.05$ *versus* normoxic hPASMC, Student's *t*-test). The PD3A/G3PDH transcript ratio in normoxic and hypoxic hPASMC respectively were: 1.01 ± 0.06, 2 ± 0.1 (n=3, $P<0.05$ *versus* normoxic, Student's *t*-test). The corresponding changes in PDE3B, PDE5A, and G3PDH transcript levels with chronic hypoxia versus normoxia in hPASMC were 0 ± 4%, 0 ± 1% and –2 ± 1% respectively (n=3, NS, *versus* normoxic hPASMC, Student's *t*-test). For PDE3B and PDE5A, the ratios to G3PDH were: PDE3B/G3PDH, 1.01 ± 0.05% and 1 ± 0.07%, PDE5A/G3PDH, 1 ± 0.03 and 0.99 ± 0.05 respectively (n=3, NS, *versus* normoxic hPASMC, Student's *t*-test). Alignment of the PDE3A, PDE3B, and PDE5A (300bp) with the corresponding published sequence to which the primers were designed against and the rat PA sequence (obtained experimentally) can be seen in

figures 3.3.8, 3.3.9, and 3.3.10, all revealed ≥90% similarity in their nucleotide sequences.

The increase in PDE3A transcript levels with hypoxia could be correlated with an ~1.5 fold increase in total cAMP PDE activity, measured in tissue homogenates at 0.5μM cAMP (47 ± 7%, n=3, P<0.05 versus normoxic hPASMC, Student's *t*-test, figure 3.3.14A). On the addition of SKF94836 (10μM) to the PDE assay, the increased total cAMP PDE activity was substantially reduced to 15 ± 25% (n=3, P<0.05 versus normoxic hPASMC, Student's *t*-test, figure 3.3.14A). Figure 3.3.14 shows there a 2.57 fold increase in SKF94836 sensitive PDE3 activity in response to chronic hypoxia (control *versus* hypoxic: 7.9 ± 2.5 pmol min mg^{-1} protein *versus* 20.3 ± 1.4 pmol min mg^{-1} protein, n=3, P<0.05, *versus* normoxic hPASMC, Student's *t*-test, figure 3.3.14B). Data showing that chronic hypoxia has no effect on PDE3B transcript levels suggests that the increase in PDE activity is attributed to PDE3A, since it was substantially reduced by addition of the PDE3-selective inhibitor SKF94836 (10μM) to the PDE assay. The Ki for PDE3 inhibition by, SKF94836, is approximately 2μM (Murray *et al.*, 1990). 10μM was used to ensure complete inhibition of PDE3. It is accepted that at this concentration SKF94836 is highly selective for PDE3.

Not only was PDE5A transcript unaffected with chronic hypoxia, neither was protein expression (figure 3.3.13) or cGMP PDE activity, measured at 0.5μM cGMP (figure 3.3.15). Western blot analysis, showed that the PDE5A2 isoform was expressed in hPASMCs, however chronic hypoxic exposure resulted in no significant increase in PDE5A2 protein (2 ± 3%, n=3, NS *versus* normoxic hPASMC, Student's *t*-test). The cGMP PDE activity measured at 0.5μM cGMP (2.2.3.7) was also unaltered in hPASMC treated with chronic hypoxia (9.9 ± 11.1%, n=4, NS *versus* normoxic hPASMC, Student's *t*-test, figure 3.3.15). High variability in the cGMP PDE activity measurements is shown by the large standard error.

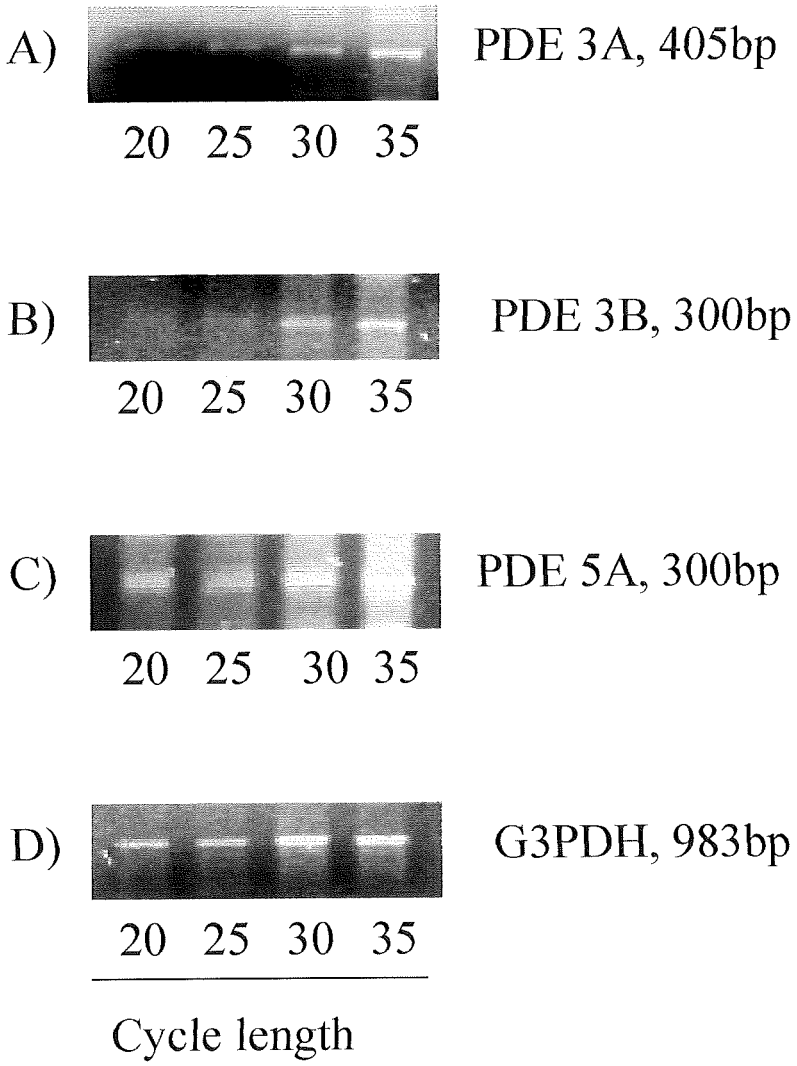


Figure 3.3.7. RT-PCR of the linear amplification of PDE3A, PDE3B, PDE5A, and G3PDH transcripts in hPASMC

RT-PCR amplification using specific primers, as described in 2.2.2.4, of: (A) PDE3A, 405bp; (B) PDE3B, 300bp; (C) PDE5A, 300bp; and (D) G3PDH, 983bp from control hPASMC. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Each reaction was carried out using 20, 25, 30, and 35 cycles of amplification. Above is a representative result of 3 individual experiments, quantified by densitometry.

```
HS PDE3A : CTGGCCAACCTTCAGGAATCCTTCATCTCTCACATTGTGGGGCCT
hPASMC   : CTGGCCAACCTTCAGGAATCCTTCATCTCTCACATTGTGGGGCCT
Rat      : CTGGCCAACCTTCAGGAATCCTCCATCTCTCACATTGTGGGGCCG

HS PDE3A : CTGTGCAACTCCTATGATTCAGCAGGACTAATGCCTGGAAAATGG
hPASMC   : CTGTGCAACTCCTATGATTCAGCAGGACTAATGCCTGGAAAATGG
Rat      : CTGTGCAACTCCTATGACTCAGCAGGACTCATGCCAGGAAAGTGG

HS PDE3A : GTGGAAGACAGCGATGAGTCAGGAGATACTGATGACCCAGAAGAA
hPASMC   : GTGGAAGACAGCGATGAGTCAGGAGATACTGATGACCCAGAAGAA
Rat      : GTTGAAGACAGCGATG AATCGGGAGATACTGATGACCCCGAAGAG

HS PDE3A : GAGGAGGAAGAAGCACCAGCACCAAATGAAGAGGAAACCTGTGAA
hPASMC   : GAGGAGGAAGAAGCACCAGCACCAAATGAAGAGGAAACCTGTGAA
Rat      : GAAGAGGAAGAAGCTACAGTGGTGAATGAAGAAGAAACCTGTGAA

HS PDE3A : AATAATGAATCTCCAAAAAAGAAGACTTTCAAAAGGAGAAAAATC
hPASMC   : AATAATGAATCTCCAAAAAAGAAGACTTTCAAAAGGAGAAAAATC
Rat      : AATGATGAATCCCTAAAAAAGAAAACTTTTGAAAGGAGGAAAATC

HS PDE3A : TACTGCCAAATAACTCAGCACCTCTTACAGAACCACAAGATGTGG
hPASMC   : TACTGCCAAATAACTCAGCACCTCTTACAGAACCACAAGATGTGG
Rat      : TACTGCCAAATAACTCAGCACCTCCTACAGAACCATAAGATGTGG

HS PDE3A : AAGAAAGTCATTGAAGAGGAGCAACGGTTGGCAGGCATAGAAAAT
hPASMC   : AAGAAAGTCATTGAAGAGGAGCAACGGTTGGCAGGCATAGAAAAT
Rat      : AAGAAAGTCATTGAAGAGGAGCAGCGATTGACCGGCAT TGGAAAT

HS PDE3A : CAATCCCTGGACCAGACCCCTCAGTCGCACT CTTCAGAACAGATC
hPASMC   : CAATCCCTGGACCAGACCCCTCAGTCGCACT CTTCAGAACAGATC
Rat      : CCAGGCCTGGACCAGTCTGCTCAGACGCACACCTCAGAGCAAATC

HS PDE3A : CAGGCTATCAAGGAAGAAGAAGAAGAGAAAGGGAAACCAAGAGGC
hPASMC   : CAGGCTATCAAGGAAGAAGAAGAAGAGAAAGGGAAACCAAGAGGC
Rat      : CAGGCTATCAAGGAAGAAGAAGAAGAGAAAGGGAAACCAAGAGGC
```

Figure 3.3.8.PDE3A cDNA sequence

PDE3A cDNA sequence from rat pulmonary arteries and cultured human pulmonary smooth muscle cells aligned with corresponding human PDE3A to which the primers were designed to (using the primers as in 2.2.2.4). The conserved amino acids across all three sequences are shaded black.

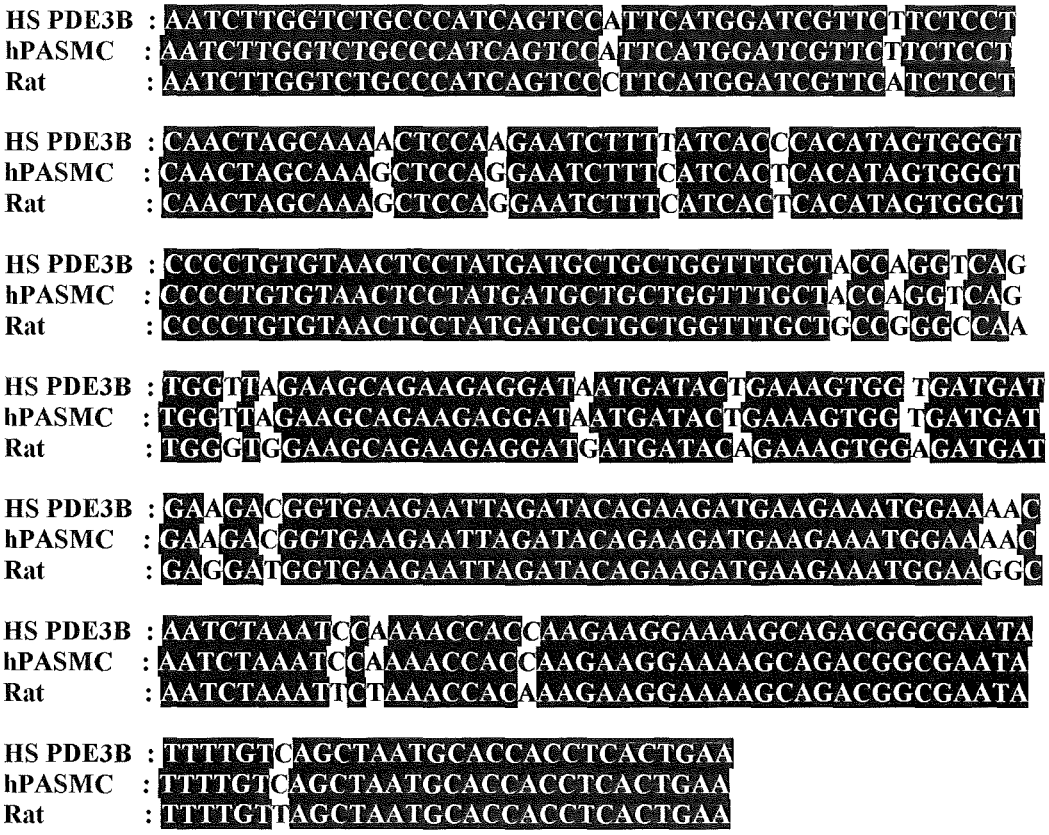


Figure 3.3.9.PDE3B cDNA sequence

PDE3B cDNA sequence from rat pulmonary arteries and cultured human pulmonary smooth muscle cells aligned with corresponding human PDE3B to which the primers were designed to (using the primers as in 2.2.2.4). The conserved amino acids across all three sequences are shaded black.

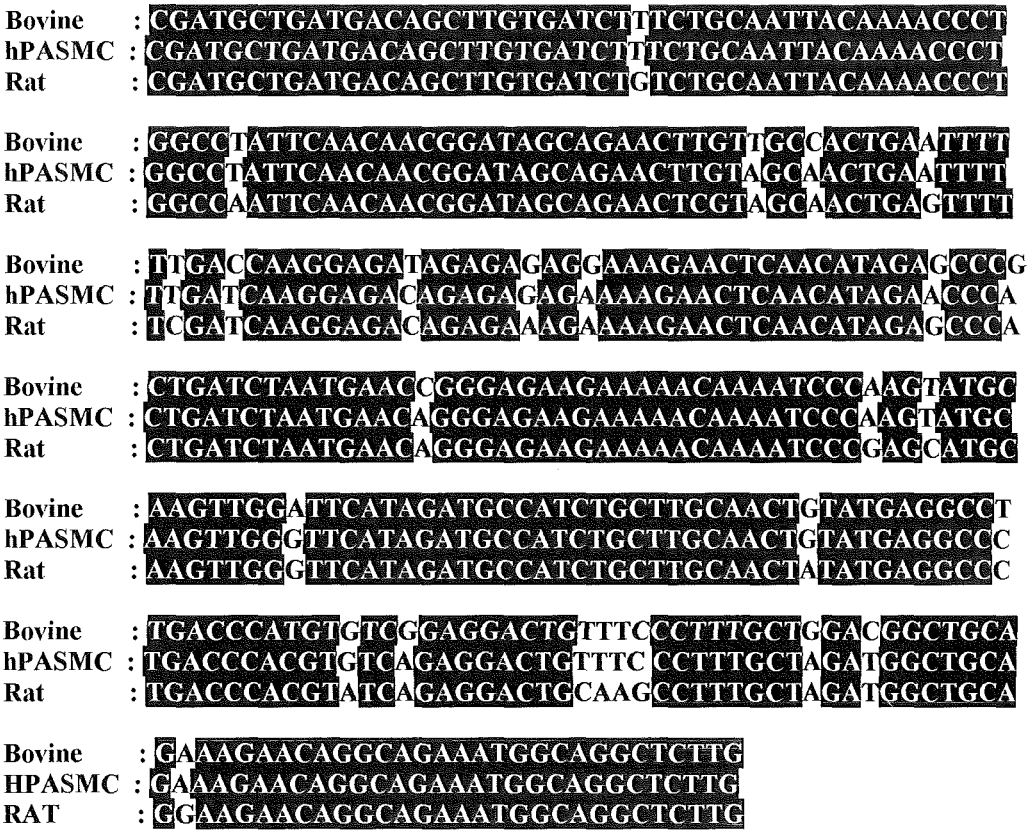


Figure 3.3.10 PDE5A cDNA sequence

PDE5A cDNA sequence from rat pulmonary arteries and cultured human pulmonary smooth muscle cells aligned with corresponding bovine PDE5A to which the primers were designed to (using the primers as in 2.2.2.4). The conserved amino acids across all three sequences are shaded black.

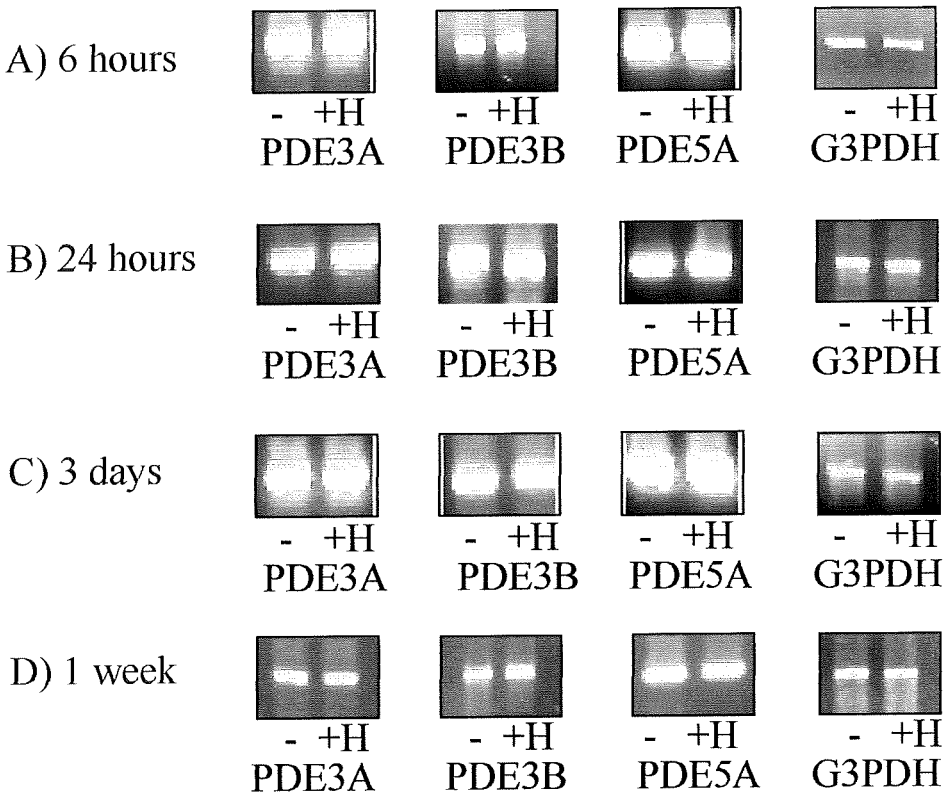


Figure 3.3.11 RT-PCR of PDE3A, PDE3B, PDE5A, and G3PDH transcripts in hPASMC exposed to hypoxia (10% O2) or normoxia for 6 hours, 24 hours, 3 days, and 7 days

RT-PCR amplification using specific primers as described in 2.2.2.4. of: PDE3A, 405bp; PDE3B, 300bp; PDE5A, 300bp; and G3PDH, 983bp from hPASMC maintained under normoxic (-) and chronic hypoxic (+H) conditions for (A) 6 hours, (B) 24 hours, (C) 3 days, or (D) 7 days. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 3 individual experiments, quantified by densitometry.

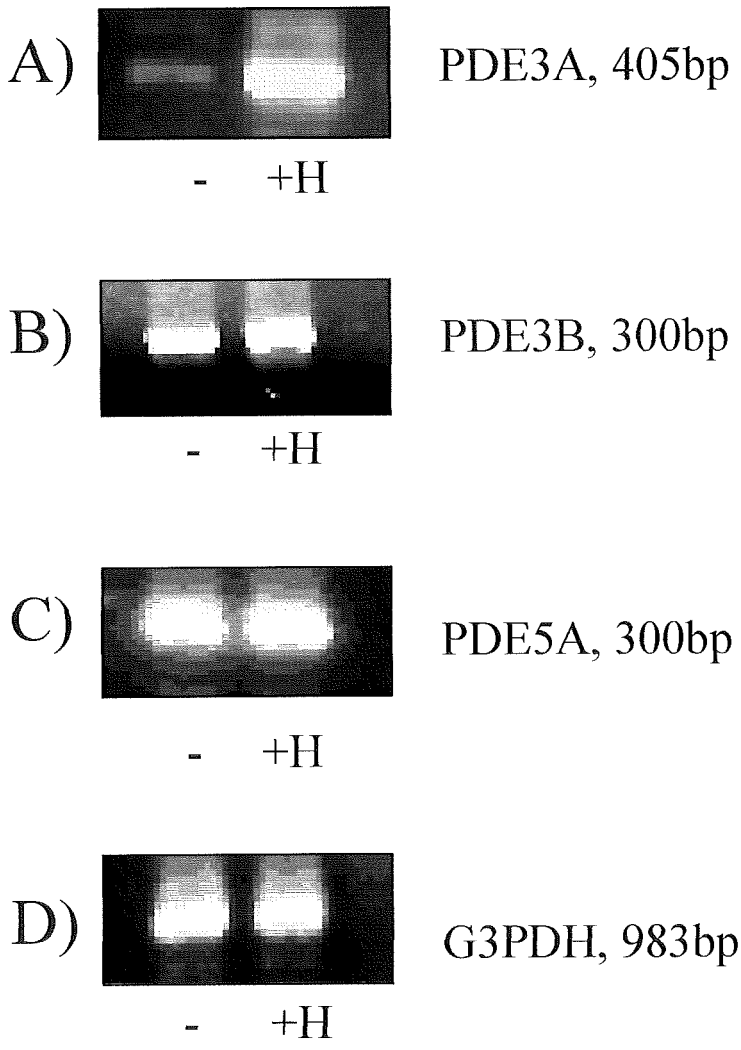


Figure 3.3.12 RT-PCR of PDE3A, PDE3B, PDE5A, and G3PDH transcripts in hPASMC exposed to hypoxia (10% O2) or normoxia for 14 days

RT-PCR amplification using specific primers, as described in 2.2.2.4. of: (A) PDE3A, 405bp; (B) PDE3B, 300bp; (C) PDE5A, 300bp; and (D) G3PDH, 983bp from hPASMC maintained under normoxic (-) and chronic hypoxic (+H) conditions for 14 days. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 3 individual experiments, quantified by densitometry.

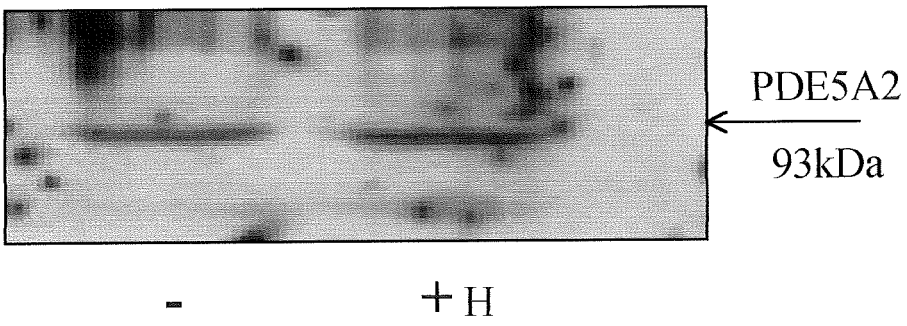
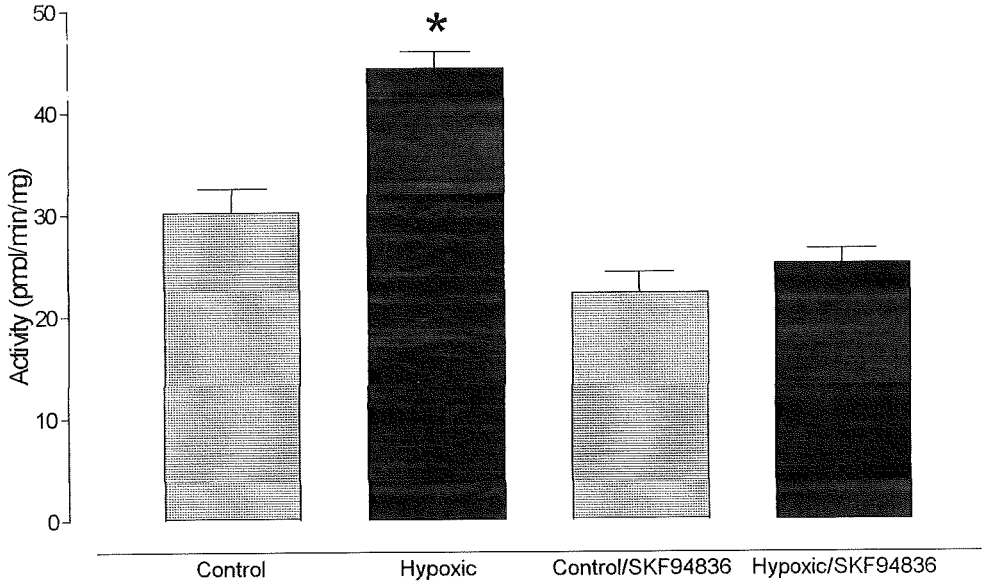


Figure 3.3.13 Western blot analysis of PDE5A from control and hypoxic hPASMC

Western blot analysis using an anti-PDE5A antibody, as described in 2.2.3.3, showing the expression level of PDE5A1 and PDE5A2 in homogenates from hPASMC maintained under normoxic (-) and chronic hypoxic (+H) conditions for 14 days. 10μg protein/sample were loaded onto SDS-PAGE. Above is a representative result of 3 individual experiments, quantified by densitometry.

A)



B)

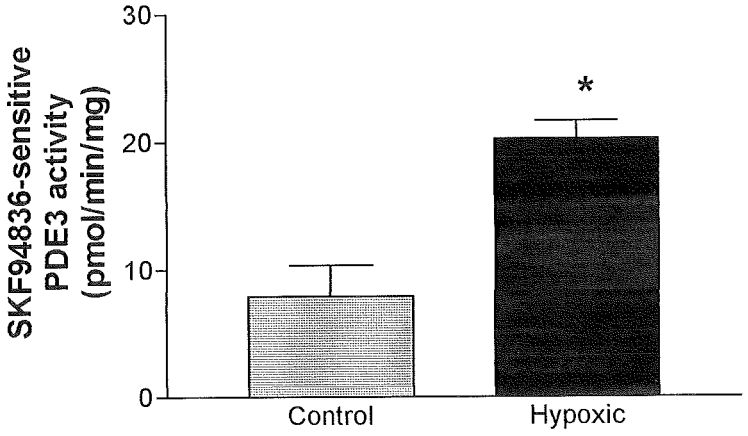


Figure 3.3.14 Total cAMP PDE activity in the presence and absence of SKF94836 from hPASMC after prolonged exposure to hypoxia

A) Histogram showing the increase in cAMP activity (PDE assay described in 2.2.3.7), in the presence and absence of the PDE3 inhibitor 10μM SKF94836, in cells treated with normoxic (control) and hypoxic (hypoxic) conditions. B) Histogram showing the hypoxic-dependant increase in SKF94836-sensitive PDE3 activity. All results are means ± s.d, expressed as pmol/min/mg, * denotes the data are significantly different (n=3 separate cell preparations, $P<0.05$, by Student's *t*-test).

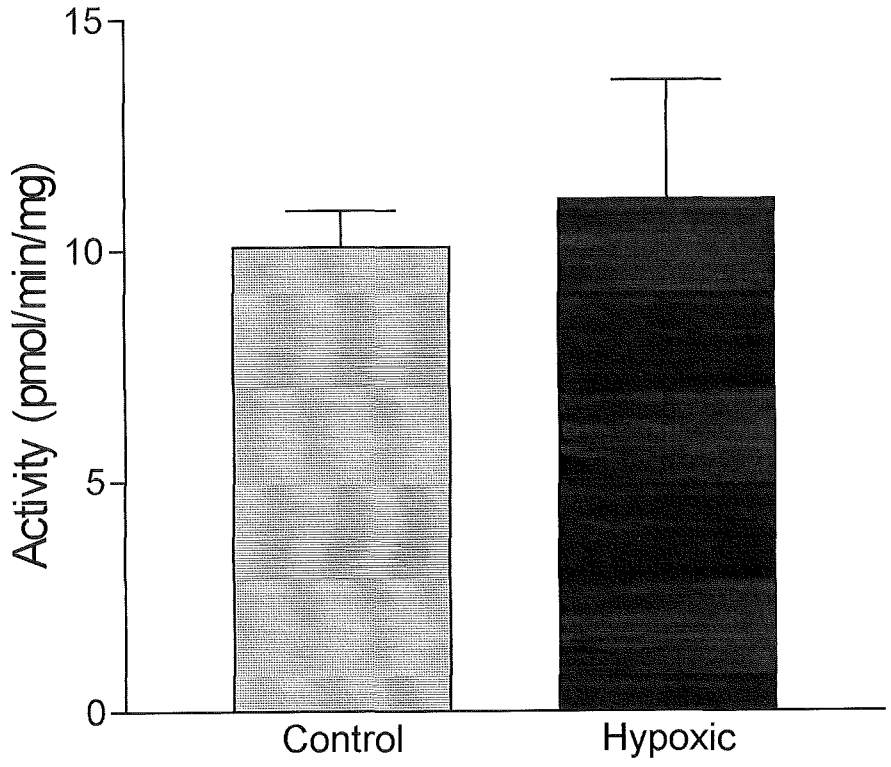


Figure 3.3.15 Total cGMP PDE activity from hPASMC after prolonged exposure to hypoxia

Histogram showing the increase in cGMP activity (PDE assay described in 2.2.3.7), in cells treated with normoxic (control) and hypoxic (hypoxic) conditions. Results are means ± s.d, expressed as pmol/min/mg (n=3 separate cell preparations, NS, by Student's *t*-test).

3.3.7 Pathways involved in the regulation of PDE3A/B and PDE5A

3.3.7.1 Role of the cAMP pathway in regulating PDE3A/B and PDE5A

The mechanism underlying the hypoxic-dependant increase in PDE3A expression in the hPASMCs was investigated. The membrane permeable analogue of cAMP, 8-Br-cAMP and the protein kinase A (PKA) inhibitor H-8 were both used to study the role of the cAMP pathway in regulating PDE3A expression in hPASMC. Basal cAMP levels in most cells are roughly 1μM, and the threshold activation for PKA is approximately 10μM (Houslay and Milligan, 1997), therefore 100μM 8-Br-cAMP is an appropriate concentration to activate PKA. hPASMC were initially treated with 100μM 8-Br-cAMP for 24 hours. Figure 3.3.16 shows that 8-Br-cAMP mimics the effect of hypoxia by increasing PDE3A transcript level (50 ± 8%, n=3, $P<0.05$ *versus* control hPASMC, Student's *t*-test), but not PDE3B (3 ± 2%, n=3, NS *versus* control hPASMC, Student's *t*-test), PDE5A (0 ± 2%, 0 ± 2%, n=3, NS *versus* control hPASMC, Student's *t*-test), or G3PDH (0 ± 2%, n=3, NS *versus* control hPASMC, Student's *t*-test). The ratios to G3PDH were: PDE3A/G3PDH, 0.99 ± 0.03% and 1.5 ± 0.06%; PDE3B/G3PDH, 0.99 ± 0.04% and 1 ± 0.02%; PDE5A/G3PDH, 0.98 ± 0.07% and 0.98 ± 0.11%, respectively (n=3, Br-cAMP treated *versus* control hPASMC, $P<0.05$ for PDE3A/G3PDH only, Student's *t*-test).

An increase in total cAMP PDE activity by 95 ± 42% (n=3, $P<0.05$ *versus* control hPASMC, Student's *t*-test), which was reduced by 10μM SKF94836 to 7 ± 15% (n=3, $P<0.05$ *versus* normoxic hPASMC, Student's *t*-test) was also seen with the treatment of cells with 8-Br-cAMP (figure 3.3.17A). The increase in PDE3A transcript was therefore correlated with a 1.8 fold increase in total cAMP PDE activity measured at 0.5μM cAMP, which was completely ablated by addition of the type-selective PDE3 inhibitor, SKF94836, to the PDE assay. There was a 2.41 fold increase in SKF94836-sensitive PDE3 activity in response to Br-cAMP (control versus Br-cAMP-treated, 9.8 ± 0.3 pmol min mg^{-1} protein *versus* 23.6 ± 1 pmol min mg^{-1} protein, n=3, $P<0.05$, *versus* control hPASMC, Student's *t*-test, figure 3.3.17B). As Br-cAMP has no effect on PDE3B transcript levels and as SKF94836 abolishes the increase in PDE activity, this suggests that the increase in response to Br-cAMP can be attributed to PDE3A.

To further investigate the cAMP pathway 50μM H8 peptide (N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide. 2HCL) was added to the cells under normoxic and hypoxic culture conditions for 2 weeks. H8 peptide attenuated the hypoxic dependent increase in PDE3A transcript (figure 3.3.17). The % change in PDE3A transcript levels versus normoxic treated cells were: hypoxic, 80 ± 15%; H8 peptide/normoxic, 18 ± 7%; H8 peptide/hypoxic, -18% ± 8% (n=3, $P<0.05$ for hypoxic *versus* normoxic hPASMC, Student's *t*-test). The PDE3A/G3PDH transcript ratios were: control, 1 ± 0.09%; hypoxic, 1.84 ± 0.12%; control/H8 peptide, 1.11 ± 0.15%; hypoxic/H8 peptide, 0.92 ± 0.1% (n=3, $P<0.05$ for hypoxic *versus* normoxic hPASMC, Student's *t*-test). H8 peptide however had no significant effect on PDE3B, PDE5A, or G3PDH (see figure 3.3.18, densitometry not shown). These results suggest the cAMP pathway may have a role in regulating PDE3A expression, leading to the de-novo synthesis of PDE3A protein.

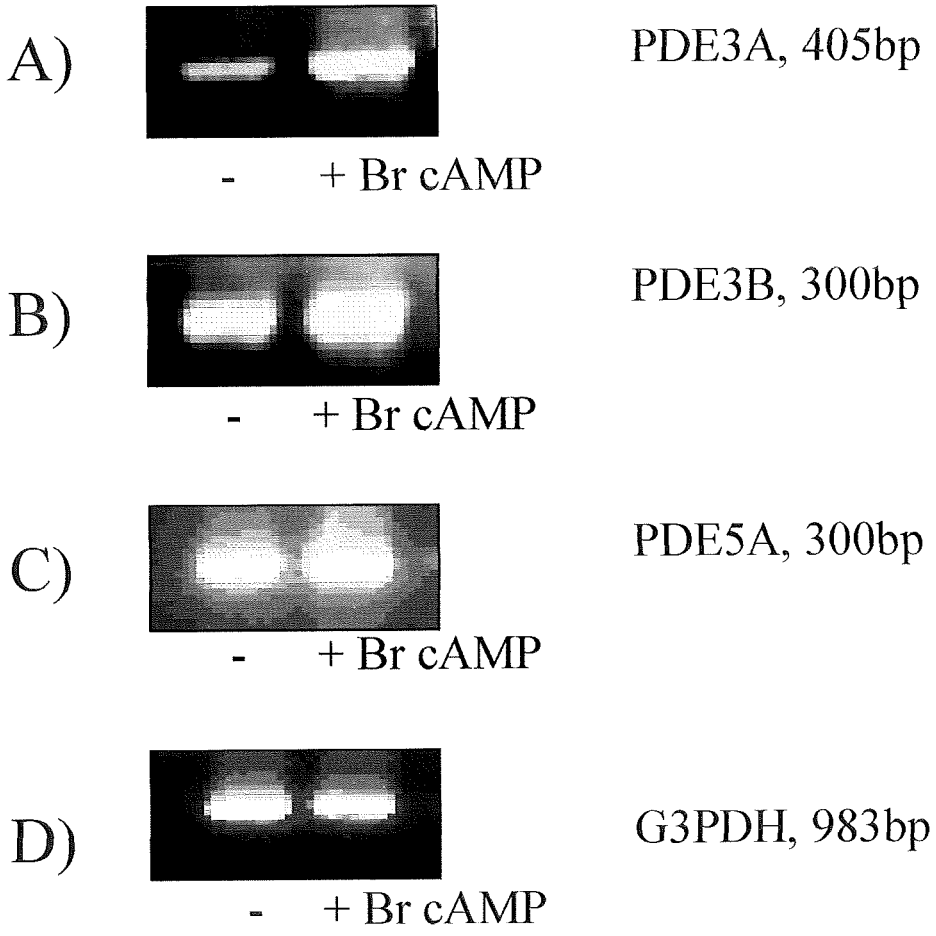
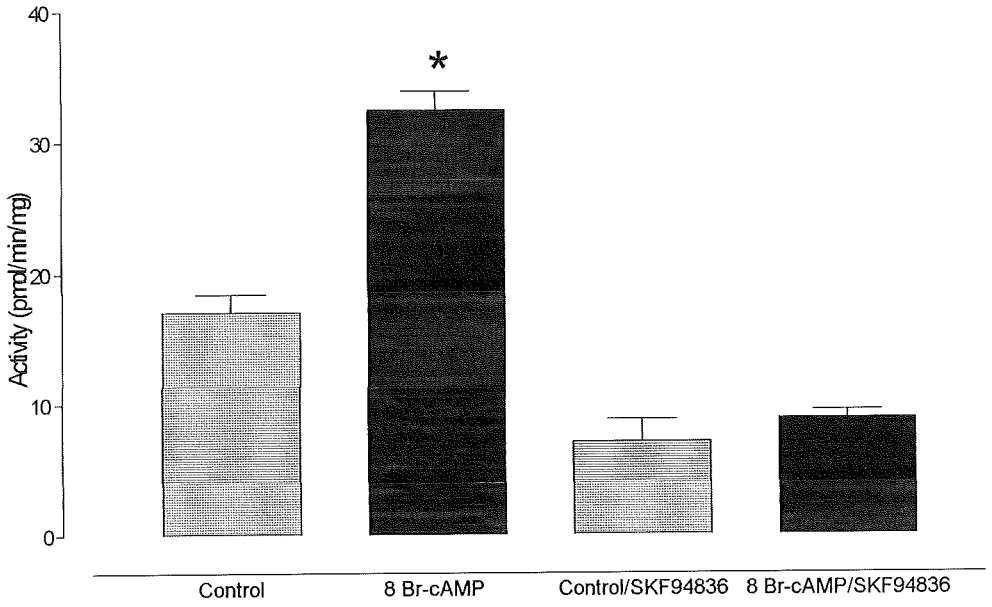


Figure 3.3.16 RT-PCR of PDE3A, PDE3B, PDE5A, and G3PDH transcripts in hPASMC treated with 100μM 8-Br-cAMP for 24 hours

RT-PCR amplification using specific primers, as described in 2.2.2.4, of: (A) PDE3A, 405bp; (B) PDE3B, 300bp; (C) PDE5A, 300bp; and (D) G3PDH, 983bp from hPASMC treated with vehicle (-) or 100μM 8-Br-cAMP (+ Br cAMP) for 24 hours. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 3 individual experiments, quantified by densitometry.

A)



B)

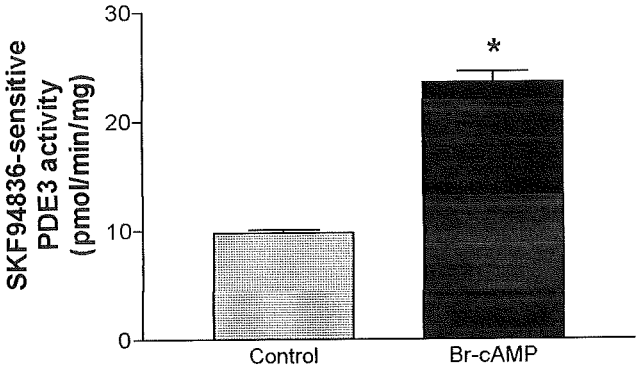


Figure 3.3.17 Total cAMP PDE activity in the presence and absence of SKF94836 from hPASMC after 24-hour exposure to 8-Br-cAMP

A) Histogram showing the increase in cAMP activity (PDE assay described in 2.2.3.7), in the presence and absence of the PDE3 inhibitor 10μM SKF94836, in cells treated with vehicle (control) and 100μM 8-Br-cAMP for 24 hours (8 Br-cAMP). B) Histogram showing the increase in SKF94836-sensitive PDE3 activity in cells treated with 100μM Br-cAMP for 24h. All results are means ± s.d, expressed as pmol/min/mg, * denotes the data are significantly different (n=3 separate cell preparations, $P<0.05$, by Student's *t*-test).

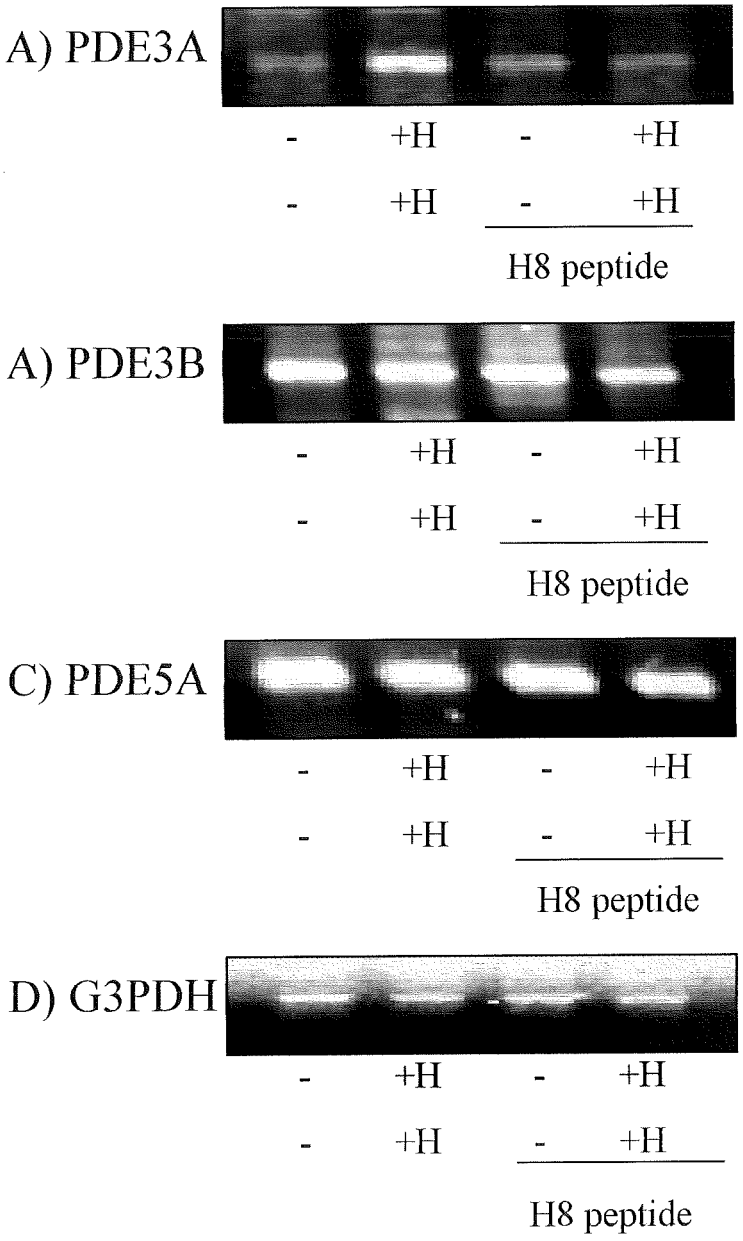


Figure 3.3.18 RT-PCR of PDE3A, PDE3B, PDE5A, and G3PDH transcripts in control and hypoxic hPASMC treated with 50μM H8 peptide for 14 days

RT-PCR amplification using specific primers, as described in 2.2.2.4, of: (A) PDE3A, 405bp; (B) PDE3B, 300bp; (C) PDE5A, 300bp; and (D) G3PDH, 983bp from control(-) and chronic hypoxic (+H) hPASMC treated with 50μM H8 peptide for 14 days. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 3 individual experiments, quantified by densitometry.

3.3.7.2 Role of the NF-κB pathway in regulating PDE3A/B and PDE5A

Nuclear factor-kappa B (NF-κB) is a transcription factor, which has been shown to be induced by stress responses and inflammation (reviewed by Makarov, 2000, Balwin, 1996; Ghosh *et al.*, 1998). It was therefore thought appropriate to investigate the inhibition of NF-κB in regulating the hypoxic-dependant increase in PDE expression. Normoxic and hypoxic hPASMC were treated chronically with 100μM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), an inhibitor of IκB degradation. TLCK inhibits trypsin-like serine proteinases and blocks interferon- and LPS-induced NF-κB–dependent nitric oxide synthase induction with an EC_{50} = 80μM (Schini-Kerth *et al.*, 1997). TLCK (100μM, 14 days) had no significant effect on PDE3A transcript and the increase observed with hypoxia was maintained (figure 3.3.19). The corresponding % changes in PDE3A transcript levels versus normoxic cells were: hypoxic 87 ± 3%; TLCK/normoxic, 2 ± 7%; TLCK/hypoxic, 95 ± 9% (n=3, $P<0.05$ for hypoxic and TLCK/hypoxic *versus* normoxic hPASMC, Student's *t*-test). The PDE3A/G3PDH transcript ratios were: control, 1 ± 0.03%; hypoxic, 2.04 ± 0.06%; control/TLCK, 1.02 ± 0.05%; hypoxic/TLCK, 2.1 ± 0.1% (n=3, $P<0.05$ for normoxic/TLCK and hypoxic/TLCK *versus* normoxic hPASMC, Student's *t*-test). PDE3B or G3PDH transcript levels were not significantly affected by the addition of TLCK (figure 3.2.20). The changes in PDE3B and G3PDH were: PDE3B, hypoxic 4 ± 5%; TLCK/normoxic, 2 ± 3%; TLCK/hypoxic, 3 ± 5%; G3PDH, hypoxic -1 ± 1%; TLCK/normoxic, 1 ± 1%; TLCK/hypoxic, -2 ± 1% (n=3, NS *versus* normoxic hPASMC, Student's *t*-test). The PDE3A/G3PDH transcript ratios were: control, 1.01 ± 0.06%; hypoxic, 0.99 ± 0.03%; control/TLCK, 1 ± 0.02%; hypoxic/TLCK, 0.98 ± 0.1% (n=3, NS, *versus* normoxic hPASMC, Student's *t*-test).

In contrast, TLCK substantially reduced the basal levels of PDE5A transcript in both the hypoxic and normoxic treated hPASMC (figure 3.3.20A). The % changes in PDE5A transcript levels versus normoxic cells were: hypoxic –4 ± 3%; TLCK/normoxic, -42 ± 7%; TLCK/hypoxic, -44 ± 9% (n=3, $P<0.05$ for TLCK/normoxic and TLCK/hypoxic *versus* normoxic hPASMC, Student's *t*-test). The PDE5A/G3PDH transcript ratios were: control, 1.02 ± 0.03%; hypoxic, 0.98 ± 0.05%; control/TLCK, 0.58 ± 0.05%; hypoxic/TLCK, 0.59 ± 0.1% (n=3, $P<0.05$ for normoxic/TLCK and hypoxic/TLCK *versus* normoxic hPASMC, Student's *t*-test).

There was also a correlation between the reduction in PDE5 transcript seen in cells treated with TLCK, with a reduction in PDE5A2 protein expression detected on Western blots (figure 3.2.20B). The TLCK-dependant changes in PDE5A2 expression in hPASMC lysates were: hypoxic 2 ± 3%; TLCK/normoxic, -22 ± 7%; TLCK/hypoxic, -29 ± 9% (n=3, $P<0.05$ for TLCK/normoxic and TLCK/hypoxic *versus* normoxic hPASMC, Student's *t*-test).

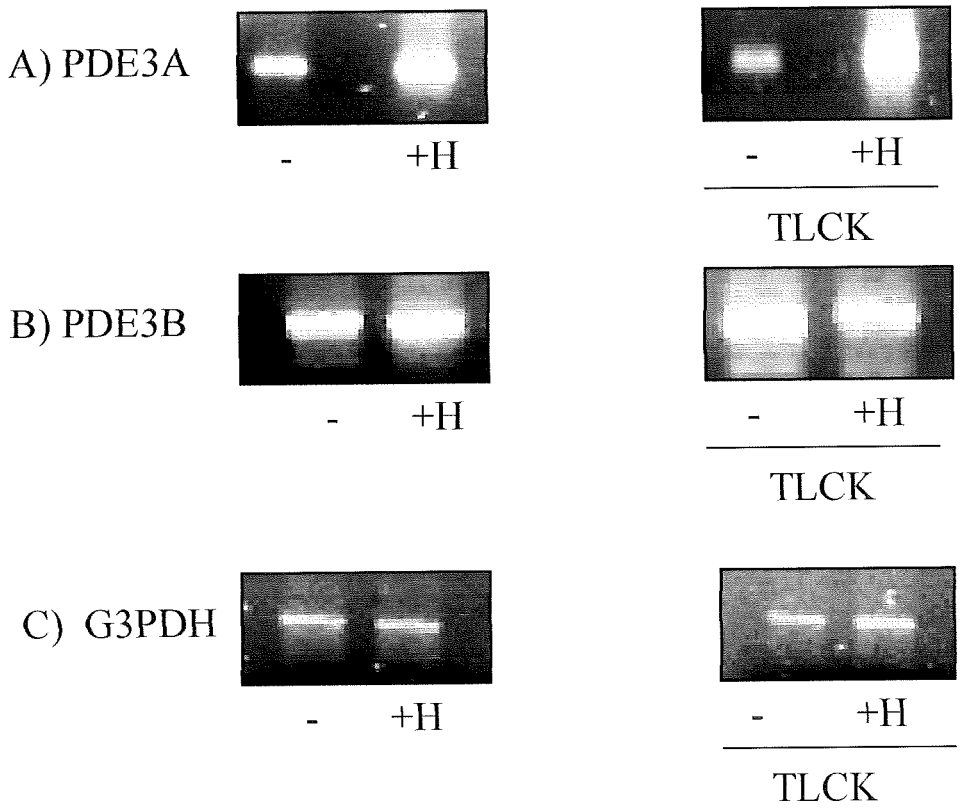


Figure 3.3.19 RT-PCR of PDE3A, PDE3B, and G3PDH transcripts in control and hypoxic hPASMC treated with 100μM TLCK for 14 days

RT-PCR amplification using specific primers, as described in 2.2.2.4, of: (A) PDE3A, 405bp; (B) PDE3B, 300bp; and (C) G3PDH, 983bp from control (-) and hypoxic (+H) hPASMC treated with 100μM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) for 14 days. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 3 individual experiments, quantified by densitometry.

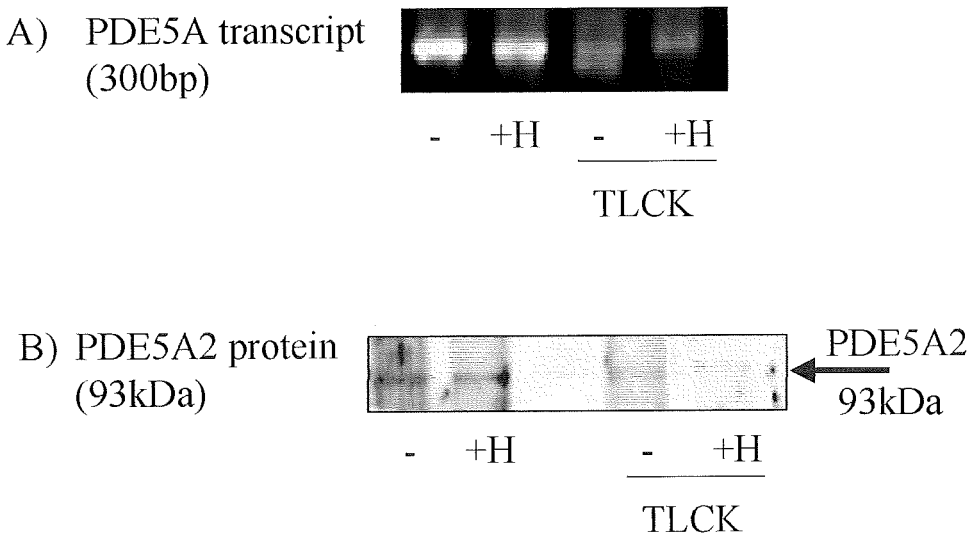


Figure 3.3.20 RT-PCR of PDE5A transcript and Western blotting of PDE5A protein from control and hypoxic hPASMC treated with 100μM TLCK for 14 days

(A) RT-PCR amplification using specific primers, as described in 2.2.2.4, of PDE5A, 300bp, from control and hypoxic hPASMC treated with 100μM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) for 14 days. 1μg total RNA/sample was used to make cDNA, of which one fifth was used for each RT-PCR. (B) Western blot (2.2.3.5) with anti-PDE5 antibodies showing the expression of PDE5A2 in homogenates from control (-) and hypoxic (+H) hPASMC treated with 100μM TLCK for 14 days. 10μg protein/sample were loaded onto SDS-PAGE. Above is a representative result of 3 individual experiments, quantified by densitometry.

3.4 Discussion

These experiments aimed to provide a molecular mechanism to explain the increased PDE3 and PDE5 activity, and the subsequent decrease in cyclic nucleotides previously observed in the branches of the pulmonary arteries from the chronic hypoxic rats (MacLean *et al.*, 1997; MacLean *et al.*, 1996)

3.4.1. Mechanism of the increased PDE3 activity seen in the PAs from CH rats

PDE3 activity is increased in the main, first branch and intrapulmonary vessels from rats maintained under chronic hypoxia for 14 days (MacLean *et al.*, 1997). It can be concluded from these studies that the increased PDE3 activity in these PAs appears, in part, to be accounted for by the increase in the *de-novo* synthesis of both PDE3A and PDE3B from their respective genes. This is in agreement with Wagner *et al.* (1997), who also reported that PDE3A transcript was increased in the first branch from hypoxic treated rats compared with controls. Surprisingly, both PDE3A and PDE3B transcripts were both seen to increase in the resistance vessels from the CH rat, even though in the previous study by MacLean *et al.* (1997), no increase in PDE3 activity was observed. It may be that the increased transcript is not translated into a corresponding PDE3A/B protein in the resistance vessels, or that PDE3 represents a small fraction of the total activity in these vessels and, therefore the increase in PDE3 activity is too small to detect. It may also be possible that variations in translation efficiency or protein turnover may render RT-PCR results misleading.

An interesting observation is the presence of both PDE3A and PDE3B transcripts in the PAs. The PDE3 family includes two genes, PDE3A and PDE3B, whose products possess similar kinetic and regulatory properties (Dergerman *et al.*, 1996). PDE3A has historically been thought of as the cardiovascular PDE3 (Meacci *et al.*, 1992), whereas PDE3B is often referred as adipocyte PDE3 as it was cloned from rat adipocytes (Dergerman *et al.*, 1996, Taira *et al.*, 1993). The differential tissue expression of PDE3A, compared with PDE3B, was initially thought to be one of the defining characteristics between the two sub-types (Reinhardt *et al.*, 1995). However, PDE3A and PDE3B expression have both shown to be expressed in rat aortic smooth muscle cells (rASMC) and human aortic smooth muscle cells (hASMC) (Lui and Maurice,

1998, Palmer and Maurice, 2000). Together with the results presented, the co-expression of both PDE3A and PDE3B in blood vessels from both the systemic and pulmonary circulation has now clearly been shown.

The results from the current study may also offer an explanation to why PDE3 inhibitors reduce pulmonary vasoconstriction in models of PHT. PDE3 inhibitors may be effective in the pulmonary circulation, as they inhibit a target that appears to play a significant role in altering vasoactive responsiveness. Inhibition of increased PDE3A/B expression may account for why cilostamide, the PDE3 inhibitor, attenuated acute and chronic hypoxia induced PHT (Phillips *et al.*, 2000), or why PDE3 inhibitors amplify the vasodilatory response in the pulmonary circulation to prostacyclin (Schermuly *et al.*, 1999). Furthermore, PDE3 inhibition has also been shown to inhibit serum-stimulated [^{3}H] thymidine incorporation and proliferation of rat vascular smooth muscle cells, showing that the increase PDE3 transcript and activity may also have a role in the PA remodelling seen with PHT (Pan *et al.*, 1994; Polson and Strada, 1996). Inhibition of PDE3 would increase cAMP, which *via* PKA activation, attenuates proliferation by inhibiting p42/p44 MAPK activation (Graves *et al.*, 1993; Bornfeldt and Krebs, 1999; Bonisch *et al.*, 1998).

3.4.2 Regulation of the hypoxic-induced increase in PDE3A activity

Another major finding of this study was that PDE3A transcript levels increased in cultured hPASMC maintained under chronic hypoxic conditions for 14 days. The PASMCs are derived from human main and first branch PA (Clonetics). The increase in PDE3A transcript was associated with a substantial increase in PDE3 activity in these cultured cells. In accordance with this, it has previously been observed that exposure of cultured vascular smooth muscle cells to hypoxia resulted in a time-dependant increase of the soluble fractions of PDE3 and 4 activity (Pinsky *et al*, 1993).

Further experiments using this cellular model showed that the hypoxic-dependant increase in PDE3A expression was mediated via a cAMP-dependant mechanism. Hypoxia was mimicked by exposing the cells to 8-Br-cAMP (a membrane permeable analogue of cAMP, with reduced metabolic turnover by PDEs), and ablated by chronically treating hPASMC with the PKA inhibitor, H8 peptide. Similarly, it has been reported that 8-Br-cAMP augments both PDE3 transcript and ORG 9935-inhibitable PDE activity (PDE3 inhibitor) in human T lymphocytes (Seybold *et al.*,

1998). As 8-Br-cAMP increases PDE3A expression, and H8 peptide reduces the hypoxic-dependant increase, a role for cAMP/PKA pathway in the up-regulation of PDE3A can be proposed.

The results presented in this study suggest cAMP may be initially increased in response to hypoxia in the pulmonary circulation. An initial increase in the production of cyclic nucleotides, may be an attempt of the pulmonary circulation to restore normal tone. Evidence for an increase in cAMP also stems from reports that prostacyclin (PGI_2), a known activator of cAMP, is also increased in response of the pulmonary circulation to hypoxia (Peterson *et al.*, 1982; Shaul *et al.*, 1991; Martin *et al.*, 1992). However, desensitisation of the cyclic nucleotide pathways may occur after prolonged exposure to hypoxia, which would explain the increased vasoconstriction seen with PHT. Excess cAMP may result in desensitisation (accumulated after 2 weeks), possibly through the activation of PKA, which may subsequently induce the *de-novo* synthesis of PDE3A. The increase in PDE3 expression would then result in a time-dependant decrease in the intracellular concentration of cAMP, which would explain results by MacLean *et al.*, (1996), who demonstrated reduced cAMP in response to chronic hypoxia (14 days). In fact, it has been reported that prolonged exposure of cells to cAMP analogues, or stimuli that activate adenylyl cyclase, and raise intracellular cAMP levels, elicit a variety of adaptive responses that subsequently down regulate cAMP-mediated signal transduction (Gettys *et al.*, 1987; Moon *et al.*, 2002). One mechanism for such desensitisation proposed by Moon *et al.*, 2002, was the up-regulation of PDE activity, which would allow the tight regulation of cAMP. A similar negative feedback control of cAMP involving the PKA phosphorylation of PDE3 has also previously been demonstrated (Corbin *et al.*, 1985, Degerman *et al.*, 1997). These authors proposed that PKA acts directly on multi-phosphorylation sites of PDE3A, to increase transcription, and subsequently increased PDE activity (Corbin *et al.*, 1985, Degerman *et al.*, 1997). The PDE3 family may therefore provide a route for prolonged elevated cAMP to subsequently attenuate the cAMP signalling process.

PKA may also have a more indirect role in the increase in PDE3 activity. It is possible that PKA may increase PDE3A transcript indirectly through activation of the cAMP response element binding protein (CREB). CREB is a member of a large family of transcription factors, which is phosphorylated by PKA *via* a specific phosphorylation on serine 133 (reviewed by Shaywitz and Greenberg, 1999; Yamamoto *et al.*, 1988;

Gonzalez and Montminy, 1989; Montminy, 1997; Roesler, 2000). Upon phosphorylation, CREB initiates gene expression that is known to persist long after the original stimulating cAMP that has been degraded. It is therefore been speculated that activation of CREB-mediated protein synthesis is a mechanism of transforming short-term effects of cAMP into long-term durable changes of the cell (Schwartz, 2001). This model may explain the increased PDE3A transcript that is reported, even though the initial increase in cAMP is diminished (seen in the CH, MacLean *et al.*, 1996). In fact, hypoxia has been shown to lead to the phosphorylation of CREB at the PKA phosphorylation site Ser133, inducing transcription of a number of genes (Beitner-Johnson and Millhorn, 1998; Childa and Voelkel, 1996). A schematic diagram of the proposed model of the cAMP pathway in regulating the increased PDE3 expression with hypoxia is depicted in figure 3.4.1.

The possible role of PKG cannot be excluded as it is also inhibited by H8 peptide, although at much higher concentration. Therefore, a role for PKG in the increased PDE3A transcript cannot presently be excluded. Future studies should include the chronic treatment of hypoxic hPASMC with more specific PKG inhibitors such as Rp-8-pCPT-cGMPS a membrane permeant inhibitor of PKG, or KT5823, to exclude the possible effect of PKG. Studies by Fouty *et al.* (1999), have however shown that inhibition of PKG has no effect on hypoxic pulmonary vasoconstriction, suggesting it may not have a central role. Additionally, H8 peptide also inhibits myosin light chain kinase (MLCK) and PKC. However, the involvement of MLCK can be excluded as elevation of cAMP via PKA, as seen in this study would inhibit this enzyme (Higashi *et al.*, 1983). These studies demonstrated that elevated cAMP leads to an increase PDE expression. Thus, if MLCK was involved, its inhibition by H8 peptide should lead to an increase in PDE3A, and not reduced as observed. As there is no evidence that PKA directly mediates PKC activity, PKC can also be excluded.

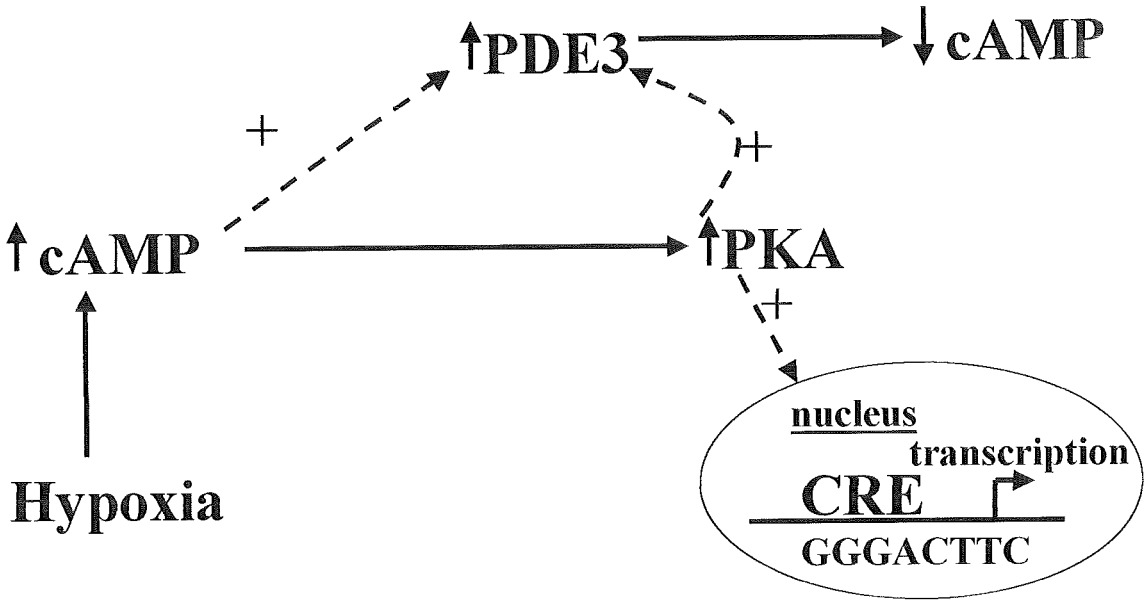


Figure 3.4.1 Proposed model of the increased PDE3 transcript and activity with chronic hypoxia

Above shows a schematic diagram showing the proposed theory for negative feedback of the cAMP pathway in response to chronic hypoxia. On exposure to hypoxia it is anticipated that an increase in cAMP would be observed, possibly through increased prostacyclin. This increase in cAMP would induce the activation of cAMP-specific phosphodiesterases, which would catalyse its hydrolysis. Results indicate a role for protein kinase A (PKA), which after a threshold level of cAMP is reached (accumulation after 2 weeks) is activated, increasing the transcription of PDE3. PKA could phosphorylate PDE3 directly to increase activity, or indirectly by activating the cAMP response element (CRE), which induces transcription through the phosphorylation of the cAMP response element binding protein (CREB). Increasing PDE3 activity would then result in a corresponding reduction in cAMP levels. This feedback pathway appears to allow increased cAMP in response to hypoxia to subsequently attenuate the cAMP pathway.

3.4.3 Mechanism of the increased PDE5 activity seen in the PAs from CH rats

PDE5 activity is increased in the first branch and intrapulmonary artery from rats maintained under chronic hypoxic conditions (MacLean *et al.*, 1997). The increased PDE5 activity previously observed in the first branch PA by MacLean *et al.* (1997), can now be explained by the increased transcript and *de novo* synthesis of PDE5A2. These results are consistent with previous findings by Black *et al.* (2001), showing PDE5 protein expression is increased in lambs with PHT, induced by aorta-pulmonary vascular graft replacement. Although increased PDE5 activity was not observed in the main pulmonary artery by MacLean *et al.* (1997), increased transcript and corresponding PDE5A2 protein levels were found in these arteries from the CH. It may be that in the main branch, PDE5 represents a smaller fraction of the total cGMP PDE hydrolysing activity, and that a significant increase in PDE5 activity may be difficult to detect using the pharmacological approach by MacLean *et al.* (1997), where selective PDE inhibitors were used in the assay to identify the activity of each PDE in the homogenates studied. These results may suggest that RT-PCR and Western blotting are more sensitive than the PDE assays employed. Alternatively, it is possible that PDE5 is subject to additional regulation in response to hypoxia, which might explain the discrepancies between results. For instance, phosphorylation of PDE5 by PKA markedly increases its activity, however reduces the sensitivity of PDE5 to inhibition by zaprinast (Burns *et al.*, 1992). Hence, it may be that PDE5 in the main branch is insensitive to inhibition by zaprinast, the PDE5 inhibitor used in the assay by MacLean *et al.* (1997).

As postulated with the cAMP pathway, the increased PDE5 expression in the main and first branch PAs may be due to a negative feedback pathway activated by an initial increase in cGMP in response to hypoxia. This hypothesis appears plausible, as the formation of NO, which exerts most of its biological effects *via* cGMP production in smooth muscle cells, is actually enhanced in lungs with the development of PHT (Archer *et al.*, 1996; Forrest *et al.*, 1999; Isaacson *et al.*, 1994, Xue *et al.*, 1994; Le Cras *et al.*, 1998; Le Cras *et al.*, 1996, Resta *et al.*, 1997). Increases in cellular cGMP levels have been shown to activate PDE5 both by activating PKG, and by binding to the allosteric sites of PDE5, increasing phosphorylation (Corbin *et al.*, 2000; Turko *et al.*, 1998; Thomas *et al.*, 1990; Venkatesh *et al.*, 2001). Venkatesh *et al.* (2001), concluded

that cGMP elevation would cause increase sequestration of cGMP by PDE5, resulting in dampening of the cGMP signal and rendering it unavailable to target proteins, such as PKG. The increase in PDE5 expression would therefore explain the reduced cGMP levels in CH rats (14 days, MacLean *et al.*, 1996).

Cross-talk between the cGMP and the cAMP pathways in these vessels may be possible, as PDE5 is activated not only by PKG, but also by PKA in smooth muscle (Burns *et al.*, 1992, Corbin *et al.*, 2000, Murthy, 2001). Therefore, the proposed increase in PKA in response to the hypoxic-induced elevation in cAMP, may contribute not only to the activation of PDE3, but also for the increase in PDE5 in these branches. Thus, when both cyclic nucleotides are present, cAMP could enhance the breakdown of cGMP *via* the PKA-dependant activation of PDE5 (Murphy *et al.*, 2001a/b). The subsequent decrease in cGMP could then reduce its inhibitory action over PDE3, thereby leading to an increase in PDE3 activity.

PDE5A1 nor PDE5A2 protein or transcript levels were not however significantly altered with hypoxia in either the intrapulmonary or resistance vessels. As increases in transcript or protein were not detected, therefore the hypoxic-dependant increase in PDE5 activity cannot be explained by changes in the expression of PDE5A1 or PDE5A2. The observed changes in PDE5 activity in the intrapulmonary artery may be due to post-translational modifications, such as increased phosphorylation. This seems feasible, as Hanson *et al.*, (1998) showed that increased PDE5 activity in the ovine model of PHT was correlated with increased phosphorylation of the enzyme. Future studies could use specific antibodies for the phophorylated form of PDE5 to investigate this hypothesis.

The increased expression of PDE5 in the main and first branch PA may explain why the PDE5 inhibitors E-4010 and E-4021 cause selective pulmonary vasodilation, and attenuate the increase in pulmonary arterial pressure (PAP), right ventricular hypertrophy, and pulmonary arterial remodelling seen in rat models of PHT (Takahashi *et al.*, 1996, Hanasato *et al.*, 1999). PDE5 inhibitors would specifically inhibit PDE5, which appear to be an important target in pulmonary vasoreactivity, to increase cGMP levels in pulmonary vascular smooth muscle, promoting relaxation. In fact long-term administration of E4010 improved the survival of pulmonary hypertensive rats (Kodama and Adachi, 1999). Furthermore, a number of groups have shown that

PDE5A inhibitors potentiate the vasodilatory effects of inhaled NO (Ishihara *et al.*, 1998, Ohnishi *et al.*, 1999). The results of this study are in fact timely in the light of recent studies showing that the PDE5 inhibitor sildenafil inhibits acute hypoxia-induced PHT in humans (Zhao *et al.*, 2001; Ghofrani *et al.*, 2002; Sanjay *et al.*, 2000). Together these results show evidence for using selective PDE5 inhibitors in the treatment of PHT

3.4.4 Difference in PDE5 expression between the proximal and distal pulmonary artery

Previously it has been reported that in the PAs from CH rats and from patients with PHT, endothelium-dependant relaxation is decreased in the conduit (main and first branch) PAs, however increased in the resistance vessels (MacLean *et al.*, 1995; MacLean and McCulloch, 1998; Dinh-Xuan, *et al.*, 1991). Oka, *et al.* (2001), also reported that ACh- and nitroprusside-induced relaxation via a NO/cGMP mediated pathways were only impaired in the larger PAs, but not in the smaller vessels from CH. Consistent with this, cGMP levels are decreased in the main and first branch PA, but unchanged in the resistance vessels from CH (MacLean *et al.*, 1996; Oka, *et al.*, 2001). As endothelial NO synthase is known to be increased in both the large and small PA from CH (Le Cras *et al.*, 1996), this current study can therefore provide a molecular mechanism to explain these previous results. The increased PDE5 activity is increased in the large PAs through synthesis of PDE5A2 protein, which can account for the decrease in cGMP, and the subsequent decrease in acetylcholine-induced relaxation. In parallel, PDE5 levels were unchanged in the resistance vessels, which would preserve the ability of ACh to induce vasodilation. In fact, due to increased levels of guanylyl cyclase in these vessels, ACh-induced relaxation could even be enhanced (Li *et al.*, 1999a).

Additionally, the PDE5 inhibitor sildenafil has been shown to selectively vasodilate the large PAs, but not the resistance vessels (Oka *et al.*, 2001). Sildenafil has been shown in mice to protect against the development of PHT (Zhao *et al.*, 2001). The remodelling seen in the pulmonary circulation with PHT, is more significant in the intrapulmonary and resistance vessels (distal), which are also the main site of resistance to flow. The role of the large PA is to maintain peripheral flow by dilating to accommodate stroke volume then recoiling during diastole. Pulsatile load is increased with PHT, due to the decreased compliance of the large PA. Reduced pulmonary artery compliance has been

speculated to be as important as increased resistance in elevating right ventricular afterload (Milnor *et al.*, 1969). These results suggest that the anti-pulmonary hypertensive effects of PDE5 inhibitors such as sildenafil are due to increasing the compliance of the large PA, thereby preventing right ventricular hypertrophy.

3.4.5 Regulation of PDE5-Role of the NF-κB pathway

Although an increase in PDE3 transcript and activity were both seen with chronic hypoxia in the hPASMC, no changes were detected in PDE5 transcript, protein, or activity. As the hPASMC are derived from human main and first branch PA the reason for this difference is not known. However, it may be that PDE5 is unaltered in the cellular model with hypoxia due, to the relative difference in PKG content. As outlined above it is possible that PDE5A2 expression may be regulated by cGMP and PKG under chronic hypoxia. It is well known that PKG expression is attenuated when vascular smooth muscle cells are cultured (Cornwell *et al.*, 1994; Cornwell and Lincoln, 1989). Therefore, the inability of chronic hypoxia to modulate the expression of PDE5A2 in cultured hPASMC may be the due to the absence of PKG.

In response to stress stimuli, the ubiquitous, dimeric transcription factor nuclear factor-kappa B (NF-κB) is known to be activated controlling the transcription of genes encoding for growth factors and other mediators that can influence vasodilation and proliferation (reviewed by Makarvo, 2000; Shulze-Osthoff, 1997; Sibenlist *et al.*, 1994; Thanos and Maniatis, 1995 Faller 1999). It was of particular interest to investigate NF-κB in hypoxia, as the promoter region of PDE3A/3B genes contain transcriptional sites for NF-κB, and it is controlled by the intracellular redox state (Wattanapitayakul and Bauer, 2001). However, the chronic treatment of hPASMC with the NF-κB inhibitor Nα-p-tosyl-L-lysine chloromethyl ketone (a selective inhibitor of trypsin-like serine proteases, TLCK), which prevents IκB degradation, had no significant effect on basal PDE3A or the hypoxic-dependant increased PDE3A transcript.

The final finding of this study was that the chronic treatment of normoxic/hypoxic hPASMC with TLCK reduced the basal expression of PDE5A. These results therefore suggest that NF-κB may have a role in controlling pulmonary vascular tone. While TLCK also inhibits other proteases, these do not have specificity against transcription factors, such as NF-κB that could alter PDE5 expression. NF-κB could directly control

transcription of PDE5, suggesting that the PDE5 gene may have NF-κB binding sites in its promoter region. However, it is also possible that NF-κB acts through a more indirect method involving inducible nitric oxide (iNO), to reduce PDE5 expression. Inducible NOS is present in the vasculature and contributes to nitric oxide production (NO). NF-κB is known to regulate iNOS, as it is well established that the 5' flanking region of the iNOS gene contains a consensus sequence that binds to NF-κB (Xie *et al.*, 1993). Additionally, TLCK can prevent the LPS-inducible expression of the iNOS gene in rat alveolar macrophages, and also by preventing the transcription of the iNOS gene, inhibit NO production (Griscavage and Ignarro, 1995; Schini-Kerth *et al.*, 1997). NF-κB may therefore control iNOS, which in turn regulates vascular tone through the action of NO and cGMP. Through the activation of soluble guanylyl cyclase, and the subsequent activation of protein kinase G (PKG), NO can increase intracellular cGMP levels, which in turn may govern PDE5 expression. Thus, a decrease in NOS would decrease cGMP, therefore decreasing PDE5. These results together suggest that NF-κB may control iNOS expression, which may in turn regulate NO, cGMP, PKG, and consequentially PDE5 levels. It can therefore be proposed that increased PDE5 expression with hypoxia may, in part, be due to the activation of NF-κB.

Evidence for changes in the components of the NF-κB/PDE5 pathway and there importance in hypoxia can be taken from the following studies. With respect to a role for NF-κB in PHT, NF-κB activation has been associated with the stimulated oxidative stress, which is related to monocrotaline-induced PHT (Aziz *et al.*, 1997). Furthermore, treatment of spontaneously hypertensive rats with the NF-κB inhibitor, pyrrolidinedithiocarbamate (PDTC) and the iNOS inhibitor, aminoguanidine, reduced the development of hypertension and improved the reduced vascular responses to ACh (Hong *et al.*, 2000). NF-κB has also been shown to modulate proliferation, branching, and morphogenesis in lung epithelium (Muraoka *et al.*, 2000), therefore inhibition may reduce the remodelling seen with PHT.

Following on from this, increased *de novo* expression of iNOS mRNA and protein expression has been noted in whole lung extracts and in large and small PA from rats and mice with chronic hypoxia induced-PHT (Carville *et al.*, 1997; Le Cras 1996; Palmer *et al.*, 1998; Xue *et al.*, 1996; Kinnula *et al.*, 1995). Additionally, shear stress, of which hypoxia may be a stimulant, is a potent inductor of iNOS expression in smooth muscle cells, which is in part mediated by NF-κB (Gosgnach *et al.*, 2000). Increased

iNOS with hypoxia would induce the formation of increased NO. Increased NO has also been shown to have a significant role in PHT (Le Cras *et al.*, 1996). Studies in animal models support the hypothesis that NO production may be increased with the development of PHT, possible through increased nitric oxide synthase expression (Shaul *et al.*, 1995). Isaacson *et al.*, 1994, measured the NO oxidation product nitrite in lung perfusate of rats as an index of NO production, and found an increase from 0.4nM in normotensive to 24.3nM in pulmonary hypertensive rats. Finally, cGMP production has been shown to be initially increased in the CH in response to hypoxia, which could explain the subsequent increase in PDE5 activity (Li *et al.*, 1999). The increase in PDE5 activity would explain the net decrease in intracellular cGMP levels in the PAs from the CH after 14 days (MacLean *et al.*, 1996).

Together these findings suggest that inhibiting NF-κB could be a potential new strategy to reduce PDE5 activity indirectly, possibly by improving the vasodilatory action of agents such as nitrates or even PDE5 inhibitors in patients with PHT. Anti-inflammatory drugs such [non-steroid anti-inflammatory drugs (NSAIDs), immunosuppressants] and glucocorticoids can all inhibit NF-κB (Epinat and Gilmore, 1999; Wissink *et al.*, 1998). However, these drugs are non-specific for NF-κB and also inhibit a number of other important inflammatory mediators. Additionally, due to the wide role of NF-κB in cell regulation, adverse side effects could limit the use of more specific NF-κB inhibitors as a therapeutic target in PHT. Potential future work would be to administer TLCK chronically to the CH, in order to prevent the development of PHT.

3.4.6 Conclusion

Together these results suggest that phenotypic changes in PDE3 and PDE5 expression could account at least in part for the reduced sensitivity of PA to vasodilators. The increase in PDE3 and PDE5 expression with hypoxia would accelerate the rate of cAMP and cGMP degradation, blunting the relaxant effects of agents such as isoprenaline and nitric oxide. These data provide a molecular mechanism of why PDE3 and PDE5 inhibitors exert favourable effects.

CHAPTER 4

EFFECT OF PDE3 AND PDE5 INHIBITORS ON ISOLATED PULMONARY ARTERIAL RINGS

Chapter 4 – Effect of PDE3 and PDE5 inhibitors on isolated pulmonary arterial rings

4.1 Introduction

The relaxation of smooth muscle via the elevation of cAMP and/or cGMP is well known and understood (reviewed by Schwede *et al.*, 2000; Schmidt *et al.*, 1993; Koyama *et al.*, 2001). An increase in cAMP and cGMP can be achieved by inhibiting phosphodiesterases (PDEs), the enzymes responsible for their conversion to their corresponding 5'-monophosphate inactive counterparts. The high diversity of PDE families, such as in their tissue distribution, and functional roles, make these enzymes likely targets for therapeutic application (reviewed by Thompson, 1991; Beavo *et al.*, 1994; Beavo, 1995: Soderling *et al.*, 1998, 1999; Corbin and Francis, 1999; Fawcett *et al.*, 2000; Conti, 2000; Soderling and Beavo 2000; Yuasa *et al.*, 2000; Koyama *et al.*, 2001). Due to the rapid development of pharmacological and biochemical research in the field of PDEs, family-specific inhibitors for many of the PDEs are presently available.

In human pulmonary arteries (PAs), PDE 1, 3, 4, and 5 are present in the cytosolic and particulate phases of the homogenised tissue (Rabe *et al.*, 1994). MacLean *et al,* (1997), reported that the activity of both cAMP and cGMP-PDEs are increased in the PA branches from chronic hypoxic rats (CH). The most significant increases in activity with hypoxia were associated with PDE1, PDE3 and PDE5 (MacLean *et al.*, 1997). Results from chapter 3 show increased transcript levels for PDE3 and PDE5 in both the main and first PA from CH. Together, these studies suggest a possible therapeutic application of PDE3 and PDE5 inhibitors in PHT. Studies outlined below give further evidence for examining the functional consequence of selective PDE3 and PDE5 inhibitors in isolated conduit PA rings.

PDE3 is known as the cAMP-specific, cGMP-inhibited PDE. Two genes encode for the PDE3 family, namely PDE3A and PDE3B. Commonly used PDE3 inhibitors include milrinone, amrinone, and cilostamide. Clarke *et al.* (1991), showed that inhibition of PDE3 by amrinone reduced pulmonary vascular resistance (PVR) in isolated perfused lung. Also, the PDE3 inhibitor SCA40 has been shown to relax main and intrapulmonary PAs precontracted with phenylephrine (PE, Crilley *et al.*, 1998).

SCA40 reversed the vasoconstriction induced by PE, and was 4.9-fold more potent in the PAs from the CH than from the control. These studies suggest PDE3 inhibitors may be useful in treating PHT. The pulmonary vasodilatory ability of SKF94836 [2-cyano-1-methyl-3- [4-(-methyl-6-oxo-1, 4,5,6-tetrahydropyridazine-3-yl) phenyl] guanidine], a new potent PDE3 inhibitor (Ki = 1-3μM, Murray *et al.*, 1991; Souness *et al.*, 1992), was investigated in this study.

PDE5 is the main cGMP PDE in the lung (Francis *et al.*, 1980; Thomas *et al.*, 1990). Drugs such as zaprinast, dipyridamole, and DMPPO are commonly used specific inhibitors of PDE5. These inhibitors act by competing with cGMP to bind to the catalytic sites, but not the allosteric sites, of PDE5 (Francis *et al.*, 1990; Corbin and Francis, 1999). Not surprisingly, due to the high concentration of PDE5 in the lung, PDE5 inhibitors such as zaprinast have been shown to reduce PVR in both isolated lungs and in the pulmonary circulation of foetal animals (McMahon *et al.*, 1993; Ichinose, 1995a; Ichinose *et al.*, 1995b; Jeffery and Wanstall, 1998; Black *et al.*, 2001). In addition, DMPPO and the potent PDE5 inhibitor E4010 have both been shown to attenuate the development of pulmonary vascular remodelling when administered chronically to chronic hypoxic rats, with no significant systemic effects (Eddahibi *et al.*, 1998; Hanasato *et al.*, 1999; Kodama and Adachi, 1999). These studies suggest a positive role for PDE5 inhibitors in the treatment of PHT.

A recently developed, highly specific PDE5 inhibitor is sildenafil, Ki of ~3.5nM (Ballard *et al.*, 1998). Sildenafil citrate (Viagra®) is currently successfully used to treat male impotence at doses of 50mg to 100mg (reviewed by Moreland *et al.*, 1999). Following sexual stimulation sildenafil has been shown to enhance NO-mediated smooth muscle relaxation in blood vessels, thereby improving penile erection by increasing blood flow (Boolell *et al.*, 1996; Ballard *et al.*, 1998; Stief *et al.*, 1998). The order of potency of sildenafil compared to some commonly known PDE5 inhibitors is; sildenafil (most potent) > zaprinast > dipyridamole > IBMX > cilostamide > theophylline > caffeine (Thomas *et al.*, 1990; Ballard *et al.*, 1998). The clinical use of sildenafil opened up the possibility that this new potent PDE5 inhibitor may be effective in the treatment of PHT. In fact, Zhao *et al.* (2001), investigated the effects of sildenafil on hypoxia-induced PHT in mice and healthy human volunteers. These authors found that 100mg of sildenafil inhibited the hypoxic rise in PAP in human subjects. In addition, sildenafil was shown to attenuate the increase in RV hypertrophy and

remodelling in mice chronically exposed to hypoxia. Furthermore, a randomised controlled trial showed sildenafil caused selective pulmonary vasodilation and improved gas exchange in individuals with PHT secondary to lung fibrosis (Ghofrani *et al.*, 2002). Sildenafil was the PDE5 inhibitor used throughout the following investigation.

The action of vasodilators in the pulmonary circulation is dependent on the type of vasoconstrictor, and the size of the PA used (Plane and Garland 1996; Frid *et al.*, 1997; Wanstall, 1996). Several factors are considered to be mediators or modulators of the hypoxia-induced vasoconstriction associated with PHT. In this study three well-characterised pulmonary vasoconstrictors with differing mechanisms of action were used to study the effects of the PDE3 and PDE5 inhibitors. These were the G-protein coupled receptor (GPCR) agonists, phenylephrine (PE), 5-hydroxytryptamine (5-HT), and endothelin-1 (ET-1), of which brief outlines of their mode of action and evidence of a role in PHT are given below.

Sympathetic stimulation causes changes in pulmonary vascular resistance, mediated via noradrenaline and α- and β-adrenoreceptors (reviewed by Barnes and Liu *et al.*, 1995; Bevan, 1989). The α_1-adrenoreceptors appear to mediate both the vasoconstrictive and proliferative actions of sympathetic nerve stimulation in the pulmonary circulation (Hyman, 1986; Nakaki *et al.*, 1990). PE is a commonly used α_1-adrenoreceptor ligand/agonist known to vasoconstrict isolated PA rings. Vasoconstriction of vascular smooth muscle by activation of α_1-adrenoreceptors is believed to occur through coupling to phospholipase C (PLC), leading to an increase in the release of intracellular calcium and activation of protein kinase C (PKC), mediated by diacylgycerol (DAG) and inositol trisphosphate (IP_3). An increase in α_1-adrenoreceptor activation occurs in response to hypoxic induced PHT (Sundeep, 1999; Eckhart *et al.*, 1996; Mardon *et al.*, 1998).

ET-1, a 21 amino acid peptide, is also known to be a potent vasoconstrictor in large PAs, and to increase pulmonary vascular resistance (for review, see MacLean, 1998b; MacLean, 1999a). ET-1 has a multifactorial action in the pulmonary circulation mediated by the two ET receptors. ET-1, acting via ET_A receptors, has been shown to cause a concentration-dependent contraction in the conduit PAs of the rat (MacLean *et al.*, 1998b). In parallel, evidence exists for the role of ET_B receptors in the ET-1 induced vasoconstriction in both rat and human small PAs (MacLean *et al.*, 1994b;

McCulloch *et al.*, 1998). ET-1, in common with α_1-adrenoreceptors-agonists, initiates smooth muscle contraction via induction of phospholipase C (PLC) and subsequent generation of the second messengers inositol trisphosphate ($InsP_3$) and diacylgycerol (DAG). In the lung both ET-1 and ET-3 are abundantly expressed providing further evidence for a role in the control of pulmonary tone (Firth *et al.*, 1992).

ET-1 has been implicated in the development of PHT (for review, see MacLean, 1998; MacLean, 1999b). This is supported by Griaid *et al*, 1993 who showed an increased expression of ET-1 mRNA in patients with both primary and secondary PHT. Also, an increase in ET-1 concentration, and ET-1, ET_A receptor and ET_B receptor mRNA has been observed in the lungs from CH (Li *et al.*, 1994). In fact, a common pathophysiological feature of PHT, regardless of the etiology, appears to be an increase in the circulating levels of ET-1 (MacLean, 1998b, Stewart *et al.*, 1993). Additionally, ET-1 has been shown not only to be a potent pulmonary vasoconstrictor, but to also stimulate DNA synthesis and proliferation of PASMC (Janakidevi *et al.*, 1992). Hence increased levels may not only lead to the increased tone but also the increased remodelling seen with PHT. Therefore inhibition of ET-1 would be of therapeutic value in the treatment of PHT. In fact, bosentan (Tracleer®), which is presently used in the treatment of PHT, is a dual endothelin-receptor antagonist (ET_A/ET_B).

Another potent vasoconstrictor in the pulmonary circulation is 5-HT (also known as serotonin). Recent evidence would suggest there are at least 17 genetically different 5-HT receptors (reviewed by, Hoyer *et al.*, 2001; MacLean, 1999b). These include 5-HT_{1A-F}, $5HT_{2A-C}$, 5-HT_3 and 5-HT_4 (MacLean *et al.*, 1999b). In the pulmonary circulation the vasoconstrictive effect of 5-HT appears to be mediated via 5-$HT_{1B/1D}$ and 5-HT_{2A} receptors depending on the level of pre-existing tone and the species (Morecroft and MacLean, 1998). 5-HT induces vasoconstriction via 5-$HT_{1B/1D}$ receptors through a $G_{\alpha}i$ dependent pathway leading to the inhibition of adenylyl cyclase, and a subsequent decrease in cAMP. In parallel, 5-HT initiates vasoconstriction via the 5-HT_{2A} receptors through a $G_{\alpha}q$ dependent pathway, leading to the hydrolysis of IP_3 and DAG from PLC, and a subsequent increase in intracellular calcium and activation of PKC (Sumnner and Humphrey, 1990).

As with ET-1, increased 5-HT is thought to have a role in the increased tone and vascular remodelling associated with all forms of PHT (for review, see MacLean,

1999b/c). Circulating levels of 5-HT were shown to increase from 1-2nmol/L to about 30nmol/L in PPHT (Anderson *et al.*, 1987). Herve *et al.* (1995), reported an increase in plasma 5-HT levels in PPHT. Furthermore, platelet and plasma 5-HT levels are increased in PHT that is secondary to anorexigen intake (Herve *et al.*, 1995). Eddahibi *et al.*, 1999, demonstrated that 5-HT also has a co-mitogenic action on pulmonary vascular smooth muscle cells. Together these studies would suggest a role for 5-HT in the development of PHT.

The aim of this study was to investigate the effect of SKF94836 (PDE3 inhibitor), and sildenafil (PDE5 inhibitor) on PE, ET-1, and 5-HT mediated vasoconstriction in both the main and first branch PA of control and hypoxic rats. In addition the vasodilatory effects of the PDE inhibitors will be assed in endothelium-denude PA.

4.2 Materials and Methods

4.2.1 Materials

All reagents, unless otherwise stated, were obtained from Sigma chemical company (U.K.), or BDH (U.K.). PDE3 inhibitor SKF94836 (M.W. 270) was from GalaxoSmithkline (U.K.). PDE5 inhibitor sildenafil (M.W. 430) was from Pfizer (U.K.).

4.2.2 Animal Studies – Chronic Hypoxic Rat

Male Wistar rats of 28-30 days old (at start of experiment) were housed in a specially designed perspex hypobaric chamber (Royal Hallamshire Hospital, Sheffield). The pressure within the chamber was decreased to 550mbar, this reduced the inspired pO_2 to approximately 110mmHg (~10% equivalent). The temperature of the chamber was maintained at 21-22°C and the chamber was ventilated with air at approximately 451 min^{-1}. Animals were maintained in these hypoxic/hypobaric conditions for 14 days. Age-matched controls were housed under normoxic/normobaric room conditions (20% v/v oxygen). Following sacrifice the right ventricle of the heart was dissected free of the septum and left ventricle and these were blotted and weighed. PHT was assessed by measuring the ratio of right ventricle (RV)/total ventricular (TV) weight. This is a well-established index of the degree of PHT in the rats (Hunter, *et al.*, 1974). Pulmonary arteries were dissected out then either kept in cold gassed Krebs-Heinslet (Krebs)

solution [118.4mM NaCl, 25mM $NaHCo_3$, 47mM KCl, 1.2mM KH_2PO4, 1.2mM $MgSo_4$, 2.5mM $CaCl_2$, 11mM, pH 7.4] at 4^0C (for no more than 24hrs) for use in organ bath experiments.

4.2.3 10ml Organ Bath Set-up for isolated main and first branch pulmonary artery

Standard organ bath procedures were used. Each PA was suspended by two wire supports. The top wire support (hook shaped) was then connected by thread to a force displacement transducer, while the bottom wire support, which was attached to a glass rod, was clamped in place. The isometric force transducer was connected via an amplifier to Mac lab (Chart V3.5, MacLab Data Acquisition System, Version 8E, AD Instruments Pty Ltd, Australia), a computer based data handling system which recorded vessel contraction/relaxation as in a pen chart recorder. The rings were mounted in 10ml organ baths containing Krebs as and continuously oxygenated with 16% O_2, 5% CO_2, and 79% N_2, at 37°C.

Rings were placed under a resting tension of 1.5g, which was maintained throughout all experiments. A tension of 1.5g was set, as it is known to be the optimal tension to produce a maximal contraction to 50mM potassium chloride (KCl) in control PA, and can be said to mimic the *in vivo* tension. Initially all PA were equilibrated for 45 minutes after which each vessel was contracted with 50mM potassium chloride (KCl). After washing with Krebs, this procedure was repeated to ensure maximal contraction. After a further equilibration period of approximately 45 minutes, endothelium function was checked. Functional endothelium was assessed by the ability of 10^{-6}M acetylcholine (ACh) to significantly relax PA rings pre-contracted with 10^{-6}M phenylepherine (PE). In selected experiments the endothelium was removed by gently rubbing the luminal surface of the rings with ridged forceps. When no response was achieved with 10^{-6}M ACh the vessels were considered to be denude of functional endothelium.

4.2.4 Preconstrictors

Cumulative concentration response curves (CCRCs) were constructed for PE (10^{-9}-10^{-5}M), 5-HT (10^{-9}-10^{-5}M), and ET-1 (10^{-11}-10^{-7}M) in half log steps for both the main and first branch. The periods between additions were dictated by the time taken for the

responses to stabilise, which was usually 5 minutes. In further experiments a concentration of each vasoconstrictor was used that consistently produced contractions with a magnitude similar to that of 50mM KCl in the PA rings.

4.2.5 Effect of PDE3 and PDE5 inhibitors on preconstrictor responses

Both the PDE5 inhibitor sildenafil, and the PDE3 inhibitor SKF94836 were stored at a stock concentration of 10^{-2}M in 1% DMSO. CCRCs were constructed using the PDE5 inhibitor sildenafil (10^{-9}-10^{-5}M in 1% DMSO), and the PDE3 inhibitor SKF94836 (10^{-9}-10^{-5}M in 1% DMSO) in half log steps for each of the three preconstrictor agents. CCRCs were carried out for each PDE inhibitor in the main and first branch pulmonary artery from both control (+/-endothelium) and hypoxic animals. Additions of both inhibitors only commenced once a stable plateau had been reached for the preconstrictor used. In all experiments, in order to show the true effect of the inhibitors, one half of the branch was always used as a time control where only 1% DMSO was added in to the bath (final concentration 0.01% DMSO).

4.2.6 Data Analysis

EC_{50} values could generally not be calculated for each individual CCRC as a maximum plateau was not reached, even on the addition of the stock concentration of each inhibitor. Therefore throughout this study the maximum relaxation in response to the PDE inhibitor (3 x 10^{-5}M for both SKF94836 and sildenafil) were compared between groups (the efficacy). All data were expressed as percentage of the reference response to 50mM KCl in each vessel. Each point is the mean ± s.e.m. Statistical comparisons of the means of groups of data were made by use of Student's *t*-test for paired or unpaired data where appropriate. Student's *t*-test was used to compare the maximum response of each PDE inhibitor between control/control endothelium-denuded and control/hypoxic PA. A level of probability of $P<0.05$ was taken to indicate statistical significance. As above, "n" equals the number of different animals used.

To take into account a possible effect of the vehicle (DMSO), the response of the PA to DMSO alone was subsequently subtracted from the parallel response to the PDE inhibitor. These calculations allowed results to be obtained for relaxations that could only be attributable to the PDE inhibitors.

4.3 Results

4.3.1 The Chronic Hypoxic rat (CH)

The animals used throughout these experiments correspond to the results in 3.2.1 and figures 3.3.1 and 3.3.2. The exposure of male Wistar rats to 10% O_2 for 2 weeks resulted in a significant decrease ($P<0.05$) in body weight from 221.3g ± 2.6 to 199.7g ± 2.4 ($n=80$, $P<0.05$, Student's *t*-test, see figure 3.3.1).

RV/TV ratio was significantly increased with hypoxia, confirming that right ventricular hypertrophy had occurred (figure 3.3.2). RV/TV ratios were 0.202 ± 0.001 and 0.336 ± 0.006 for normoxic and hypoxic rats respectively ($n=80$, $P<0.05$, Student's *t*-test, see figure 3.2.2). There was a 66.3% increase in the RV/TV ratio in CH compared to control rats, indicating the development of severe PHT in the rats used throughout the study.

4.3.2 Optimising PE, 5-HT, ET-1 and ACh concentrations

Age matched controls were used to optimise the concentrations of vasoconstrictors used throughout this study, as more were readily available.

Before each experiment the response to 50mM potassium chloride (KCl) was determined. This provided a control, to show firstly the integrity of the tissue, and secondly to allow direct comparison of the response of each precontractile agent (by expressing data as a % of the reference response to 50mM KCl). 50mM KCl was used, as this concentration is known to produce a maximal contractile response in this preparation, as higher concentrations have been shown to result in a decrease in response (MacLean *et al.*, 1994b). KCl causes contraction of smooth muscle by depolarising the membrane, resulting in the stimulation of L-type voltage operated Ca^{2+} channels. Hence, KCl acts directly on smooth muscle, not involving receptor activation.

Figure 4.3.1A/B and 4.2.3A show the cumulative concentration response curves (CCRC) constructed for phenylephrine (PE, 1 x 10^{-9}M to 3 x 10^{-4}M, figure 4.1A), 5-hydroxtryptamine (5-HT, 1 x 10^{-9}M to 3 x 10^{-5}M, figure 4.3.1B), and endothelin-1 (ET-1, 1 x 10^{-13}M to 3 x 10^{-8}M, figure 4.3.2A). PE, ET-1 and 5-HT all produced concentration-dependent contractions in both the main and first branch pulmonary

arteries (PA). The CCRCs produced by each of the preconstrictors were not significantly different in the main versus the first branch pulmonary artery (figure 4.3.1A/B and 4.2.3A). The concentrations of each preconstrictor that produced 90-100% (a sustainable and reproducible contraction) of the reference KCl response were extrapolated from the CCRCs. These concentrations were as follows; PE, 1 x 10^{-6}M; ET-1, 3 x 10^{-9}M; and 5-HT, 3 x 10^{-5}M. These concentrations were used in each subsequent experiment to produce a level of active tension in both PA branches before the addition of the PDE inhibitor.

Each PA was shown to have intact functioning endothelium before commencing the experimental protocol. This was determined by the ability of the endothelium-dependent agonist acetylcholine (ACh) to cause significant relaxation of the PA after preconstriction with 1μM PE. In order to determine the optimal concentration of ACh to use in all subsequent experiments, a CCRC to ACh (ACh, 1 x 10^{-9}M to 3 x 10^{-4}M) was constructed in both control main and first branch PA preconstricted with 1μM PE. In both the control main and first branch PA, ACh caused a similar concentration-dependent relaxation (figure 4.3.2B). From figure 4.3.2B, it can be seen that 1 x 10^{-6}M ACh produced a significant sustainable relaxation, approximately 50% of the reference KCl response in both the main and first branch. Therefore, 1 x 10^{-6}M ACh was used in all subsequent experiments to indicate the presence of a functional endothelium.

4.3.3 Response of hypoxic and endothelium-denuded main and first branch pulmonary arteries to PE, ET-1, and 5-HT

Initially, it was required to investigate whether hypoxia or removal of the endothelium altered the response of the PAs to each preconstrictor. Figures 4.3.3 and 4.3.19 show the level of preconstriction produced in the control, hypoxic and endothelium-denuded (A) main and (B) first branch PAs used in the SKF94836 and the sildenafil studies respectively. It can be seen in figure 4.3 and 4.19 that the maximum response to 1 x 10^{-6}M PE in the main PA, and also in the first branch PAs, were not significantly different when comparing the control, hypoxic, and endothelium-denuded vessels (data not listed, n=5 for all, NS). Likewise, it can be seen from figures 4.3.3 and 4.3.19 that the responses to 3 x 10^{-9}M ET-1 were not significantly different when comparing the control, hypoxic, or endothelium-denuded conduit PAs (data not listed, n=5 for all, NS).

In contrast, the magnitude of the contraction induced by 3 x 10^{-5}M 5-HT increased significantly in both the main and first branch PAs from CH compared to those from the control, and those that were endothelium-denuded (figures 4.3.3 and 4.3.19). The response to 3 x 10^{-5}M 5-HT, as a % of the reference KCl, were as follows in the main PAs in the SK94836 study: main control; 96.4 ± 5.5%; main PA endothelium-denuded; 94.7 ± 4.8%, main PA hypoxic; 115 ± 7.1% (n=5, P<0.05, hypoxic *versus* control with/without endothelium, Student's *t*-test, figure 4.3.3). In parallel, the response to 3 x 10^{-5}M 5-HT in the first branch, as a % of the reference KCl, were as follows: first branch PAs used in the SKF94836 study: first branch PA control; 95.9 ± 4.2% (n=5); first branch PA endothelium-denuded; 97.6 ± 4.4% (n=5), first branch PA hypoxic; 115.1 ± 9.9% (n=5, P<0.05, hypoxic *versus* control with/without endothelium, Student's *t*-test, figure 4.3.3). Likewise, the response to 3 x 10^{-5}M 5-HT, as a % of the reference KCl, were as follows in the main PAs used in the sildenafil study (figure 4.3.19): main control; 91.8 ± 7.5%; main PA endothelium-denuded; 91.5 ± 4.5%, main PA hypoxic; 113 ± 7.3% (n=5, P<0.05, hypoxic *versus* control with/without endothelium, Student's *t*-test). Again in parallel, the response to 3 x 10^{-5}M 5-HT in the first branch, as a % of the reference KCl, were as follows: first branch PAs used in the sildenafil study (figure 4.3.19): control; 92.8 ± 5.8% (n=5); first branch PA endothelium-denuded; 93.2 ± 5.4% (n=5), first branch PA hypoxic; 111.7 ± 4.8% (n=5, P<0.05, hypoxic *versus* control with/without endothelium, Student's *t*-test).

These results suggest chronic hypoxia enhances the maximum response to and therefore sensitivity to 5-HT in the branches of the pulmonary circulation studied. In fact, the maximum response to 5-HT has previously been shown to be enhanced in PAs obtained from both the monocrotaline-induced and hypoxia-induced models of PHT (Wanstall and Donnell, 1990; MacLean *et al.*, 1996). MacLean *et al.* (1996), found exposure to chronic hypoxia increased the sensitivity (pEC_{50}) of the main and first branch PA to 5-HT from 5.0 ± 0.2 to 6.4 ± 0.2, and 5.3 ± 0.1 to 6.3 ± 0.2, respectively

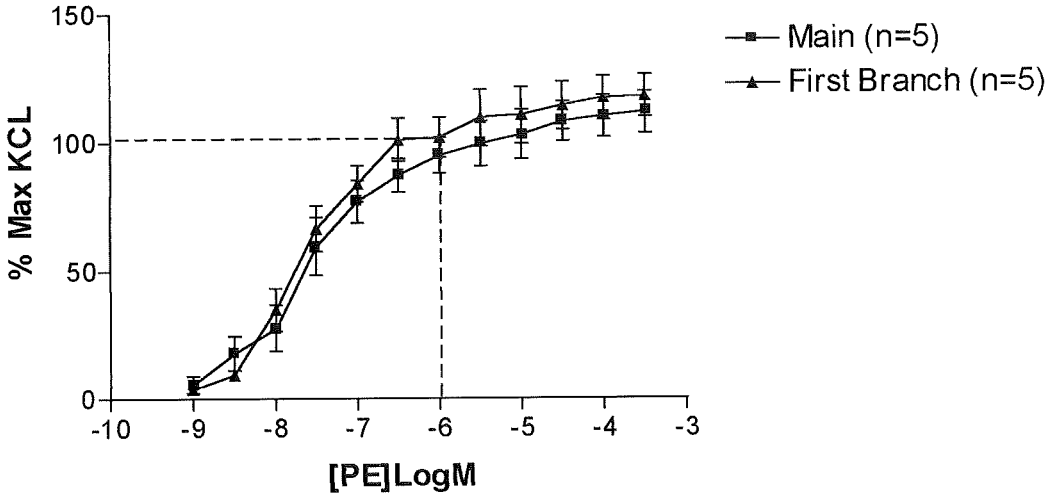
4.3.4 Response of hypoxic and endothelium-denuded main and first branch pulmonary arteries to ACh

Figures 4.3.4 and 4.3.20 compare the response to 1μM ACh in the main and first branch PAs used in both the SKF94836 and sildenafil studies. Exposure to chronic hypoxia markedly attenuated the response of 1μM ACh in both the main and first branch PAs.

The maximum relaxant response to 1μM ACh in the main and first branch PAs used in the SKF94836 study (figure 4.3.4), as % reversal of KCl induced contraction, were as follows: main PA control 50.1 ± 4.1% (n=15); main PA hypoxic 31.8 ± 2.8% (n=15 $P<0.05$, Student*t*-t test); first branch PA control 54.6 ± 3.6% (n=15); first branch PA hypoxic 33.9 ± 3.2% (n=15 $P<0.05$, Student*t*-t test). Likewise, the maximum relaxation to 1μM ACh in the main and first branch PAs used in the sildenafil study (figure 4.3.20), as % reversal of KCl induced contraction, were as follows: main PA control 47.9 ± 3.6% (n=15); main PA hypoxic 33.5 ± 3.6% (n=15 $P<0.05$, Student*t*-t test); first branch PA control 50.8 ± 4.0% (n=15); first branch PA hypoxic 34.4 ± 4.6% (n=15 $P<0.05$, Student*t*-t test). These results show the response to ACh is attenuated with hypoxia in the vessels studied.

To investigate the effect of endothelal dysfunction, the endothelium was removed by gently rubbing the lumen of the PA with forceps. The ability of 1×10^{-6}M ACh to relax preconstricted PAs was abolished on removal of the vascular endothelium (data not shown). Hence, PAs in this study were classified as endothelium-denuded if they did not significantly relax in response to 1×10^{-6}M ACh.

A)



B)

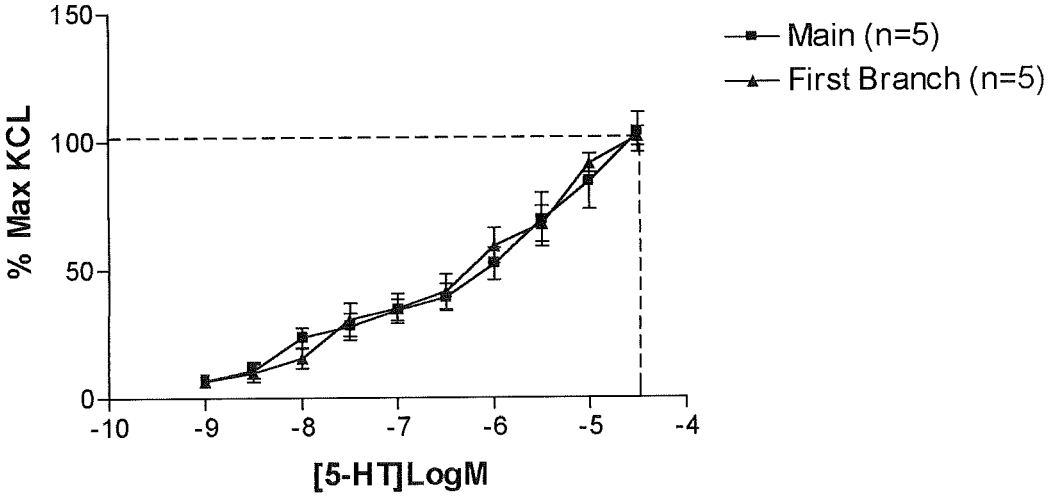
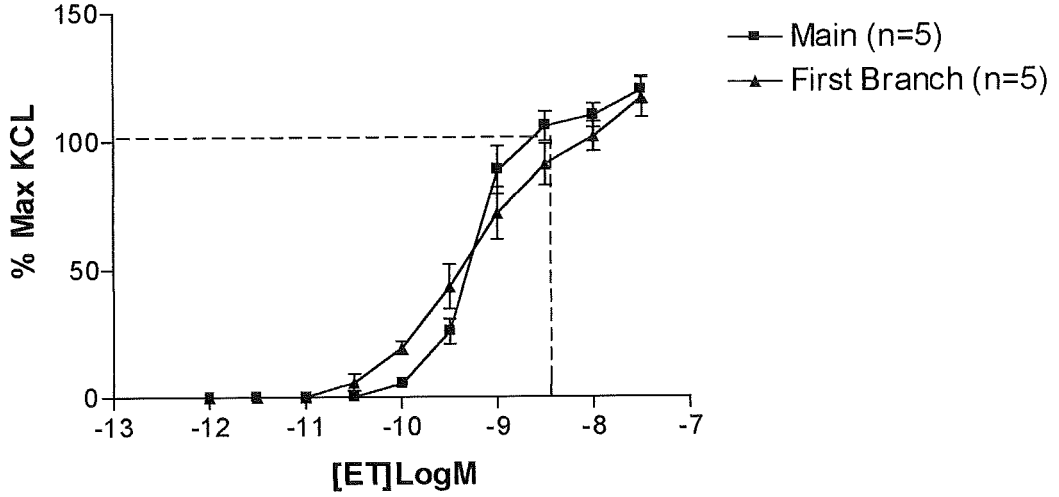


Figure 4.3.1. CCRC to PE and 5-HT in rat control main and first branch pulmonary artery

Cumulative concentration response curves to A) phenylephrine (PE), and B) 5-hydroxytryptamine, in main (■) and first branch pulmonary arteries (▲). Data are expressed as percentage of the response to 50mM KCl. Each point represents mean ± s.e.m., where n = number of different animals.

A)



B)

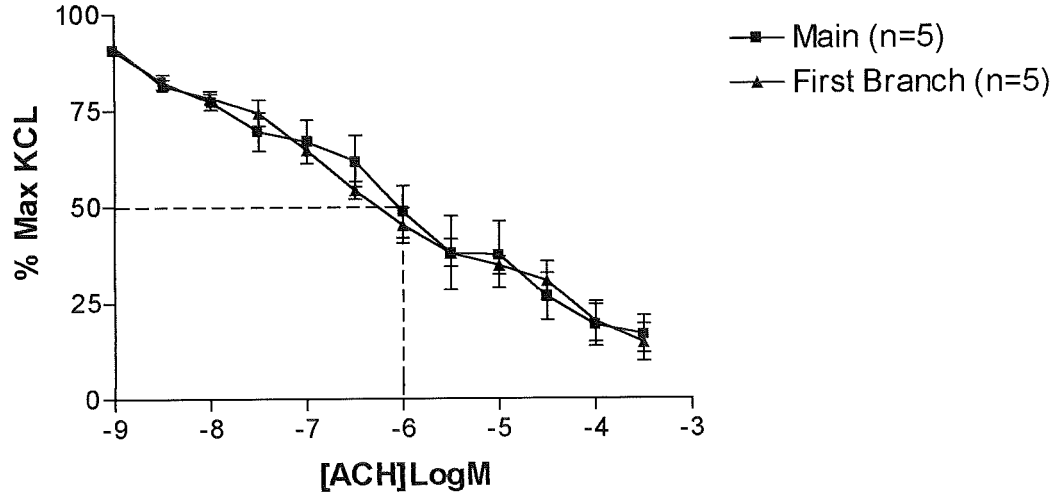
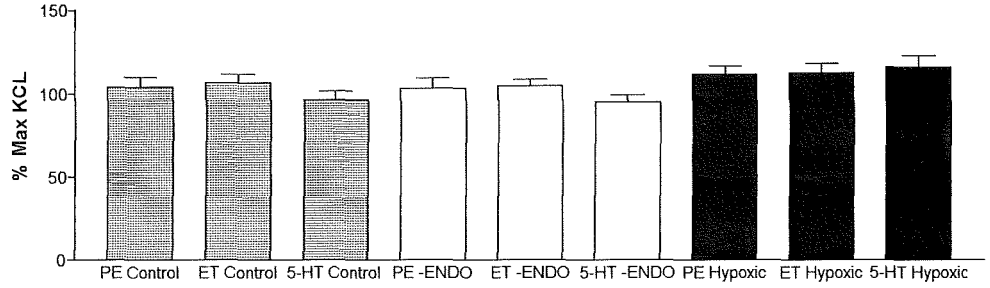


Figure 4.3.2. CCRC to ET-1 and ACh in rat control main and first branch pulmonary artery

Cumulative concentration response curves to A) endothelin-1 (ET-1) and B) acetylcholine (ACh) in main (■) and first branch pulmonary arteries (▲). To construct the CCRC for ACh tone was raised in each vessel with 1μM PE. Data are expressed as percentage of the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A) Main PA



B) First Branch PA

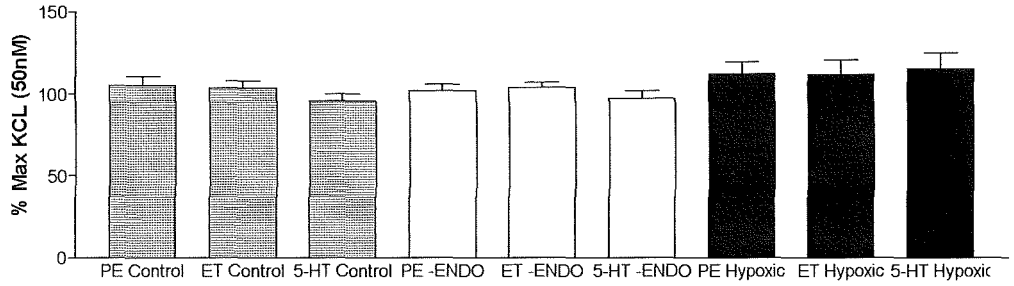


Figure 4.3.3 Maximum response to 1 x 10^{-6}M PE, 3 x 10^{-9}M ET-1 and 3 x 10^{-5}M 5-HT in rat control, endothelium-denuded and hypoxic A) main and B) first branch pulmonary arteries

Maximum response to 1 x 10^{-6}M phenylephrine (PE), 3 x 10^{-9}M endothelin-1 (ET-1) and 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) in control, endothelium-denuded (-endo) and hypoxic rat A) main and B) first branch (FB) pulmonary arteries. Data are expressed as percentage of the response to 50mM KCl, mean ± s.e.m,. where n=5 for all, (n = number of different animals). Data in the above figure represent those vessels in the SKF94838 study.

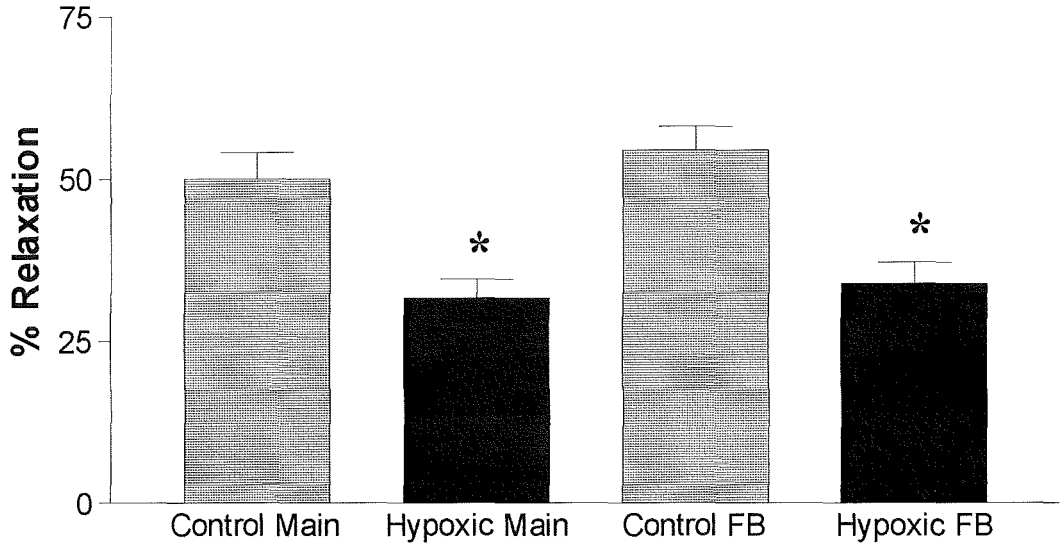


Figure 4.3.4. Maximum relaxation to 1 x 10^{-6}M ACh in rat control and hypoxic main and first branch pulmonary arteries

Maximum relaxation to 1 x 10^{-6}M acetylcholine (ACh) in control and hypoxic rat main and first branch (FB) pulmonary arteries precontracted with 1μM phenylephrine. Data are expressed as percentage of the response to 50mM KCl, mean ± s.e.m,. where n=15 for all, (n = number of different animals), and * denotes significance ($P<0.05$). The data in the above figure represent those vessels used in the SKF94838 study.

4.3.5 Vehicle DMSO

The vehicle for both SKF94836 and sildenafil in this study was dimethyl sulfoxide (DMSO). Therefore, in each experiment the response of the PA to DMSO alone over time was investigated (as a control). In every experiment the vehicle DMSO demonstrated a significant drop in tension in the PA (see figures 4.3.5A-4.3.34A). These results may account for the effect of the vehicle DMSO, or merely a drop in tension due to time. A time control with no DMSO could not also have been included as each PA could merely be cut into two equal sized rings. The effect of DMSO appeared dependent on the preconstrictor used, and differed when comparing the control, hypoxic and the endothelium-denuded PAs (data not listed, illustrated on figures 4.3.4A-4.3.34A). The DMSO alone control was always subtracted from the parallel measured response to the PDE inhibitor from the same tissue. This allowed the effects attributable to the PDE inhibitor alone to be obtained (see figures 4.3.5B-4.3.34B). Each figure (4.3.5 to 4.3.34) includes the possible effects of DMSO (top section, A), merely to demonstrate how the results may have been misinterpreted if this control had not been included.

4.3.6 Effect of the PDE3 inhibitor SKF94836 in control, hypoxic and endothelium-denuded main and first branch PA

The effect of the selective PDE3 inhibitor, SKF94836, on the active tone developed in response to three different preconstrictors was investigated. Cumulative concentration-response curves for SKF94836 (1×10^{-9}M to 3×10^{-5}M) were performed in control, endothelium-denuded, and hypoxic main, and first branch PAs (figures 4.3.5-4.3.18). The PAs were precontracted with either 1×10^{-6}M PE, 3×10^{-5}M 5-HT, or 3×10^{-9}M ET-1 and any changes in responsiveness of vessels were observed. EC_{50} values could generally not be calculated for each individual CCRC, as a maximum plateau was not reached. Throughout this study the maximum relaxation in response to 3×10^{-5}M SKF94836 (maximum concentration that could be added to the bath) was compared between groups. Therefore the maximal efficacy of the drug in each group was compared. Data are expressed throughout as % relaxation to the reference response to KCl (mean ± s.e.m). Statistical comparisons of the means of groups of data (maximum response) were made by use of Students *t*-test for paired or unpaired data where

appropriate. A level of probability of $P<0.05$ was taken to indicate statistical significance.

4.3.6.1 Preconstricted with 1 x 10^{-6}M PE

SKF94836 (1 x 10^{-9}M to 3 x 10^{-5}M) produced a small but concentration-dependent relaxation in control main and first branch PAs preconstricted with 1 x 10^{-6}M PE (figures 4.3.5B and 4.3.6B). The highest concentration of SKF94836 (3 x 10^{-5}M) that could be added did not completely relax either the main or first branch control PAs. The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 in the main and first branch control PAs precontracted with 1 x 10^{-6}M PE were; 16.8 ± 6.6% and 20.3 ± 5.1% respectively (% relaxation of reference KCl, figures 4.3.5B, 4.3.6B).

In control endothelium-denuded PAs precontracted with 1 x 10^{-6}M PE the response of SKF94836 was dependent on the PA branch studied. In the main PA it can be seen removal of the endothelium had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M SKF94836 when compared to control (figure 4.3.5B). The maximum relaxation achieved by SKF94836 in the main control and endothelium-denuded PA precontracted with 1 x 10^{-6}M PE, were: control, 16.8 ± 6.6% (n=8), endothelium-denuded, 7.98 ± 8.9% (n=6, NS, control *versus* endothelium-denuded, Student's *t*-test). In contrast, in the first branch PA preconstricted with 1 x 10^{-6}M PE, removal of the endothelium significantly reduced the relaxant effect of SKF94836 (figure 4.3.6B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 in the control and endothelium-denuded first branch PA preconstricted with 1 x 10^{-6}M PE, were: control, 20.3 ± 5.1% (n=8), endothelium-denuded, 5.49 ± 7.3% (n=5, $P<0.05$, control *versus* endothelium-denuded, Student's *t*-test). The data presented here suggests that when tone is raised by 1 x 10^{-6}M PE, the PDE3 inhibitor SKF94836 causes a significant dose-dependent relaxation of isolated PAs, predominantly through an endothelium-independent effect in the main branch, however through an endothelium-dependent effect in the first branch PAs.

Furthermore, SKF94836 (1 x 10^{-9}M to 3 x 10^{-5}M) caused a markedly enhanced concentration-dependent relaxation in both main and first branch hypoxic PAs preconstricted with 1 x 10^{-6}M PE (figures 4.3.7B and 4.3.8B). In the main PA, preconstricted with 1 x 10^{-6}M PE, hypoxia increased the maximum relaxation produced

by 3 x 10^{-5}M SKF94836 when compared to control (figure 4.3.7B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 were: control 16.8 ± 6.6% (n=8), hypoxic 79 ± 4.6% (n=6, P<0.05, control *versus* hypoxic, Student's *t*-test). Similarly, in the first branch PAs preconstricted with 1 x 10^{-6}M PE, hypoxia increased the relaxant effect of SKF94836. The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 seen in figure 4.3.8B was: control 20.3 ± 5.1% (n=8), hypoxic 85.1 ± 7.9% (n=6, P<0.05, control *versus* hypoxic, Student's *t*-test). These results suggest that hypoxia uncovers a significantly greater response to SKF94836 in both the main and first branch PA preconstricted with 1 x 10^{-6}M PE.

4.3.6.2 Preconstricted with 3 x 10^{-5}M 5-HT

After the addition of 3 x 10^{-5}M 5-HT, cumulative concentration-response curves for SKF94836 (1 x 10^{-9}M to 3 x 10^{-5}M) were performed in control, endothelium-denuded, and hypoxic main and first branch PAs. (Figures 4.3.9B-4.3.12B). As above the maximum relaxation in response to 3 x 10^{-5}M SKF94836 (efficacy) was compared between groups.

SKF94836 (1 x 10^{-9}M to 3 x 10^{-5}M) produced a small concentration-dependent relaxation in control main and first branch PAs preconstricted with 3 x 10^{-5}M 5-HT (figure 4.3.9B-4.3.10B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 in the main and first branch control PAs preconstricted with 3 x 10^{-5}M 5-HT was 29.9 ± 5.3%, and 40.3 ± 9.5%, respectively (% relaxation of reference KCl).

In control endothelium-denuded PAs precontracted with 3 x 10^{-5}M 5-HT the response of SKF94836 was again dependent on the PA branch studied. In the main PA it can be seen removal of the endothelium had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M SKF94836 when compared to control (figure 4.3.9B). The maximum relaxation achieved by SKF94836 in the main control and endothelium-denuded main PA precontracted with 3 x 10^{-5}M 5-HT, were: control, 29.9 ± 5.3% (n=5), endothelium-denude 16.52 ± 8.9% (n=5, NS, control *versus* endothelium-denuded). In contrast, in the first branch PA preconstricted with 3 x 10^{-5}M 5-HT, removal of the endothelium significantly abolished the effect of SKF94836 (figure 4.3.10B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 in the control and endothelium-denuded first branch PA preconstricted with 3 x 10^{-5}M 5-HT, were: control 40.3 ±

9.54% (n=7), endothelium-denude −10.8 ± 8.2% (n=5, P<0.05, control *versus* endothelium-denuded control). The data presented here suggests that when tone is raised by 3 x 10^{-5}M 5-HT, the PDE3 inhibitor SKF94836 causes a significant dose-dependent relaxation of isolated PAs, predominantly through an endothelium-independent effect in the main branch, however through an endothelium-dependent effect in the first branch PAs.

SKF94836 (1 x 10^{-9}M to 3 x 10^{-5}M) caused a greatly enhanced concentration-dependent relaxation in both main and first branch hypoxic PAs preconstricted with 3 x 10^{-5}M 5-HT (figures 4.3.11B and 4.3.12B). In the main PA preconstricted with 3 x 10^{-5}M 5-HT it can be seen from figure 4.3.11B that hypoxia potentiates the maximum relaxation produced by 3 x 10^{-5}M SKF94836 when compared to control. The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 was: control 29.9 ± 5.3% (n=5), hypoxic 83.9 ± 8.1% (n=5, P<0.05, control *versus* hypoxic). Similarly, in the first branch PAs preconstricted with 3 x 10^{-5}M 5-HT, hypoxia increased the relaxant effect of SKF94836 (figure 4.3.12B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 was: control 40.3 ± 9.5% (n=7), hypoxic 88.5 ± 5.9% (n=5, P<0.05, control *versus* hypoxic control). These results suggest that hypoxia uncovers a significantly greater response to SKF94836 in both the main and first branch PA preconstricted with 5-HT.

4.3.6.3 Preconstricted with 3 x 10^{-9}M ET-1

After the addition of 3 x 10^{-9}M ET-1, cumulative concentration-response curves for SKF94836 (1 x 10^{-9}M to 3 x 10^{-5}M) were performed in control, endothelium-denuded, and hypoxic main and first branch PAs. (Figures 4.3.13B-4.3.16B). As above the maximum relaxation in response to 3 x 10^{-5}M SKF94836 (efficacy) was compared between groups.

SKF94836 (1 x 10^{-9}M to 3 x 10^{-5}M) produced a small concentration-dependent relaxation in control main and first branch PAs preconstricted with 3 x 10^{-9}M ET-1 (figure 4.3.13B-4.3.14B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 in the main and first branch control PAs precontracted with 3 x 10^{-9}M ET-1 was, 19.9 ± 2.6%, and 39.9 ± 3.15%, respectively (% relaxation of reference KCl).

In control endothelium-denuded PAs precontracted with 3 x 10^{-9}M ET-1 the response of SKF94836 was, as with the other preconstrictors, dependent on the PA branch studied. In the main PA it can be seen from figure 4.3.13B that removal of the endothelium had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M SKF94836 when compared to control. The maximum relaxation achieved by SKF94836 in the main control and endothelium-denuded main PA precontracted with 3 x 10^{-9}M ET-1, was: control 19.9 ± 2.6% (n=7), endothelium-denuded 20.9 ± 8.9%(n=5, NS, control *versus* endothelium-denuded). In contrast, in the first branch PA preconstricted with 3 x 10^{-9}M ET-1, removal of the endothelium significantly reduced the relaxatory effect of SKF94836 (figure 4.3.14B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 in the control and endothelium-denuded first branch PA preconstricted with 3 x 10^{-9}M ET-1, were: control 39.9 ± 3.15% (n=7), endothelium-denuded, 20.4 ± 7.3% (n=5, $P<0.05$, control *versus* endothelium-denuded). The data presented here suggests that when tone is raised by 3 x 10^{-9}M ET-1, the PDE3 inhibitor SKF94836 causes a significant dose-dependent relaxation of isolated PAs, predominantly through an endothelium-independent effect in the main branch, however possibly through a more endothelium-dependent effect in the first branch PAs. Although, unlike the response seen in the PE and 5-HT precontracted first branch PAs, when active tension developed in response to ET-1 in the first branch, the response to SKF94836 was not abolished but merely attenuated.

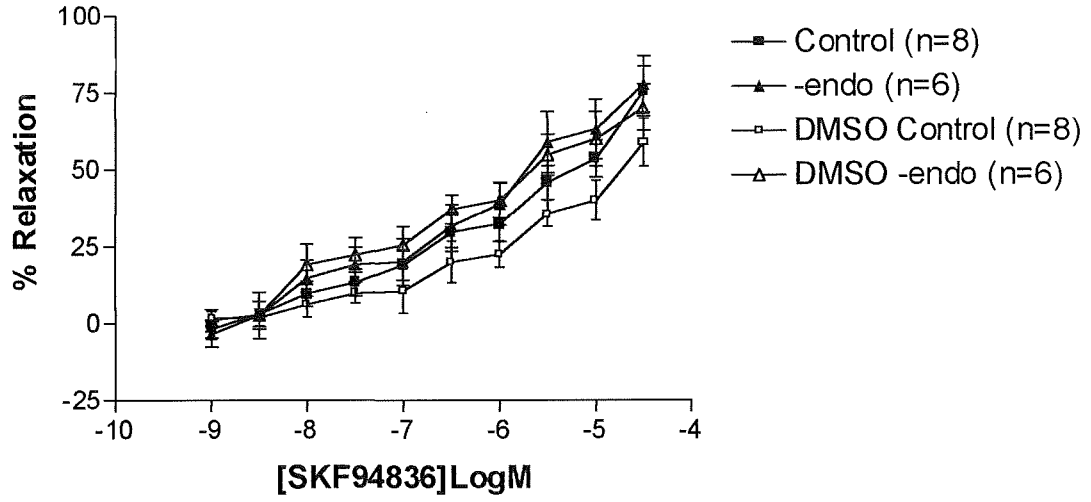
SFK94836 (1 x 10^{-9}M to 3 x 10^{-5}M) caused a markedly enhanced concentration-dependent relaxation in both main and first branch hypoxic PAs preconstricted with 3 x 10^{-9}M ET-1 (figures 4.3.15B and 4.3.16B). In the main PA preconstricted with 3 x 10^{-9}M ET-1 it can be seen hypoxia potentiates the maximum relaxation produced by 3 x 10^{-5}M SKF94836 when compared to control (figure 4.3.15B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 was: control 19.9 ± 2.6% (n=7), hypoxic 52.2 ± 7.5% (n=5, $P<0.05$, control *versus* hypoxic). Similarly, in the first branch PAs preconstricted with 3 x 10^{-9}M ET-1, hypoxia very substantially increased the relaxant effect of SKF94836 (figure 4.3.16B). The maximum relaxation achieved by 3 x 10^{-5}M SKF94836 was: control 39.9 ± 3.1% (n=7), hypoxic 220.9 ± 22.4% (n=5, $P<0.05$, control *versus* hypoxic). These results suggest that hypoxia uncovers a significantly greater response to SKF94836 in both the main and first branch PA preconstricted with ET-1.

4.3.7 Comparison of the effects of the PDE3 inhibitor SKF94836 with each preconstrictor

Figures 4.3.17 and 4.3.18 show the comparison of the relaxant effects of SKF94836 between each preconstrictor in the control and hypoxic main and first branch PAs respectively. In the control main PA the efficacy of SKF94836 was not dependent on the preconstrictor. The rank order of SKF94836 for each preconstrictor in the main branch was 5-HT=ET-1=PE (% relaxation of reference KCl: 29.9 ± 5.3%, 19.9 ± 2.6%, 16.8 ± 6.6% respectively, where = signifies an equal relaxant effect of SKF94836, figure 4.3.17A). In parallel, in the hypoxic main PA the maximum relaxation induced by SKF94836 was dependent on the preconstrictor. The level of relaxation induced by SKF94836 was significantly greater when the tone was raised with either 5-HT or PE, than when the tone was raised with ET-1. The order of efficacy of 3 x 10^{-5}M SKF9836 for each preconstrictor in the hypoxic main branch was 5-HT=PE>ET-1 (% relaxation of reference KCl: 83.9 ± 8.1%, 79 ± 4.6%, 52.2 ± 7.5% respectively, where > signifies a greater maximum relaxation with SKF94836, and = signifies an equal relaxant effect of SKF94836, figure 4.3.17B). It can be noted that the preconstrictor-dependent relaxation induced by SKF94836 alters with hypoxia.

Similarly, in the control first branch PA, the efficacy of SKF94836 was also dependent on the preconstrictor. The level of relaxation induced by SKF94836 was significantly greater when the tone was raised with either 5-HT or ET-1 than when the tone was raised with PE. The order of efficacy of 3 x 10^{-5}M SKF94836 for each preconstrictor in the first branch was 5-HT=ET-1>PE (% relaxation of reference KCl: 40.3 ± 9.5%, 39.9 ± 3.15%, 20.3 ± 5.1% respectively, where > signifies a greater maximum relaxation with SKF94836, and = signifies an equal relaxant effect of SKF94836, figure 4.3.18A). Similarly, in the hypoxic main PA the maximum relaxation induced by SKF94836 was dependent on the preconstrictor. The order of efficacy of 3 x 10^{-5}M SKF9836 for each preconstrictor in the hypoxic first branch was ET-1>5-HT=PE (% relaxation of reference KCl: 220.9 ± 22.4%, 88.47 ± 5.97%, 85.07 ± 7.89% respectively, where > signifies a greater maximum relaxation with SKF94836, and = signifies an equal relaxant effect of SKF94836, figure 4.3.18B). Again it can be noted that the preconstrictor dependent relaxation induced by SKF94836 in the first branch alters with hypoxia.

A)



B) Minus possible effects of the vehicle DMSO

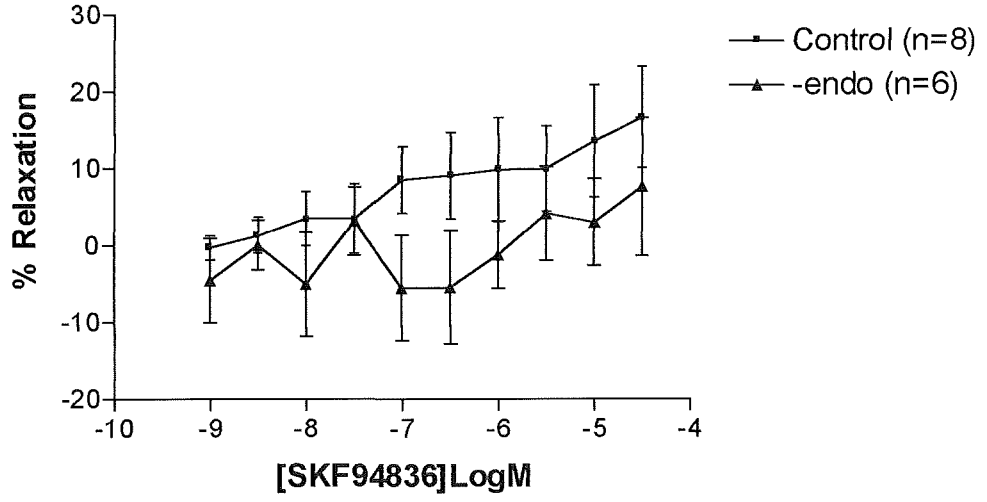
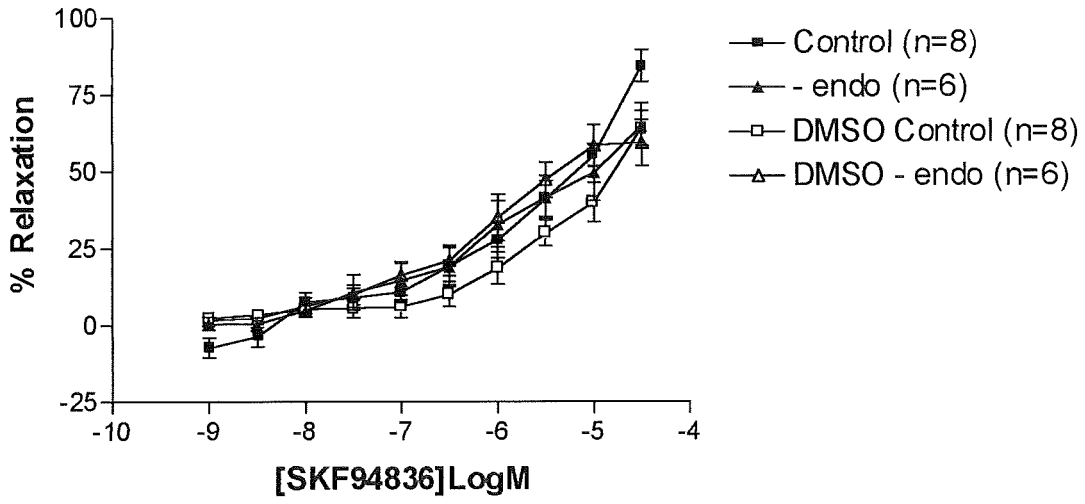


Figure 4.3.5. CCRC to SKF94836 in rat control and endothelium-denuded main pulmonary artery preconstricted with 1 x 10^{-6}M PE

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) main pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) main pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) B) Minus possible effects of the vehicle DMSO

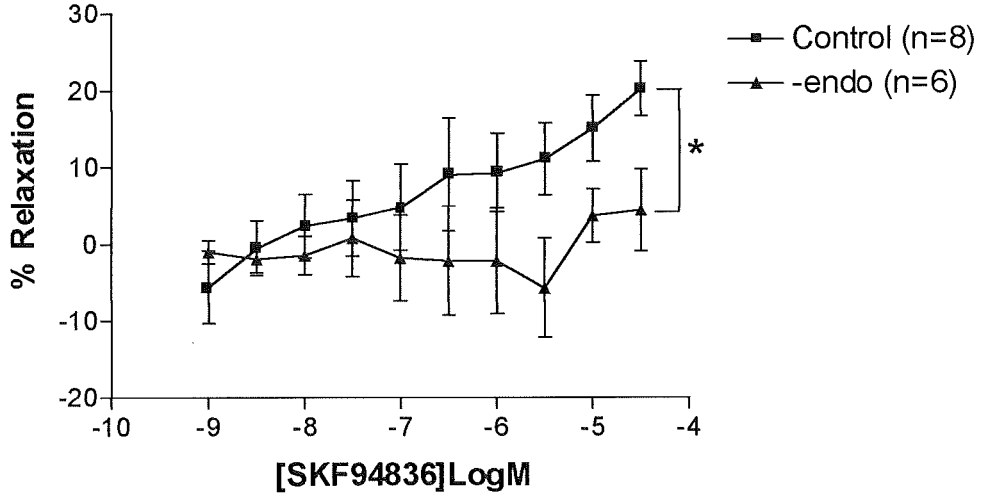
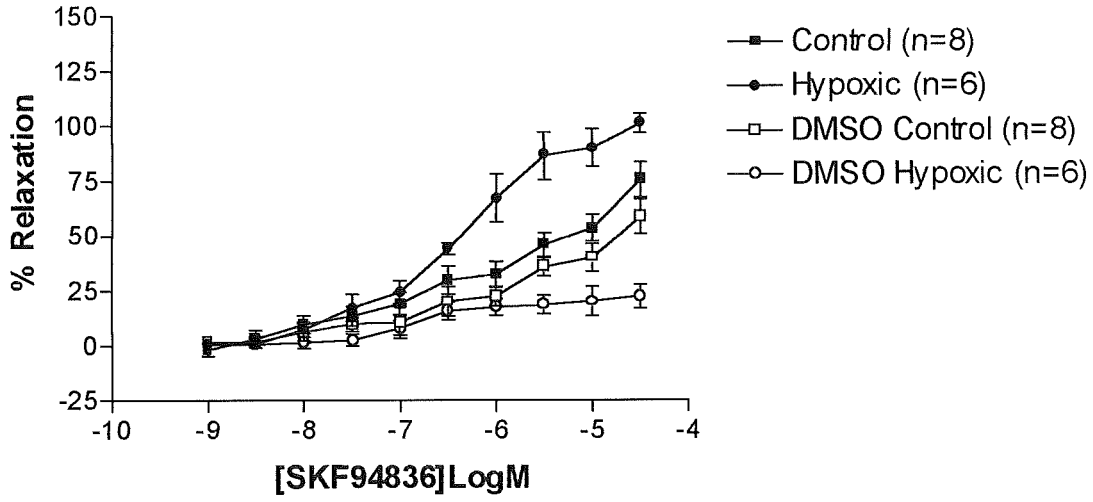


Figure 4.3.6. CCRC to SKF94836 in rat control and endothelium-denuded first branch pulmonary artery preconstricted with 1 x 10^{-6}M PE

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) first branch pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) first branch pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of the vehicle DMSO

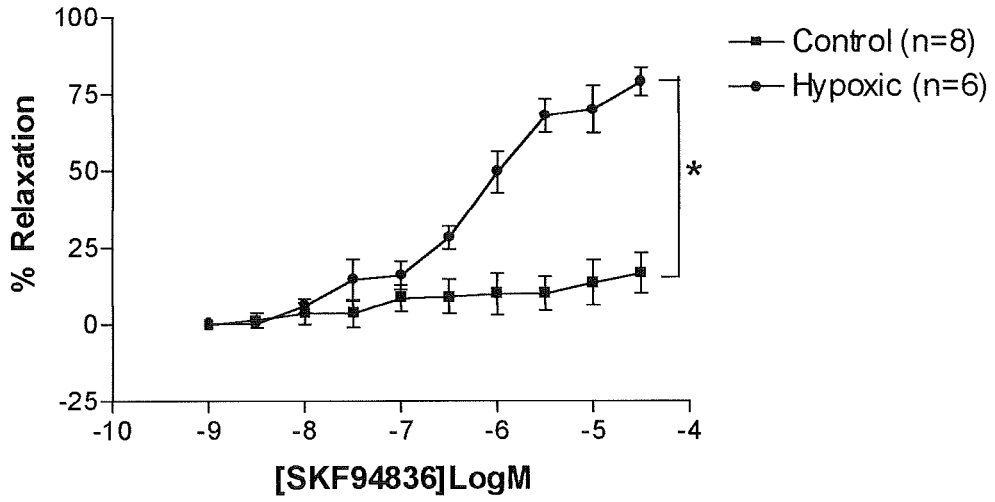
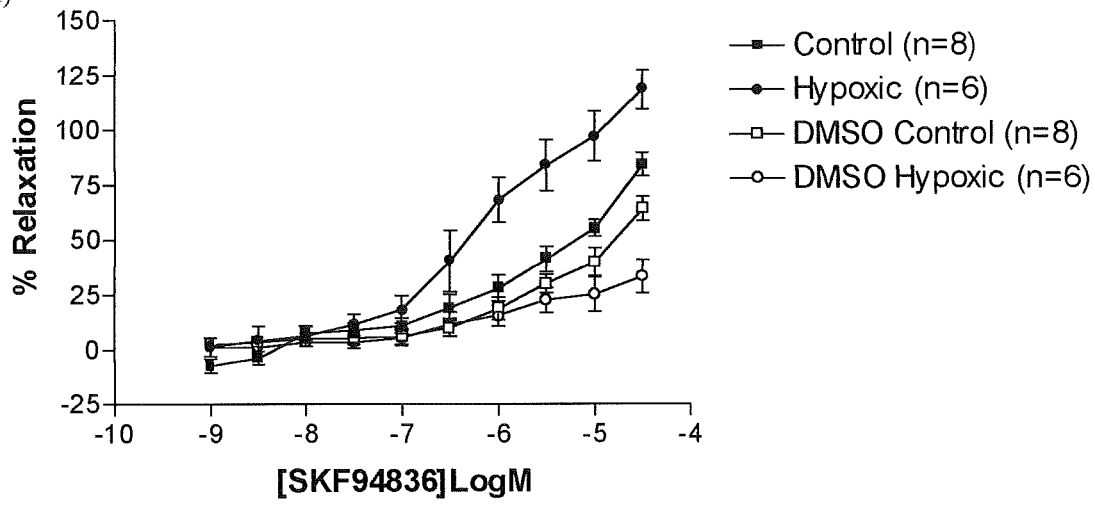


Figure 4.3.7. CCRC to SKF94836 in rat control and hypoxic main pulmonary artery preconstricted with 1 x 10^{-6}M PE

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) main pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) main pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of DMSO

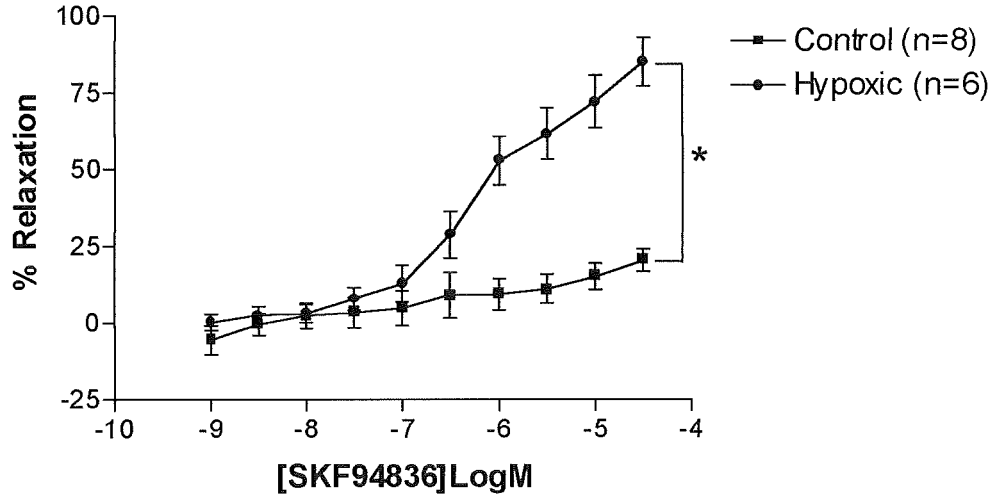
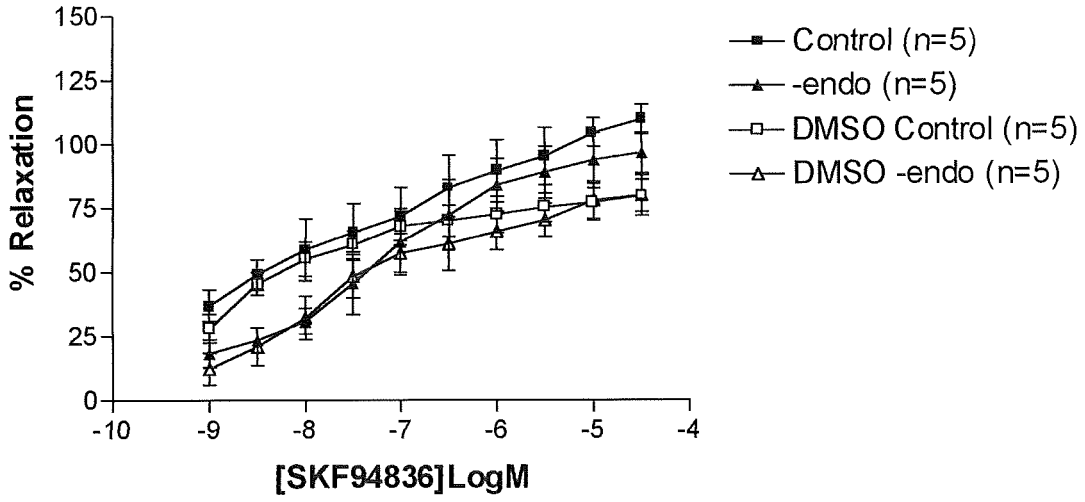


Figure 4.3.8. CCRC to SKF94836 in rat control and hypoxic first branch pulmonary artery preconstricted with 1 x 10^{-6}M PE

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) first branch pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) first branch pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of the vehicle DMSO

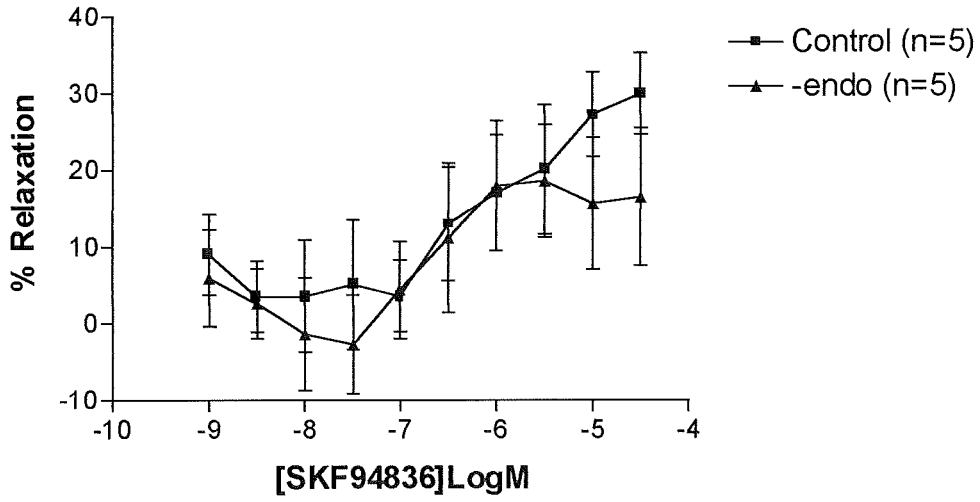
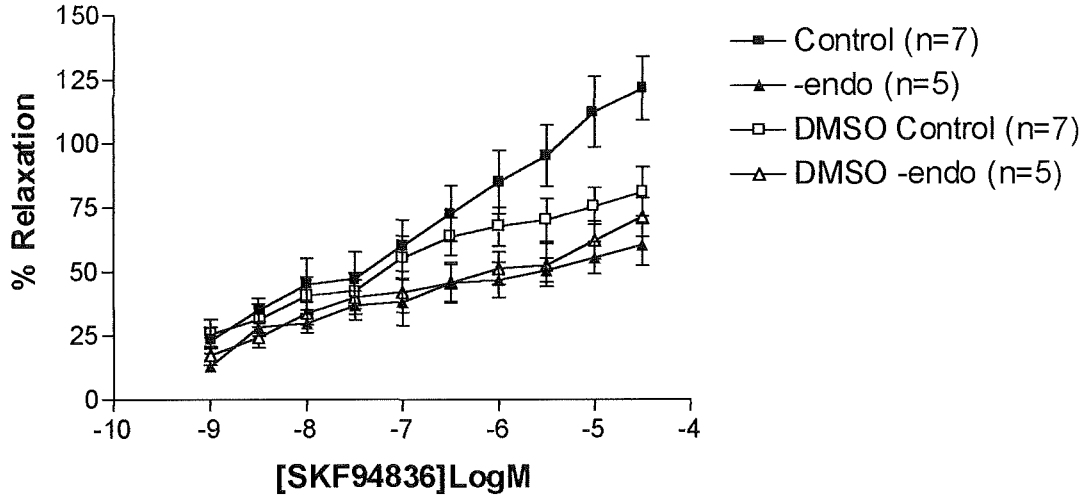


Figure 4.3.9. CCRC to SKF94836 in rat control and endothelium-denuded main pulmonary artery preconstricted with 3 x 10^{-5}M 5-HT

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) main pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) main pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) Minus possible effects of the vehicle DMSO

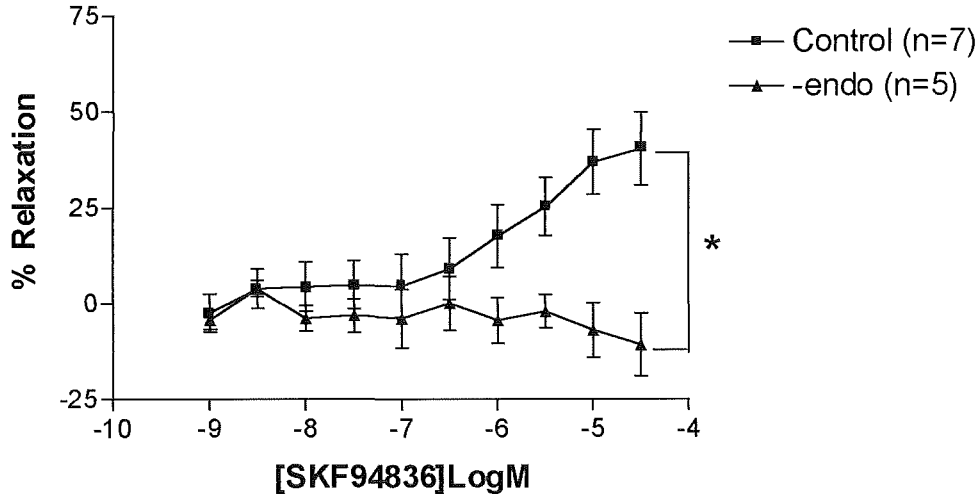
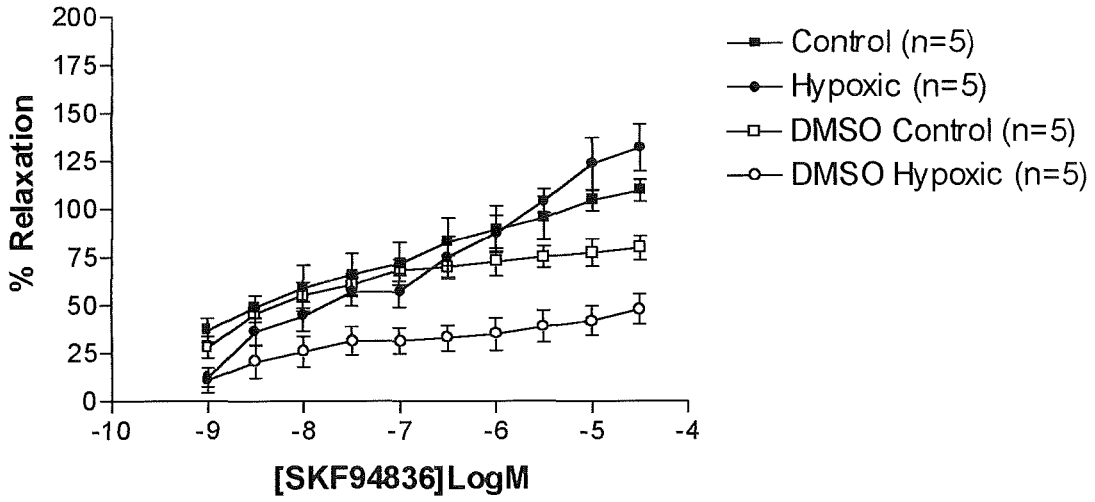


Figure 4.3.10. CCRC to SKF94836 in rat control and endothelium-denuded first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-HT

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of the vehicle DMSO

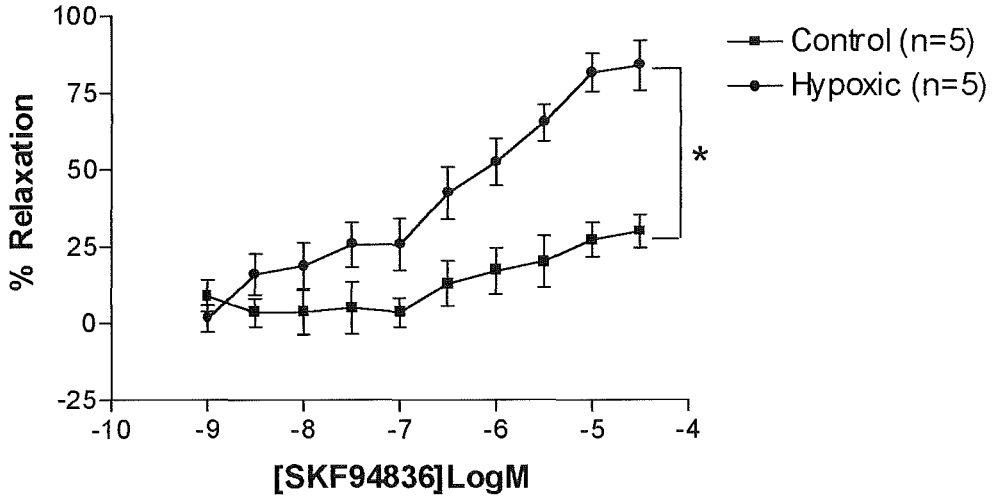
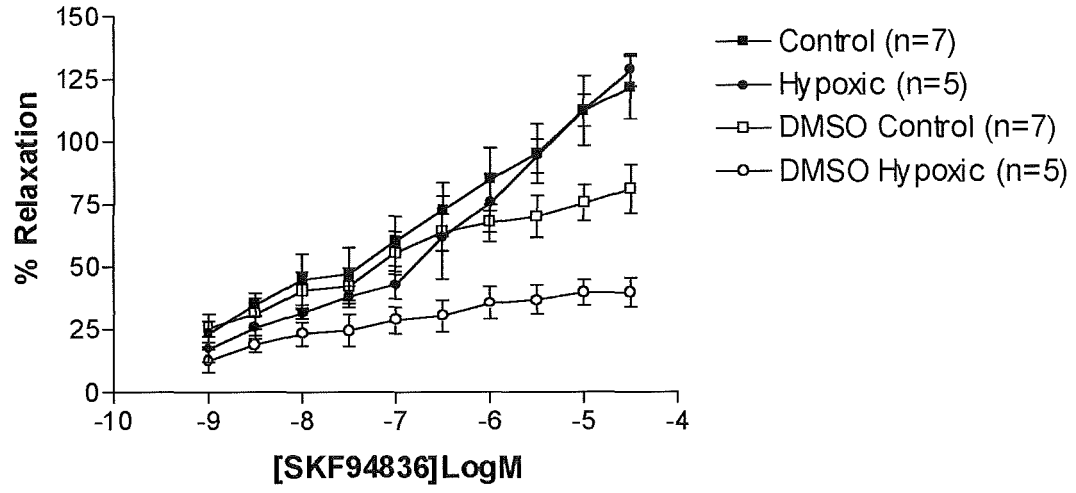


Figure 4.3.11. CCRC to SKF94836 in rat control and hypoxic main pulmonary artery preconstricted with 3 x 10^{-5}M 5-HT

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) main pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) main pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of the vehicle DMSO

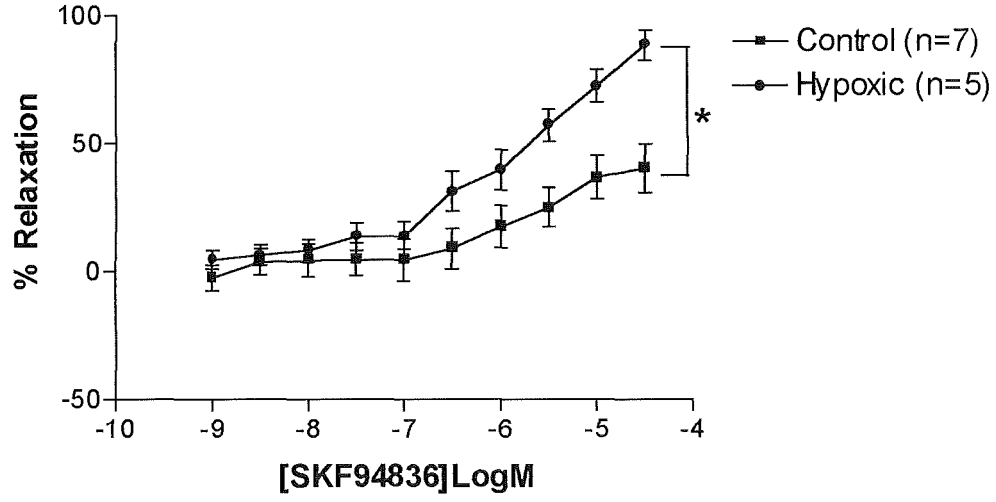
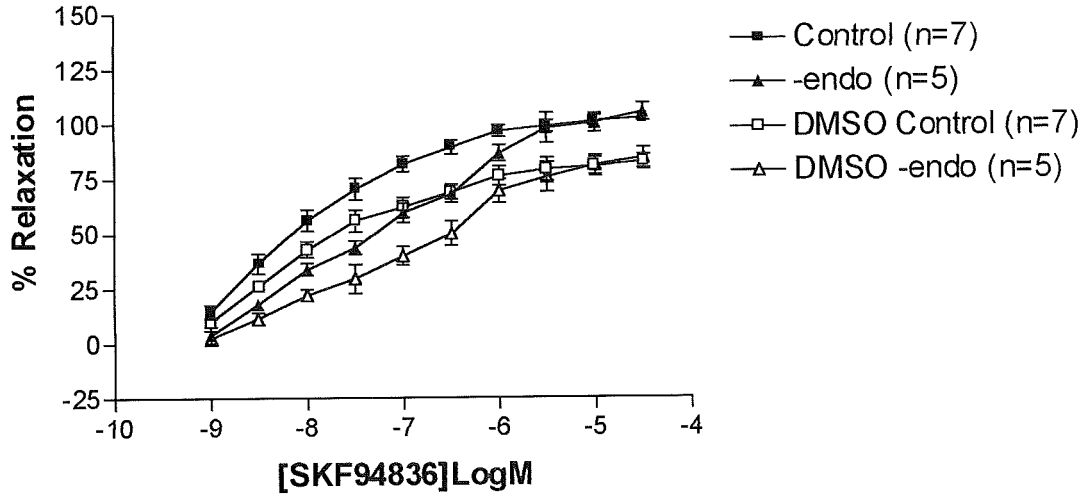


Figure 4.3.12. CCRC to SKF94836 in rat control and hypoxic first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-HT

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of the vehicle DMSO

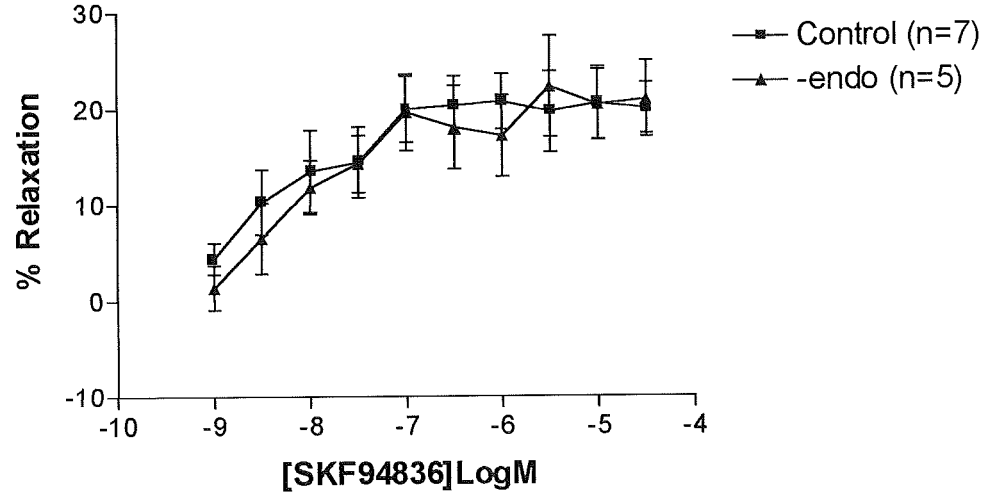
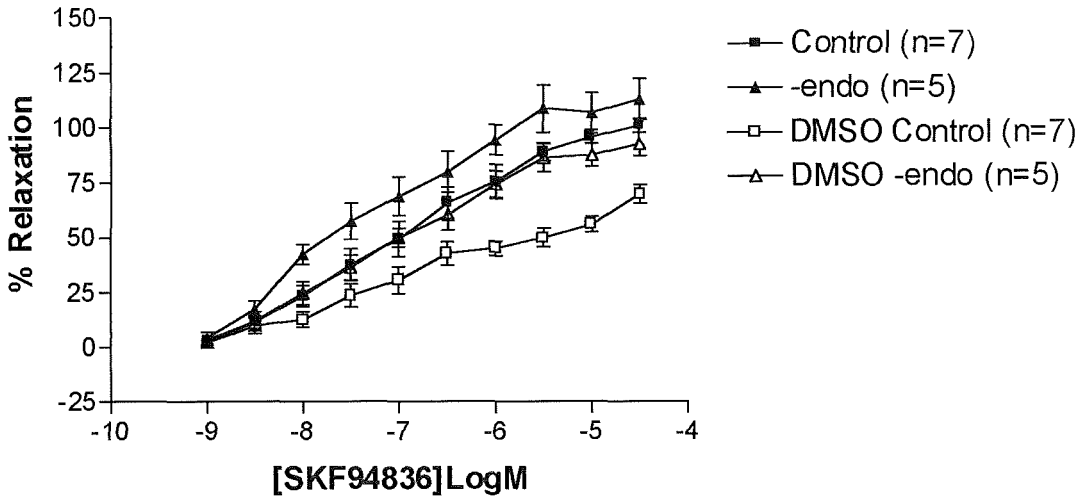


Figure 4.3.13. CCRC to SKF94836 in rat control and endothelium-denuded main pulmonary artery preconstricted with 3 x 10^{-9}M ET-1

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) main pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) main pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) Minus possible effects of vehicle DMSO

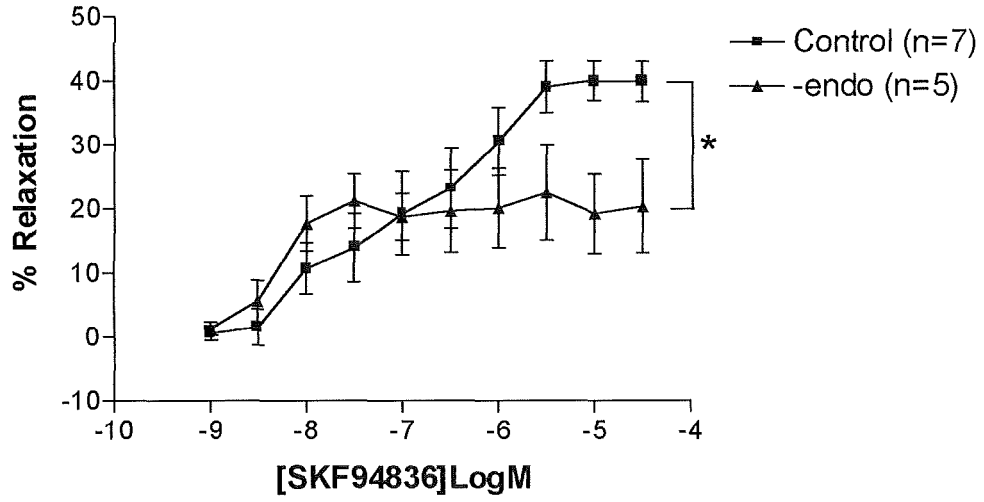
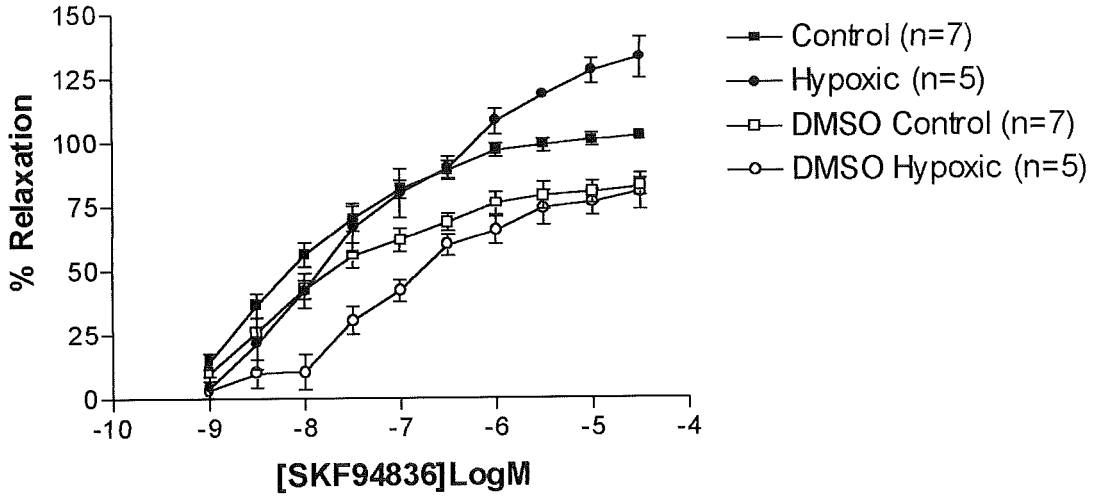


Figure 4.3.14. CCRC to SKF94836 in rat control and endothelium-denuded first branch pulmonary artery preconstricted with 3 x 10^{-9}M ET-1

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) first branch pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) first branch pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of vehicle DMSO

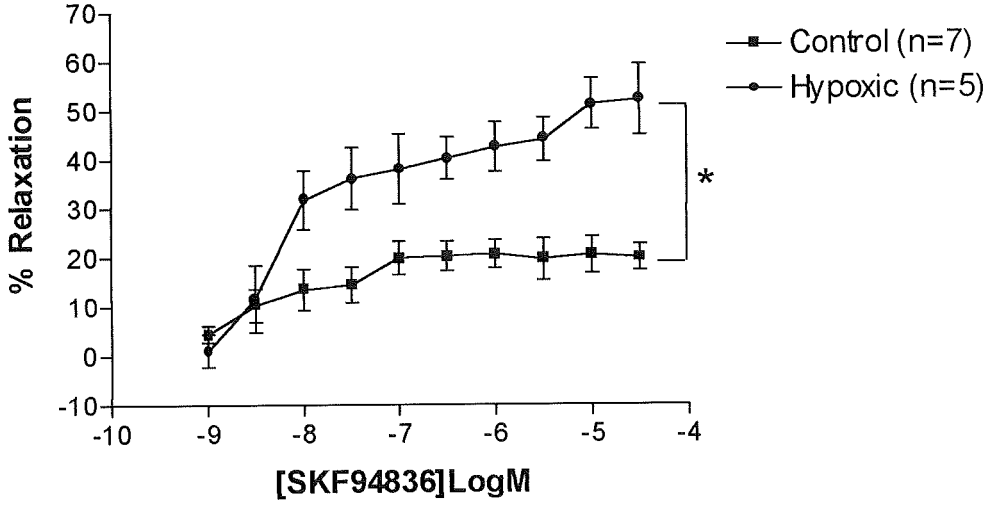
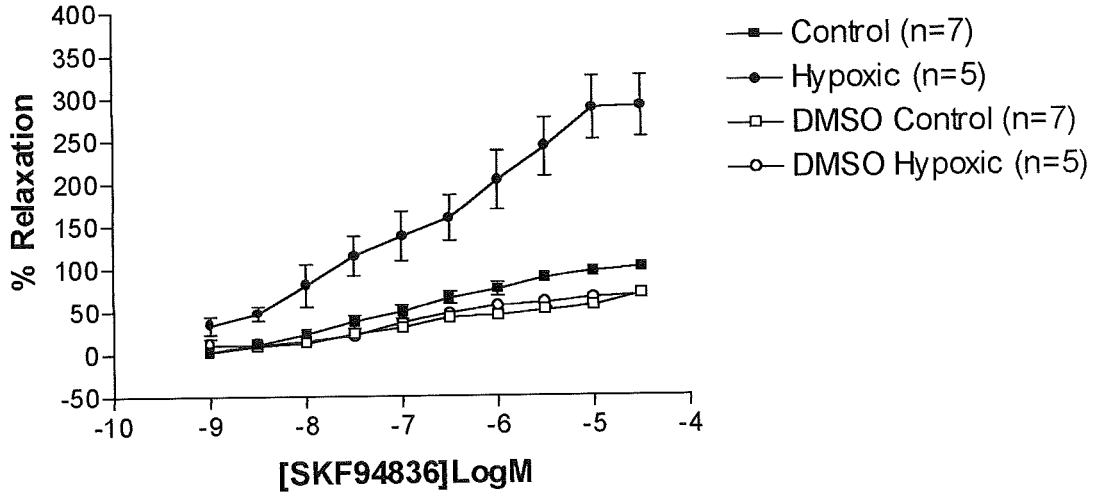


Figure 4.3.15. CCRC to SKF94836 in rat control and hypoxic main pulmonary artery preconstricted with 3 x 10^{-9}M ET-1

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) main pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) main pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of vehicle DMSO

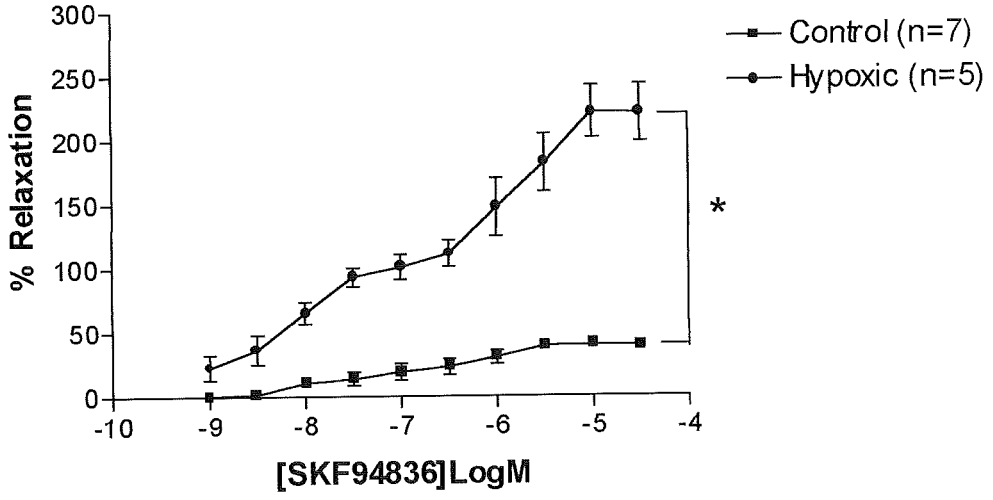
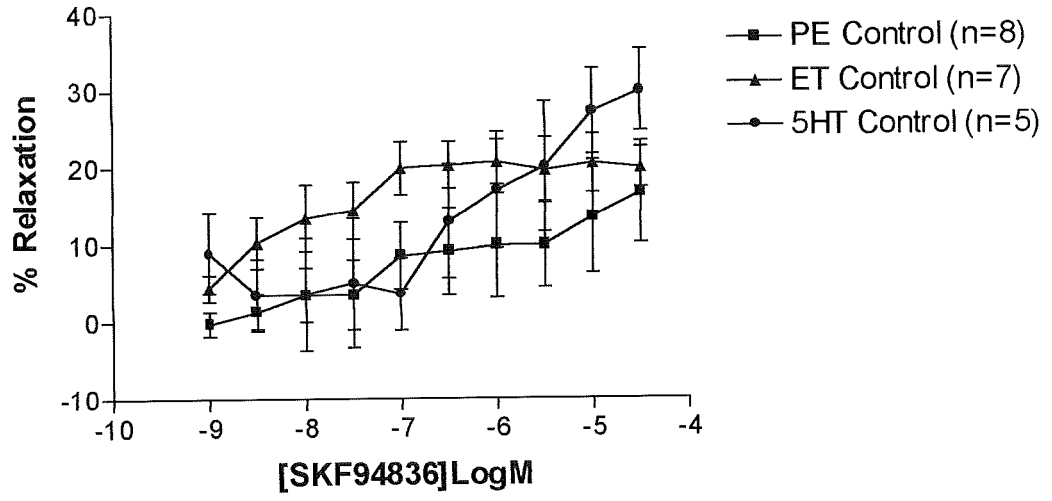


Figure 4.3.16. CCRC to SKF94836 in rat control and hypoxic first branch pulmonary artery preconstricted with 3 x 10^{-9}M ET-1

A) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) first branch pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1). B) Cumulative concentration response curves to the PDE3 inhibitor SKF94836 alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) first branch pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B)

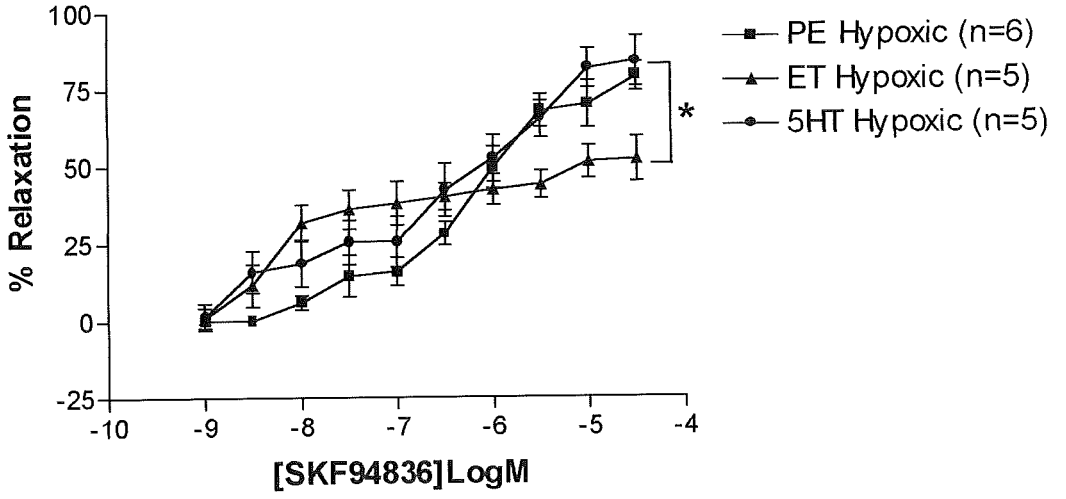
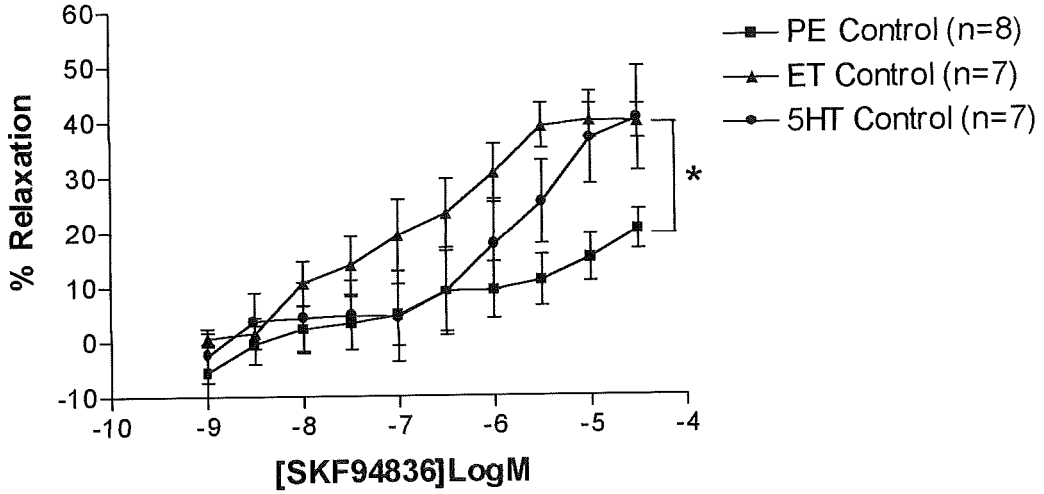


Figure 4.3.17. CCRC to SKF94836 (minus effect DMSO) in rat control and hypoxic main pulmonary artery preconstricted with 1 x 10^{-6}M PE, 3 x 10^{-5}M 5-HT and 3 x 10^{-9}M ET-1

Cumulative concentration response curves to the PDE3 inhibitor SKF94836 in A) control and B) hypoxic main pulmonary artery preconstricted with 1 x10^{-6}M phenylephrine (PE, ■), 3 x 10^{-5}M 5-hydroxytryptamine (5-HT, ●) and 3 x 10^{-9}M endothelin-1 (ET-1, ▲). In each CCRC the effect of the vehicle DMSO has been subtracted. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B)

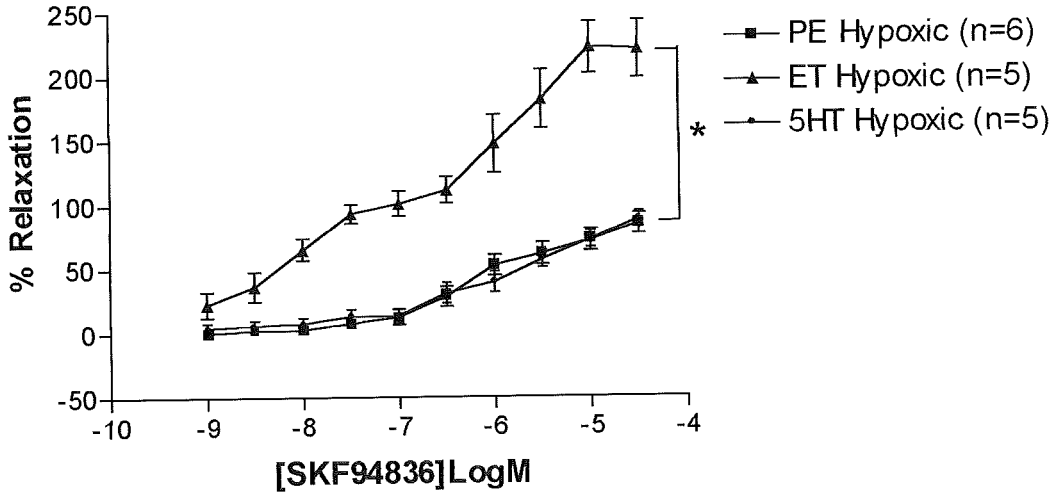


Figure 4.3.18. CCRC to SKF94836 (minus effect DMSO) in rat control and hypoxic first branch pulmonary artery preconstricted with 1 x 10^{-6}M PE, 3 x 10^{-5}M 5-HT and 3 x 10^{-9}M ET-1

Cumulative concentration response curves to the PDE3 inhibitor SKF94836 in A) control and B) hypoxic first branch pulmonary artery preconstricted with 1 x10^{-6}M phenylephrine (PE, ■), 3 x 10^{-5}M 5-hydroxytryptamine (5-HT, ●) and 3 x 10^{-9}M endothelin-1 (ET-1, ▲). In each CCRC the effect of the vehicle DMSO has been subtracted. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

4.3.8 Effect of the PDE5 inhibitor sildenafil in control, hypoxic and endothelium-denuded main and first branch PA

In these sets of experiments, the effect of the selective PDE5 inhibitor sildenafil on the tone elicited by three different preconstrictors was investigated. Cumulative concentration-response curves for sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) were performed in control, endothelium-denuded, and hypoxic main and first branch PAs (figures 4.3.21-4.3.34). The PAs were precontracted with either 1 x 10^{-6}M PE, 3 x 10^{-5}M 5-HT, or 3 x 10^{-9}M ET-1 and any changes in responsiveness of vessels were observed. Again EC_{50} values could generally not be calculated for each individual CCRC as a maximum plateau was not reached. Throughout this study the maximum relaxation in response to 3 x 10^{-5}M sildenafil (maximum concentration that could be added to the bath) was compared between groups. Therefore the maximal efficacy of the drug in each group was compared. Data are expressed throughout as % relaxation to the reference response to KCl (mean ± s.e.m). Statistical comparisons of the means of groups of data (maximum response) were made by use of Students *t*-test for paired or unpaired data where appropriate. A level of probability of $P<0.05$ was taken to indicate statistical significance.

4.3.8.1 Preconstricted with 1 x 10^{-6}M PE

Sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) produced a concentration-dependent relaxation in control main and first branch PAs preconstricted with 1 x 10^{-6}M PE (figures 4.3.21B and 4.3.22B). The maximum concentration of sildenafil (3 x 10^{-5}M) did not completely relax these main or first branch control PAs. Maximum relaxation achieved by 3 x 10^{-5}M sildenafil in the main and first branch control PAs precontracted with 1 x 10^{-6}M PE were 45.3 ± 5.2%, and 38 ± 2.5%, respectively (% relaxation of reference KCl, figures 4.3.21B and 4.3.22B).

Sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) caused a concentration-dependent relaxation in control endothelium-denuded main PAs precontracted with 1 x 10^{-6}M PE (figure 4.3.21B and 4.3.22B). In the main PA it can be seen removal of the endothelium had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M sildenafil when compared to control. The maximum relaxation achieved by sildenafil in the main control and endothelium-denuded main PA precontracted with 1 x 10^{-6}M PE, were:

control 45.3 ± 5.2% (n=5), endothelium-denuded 40.1 ± 9.2% (n=6, NS, control *versus* endothelium-denuded, Student's *t*-test, figure 4.3.21B). Likewise, in the first branch PA preconstricted with 1 x 10^{-6}M PE, removal of the endothelium also had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M sildenafil. The maximum relaxation achieved by 3 x 10^{-5}M sildenafil in the control and endothelium-denuded first branch PA preconstricted with 1 x 10^{-6}M PE, were: control, 38 ± 2.5% (n=5), endothelium-denuded 42.2 ± 5.8% (n=5, NS, control *versus* endothelium-denuded, Student's *t*-test, figure 4.3.22B). These results suggest that when 1 x 10^{-6}M PE raises tone, the PDE5 inhibitor sildenafil has a significant endothelium-independent vasodilatory effect in both the main and first branch control PAs.

In addition, sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) caused a concentration-dependent relaxation in both main and first branch hypoxic PAs preconstricted with 1 x 10^{-6}M PE (figure 4.3.23B and 4.3.24B). In the main PA preconstricted with 1 x 10^{-6}M PE it can be seen in figure 4.3.23B that hypoxia potentiates the maximum relaxation produced by 3 x 10^{-5}M sildenafil when compared to control. The maximum relaxation achieved by 3 x 10^{-5}M sildenafil were: control 45.3 ± 5.2% (n=5), hypoxic 74.4 ± 5.6% (n=5, $P<0.05$, control *versus* hypoxic, Student's *t*-test). Likewise, in the first branch PAs preconstricted with 1 x 10^{-6}M PE, hypoxia increased the relaxant effect of 3 x 10^{-5}M sildenafil (figure 4.3.24B). The maximum relaxation achieved by 3 x 10^{-5}M sildenafil in the first branch PAs were: control 16.8 ± 6.6% (n=5), hypoxic 58.7 ± 10% (n=5, $P<0.05$, control *versus* hypoxic, Student's *t*-test). These data suggests that hypoxia appears to potentiate the response to 3 x 10^{-5}M sildenafil in both the main and first branch PA preconstricted with 1 x 10^{-6}M PE. However, it is important to note that hypoxia does not significantly potentiate the response to sildenafil when the concentration of sildenafil ≤ 1 x 10^{-5}M.

4.3.8.2 Preconstricted with 3 x 10^{-5}M 5-HT

Sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) produced a concentration-dependent relaxation in control PAs preconstricted with 3 x 10^{-5}M 5-HT (figures 4.3.25B and 4.3.26B). The maximum concentration of sildenafil (3 x 10^{-5}M) did not completely relax the main or first branch control PAs. Maximum relaxation achieved by 3 x 10^{-5}M sildenafil in main and first branch control PAs precontracted with 3 x 10^{-5}M 5-HT was 33.7 ± 4.9%, and 32.3 ± 3.4%, respectively (% relaxation of reference KCl, figure 4.3.25B and 4.3.26B).

Sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) caused a concentration-dependent relaxation in control endothelium-denuded main PAs precontracted with 3 x 10^{-5}M 5-HT. In the main PA it can be seen removal of the endothelium had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M sildenafil when compared to control (figure 4.3.25B). The maximum relaxation achieved by sildenafil in the main control and endothelium-denuded main PA precontracted with 3 x 10^{-5}M 5-HT, were: control 33.7 ± 4.9% (n=7), endothelium-denuded 25.6 ± 5.3% (n=6, NS, control *versus* endothelium-denuded). In parallel, in the first branch PA preconstricted with 3 x 10^{-5}M 5-HT, removal of the endothelium also had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M sildenafil (figure 4.3.26B). The maximum relaxation achieved by 3 x 10^{-5}M sildenafil in the control and endothelium-denuded first branch PA preconstricted with 3 x 10^{-5}M 5-HT, were: control 32.3 ± 3.4% (n=5), endothelium-denude 30.5 ± 5.3% (n=5, NS, control *versus* endothelium-denuded). These results suggest that when tone is raised by 3 x 10^{-5}M 5-HT the PDE5 inhibitor sildenafil has a significant endothelium-independent vasodilatory effect in both the main and first branch PAs.

In addition, sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) caused a concentration-dependent relaxation in both main and first branch hypoxic PAs preconstricted with 3 x 10^{-5}M 5-HT (figures 4.3.27B and 4.3.28B). In the main PA preconstricted with 3 x 10^{-5}M 5-HT, it can be seen in figure 4.3.27B, that hypoxia potentiates the maximum relaxation produced by 3 x 10^{-5}M sildenafil when compared to control. The maximum relaxation achieved by 3 x 10^{-5}M sildenafil in the main PAs were: control 33.7 ± 4.9% (n=7), hypoxic 77.6 ± 7.7% (n=6, $P<0.05$, control *versus* hypoxic, Student's *t*-test). Likewise, in the first branch PAs preconstricted with 3 x 10^{-5}M 5-HT, hypoxia increased the relaxant effect of 3 x 10^{-5}M sildenafil (figure 4.3.28B). The maximum relaxation achieved by 3 x 10^{-5}M sildenafil in the first branch PAs were: control 29.9 ± 5.3% (n=5), hypoxic 58.8 ± 5.3% (n=6, $P<0.05$, control *versus* hypoxic, Student's *t*-test). These data suggests that hypoxia appears to potentiate the response to 3 x 10^{-5}M sildenafil in both the main and first branch PA preconstricted with 3 x 10^{-5}M 5-HT. Again, it is important to note that hypoxia does not significantly potentiate the response to sildenafil when the concentration of sildenafil ≤ 1 x 10^{-5}M.

4.3.8.3 Preconstricted with 3 x 10^{-9}M ET-1

Sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) produced a concentration-dependent relaxation in control PAs preconstricted with 3 x 10^{-9}M ET-1 (figures 4.3.29B and 4.3.30B). The maximum concentration of sildenafil (3 x 10^{-5}M) did not completely relax the main or first branch control PAs. Maximum relaxation achieved by 3 x 10^{-5}M sildenafil in main and first branch control PAs precontracted with 3 x 10^{-9}M ET-1 was 18.2 ± 5.1% and 18.1 ± 4.6%, respectively (% relaxation of reference KCl, figures 4.3.29B and 4.3.30B).

Sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) caused a concentration-dependent relaxation in control endothelium-denuded main PAs precontracted with 3 x 10^{-9}M ET-1. In the main PA it can be seen from figures 4.3.29B that removal of the endothelium had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M sildenafil, when compared to control. The maximum relaxation achieved by sildenafil in the main control and endothelium-denuded main PA precontracted with 3 x 10^{-9}M ET-1, were: control 18.2 ± 5.1% (n=5), endothelium-denuded 15.2 ± 5.9% (n=7, NS, control *versus* endothelium-denuded). In parallel, in the first branch PA preconstricted with 3 x 10^{-9}M ET-1, removal of the endothelium also had no significant effect on the maximum relaxation produced by 3 x 10^{-5}M sildenafil (figure 4.3.30B). The maximum relaxation achieved by 3 x 10^{-5}M sildenafil in the control and endothelium-denuded first branch PA preconstricted with 3 x 10^{-9}M ET-1, were: control 18.1 ± 4.6% (n=6), endothelium-denuded 23.7 ± 7.3% (n=7, NS, control *versus* endothelium-denuded). These data suggests that when tone is raised by 3 x 10^{-9}M ET-1, the PDE5 inhibitor sildenafil has a significant endothelium-independent vasodilatory effect in both the main and first branch PAs.

In addition, sildenafil (1 x 10^{-9}M to 3 x 10^{-5}M) caused a concentration-dependent relaxation in both main and first branch hypoxic PAs preconstricted with 3 x 10^{-9}M ET-1 (figures 4.3.31B and 4.3.32B). In the main PA preconstricted with 3 x 10^{-9}M ET-1, it can be seen from figure 4.3.31B, hypoxia potentiates the maximum relaxation produced by 3 x 10^{-5}M sildenafil when compared to control. The maximum relaxation achieved by 3 x 10^{-5}M sildenafil in the main PAs were: control 18.2 ± 5.1% (n=5), hypoxic 37.2 ± 5.5% (n=5, $P<0.05$, control *versus* hypoxic). Likewise, in the first branch PAs preconstricted with 3 x 10^{-9}M ET-1, hypoxia increased the relaxant effect of 3 x 10^{-5}M sildenafil (figure 4.3.32B). The maximum relaxation achieved by 3 x 10^{-5}M sildenafil

in the first branch PAs were: control 18.1 ± 4.6%, (n=6), hypoxic 64.2 ± 11.5% (n=5, $P<0.05$, control *versus* hypoxic). These results suggest that hypoxia appears to potentiate the response to sildenafil in both the main and first branch PA preconstricted with 3 x 10^{-9}M ET-1. Once more, it is important to note that hypoxia does not significantly potentiate the response to sildenafil when the concentration of sildenafil ≤ 1 x 10^{-6}M.

4.3.9 Comparison of the effects of the PDE5 inhibitor sildenafil with each preconstrictor

Figures 4.3.33 and 4.3.34 show the comparison of the relaxant effects of sildenafil between each preconstrictor used in the control and hypoxic main and first branch PAs respectively. In the control main PA the efficacy of sildenafil was dependent on the preconstrictor. The rank order of efficacy of sildenafil for each preconstrictor in the main branch was PE>5HT>ET-1 (% relaxation of reference KCl: 45.3 ± 5.2%, 33.7 ± 4.9%, 18.2 ± 5.1% respectively, where > signifies a greater maximum relaxation with sildenafil, figure 4.3.33A). Likewise, in the hypoxic main PA the maximum relaxation induced by sildenafil was dependent on the preconstrictor. The level of relaxation induced by sildenafil was significantly greater when the tone was raised with either 5-HT or PE, than when the tone was raised with ET-1. The order of efficacy of 3 x 10^{-5}M sildenafil for each preconstrictor in the hypoxic main branch was 5-HT=PE>ET-1 (% relaxation of reference KCl: 77.6 ± 7.7%, 74.4 ± 5.6%, 37.2 ± 5.5% respectively, where > signifies a greater maximum relaxation with sildenafil, and = signifies an equal relaxant effect of sildenafil, figure 4.3.33B). It can be noted that the preconstrictor dependent relaxation induced by sildenafil alters with hypoxia. Hypoxia only significantly potentiated the relaxatory response to 3 x 10^{-5}M sildenafil in the first branch PA, and not to any lower concentrations of the PDE5 inhibitor, irrespective of the preconstrictor used.

Similarly, in the control first branch PA, the efficacy of sildenafil was also dependent on the preconstrictor. The level of relaxation induced by sildenafil was significantly greater when the tone was raised with either PE or 5-HT than when the tone was raised with ET-1. The order of efficacy of 3 x 10^{-5}M sildenafil for each preconstrictor in the first branch PA was PE=5-HT>ET-1 (% relaxation of reference KCl: 38 ± 2.5%, 32.3 ± 3.4%, 18.1 ± 4.6% respectively, where > signifies a greater maximum relaxation with

sildenafil, and = signifies an equal relaxant effect of sildenafil, figure 4.3.34B). In parallel, in the hypoxic main PA the maximum relaxation induced by sildenafil was not dependent on the preconstrictor. The order of efficacy of 3 x 10^{-5}M sildenafil for each preconstrictor in the hypoxic first branch PA was ET-1=5-HT=PE (% relaxation of reference KCl: 64.2 ± 11.5%, 58.8 ± 5.3%, 58.7 ± 10% respectively, where = signifies an equal relaxant effect of sildenafil, figure 4.3.34B). It can be noted that the preconstrictor dependent relaxation induced by sildenafil in the first branch becomes a preconstrictor independent response with hypoxia. Hypoxia only significantly potentiated the relaxatory response to 3 x 10^{-5}M sildenafil in the first branch PA, and not to any lower concentrations of the PDE5 inhibitor, irrespective of the preconstrictor used.

4.3.10 SKF94836 versus sildenafil

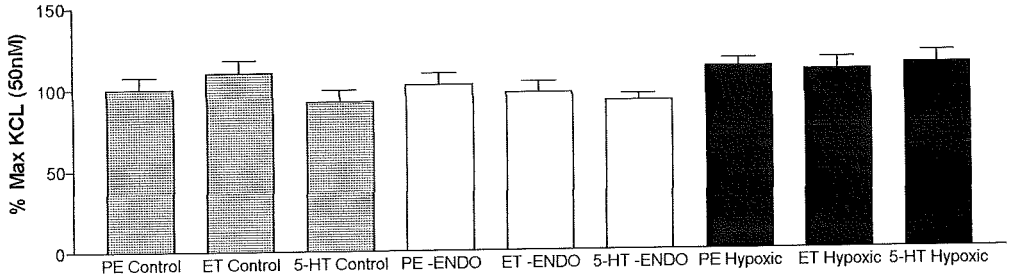
Both treatment with SKF94836 or sildenafil significantly reduced the active tension developed in response to each preconstrictor in both the control and hypoxic conduit PAs studied. The maximal efficacy is the greatest attainable response of the inhibitors, and is compared throughout this study. The maximum relaxation of both PDE inhibitors (3 x 10^{-5}M) was increased in the hypoxic vessels compared to the controls. In general, a similar pattern of relaxation occurred in response to SKF94836 and sildenafil in the control, endothelium-denuded and hypoxic main PAs. However, in general hypoxia only potentiated the relaxation observed in response to 3 x 10^{-5}M sildenafil, whereas hypoxia potentiated the relaxatory response to SKF94836 at lower concentrations (≤3 x 10^{-5}M). The main difference seen in the main PA occurred in the control and endothelium-denude vessels preconstricted with 1 x 10^{-6}M PE. Sildenafil (produced a significantly greater degree of relaxation in the control main PA preconstricted with 1 x 10^{-6}M PE, compared to SKF94836 (45.3 ± 5.2%, 16.8 ± 6.6% respectively, % relaxation of reference KCl, $P<0.05$, Student's *t*-test, figures 4.3.5B and 4.3.21B). Likewise, the maximum relaxation produced by sildenafil was greater than SKF94836 in the main PA denude of endothelium and preconstricted with 1 x 10^{-6}M PE (40.1 ± 9.2%, 7.9 ± 8.9% respectively, % relaxation of reference KCl, $P<0.05$, Student's *t*-test, figures 4.3.5B and 4.3.21B). In contrast, however both sildenafil (3 x 10^{-5}M) and SKF94836 (3 x 10^{-5}M) had a similar efficacy in the hypoxic main PA preconstricted with 1 x 10^{-6}M PE (74.4 ± 5.6%, 79 ± 4.6% respectively, % relaxation of reference KCl, NS, Student's *t*-test, figures 4.3.7B and 4.3.23B).

The major variations in the efficacy of the two inhibitors were seen in the first branch PAs. In the first branch PA SKF94836 acted in an endothelium-dependent manner. Removal of the endothelium attenuated or even prevented the vasorelaxatory effects of SKF94836 on the active tension developed in the first branch PA (figures 4.3.6B, 4.3.10B, 4.3.14B). The maximum relaxation achieved by SKF94836 was: PE, first branch PA control 20.3 ± 5.1% (n=8), endothelium-denuded 5.49 ± 7.3% (n=5, $P<0.05$, control *versus* endothelium-denuded, Student's *t*-test); 5-HT, first branch PA control 40.3 ± 9.54 (n=7), endothelium-denuded −10.8 ± 8.2% (n=5, $P<0.05$, control *versus* endothelium-denuded; ET-1, first branch PA control 39.9 ± 3.15% (n=7), endothelium-denuded 20.4 ± 7.3% (n=5, $P<0.05$, control *versus* endothelium-denuded). In contrast, sildenafil was as effective on removal of the endothelium in the first branch PA as it was in the control first branch (figures 4.3.22B, 4.3.26B, 4.3.30B). The maximum relaxation achieved by sildenafil was: PE, first branch PA control 38 ± 2.5% (n=5), endothelium-denude 42.2 ± 5.8% (n=5, NS, control *versus* endothelium-denuded, Student's *t*-test); 5-HT, first branch PA control 32.3 ± 3.4% (n=5), endothelium-denuded 30.5 ± 5.3% (n=5, NS, control *versus* endothelium-denuded); ET-1, first branch PA control 18.1 ± 4.6% (n=6), endothelium-denuded 23.7 ± 7.3% (n=7, NS, control *versus* endothelium-denuded).

Furthermore, in the first branch PA the maximum relaxation produced by SKF94836 (3×10^{-5}M) was generally significantly greater than that produced by sildenafil (3×10^{-5}M). In particular the maximum relaxation induced by SKF94836 was approximately three fold greater than the relaxation produced by sildenafil in the first branch preconstricted with 3×10^{-9}M ET-1 (220.9 ± 22.4%, 64.2 ± 11.5% respectively, % relaxation of reference KCl, $P<0.05$, Student's *t*-test, figures 4.3.16B and 4.3.32B).

The efficacy of both the PDE3 and PDE5 inhibitors was dependent on the preconstrictor used, the branch of the PA studied and was altered with hypoxia. The response of the PDE3 inhibitor SKF94836 was dependent on the presence of an intact endothelium in the first branch.

A) Main PA



B) First Branch PA

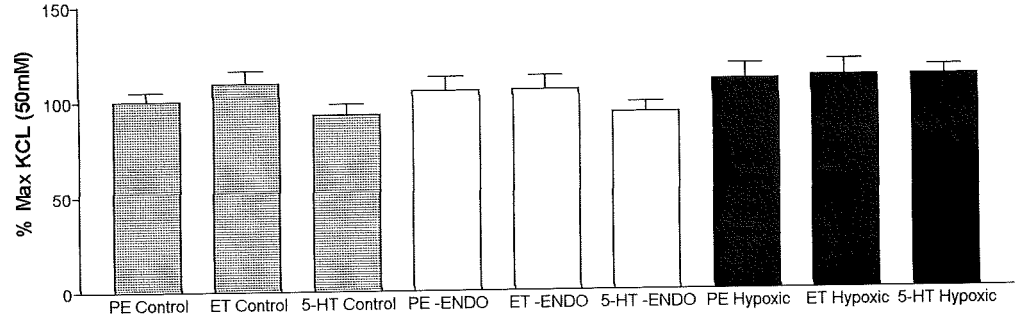


Figure 4.3.19. Maximum response to 1 x 10^{-6}M PE, 3 x 10^{-9}M ET-1 and 3 x 10^{-5}M 5-HT in rat control, endothelium-denuded and hypoxic rat A) main and B) first branch pulmonary arteries

Maximum response to 1 x 10^{-6}M phenylephrine (PE), 3 x 10^{-9}M endothelin-1 (ET-1) and 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) in control, endothelium-denuded (-endo) and hypoxic rat A) main and B) first branch pulmonary arteries. Data are expressed as percentage of the response to 50mM KCl, mean ± s.e.m,. where n= 5 for all (n = number of different animals), and * denotes significance ($P<0.05$) when comparing hypoxic *versus* control with/without endothelium in 3 x 10^{-5}M 5-HT precontacted vessels. The data in the above figure represents those vessels in the sildenafil study.

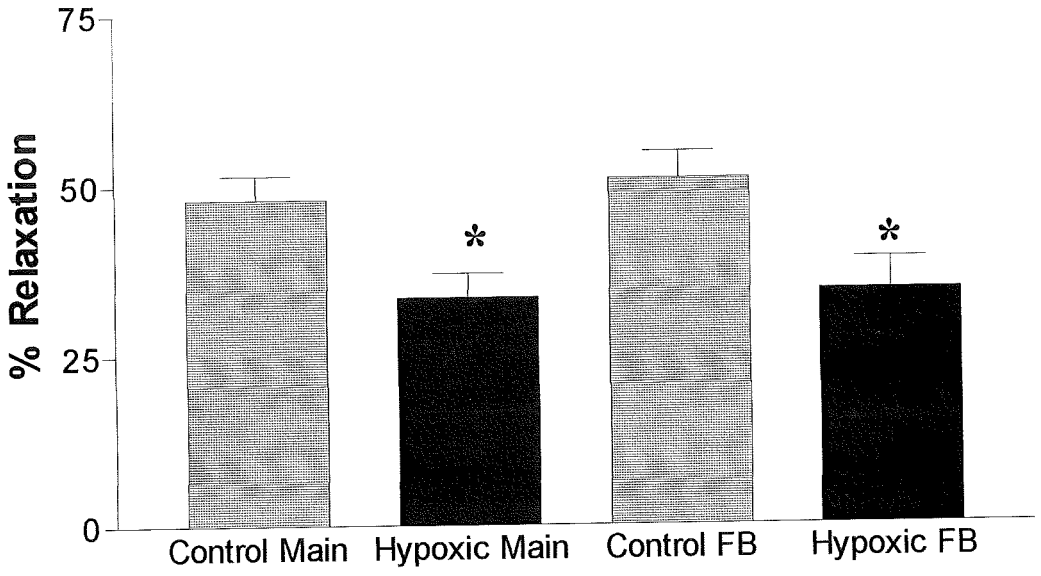
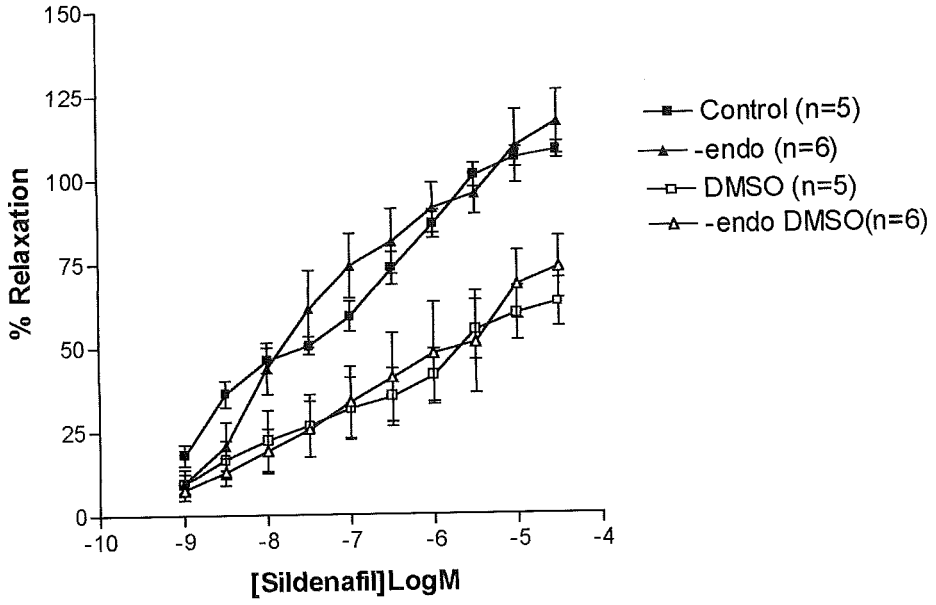


Figure 4.3.20. Maximum relaxation to 1 x 10^{-6}M ACh in rat control and hypoxic rat main and first branch pulmonary arteries

Maximum relaxation to 1 x 10^{-6}M acetlycholine (ACh) in control and hypoxic rat main and first branch (FB) pulmonary arteries precontracted with 1μM phenylephrine. Data are expressed as percentage of the response to 50mM KCl, mean ± s.e.m,. where n=15 for all (n = number of different animals), and * denotes significance ($P<0.05$). The data in the above figure represents those vessels used in the sildenafil study.

A)



B) Minus the possible effect of the vehicle DMSO

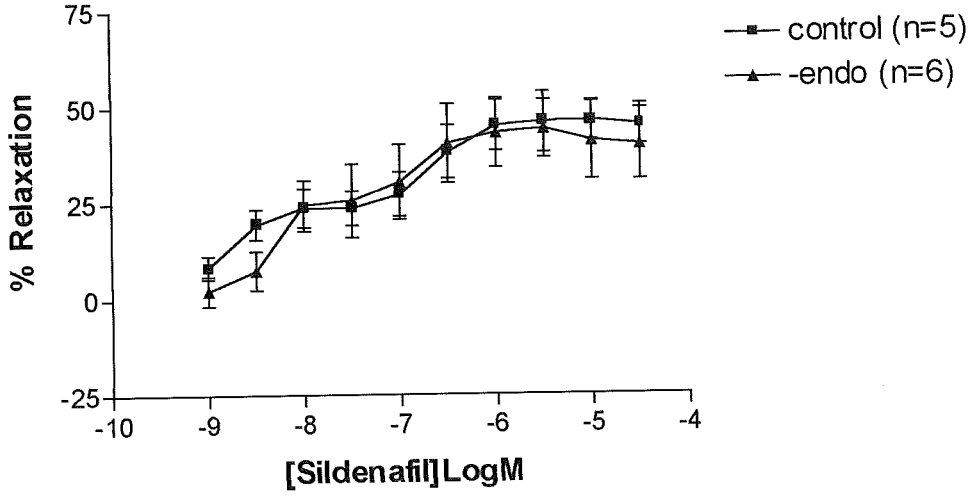
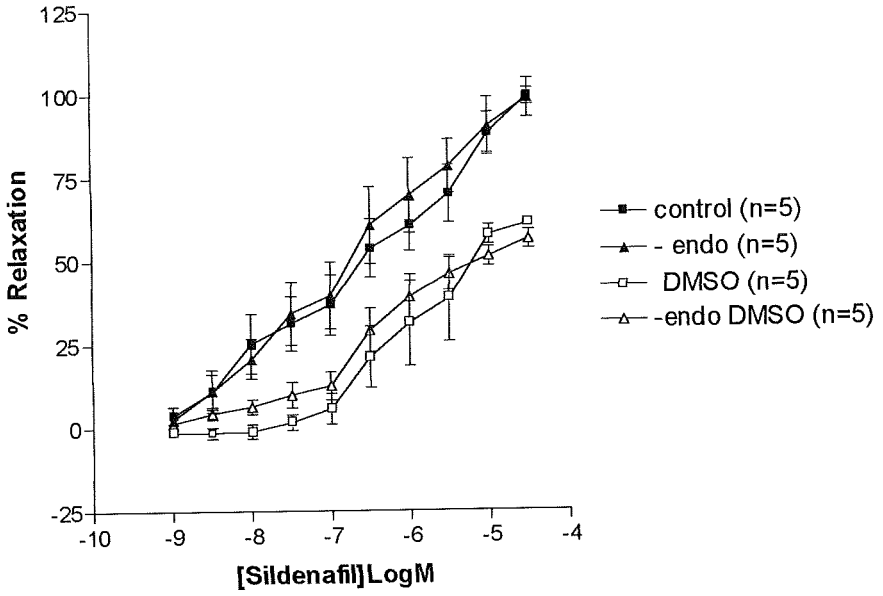


Figure 4.3.21. CCRC to sildenafil in rat control and endothelium-denuded main pulmonary artery preconstricted with 1 x 10^{-6}M PE

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) main pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) main pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) Minus the possible effect of the vehicle DMSO

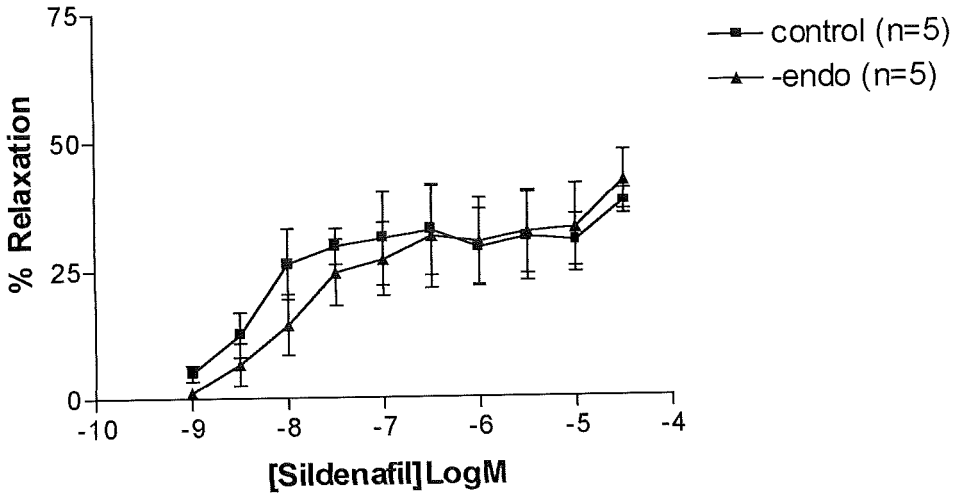
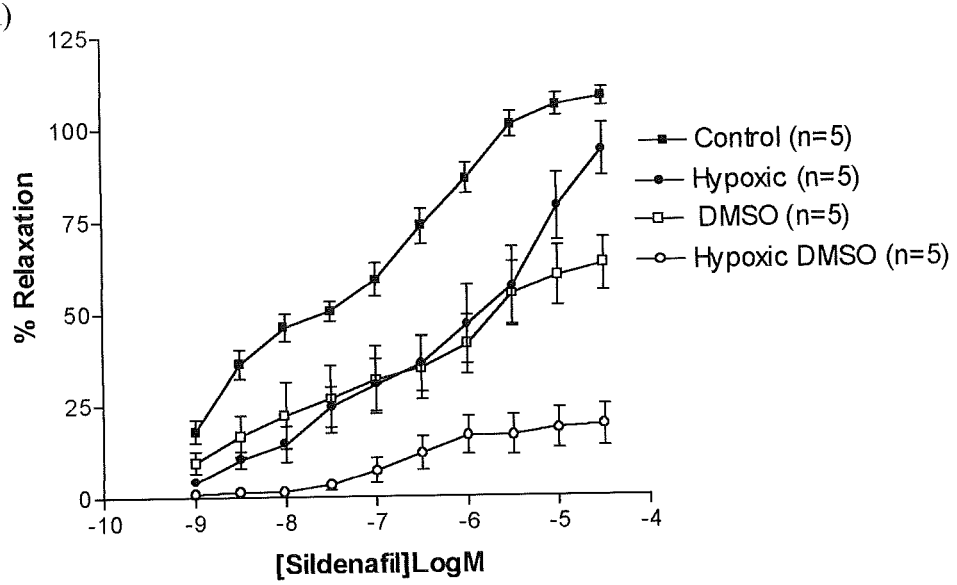


Figure 4.3.22. CCRC to sildenafil in rat control and endothelium-denuded first branch pulmonary artery preconstricted with 1 x 10^{-6}M PE

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) first branch pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) first branch pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) Minus the possible effect of vehicle DMSO

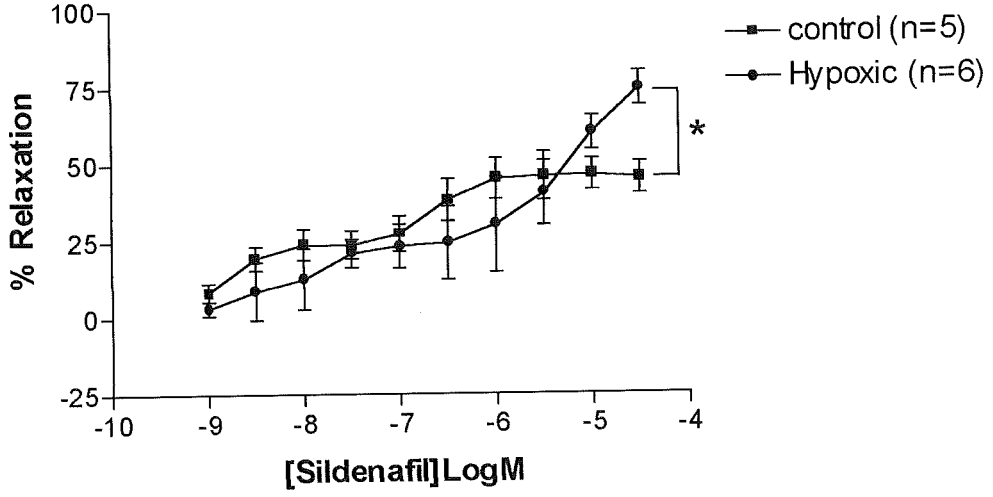
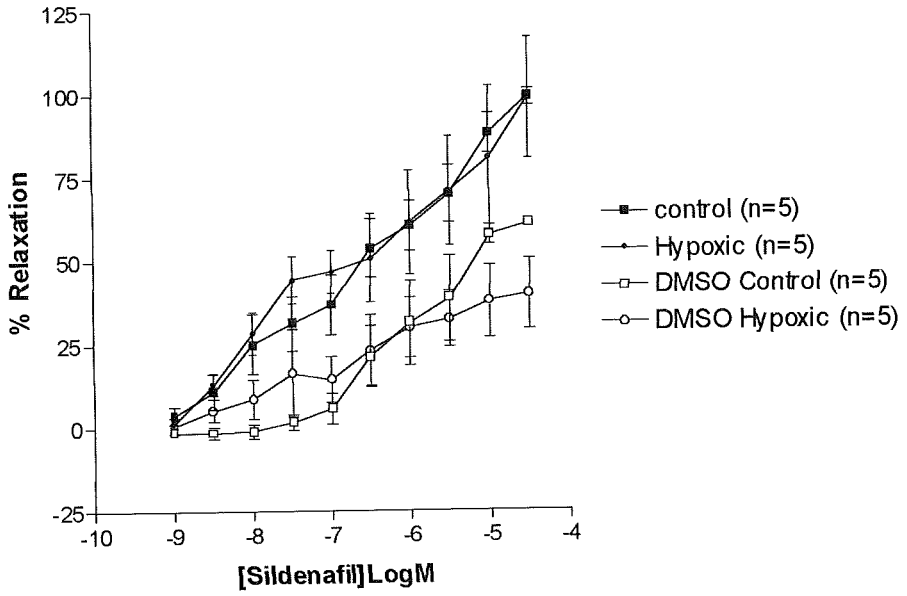


Figure 4.3.23. CCRC to sildenafil in rat control and hypoxic main pulmonary artery preconstricted with 1 x 10^{-6}M PE

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) main pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) main pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of the vehicle DMSO

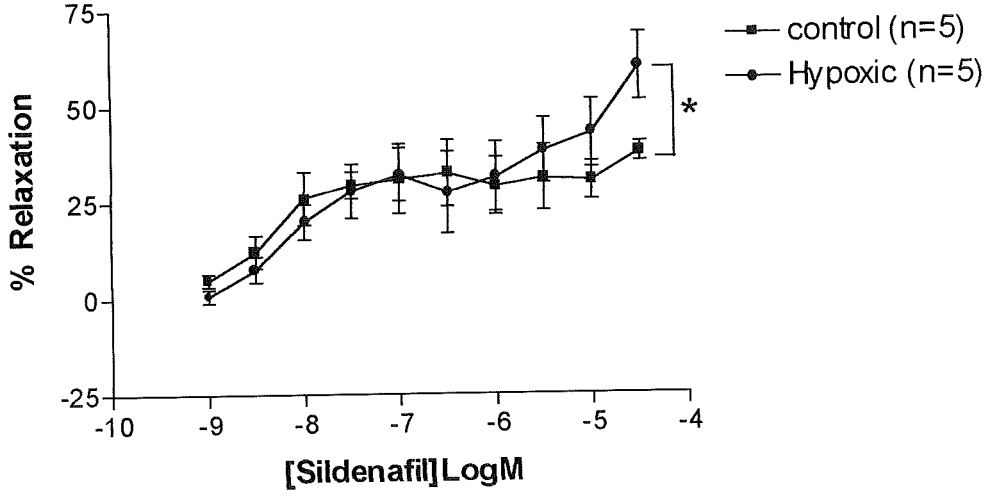
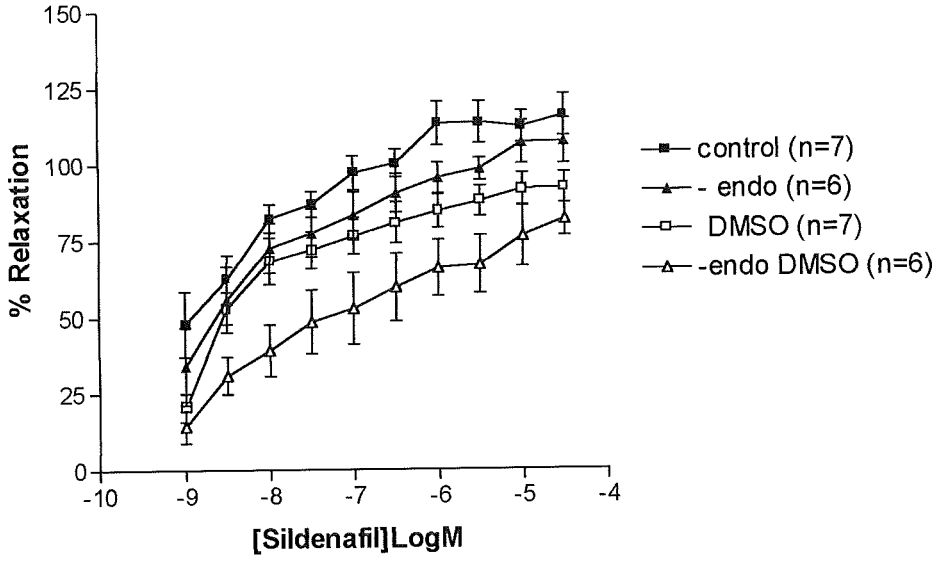


Figure 4.3.24. CCRC to sildenafil in rat control and hypoxic first branch pulmonary artery preconstricted with 1 x 10^{-6}M PE

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) first branch pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) first branch pulmonary artery preconstricted with 1 x 10^{-6}M phenylephrine (PE) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus possible effects of the vehicle DMSO

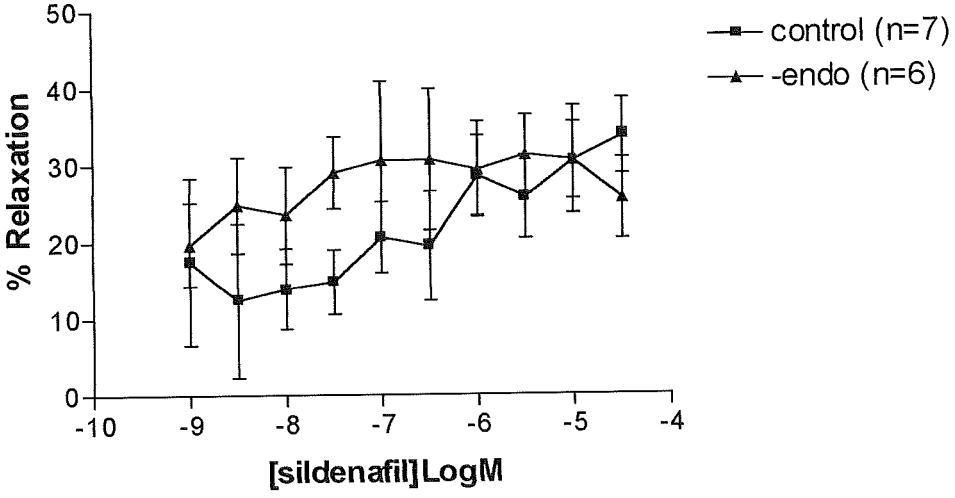
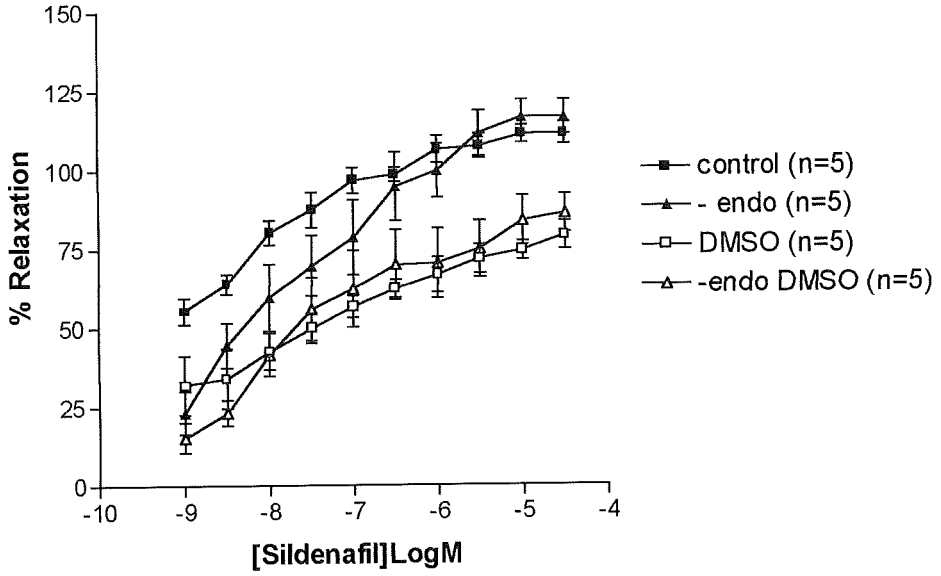


Figure 4.3.25. CCRC to sildenafil in rat control and endothelium-denuded main pulmonary artery preconstricted with 3 x 10^{-5}M 5-HT

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) main pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) main pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) Minus the possible effects of the vehicle DMSO

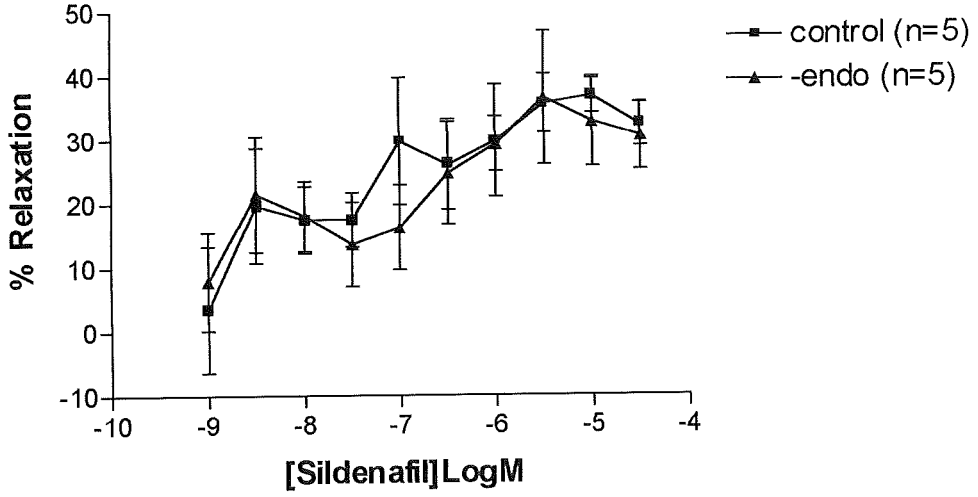
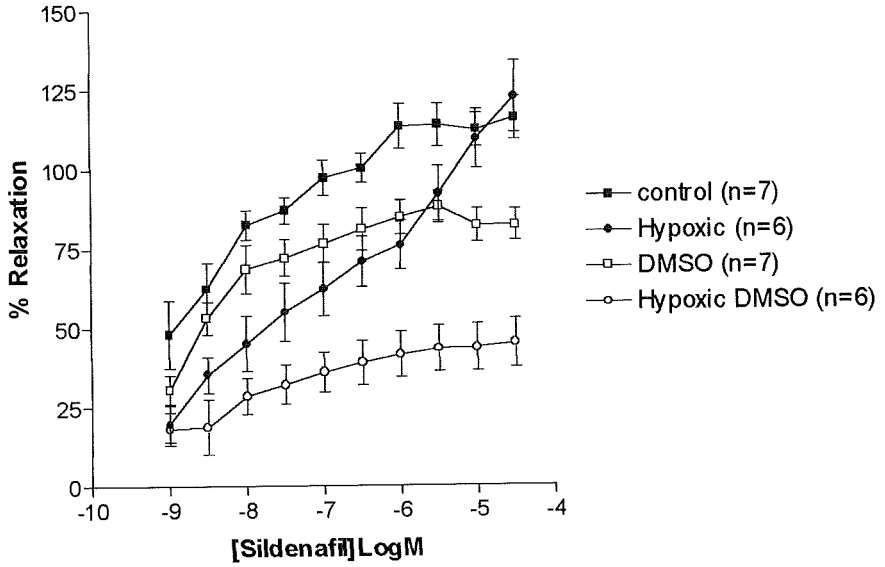


Figure 4.3.26. CCRC to sildenafil in rat control and endothelium-denuded first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-HT

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) Minus possible effects of the vehicle DMSO

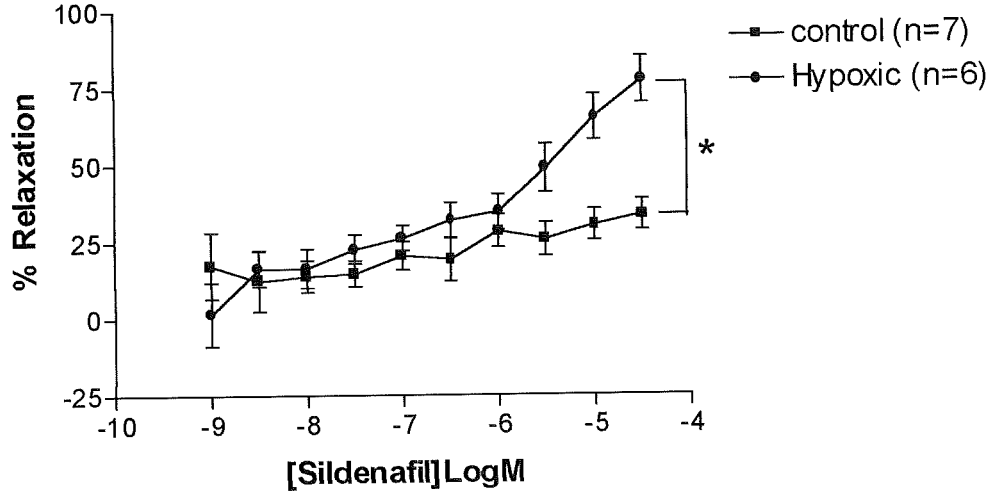
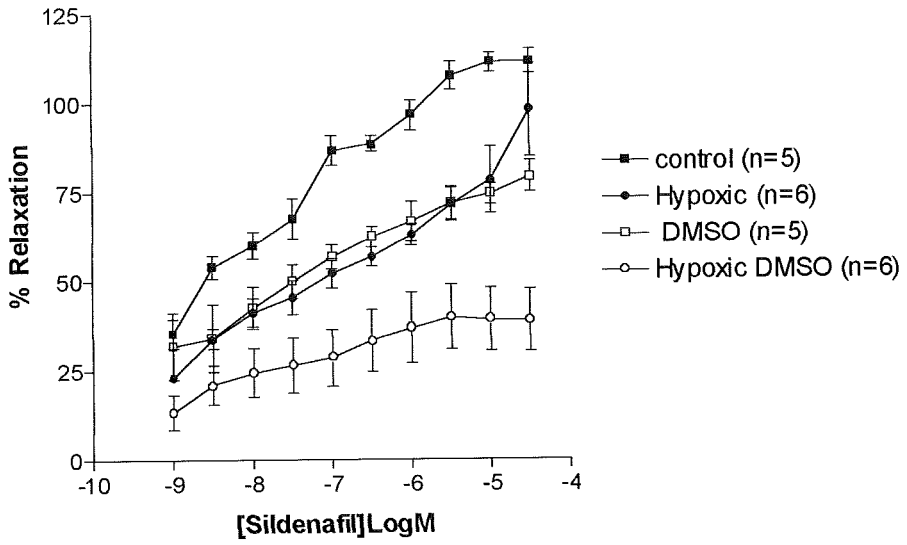


Figure 4.3.27. CCRC to sildenafil in rat control and hypoxic main pulmonary artery preconstricted with 3 x 10^{-5}M 5-HT

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) main pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) main pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus the possible effect of the vehicle DMSO

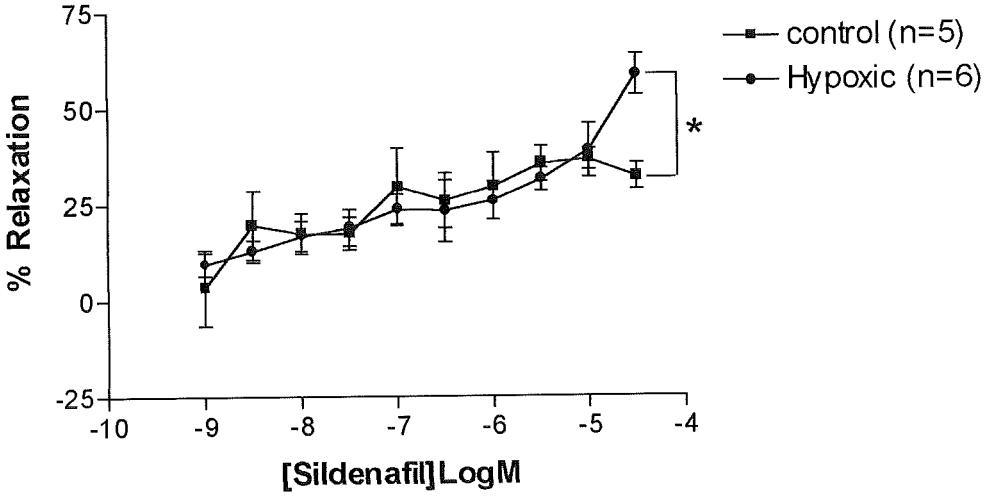
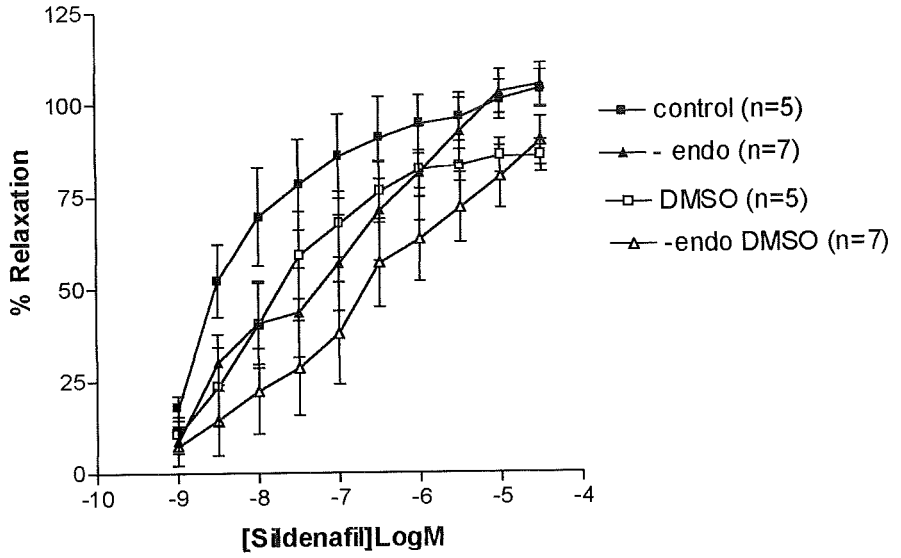


Figure 4.3.28. CCRC to sildenafil in rat control and hypoxic first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-HT

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) first branch pulmonary artery preconstricted with 3 x 10^{-5}M 5-hydroxytryptamine (5-HT) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus the possible effect of the vehicle DMSO

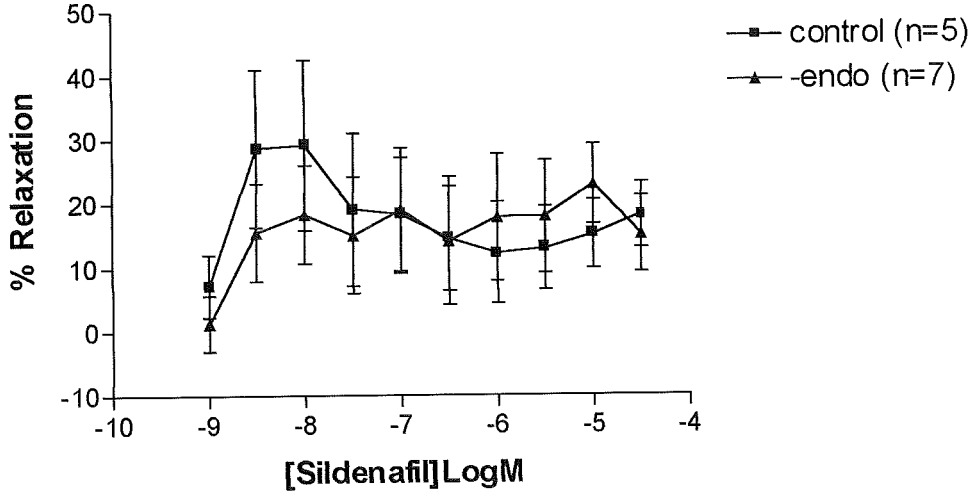
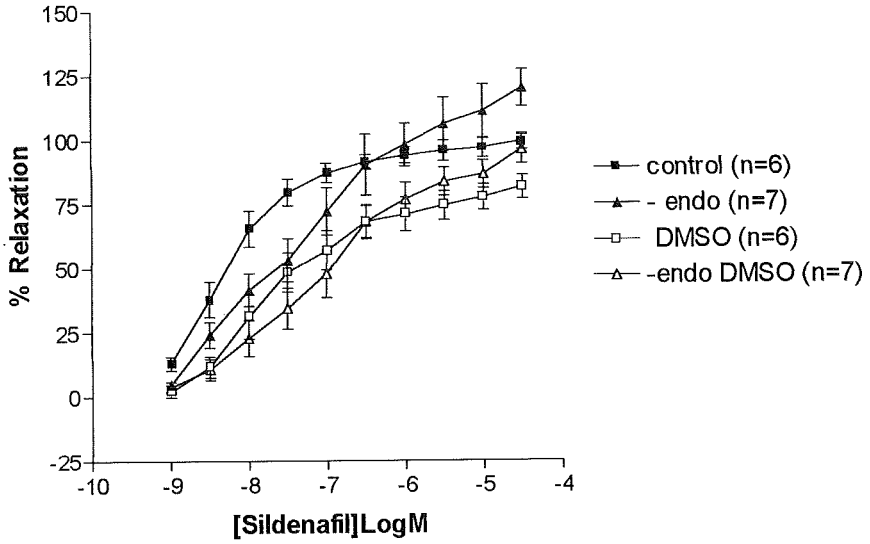


Figure 4.3.29. CCRC to sildenafil in rat control and endothelium-denuded main pulmonary artery preconstricted with 3 x 10^{-9}M ET-1

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) main pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) main pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) Minus the possible effects of the vehicle DMSO

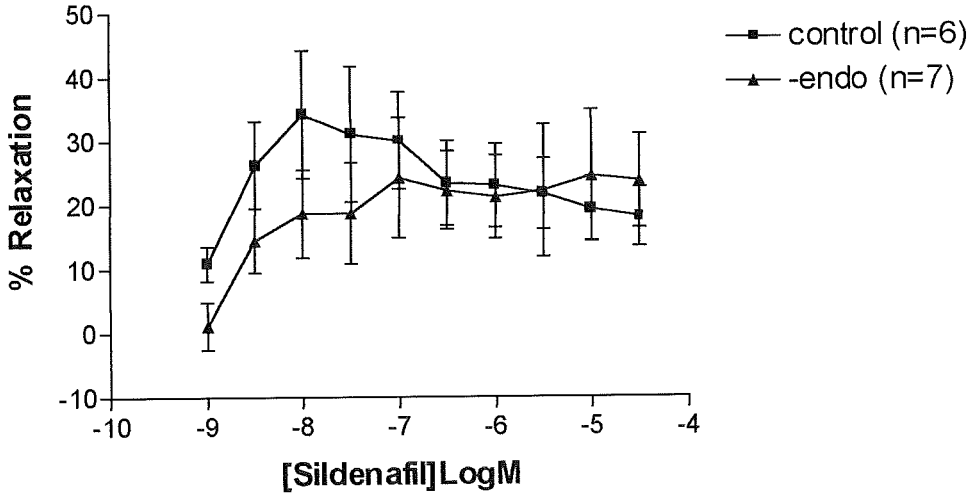
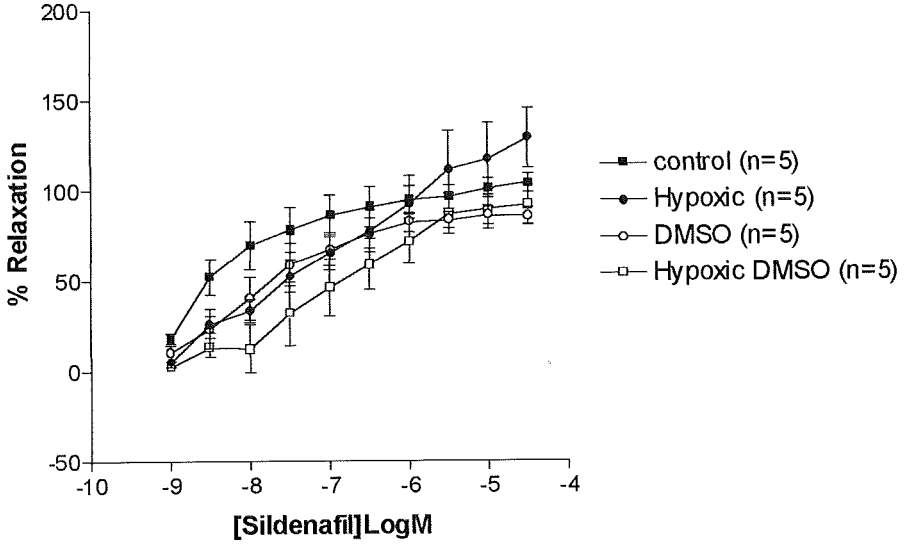


Figure 4.3.30. CCRC to sildenafil in rat control and endothelium-denuded first branch pulmonary artery preconstricted with 3 x 10^{-9}M ET-1

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and endothelium-denuded (-endo; ▲ and △ respectively) first branch pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and endothelium-denuded (- endo, ▲) first branch pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals.

A)



B) Minus the possible effect of the vehicle DMSO

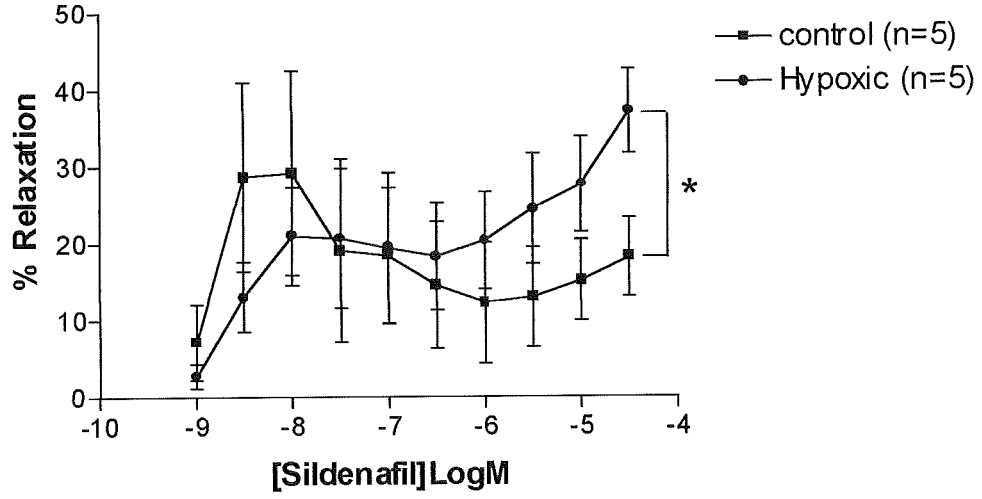
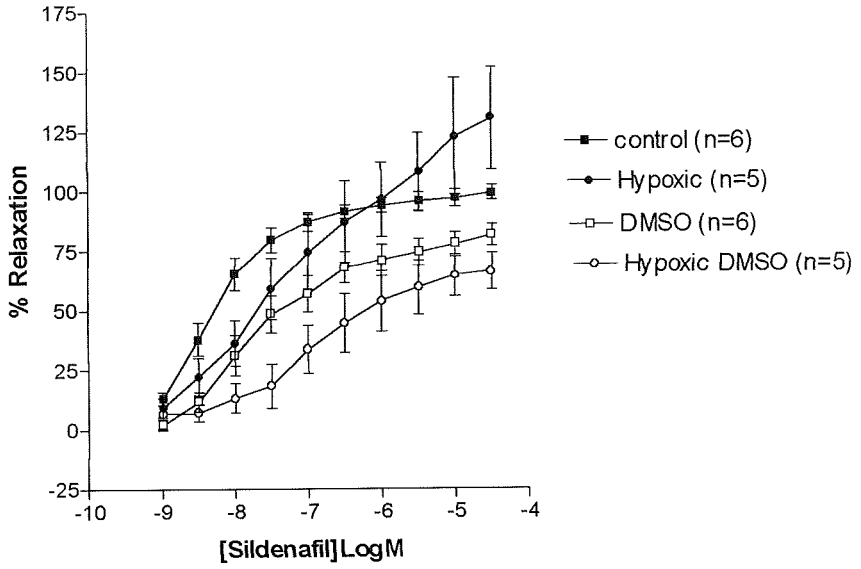


Figure 4.3.31. CCRC to sildenafil in rat control and hypoxic main pulmonary artery preconstricted with 3 x 10^{-9}M ET-1

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) main pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) main pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B) Minus the possible effect of the vehicle DMSO

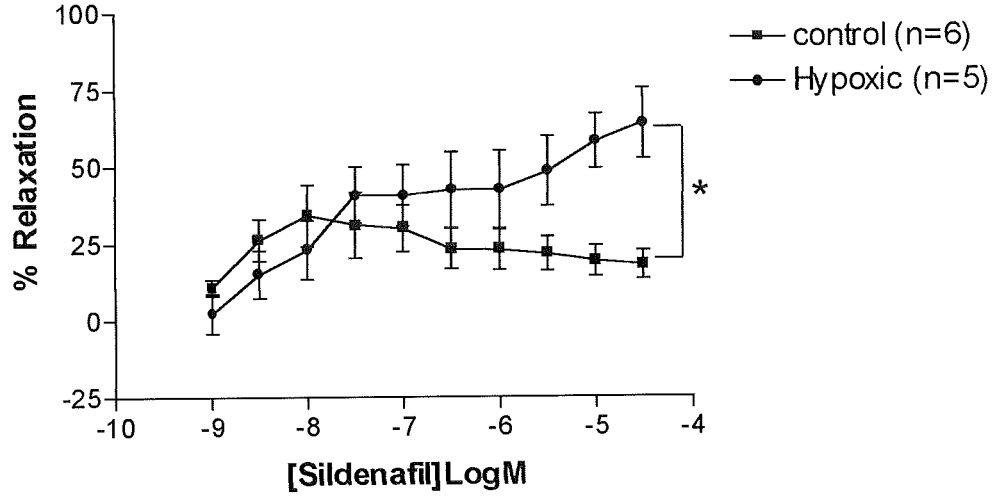
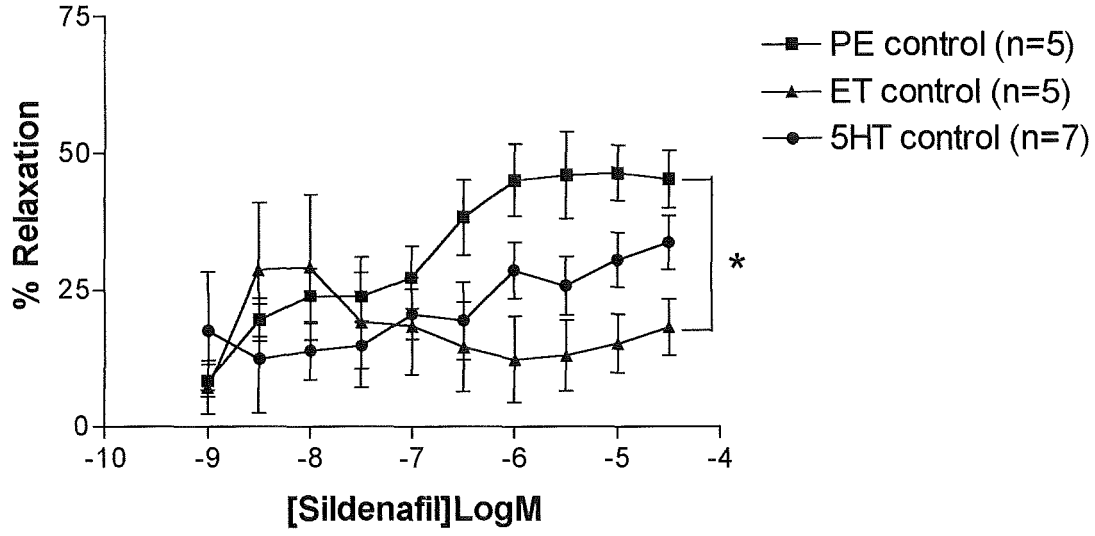


Figure 4.3.32. CCRC to sildenafil in rat control and hypoxic first branch pulmonary artery preconstricted with 3 x 10^{-9}M ET-1

A) Cumulative concentration response curves to the PDE5 inhibitor sildenafil and DMSO in control (■ and □ respectively) and hypoxic (● and ○ respectively) first branch pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1). B) Cumulative concentration response curves to the PDE5 inhibitor sildenafil alone, subtracting the effect of DMSO, in control (■) and hypoxic (●) first branch pulmonary artery preconstricted with 3 x 10^{-9}M endothelin-1 (ET-1) as in A. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B)

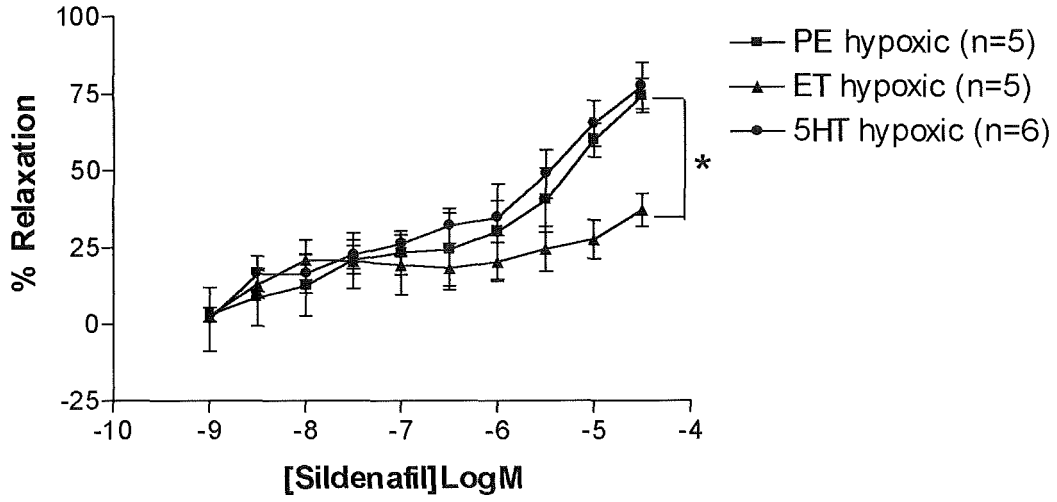
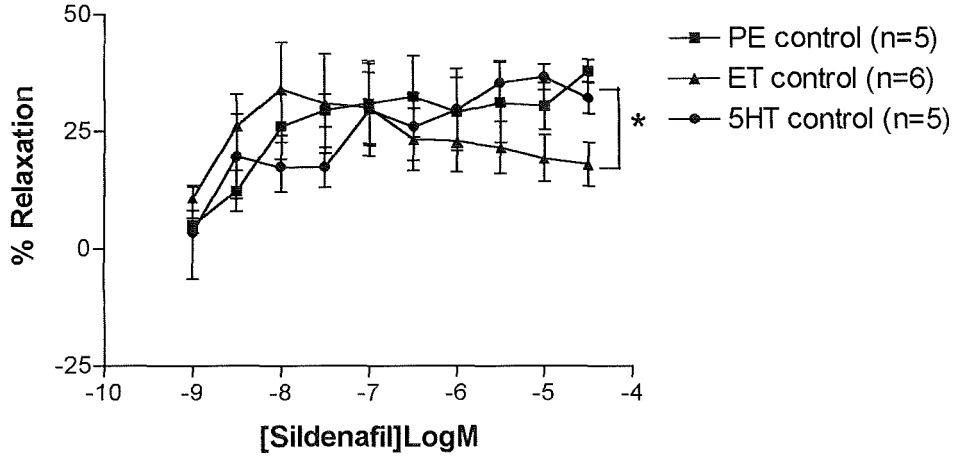


Figure 4.3.33. CCRC to sildenafil in rat control and hypoxic main pulmonary artery preconstricted with 1 x 10^{-6}M PE, 3 x 10^{-5}M 5-HT and 3 x 10^{-9}M ET-1

Cumulative concentration response curves to the PDE5 inhibitor sildenafil in A) control and B) hypoxic main pulmonary artery preconstricted with 1 x10^{-6}M phenylephrine (PE, ■), 3 x 10^{-5}M 5-hydroxytryptamine (5-HT, ●) and 3 x 10^{-9}M endothelin-1 (ET-1, ▲). In each CCRC the effect of the vehicle DMSO has been subtracted. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation.

A)



B)

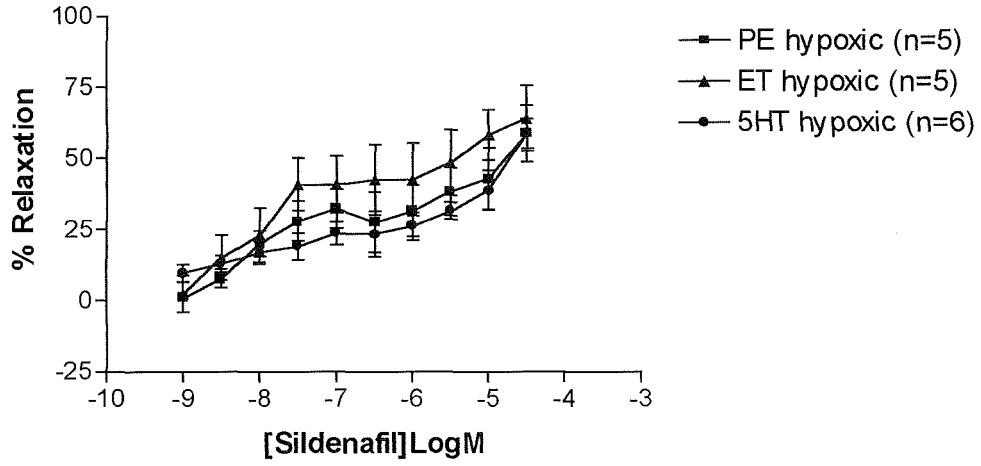


Figure 4.3.34. CCRC to sildenafil in rat control and hypoxic first branch pulmonary artery preconstricted with 1 x 10^{-6}M PE, 3 x 10^{-5}M 5-HT and 3 x 10^{-9}M ET-1

Cumulative concentration response curves to the PDE5 inhibitor sildenafil in A) control and B) hypoxic first branch pulmonary artery preconstricted with 1 x10^{-6}M phenylephrine (PE, ■), 3 x 10^{-5}M 5-hydroxytryptamine (5-HT, ●) and 3 x 10^{-9}M endothelin-1 (ET-1, ▲). In each CCRC the effect of the vehicle DMSO has been subtracted. Data are expressed as percentage relaxation to the response to 50mM KCl. Each point represents mean ± s.e.m,. where n = number of different animals and * denotes significance ($P<0.05$) between maximum relaxation..

4.4 Discussion

The purpose of this investigation was to determine the effect of SKF94836 (PDE3 inhibitor), and sildenafil (PDE5 inhibitor) on PE, ET-1, and 5-HT mediated vasoconstriction in both the main and first branch PA of control and hypoxic rats. In addition, the vasodilatory effects of each of the PDE inhibitors were assed in endothelium-denuded conduit PAs.

4.4.1 Response of hypoxic and endothelium-denuded main and first branch pulmonary arteries to PE, ET-1, 5-HT, ACh

PE, ET-1 and 5-HT all produced concentration-dependent contractions in isolated main and first branch PAs. ET-1, and the α_1-adrenoreceptors-agonist PE both initiate smooth muscle contraction by binding to the appropriate receptors and leading to the induction of phospholipase C (PLC), and subsequent generation of the second messengers inositol trisphosphate ($InsP_3$) and diacylgycerol (DAG). Production of $InsP_3$ and DAG results in the release of intracellular calcium, and activation of protein kinase C (PKC), leading to vasoconstriction. In parallel, 5-HT, via the $5\text{-}HT_{2A}$ receptor, can also lead to the formation of IP_3 and DAG by PLC. However, 5-HT can also induce vasoconstriction by binding to $5\text{-}HT_{1B/1D}$ receptors, and leading to the inhibition of adenylyl cyclase, and a subsequent decrease in cAMP. The maximum vasoconstriction induced by either PE or ET-1, were not significantly different when comparing responses in the control, hypoxic and endothelium-denuded main and first branch PAs. MacLean *et al.* (1995), also showed responses to ET-1 were not increased in CH rat capacitance PAs. However, chronic hypoxia enhanced the maximum response to 5-HT in both the main and first branch PAs. Similar increased sensitivity to 5-HT was observed in rats with monocrotaline induced PHT, in the CH, and in the pulmonary arteries of patients with PPHT (MacLean *et al.*, 1996; Brink *et al.*, 1988). These authors suggested such hypersensitivity might be due to increased plasma 5-HT, or receptor upregulation with the development of PHT. In fact, increased circulating levels of 5-HT have been reported in PPHT and in PHT that is secondary to anorexigen intake (Anderson *et al.*, 1987; Herve *et al.*, 1995).

Additionally, the endothelium-dependent relaxation induced by acetylcholine (ACh) was attenuated in both the main and first branch PA from the CH. ACh acts on vascular

endothelial cells to release nitric oxide, which relaxes smooth muscle. These results are consistent with previous reports in which endothelium-dependent relaxation by receptor agonists are impaired in the PA from the CH (Wanstall and O'Donnel, 1992; Oka *et al.*, 1988; Adnot *et al.*, 1991, MacLean *et al.*, 1995; MacLean *et al.*, 1996; MacLean *et al.*, 1998a; McCulloch *et al.*, 1995; Altiere *et al.*, 1985, Ito *et al.*, 1998; Mathew *et al.*, 1995). The vascular endothelium in the PA from chronic hypoxic and MCT-treated rats, have actually been shown to have an increased density of microfilament bundles, an increased density of rough endoplasmic reticulum, and cell swelling (Rabinovitch, 1996). These alterations in the structure of the endothelium with hypoxia are thought to lead in part to the reduced responsiveness of endothelium-dependent agonist. Additionally, it can be hypothesised that the impaired endothelium-dependent relaxation in the conduit PA associated with hypoxia may be due to the decrease in NO production and/or the decrease in cGMP production observed in these vessels (Shaul *et al.*, 1993; MacLean *et al.*, 1996).

4.4.2 Effect of the PDE3 inhibitor SKF94836 and PDE5 inhibitor sildenafil in control main and first branch PA

It was established in age-match control rats, that inhibition of PDE3 activity by SKF94836 and inhibition of PDE5 activity by sildenafil, caused a dose-dependent relaxation in the main and first branch PAs preconstricted with 1 x 10^{-6}M PE, 3 x 10^{-5}M 5-HT and 3 x 10^{-9}M ET-1. Inhibiting PDE3 activity by SKF94836 and PDE5 activity by sildenafil would increase cAMP and cGMP respectively, thereby activating signal transduction pathways controlled by these cyclic nucleotides that initiate relaxation. Also, there is the potential for "crosstalk" between cAMP and cGMP in the vasoactive effects of the PDE inhibitors. For example, sildenafil may indirectly inhibit PDE3 (cGMP-inhibited, cAMP-specific PDE), due to increasing the circulating levels of cGMP as a result of PDE5 inhibition.

The stock concentration of SKF94836 (3 x 10^{-5}M) or sildenafil (3 x 10^{-5}M) did not completely relax the control main or first PAs. In fact, the relaxation induced by SKF94836 and sildenafil appeared somewhat disappointing, however other investigators have also found this to be true for selected PDE inhibitor. For example, Wagner *et al.* (1997), found the treatment of PA rings from rats with 1μM milrinone did not significantly reduce the active tension developed in response to U46619 in the main

PA. Even in primary cultures of smooth muscle cells Kim *et al.* (2000), demonstrated that sildenafil could only produce a small change in intracellular cGMP levels.

The smaller than anticipated vasodilatory effects of both the PDE inhibitors may be due to the low inherent tone that is present in the control PAs (Jeffery and Wanstall, 1998). Under resting tension there is little tonic release of vasoactive agents such as NO, which would lead to a low basal level of cyclic nucleotides (target for PDE inhibitors). However, both PDE3 and PDE5 did initiate a significant relaxation in precontracted control main and first branch PAs, therefore these results still enforce a role for cAMP/cGMP-controlled pathways in the regulation of the pulmonary vasculature.

Both PDE3 and PDE5 inhibitors have been show to relax systemic arteries. Precontracted rat aorta has been shown to relax in a concentration-dependent manner in response to an array of PDE3 inhibitors including cilostamide, amrinone, cilostazol (Nakamura *et al.*, 2001; Van der Zypp *et al.*, 2000; Delpy *et al.*, 1996). Additionally, PDE5 inhibitors such as E4021, DMPPO, zaprinast, and sildenafil all relax systemic arteries in a concentration-dependent manner. As in the PA, sildenafil and zaprinast (at concentrations 1 x 10^{-7}- 3 x 10^{-6}M) only had a small relaxant effect on U46619 precontracted coronary arteries (Medina *et al.*, 2000). These authors found sildenafil only had a highly significant relaxant effect when used at the highest concentrations (3 x 10^{-5}M). PDE5 inhibitors were shown to induce relaxation by increasing intracellular cGMP.

In general both PDE3 and PDE5 inhibitors are only very effective in relaxing systemic arteries if smooth muscle inhibitors such as NO donor sodium nitroprusside (SNP) or isoprenaline are present. Sildenafil has been shown to amplify the relaxation induced by SNP in human coronary arteries by enhancing cGMP levels (Medina *et al.*, 2000). These authors found sildenafil (1 x 10^{-6}M) enhanced the sodium nitroprusside induced relaxation in coronary arteries by approximately 8 times. These results indicate the action of sildenafil in the coronary artery is dependent on the pre-existing activation of the N0-cGMP pathway. Such findings in the systemic circulation could be relevant in understanding the action of PDE in the pulmonary artery. The role of the cAMP and cGMP pathways in the effect of SKF94836 and sildenafil will be discussed in more detail in section 4.4.4.

4.4.3. Action of the PDE3 inhibitor SKF94836 and PDE5 inhibitor sildenafil in control main and first branch PA on removal of the endothelium

Endothelial control is known to be important in both the systemic and pulmonary vasculature. A decrease in endothelium-dependent relaxation was reported to occur in the conduit PAs from CH in this study. It was therefore important to investigate if the response to either SKF94836 or sildenafil is attenuated if an intact endothelium is not present, as this may reduce their effectiveness as a therapy for PHT. Sildenafil was shown to act in an endothelium-independent manner in both the main and first branch PAs preconstricted with either PE, 5-HT, or ET-1. No significant difference was seen in the efficacy of sildenafil on removal of the endothelium compared to control main and first branch PAs. In contrast, SKF94836 acted in both an endothelium-dependent and endothelium-independent manner depending on the PA branch studied. In the main PA, in common with sildenafil, the efficacy of SKF94836 was not significantly different on removal of the endothelium, when compared to the control PA. However, the efficacy of SKF94836 was attenuated or even abolished on removal of the endothelium in the first branch control PAs. Therefore, these results show SKF94836 acts in an endothelium-dependent manner in the first branch PA.

MacLean *et al.* (1994), showed that the removal of the endothelium in the conduit PAs, although abolishing the action of ACh, had no significant effect on either cGMP or cAMP basal levels. These authors suggested that the main site of basal cyclic nucleotide generation, in the conduit PAs, was the vascular smooth muscle. Therefore, PDE inhibitors that target cyclic nucleotides may not necessarily require an intact endothelium. This may explain the endothelium-independent action of sildenafil in the main and first branch PAs. In fact, the endothelium-independent action of sildenafil in the conduit PAs is of advantage in the treatment of PHT, as it potency and efficacy would not be reduced in patients where the endothelium is known to be damaged (Dinh-Xuan *et al.*, 1991: 1993).

SKF94836 has a site dependent effect on removal of the endothelium. The pulmonary circulation is composed of a heterogeneous population of smooth muscle cells, expressing different cytoskeletal and contractile proteins, channels and receptors (Frid *et al.*, 1997). Regional differences in the response of each branch of the PA to vasoactive agents are therefore likely. These results suggest that the major pathways

involved in the regulation of cAMP may differ between branches of the PA tree. It may be that the generation of cAMP in the first branch compared to that in the main branch is more dependent or sensitive to agents released from the endothelium. This does not eliminate the possibility of SKF94836 as a therapy for PHT, but may merely suggest for its optimal effect fully functional endothelium is required. SFK94836 may still be useful in some forms of PHT where endothelium function is not severely impaired. For example, the PA from CH and MCT-treated rats all have reduced yet still functioning endothelium, as they responded to ACh (see figures 4.3.4 and 4.3.20).

Removal of the endothelium in systemic arteries has been shown to attenuate the response of both PDE3 and PDE5 inhibitors (Deply *et al.*, 1996; Saeki *et al.*, 1995). For the maximal effect of PDE inhibitors to be observed in systemic arteries, an intact endothelium appears necessary. For example, cilostazol induced the relaxation of the thoracic aorta precontracted with phenylephrine in a concentration-dependent manner. On removal of the endothelium the concentration-dependent relaxation was shifted to the right, suggesting the response was in part endothelium-dependent (Nakamura *et al.*, 2001). Removal of the endothelium in the systemic arteries is known to be accompanied by decrease in the basal levels of cyclic nucleotides, in particular cGMP (Schini *et al.*, 1989). PDE inhibitors have been shown to increase the intracellular levels of cAMP/cGMP even in the endothelium-denuded arteries (Delpy *et al.*, 1996). The PDE5 inhibitor E4021 caused a significant increase in intracellular cGMP in endothelium-denuded coronary artery, but had no effect on cAMP (Saeki *et al.*, 1995). In both the pulmonary and systemic arteries PDE3 and PDE5 inhibitors have both an endothelium-dependent component of relaxation and an endothelium-independent component of relaxation. This is evident as even on removal of the endothelium, relaxation is generally attenuated and not completely abolished, suggesting PDE inhibitors act directly on smooth muscle.

4.4.4. Action of the PDE3 inhibitor SKF94836 and PDE5 inhibitor sildenafil in hypoxic main and first branch PAs

Development of PHT can be accompanied by a change in responsiveness of some vasodilators. Altered response to vasodilators associated with hypoxia may be due to the associated hypertrophy of the PA, endothelial dysfunction, and/or an indirect result of increased PAP. Both SKF94836 and sildenafil produced a concentration-dependent

relaxation in both main and first branch PAs preconstricted with either PE, 5-HT, ET-1. The efficacy of both SKF94836 and sildenafil increased significantly in the main and first branch PAs from CH. At low concentrations (1 x 10^{-9}M to 3 x 10^{-6}M) the relaxant effect of sildenafil was not significantly potentiated in the hypoxic main and first PAs. Relaxation at the highest concentration of sildenafil used (3 x 10^{-5}M), was increased in PAs from the chronic hypoxic rat. Increased relaxation as a result of hypoxic exposure was particularly evident with SKF94836, which induced over 200% relaxation in ET-1 preconsticted first branch PAs (discussed below in section 4.4.5). It may be that the increase in maximum relaxation induced by each PDE inhibitor is due to increased inherent tone, which is known to be present in the conduit PAs from CH (MacLean *et al.*, 1995; MacLean *et al.*, 1996; MacLean *et al.*, 1997). Increase responsiveness of the PDE inhibitors in the PAs of CH may also be due to changes in the phenotype of vascular smooth muscle cells in the larger pulmonary arteries, that is known to occur on the onset of PHT (Meyrick and Reid, 1978; Sasaki *et al.*, 1995).

Inhibition of PDE activity would have a greater significant effect if the rate of cAMP and cGMP production, or the levels of PDEs themselves were increased. The increased efficacy of SKF94836 (3 x 10^{-5}M) and sildenafil (3 x 10^{-5}M) with hypoxia may be due to the increased PDE activity in the PAs from CH, both of which have been observed. Both an increase in PDE3 and PDE5 activity occurs in response to hypoxia (MacLean *et al.*, 1997). In fact, chapter 3 demonstrates that in both the main and first branch PAs the increase in PDE3 activity correlates with an increase in the *de-novo* synthesis of PDE3. Likewise, there is an increase in transcript and protein levels of PDE5 associated with chronic hypoxic exposure in the main and first branch PAs (chapter 3). Both PDE inhibitors would attenuate the increase in PDE activity associated with hypoxia, increasing the intracellular levels of both cyclic nucleotides, leading to relaxation of the smooth muscle of the PA.

Interestingly, vasoactive agents that are known to modulate the levels of cyclic nucleotides have previously been shown to be increased with the development of PHT. For example, reports suggest that NO production is increased in PHT possibly due to increased NOS expression (Shaul *et al.*, 1995; Isaacson *et al.*, 1994, Xue *et al.*, 1994; Le Cras *et al.*, 1998; Le Cras *et al.*, 1996, Resta *et al.*, 1997). Also, the atrial natriuretic peptide (ANP), is increased in human primary and secondary PHT (Morice *et al.*, 1990; Zhao *et al.*, 1999). Both an increase NO and an increase in ANP with the development

of hypoxia would increase cGMP in the PAs. However, the increase in PDE5 activity also associated with hypoxia may mask the increase in cGMP in response to NO or ANP, by increasing its degradation. PDE5 inhibition by sildenafil would attenuate any increase in PDE5 activity, thereby uncovering the increase in circulating levels of cGMP. Inhibition of PDE5 activity may be more effective if the basal rate of cGMP production was increased. Together this hypothesis may explain why sildenafil has a higher efficacy in the hypoxic PAs compared to the control. Evidence for such an interaction between the effectiveness of PDE5 inhibitors and increased basal cGMP production, can be seen in studies by Kim *et al.* (2000). These authors showed that incubation with sildenafil in combination with the NO donor sodium nitroprusside (SNP) produced a marked increase in cGMP, significantly greater than sildenafil alone. Likewise, functional studies showed sildenafil (10-1000nM) had little effect on phenylephrine-induced tone in smooth muscle from human corpus cavernosum tissue (Ballard *et al.*, 1998). However, sildenafil (10-1000nM) significantly enhanced the amplitude and duration of electrical field stimulation-induced, nitric oxide dependent relaxation of this preconsticted smooth muscle (Ballard *et al.*, 1998). The transient, frequency-dependent relaxation induced by electrical field stimulation in smooth muscle from corpus cavernosum and PAs is thought to be mediated via the stimulation of NANC nerves and involve nitric oxide (Liu *et al.*, 1992b). NANC mediated electrical field stimulation-induced relaxation is inhibited by the nitric oxide synthase inhibitor L-NAME, and has been shown to be accompanied by an elevation in tissue cGMP concentration (Liu *et al.*, 1992b).

In common with sildenafil, the increased responsiveness to SKF94836 in hypoxic PAs, may be due increased basal cAMP that may occur in response to hypoxia. Prostacyclin, which is known to increase cAMP accumulation by mediating adenylyl cyclase activity, production and synthesis, has been shown to be increased 2.7 fold after 7 days of hypoxia (Shaul *et al.*, 1991). The stable metabolite of prostacyclin, 6-keto-prostaglandin F1 alpha, has also been seen to increase with the onset of PHT from 31 $\pm$ 3 to 842 $\pm$ 367 pg/ml (Peterson *et al.*, 1982; Martin *et al.*, 1992). These results suggest cAMP production can be increased with hypoxia, however the increase in PDE3 activity would increase its degradation. PDE3 inhibition by SKF94836 would inhibit any increase in PDE3 activity, thereby increasing the circulating levels of cAMP, increasing relaxation. Such a hypothesis may explain the increased efficacy of SKF94836 with hypoxia.

As the efficacy of SKF94836 and sildenafil were not attenuated but potentiated in the CH, this suggests that both PDE3 and PDE5 inhibitors may be useful as vasodilators in the pulmonary circulation of patients with PHT. The observation from chapter 3 can be linked to the results presented in this chapter, in that increased *de-novo* synthesis of PDE3 and PDE5 with hypoxia, may explain why SKF94836 and sildenafil are so effective in relaxing the conduit PAs from the CH.

4.4.5. Vasoconstrictor-dependent response to SKF94836 and sildenafil in the main and first branch PAs

In both the control and hypoxic PAs, SKF94836 and sildenafil produced a concentration-dependent relaxation irrespective of the preconstrictor used (1 x 10^{-6}M PE, 3 x 10^{-5}M 5-HT and 3 x 10^{-9}M ET-1). The action of vasodilators has previously been shown to be dependent on the type of preconstriction (Plane and Garland 1996). The results presented here show the efficacy of SKF94836 and sildenafil can vary depending on the preconstrictor used to increase tone in the same PA. In general, a similar pattern of relaxation occurred in response to SKF94836 and sildenafil in the control, endothelium-denuded and hypoxic main PAs. Differences in the efficacy of each PDE inhibitor, may be due to the diverse signalling pathways involved in the action of each preconstrictor, and their response to altered cyclic nucleotide levels.

The most dramatic increase in the efficacy between the PDE inhibitors was seen in the hypoxic first branch PA preconstricted with 3 x 10^{-9}M ET-1. SKF94836 resulted in a relaxation that was more than 2-fold greater than in first branch hypoxic PAs preconstricted with either PE, or 5-HT. It is possible that this may due to increased sensitivity of ET-1 that has been documented to occur in the first branch PAs in response to hypoxia (Mullaney *et al.*, 1998). A significant increase in ET-1 sensitivity was not observed in this present study, which may merely be due to the smaller n numbers. However, this would not explain why in the first branch PA the maximum relaxation produced by SKF94836 was approximately three fold greater than the relaxation produced by sildenafil in the first branch preconstricted with 3 x 10^{-9}M ET-1.

In the lungs of the CH there is an increase in ET-1 concentrations, and ET-1, ETA receptor and ETB receptor mRNA (Li *et al.*, 1994). The addition of ET-1 in the CH has been shown to result in a rise in cAMP levels in the first branch PAs (Mullaney *et al.*, 1998). This was thought to be due to the downregulation and redistribution of the

inhibitory ET-1 receptor, as a result of the increased circulating levels of ET-1 with hypoxia. It is possible that SKF94836 acts synergistically with increased cAMP induced by both preconstricting the PAs with ET-1, and with the increased ET-1 that has been shown to occur with hypoxia, leading to the significantly larger degree of relaxation that was observed. As ET-1 is increased in patients with primary and secondary PHT (Giaid et al, 1993), these results suggest SKF94836 as a possible therapy.

4.4.6 Effect of DMSO

The importance of controlling for the potential effects of the vehicle of the drug was highlighted in this study. Dimethyl Sulfoxide (DMSO) alone, the vehicle for both SKF94836 and sildenafil, caused a concentration-dependent relaxation in both the main and first branch control, hypoxic, and endothelial denude PAs. Without subtracting the relaxation seen with DMSO, both PDE inhibitors appeared to produce a much greater relaxation in the PA studied. It is possible the relaxation may merely be due to a drop in tension due to time, however McAuley *et al.* (2001), have also observed an effect of DMSO. These authors found the relaxation to sildenafil was enhanced in the presence of DMSO. Other investigators found increasing concentrations of DMSO (0.1% v/v) induced an endothelium-independent relaxation in precontrated porcine and rabbit PA (Lawrence *et al.*, 1998; Murtha *et al.*, 1999). The effect does not appear to be selective to the pulmonary circulation as DMSO also decreases systemic vascular resistance and was shown to relax coronary arteries (Hameroff *et al.*, 1981; Lawrence *et al.*, 1998).

DMSO scavenges hydroxyl radicals, is an organic solvent, and an antioxidant. It has been suggested that reactive oxygen species (ROS) may play both a physiological and pathophysiological role in vascular homeostasis. Studies have shown the production of reactive oxygen species can lead to vasoconstriction (Jones *et al.*, 1997; Rhoades *et al.*, 1990). It can be hypothesised that by scavenging hydroxyl radicals, DMSO may cause relaxation. Also, DMSO has been shown to inhibit tension and ATP hydrolysis in skeletal muscle myofibrils, which may occur in the isolated PAs (Mariano *et al.*, 2001). Lawrence *et al.* (1998), studied the possible cardiovascular effects of drug vehicles. These authors found the least active solvent to be methanol. If possible it may be of advantage to repeat some of the experiments using methanol as the vehicle, to confirm

the degree of relaxation seen by the PDE inhibitors is not due to a synergistic action with DMSO.

4.4.7. Possible therapeutic potential of PDE3 inhibitors

Further evidence exists enforcing a role for the therapeutic use of PDE3 inhibitors in PHT. The PDE3 inhibitor SCA40 has been demonstrated to relax main and intralobar PA precontacted with PE (Crilley *et al.*, 1998). Milrinone has been shown to significantly decrease the mean PAP and PVR in the hypoxic dog, and in early clinical trials to lower the pulmonary capillary pressure in patients with heart failure. (Kato *et al.*, 1998; Baim *et al.*, 1983, Jaski *et al.*, 1985). Additionally, PDE3 inhibitors have also been shown to inhibit airway smooth muscle proliferation. Billington *et al.* (1999), showed that siguazodan (PDE3 inhibitor) inhibited both [^{3}H] thymidine incorporation and the increase in cell number induced by PDGF-BB (20ng/ml).

However, one major concern of the use of PDE3 inhibitors in the therapy of PHT is their likely side effects, especially in the cardiovascular system. Unfortunately PDE3 inhibitors have been shown to induce positive inotropism and vasodilation in the systemic circulation (Nicholson *et al.*, 1995). In long term clinical trials with PDE3 inhibitors the hemodynamic improvements seen early in therapy were typically not sustained, and an increase in mortality of about 40% after several months of treatment was observed (Uretsky *et al.*, 1990, Parker *et al.*, 1991). Relevant to this study, SKF94836 does have inotrope/vasodilator activity with sustained duration *in vivo* in the systemic circulation in both cats and dogs (Gristwood *et al.*, 1988). This demonstrates that SKF94836 is not selective for the pulmonary circulation, as systemic vascular resistance declined in a corresponding manner.

The lack of selectivity of PDE3 inhibitors for the pulmonary circulation does not completely rule them out in the treatment of PHT. It may be possible that co-administration with other PDE inhibitors would allow the use of subthreshold doses, which under clinical conditions do not have cardiovascular side effects. Wagner *et al.*, (1997), demonstrated that the co-application of subthreshold doses of PDE3 (milrinone) and PDE4 (rolipram) inhibitors enhanced isoproterenol and forskolin-induced relaxation of isolated PA from CH. In addition, the greatest amplification of the pulmonary vasodilator effect of PGI2 was achieved using the dual-selective PDE3/PDE4 inhibitors zardaverine and tolafentrine (Schermuly *et al.*, 1999). Co-administration of PDE3 and

PDE4 inhibitors also demonstrated a synergistic action in attenuating cellular proliferation in cultured vascular SMCs (Pan *et al.*, 1994).

A close interaction between cAMP and cGMP mediated vasorelaxant effects have always been thought to occur. It has been postulated that the inhibition of PDE5 would lead to vasorelaxation by increasing cGMP, which can subsequently further inhibit PDE3, potentiating the cAMP-mediated vasorelaxation. Clarke *et al.*, (1994) demonstrated such a synergistic effect, "a low dose" of milrinone (0.17μM) yielded a 4.6 ± 2.4% reduction of elevated PVR, and a "low dose" of dipyridamole (0.06μM) yielded a 8.2 ± 2.8% reduction of elevated PVR. Administration of both the "low doses" of milrinone and dipyridamole yielded a 41.9 ± 7.3% reduction of elevated PVR. This data supports a synergistic and not merely additive effect of the co-application of PDE3 and PDE5 inhibitors to reduce elevated PVR. This is of clinical significance, as patients with PHT would benefit from such a combination of a marked reduction of PVR with a far smaller reduction in SVR.

4.4.8. Possible therapeutic potential of PDE5 inhibitors

These results have demonstrated that sildenafil can relax isolated preconstricted main and first branch PAs. There is a great deal of literature that shows PDE5 inhibitors may be useful in the treatment of PHT. Several other researchers have reported the efficacy of PDE5 inhibitors as pulmonary vasodilators in animal models of PHT. PDE5 inhibitors such as DMPPO, zaprinast, and dipyridamole have all been shown to induce selective vasodilation of the pulmonary vascular bed, and also found to protect against the development of pulmonary vascular remodelling (Eddahibi *et al*, 1998, Ichinose *et al.*, 1995b, Thebaud *et al.*, 1999, Hanson *et al.*, 1998). More specific PDE5 inhibitors such as E4010 have also been shown to improve mortality in MCT-induced pulmonary hypertensive rats (Kodama and Adachi, 1999). In this particular study it was shown that rats treated chronically with 0.1% E4010 in their diet showed reduced right ventricular hypertrophy and increased plasma cGMP levels compared to rats treated with vehicle. Likewise in the CH a single oral dose of 1.0mg/kg E4010 caused a long-lasting reduction in PAP, with no significant systemic side effects on SAP, cardiac output, and heart rate (Hanasato *et al.*, 1999).

Together these studies suggest PDE5 inhibitors would be useful in treating PHT, but PDE inhibitors such as E4021 and E4010 although more selective are not yet available for humans. Sildenafil citrate (Viagra®) has already however been successfully launched by Pfizer in 1998 for the treatment of male erectile dysfunction (Boolell *et al.*, 1996). Zhao *et al.* (2001), examined the effect of sildenafil on hypoxia-induced PHT in mice and healthy human volunteers. They found in humans 100mg of sildenafil inhibited the hypoxic rise in PAP without significantly effecting the systemic circulation. This result was reproduced in isolated mouse lung, where sildenafil attenuated the increase RV hypertrophy and remodelling in mice chronically exposed to hypoxia.

There is an unresolved debate concerning potential side effects of sildenafil. A clinical trial by Goldstein *et al.* (1994), found the main side effects to be headache, flushing, dyspepsia, rhinitis and visual disturbances. The transient visual disturbances may be caused by nonselective inhibition of other PDEs such as PDE6, which is structurally closely related. A further placebo-controlled and open-label phase 2/3 trial including men with ischemic heart disease did not show an increase in myocardial infarction or serious cardiovascular events in patients treated with sildenafil versus placebo (Kloner, 2000). Together, these studies show sildenafil would appear to be extremely beneficial as a treatment for PHT. In addition, researchers have developed PDE5 inhibitors that are more selective than sildenafil, such as vardenafil hydrochloride, which may be even better in reducing PAP with fewer unwanted side-effects (Bischoff *et al.*, 2001).

4.4.9. Conclusion

These results demonstrate that both SKF94836 and sildenafil are effective pulmonary vasodilators in isolated main and first branch PAs. It may be that PDE inhibitors block the development of hypoxic pulmonary vasoconstriction by increasing the intracellular levels of cyclic nucleotides. As SKF94836 and sildenafil inhibited the vasoconstriction induced by PE, 5-HT, and ET-1, and hypoxia did not attenuate the relaxation, this provides evidence for the use of these inhibitors in the clinical treatment of PHT.

The magnitude of the response to both SKF94836 and sildenafil were dependent on the preconstrictor used, the branch of the PA studied, the presence of an intact endothelium, and enhanced by the vehicle DMSO. These results reinforce that caution must be taken

when interpreting the responsiveness of the pulmonary vasculature to inhibitors, and when comparing data from various studies. Caution must also be taken in generalising the response seen in the main and first branch PAs would occur in the whole lung or/and in the resistance vessels. Further studies would intend to define whether the effect observed in isolated PA segments *in vitro* is also seen in the entire pulmonary vasculature *in vivo*. For example, to further assess the role of PDE5 on pulmonary vascular tone and development of PHT, sildenafil could be administered to rats in their drinking water from the first day of the hypoxic exposure. PDE-5 inhibition by sildenafil would be expected to reduce the development of PHT. In addition, these results suggest it plausible to examine the functional consequence of co-administration of SKF94836 and sildenafil in isolated PA rings. It is proposed that PDE3 and PDE5 inhibitors may act synergistically to reduce pulmonary arterial vasoconstriction, leading to a new approach in the treatment of PHT.

CHAPTER 5

THE ROLE OF PDEγ IN CHRONIC HYPOXIA

Chapter 5: The role of PDEγ in chronic hypoxia

5.1 Introduction

Pulmonary hypertension (PHT) appears not only to be due to an imbalance between the vasoconstrictor/vasodilator actions of endogenous mediators, but also due to changes in their mitogenic/anti-mitogenic effects (MacLean, 1999). Chronic hypoxia causes abnormal cell proliferation and increased hypertrophy in pulmonary arteries (PAs), which results in remodelling. In response to chronic hypoxia PAs show increased wall thickness, progression of muscularisation into normally non-muscular vessels, and the formation of neointima and plexiform lesions (for review see Jeffery and Wanstall, 2001). Remodelling of PAs is thought to reduce the ability of vasodilators to lower resistance and pressure in the pulmonary vasculature. Therefore, targeting pulmonary vascular remodelling may be important in finding new therapies for PHT.

Cellular proliferation is initiated by mitogenic stimuli such as growth factors and hormones acting via cell surface receptors, that include growth factor tyrosine kinase receptors (RTK) and G-protein coupled receptors (GPCR). Activation of these receptors stimulates p42/p44 mitogen-activated protein kinase (p42/p44 MAPK or ERK1/2), a serine/theronine kinase belonging to the MAPK super family (Marshall, 1995 and Van Biesen *et al.*, 1995). Other subfamilies of MAPKs include the c-Jun N-terminal kinases also known as stress activated protein kinases (JNKs/SAPKs), and the p38 MAPKs. JNK and p38 MAPK appear to be more involved in response to stress such as cytotoxic insults, and not in mitogenesis (Orsini *et al.*, 1999). The general sequence of activation for MAPK is, MAPK kinase kinase (MAPKKK) $\Rightarrow$ MAPK kinase (MAPKK) $\Rightarrow$ MAPK. These signalling cascades are deactivated by the dephosphorylation of MAPKs by MAPK phosphatases (MKPs) (for review see Keyse, 2000). There is substantial evidence showing that hypoxia activates MAPK pathways in cells from the PA (Jin *et al.*, 2000; Welsh *et al.*, 2001). Furthermore, MKPs are induced in response to hypoxic stimuli in various cell types (Seta *et al.*, 2001; Laderoute *et al.*, 1999). Together these studies suggest a role for MAPK pathways in the increased cellular proliferation in the PA that can lead to the development of hypoxic induced PHT.

Mitogens including epidermal growth factor (EGF), platelet derived growth factor (PDGF), and thrombin have previously been shown to produce a robust and sustained activation of p42/p44 MAPK, which could be correlated with increased DNA synthesis in human airway smooth muscle cells (Orsini *et al.*, 1999). Furthermore, this increase in growth was inhibited by the MEK1 (the MAPKK activator of p42/p44 MAPK) inhibitor PD098055. Studies such as these and others, for example by Karpova *et al.* (1997), in bovine airway smooth muscle, suggest the p42/p44 MAPK pathway appears to be a key signalling event mediating mitogen-induced proliferation. In relation to the present study, many growth factors including PDGF-A, PDGF-B, VEGF, TGF-β, bFGF, IGF-1, and EGF have been documented to be elevated in PHT (for review see Jeffery and Wanstall, 2001), providing evidence for a role of growth factor induced MAPK pathways in remodelling of the PA.

Another well-documented and studied GPCR pathway is the phototransduction cascade, which is similar to signalling by growth factors and GPCRs in other mammalian cell systems. Photoexcitation of the GPCR rhodopsin, results in the GDP-GTP cycle activation of the heterotrimeric G protein, transducin, and G-protein receptor coupled kinase (GRK) and β-arrestin (Stryer, 1991). GTP-bound transducin stimulates PDE6 [expressed as a tetrameric protein composed of catalytic heterodimers (αβ), and two inhibitory γ-subunits (PDEγ)], by displacing PDEγ. Activation of PDE6αβ leads to the hydrolysis of cGMP to 5'GMP resulting in the closure of cGMP-gated Na^{+}/Ca^{2+} channels on the plasma membrane. Decrease in Na^{+} and Ca^{2+} results in hyperploarisation of the cells (for review see Yafitz and Hurley, 1994).

Two functionally similar PDEγ isoforms are known to exist, PDEγ1 (rod) and PDEγ2 (cone), differing in their amino-terminal regions and their location in the retina (Hamilton and Hurley, 1990). The carboxyl-terminal domains, which are involved in the interaction with transducin, are almost identical. Furthermore, as a result of a 41 base pair deletion, two isoforms of PDEγ2 exist, namely long PDEγ2 and short PDEγ2. It is thought the two forms of PDEγ2 are important at different stages of embryo development. Each step of the GTP-hydrolytic cycle of transducin is closely related to molecular states of PDEγ (Morrison *et al.*, 1987). As PDEγ is an important link between rhodopsin activated transducin and effectors, it was proposed that PDEγ1 and PDEγ2 maybe expressed in other tissues where they may regulate other receptor-G-protein-mediated pathways, such as p42/p44 MAPK.

Evidence for a role of PDEγ in other systems of the body is evident primarily by results showing the expression of PDEγ1 in lung, kidney, testes, liver, heart, airway smooth muscle and human embryonic kidney (HEK) 293 cells, and its absence in all these tissues from PDEγ1 knockout mice (unpublished data from the lab, Wan *et al.*, 2001, Tate *et al.*, 1998, Tate *et al.*, 2001). Furthermore, using recombinant and antisense PDEγ in HEK293 cells Wan *et al.* (2001), showed PDEγ1 regulates the EGF and thrombin-dependent stimulation of p42/p44 MAPK through its interaction with the GRK2 signalling system in HEK 293 cells. Thrombin also stimulated the association of endogenous PDEγ1 with dynamin II, which may be required for the endocytosis of receptor signal complexes leading to the activation of p42/p44 MAPK and stimulation of cell proliferation (Wan *et al.*, 2001). These studies proposed PDEγ as a novel intermediate in p42/p44 MAPK signalling.

In addition to the role of PDEγ in regulating growth factor and GPCR stimulation of the p42/p44 MAPK pathway, it has been speculated that it may interact with other PDEs. This was proposed, as the presence of PDEγ throughout the body cannot be explained by its association with PDE6 (as PDE6 is only found in the eye). In particular PDE5 shares common structural and functional properties with PDE6. Both PDE5 and PDE6 display a high degree of amino-acid identity (45-48%) between the catalytic domain, posses cGMP binding sites, hydrolyse cGMP better than cAMP, and are both sensitive to a common set of competitive inhibitors (McAllister-Lucas *et al.*, 1993; Gillespie and Beavo, 1989; Turko *et al.*, 1999b; Gonzalez, 1999). PDE5 appears an ideal candidate to contain its own γ subunits controlling its regulation. In fact, Lochhead *et al.* (1997), identified two small molecular mass proteins termed p14 and p18 in guinea-pig airway smooth muscle cells and mouse lung (where PDE5 is the major cGMP binding protein, Burns *et al.*, 1992), which cross-reacted with antibodies raised to the polycationic mid-region and C-terminal region of PDEγ. Furthermore, recombinant PDEγ has been shown to modulate PDE5 activity by preventing its activation by PKA in a concentration-dependent manner (Tate *et al.*, 1998, and Lochhead *et al.*, 1997). It was therefore speculated that since PDEγ is a protein inhibitor of PKA-activated PDE5, this action might potentiate agonist-stimulated cGMP formation by preventing negative feedback control. It is also possible that the interaction of PDEγ with PDE-5 may be reduced under hypoxic conditions, amplifying PKA-activation of PDE5, thereby explaining, in part, the increase in PDE5 activity observed in PA (MacLean *et al.*,

1997). In addition PDEγ stimulates the proteolysis of PDE5 by caspase-3 and caspase-8 *in vitro* (Frame *et al.*, 2001). These results suggest that under conditions of cellular stress, PDEγ may convert PDE5 to a conformation that is more sensitive to attack by proteases. Together these findings suggest that PDEγ may function to localise PDE5 in signalling complexes (with dynamin II) that are organised to stimulate the p42/p44 MAPK pathway. This may protect the p42/p44 MAPK signalling pathway from the inhibitory action of cGMP. Chapter 3 reports that PDE3A/3B and PDE5 expression and activity are increased in pulmonary vessels from rats maintained under chronic hypoxic conditions (MacLean *et al.*, 1997). This would offer more protection to mitogenic signalling pathways from the inhibitory action of cyclic nucleotides and might, in part, explain the enhanced pulmonary vessel remodelling in PHT.

As PDEγ1/2 appear to regulate growth factor and GPCR stimulation of the p42/p44 MAPK pathway, it is proposed that changes in its expression may have profound effect on cellular proliferation in PA in response to hypoxia. In conjunction, PDEγ may also have a key role in modulating PDE5 activity in the pulmonary circulation, and its interaction may be altered with hypoxic stimuli. The aim of this study was therefore to investigate whether PDEγ1/2 are expressed in rat PA and hPASMC, and to determine the effect of chronic hypoxia on this expression. The effect of chronic hypoxia on the expression of PDEγ1/2 will also be correlated with any change in p42/p44 MAPK. These novel studies were intended to show a possible wider role of PDEγ in signal transduction.

5.2 Materials and Methods

5.2.1 Materials

All reagents, unless otherwise stated, were obtained from Sigma chemical company (U.K.), or BDH (U.K.). Cell culture supplies were from life Technologies (U.K.). RNeasy total RNA isolation kit and QIA shredder were from Qiagen (U.K.). Superscript II reverse transcriptase, DNase I Amplification Grade, Oligo dt (18), Taq Polymerase, and primers were from Life Technologies (U.K.). DNA Polymerase Mix (dNTPS), GFX™ PCR and Gel Purification Kit, Hybond™ECL™ Nitrocellulose Membranes were from Amersham Pharmacia Biotech (U.K.). Rat glyeraldehyde-3-phosphate dehydrogenase (G3PDH) control amplimer set was from Clonetech

laboratories Inc (USA). BigDye Dye terminator cycle sequencing kit was from PE-Applied Biosystems (U.K.). Anti-PDEγ antibody to the C-terminal domain of photoreceptor PDEγ was a kind gift from Dr. R. Cote (University of New Hampshire, USA). Phospho-p42/p44 MAPK antibody was from BD Transduction Laboratories (U.K.).

5.2.2 Animal Studies – Chronic Hypoxic Rat

Male Wistar rats of 28-30 days old (at start of experiment) were housed in a specially designed perspex hypobaric chamber (Royal Hallamshire Hospital, Sheffield). The pressure within the chamber was decreased to 550mbar; this reduced the inspired pO_2 to approximately 110mmHg (~10% equivalent). The temperature of the chamber was maintained at 21-22°C and the chamber was ventilated with air at ~451 min^{-1}. Animals were maintained in these hypoxic/hypobaric conditions for 14 days. Age-matched controls were housed under normoxic/normobaric room conditions (20% v/v oxygen). Following sacrifice the right ventricle of the heart was dissected free of the septum and left ventricle and these were blotted and weighed. PHT was assessed by measuring the ratio of right ventricle (RV)/total ventricular (TV) weight. This is a well-established index of the degree of PHT in the rats (Hunter, *et al.*, 1974). Pulmonary arteries were then dissected and taken for biochemical analysis.

5.2.3 Cell Culture

Human pulmonary artery smooth muscle cells (BioWittaker, U.K., from main and first branch PA) were maintained in smooth muscle cell growth medium (SmGM-2 bulletkit system, BioWittaker, U.K.). Following passage, flasks were split into two groups. Half the flasks were returned to the normoxic incubator (RS Biotech – Galaxy CO_2 incubator set at 5%CO_2, 95% air, humidified, set 37^{0}C), and half were transferred to the hypoxic incubator (RS Biotech – Galaxy CO_2 – oxygen control incubator 10% O_2, 5% CO_2, balanced N_2, humidified, set 37^{0}C). Cells were grown in the hypoxic incubator for 24 hours to 2 weeks to determine optimal conditions.

5.2.4 Homogenate preparation

To isolate protein from both tissue and cells, the homogenisation buffer isotonic sucrose solution (I.S.S.) was used with composition: 0.25M sucrose, 10mM Tris HCl, 1mM

EDTA, 0.1mM phenylmethylsulphonyl fluoride (PMSF) and 2mM benzamidine, pH 7.4. The PA branches were initially ground to a fine powder in liquid nitrogen using a mortar and pestle, then homogenised by adding 500µl I.S.S. and passing through a 25G syringe needle five times. After the removal of the media, cells were washed with sterile PBS, and then scraped in 600µl I.S.S. per T-75 flask. To shear the cells, the lysate was passed five times through a 25G syringe needle. Both the homogenised tissue and cells were centrifuged for 2 minutes at 12,000 rpm, and only the supernatant used in subsequent experiments.

5.2.5 Total RNA extraction

For isolation of RNA, rat pulmonary arterial branches were ground to a fine powder in liquid nitrogen with a mortar and pestle. 600µl buffer RNeasy lysis buffer (containing 14.5M β-mercaptoethanol) was then added according to manufacturers guidelines (Qiagen), and the tissue was then homogenised by passing the lysate through a 25 G needle five times. For isolation of RNA from hPASMC, the medium was aspirated and the cells washed with sterile PBS. The hPASMC were scraped in 600µl of RNeasy lysis buffer, and then passed through a 25G syringe five times. Both the tissue and cell lysates were then pipetted onto a QIAshredder (Qiagen). Total RNA was extracted according to the RNeasy protocol instruction (Qiagen). To prevent potential contamination from genomic DNA, an incubation step with 4 units of DNase at 37^0C for 15 minutes followed by a second RNA extraction (clean up protocol, Qiagen) were included. Total RNA was eluted in RNase free H_2O and stored at -20^0C.

5.2.6 RT-PCR

First strand synthesis was carried out using 1µg total RNA catalysed by the enzyme superscript II reverse transcriptase. The reaction was primed using 500ng of oligo $(dt)_{18}$. This mixture was heated to 70°C for 10 minutes and quick chilled on ice. The reverse transcriptase reaction was incubated at 42°C for 90 minutes and terminated at 70°C for 15 minutes.

The PCR was carried out using the following protocol: initial denaturation for 5 minutes at 95^0C, 15-35 cycles of amplification (each cycle consisted of denaturation for 30

seconds at 95^{0}C, annealing for 30 seconds at 50^{0}C, and extension for 1 minute 40 seconds at 72^{0}C), a final extension of 10 minutes at 65^{0}C, and storage at 4^{0}C.

RT-PCR with specific with specific forward and reverse oligonucleotide primers were used to amplify PDEγ1/2 transcripts. For PDEγ1, sense (Y00746 Forward), 5'-ATG AAC CTG GAG CCA CCC-3', and antisense, Y00746 Reverse, 5'-GCT CAC ATA GCA GGG ATC AGA-3' or C-terminal reverse, 5'-AAT GAT GCC ATA CTG GGC CAG-3'. For PDEγ2 sense, 5'-CGG GAT CCC GCC ACC ATG AGC GAC AGC CCT TGC C-3', and antisense, 5'-CCC AAG CTT GGG TCC TCA GAT GAT CCC GAA CTG-3'.

5.2.7. Sequence analysis

The purified amplicons were sequenced, in both directions, on a PE-Applied Biosystems Division Model 373A automated DNA sequencer using the PCR primers and a BigDye terminator cycle sequencing kit.

5.2.8 Western Blotting

Nitrocellulose sheets were blocked in 5% gelatin in PBS at 37°C for 1 hour and then probed with antibodies in PBS containing 1% gelatin (w/v) plus 0.05% (v/v) NP40 at 37°C for 12 hours. After this time, the nitrocellulose sheets were washed in PBS plus 0.05% (v/v) NP40. Detection of immunoreactivity was by incubating nitrocellulose sheets for 2 hours at 37°C with a reporter HRP-linked anti-rabbit antibody in PBS containing 1% gelatin (w/v) plus 0.05% (v/v) NP40. After washing the blots as described above, to remove excess reporter antibody, immunoreactive bands were detected using an enhanced chemiluminesence detection kit.

5.2.9 Quantification

RT-PCR and Western blotting results were quantified by densitometry (linear range of optical density between 0-1 arbitrary unit).

5.2.10 Statistics

In all analysis comparisons between two groups of data were made using Student's *t*-test for paired or unpaired data where appropriate. The statistical software package

Prism (Graphpad Prism, San Diego, CA, USA) was used to handle raw data, where *$P<0.05$ was considered to be statistically significant. In all experiments "n" either indicates the number of different animals used or number of different populations of cultured cells.

5.3 Results

5.3.1 The Chronic Hypoxic rat (CH)

The animals used throughout these experiments were also used to generate the results in 3.3.1 and figures 3.3.1 and 3.3.2. The exposure of male Wistar rats to 10% O_2 for 2 weeks resulted in a significant decrease ($P<0.05$) in body weight from 221.3g ± 2.6 to 199.7g ± 2.4 (n=80, $P<0.05$, Student's *t*-test) as seen in figure 3.3.1.

From figure 3.3.2 it can be seen that the RV/TV ratio was significantly increased with hypoxia, confirming that right ventricular hypertrophy had occurred. RV/TV ratios were 0.202 ± 0.001 and 0.336 ± 0.006 for normoxic and hypoxic rats respectively (figure 3.3.2, n=80, $P<0.05$, Student's *t*-test). As PHT is characterised by right ventricular hypertrophy, these results show its development in the rats exposed to hypoxia for 2 weeks. Throughout this study hypoxic refers to rats subjected to 14 days of chronic hypoxia (2.2.1.1).

5.3.2 Linear amplification of PDEγ1, PDEγ2, and G3PDH transcripts by RT-PCR

To perform semi-quantitative RT-PCR, each PDEγ transcript was amplified at various cycle lengths (conditions as in 2.2.2.4). From figure 5.3.1 linear amplification conditions for all transcripts were seen to occur using up to 25 cycles. Hence 25 cycles were used in all subsequent RT-PCR reactions.

5.3.3 The effect of hypoxia on PDEγ1, and PDEγ2 transcript levels in rat PA

Figure 5.3.2 show the RT-PCR amplification (25 cycles) of PDEγ1 (261bp product) and PDEγ2 (282bp product) from the main PA, first branch PA, intrapulmonary PA and resistance vessels of both normoxic and hypoxic rats. These results confirm that the amplicons correspond to PDEγ1/2 mRNA transcripts obtained from all the vessels. With chronic hypoxia it can be seen from figure 5.3.3 that neither PDEγ1 nor PDEγ2 transcript increased in any of the pulmonary arterial branches studied. The % effect of hypoxia on PDEγ1 and PDEγ2 transcripts in hypoxic *versus* normoxic rats were: main branch, PDEγ1, -1 ± 9%; PDEγ2, 3 ± 7%; first branch, PDEγ1, -8 ± 14%; PDEγ2, 7±

11%; intrapulmonary, PDEγ1, 5 ± 4%; PDEγ2, 12 ± 15%; resistance vessels, PDEγ1, -3 ± 14%; PDEγ2, 6 ± 9%, (n=4, P<0.05 *versus* normoxic animals, Student's *t*-test). Data can also be expressed as a ratio of G3PDH. The PDγ1/G3PDH transcript ratio in normoxic and hypoxic animals respectively were: main branch, 1 ± 0.01, 1 ± 0.03; first branch, 0.99 ± 0.01, 0.93 ± 0.1; intrapulmonary, 1.02 ± 0.06, 1.06 ± 0.08; resistance vessels, 0.98 ± 0.09; 0.94 ± 0.1 (n=4, NS, *versus* normoxic animals, Student's *t*-test, figure 5.3.3). The PDγ2/G3PDH transcript ratio in normoxic and hypoxic animals respectively were: main branch, 1 ± 0.02, 1.03 ± 0.05; first branch, 0.99 ± 0.03, 1.08 ± 0.11; intrapulmonary, 1.05 ± 0.1, 1.13 ± 0.18; resistance vessels, 0.98 ± 0.06; 1.03 ± 0.7 (n=4, NS, *versus* normoxic animals, Student's *t*-test, figure 5.3.3). Using these RT-PCR conditions, a chronic hypoxic-dependent increase in PDE3 and PDE5 transcript levels from the same samples has previously been observed (chapter 3).

Alignment of the PDEγ1 (264bp) and PDEγ2 (252bp) with the corresponding mouse and human PDEγ1 and PDEγ2 (either from the published mouse sequence or the sequence obtained experimentally using the hPASMC) can be seen in figures 5.3.7 and 5.3.8, all revealed >90% similarity in their nucleotide sequences. In both figures 5.3.7 and 5.3.8 the open reading frame (ORF) of both PDEγ1 and PDEγ2 are presented. To obtain the whole ORF for both PDEγ1 and PDEγ2, primers (2.2.2.4) were designed to include areas of the untranslated regions. Short PDEγ could not be detected or sequenced, suggesting it may not be present in the PA from rats. These results imply that PDEγ1 and PDEγ2 transcripts are not altered with hypoxia in the PA of the CH.

5.3.4 Controls in all RT-PCR reactions

5.3.4.1 G3PDH transcript levels

To verify equal loading of total RNA, and to include an internal control the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used. A product of 983bp was amplified (25 cycles) from the main, first branch, intrapulmonary and resistance vessels by RT-PCR using G3PDH specific primers (2.2.2.4), confirming its expression. No change in G3PDH transcript level was observed under normoxic and hypoxic conditions from each vessel studied (figure 5.3.2C). The percentage change in G3PDH transcripts from hypoxic versus normoxic rats were: main branch, -1 ± 2%;

first branch, -1 ± 1%; intrapulmonary arteries, -1 ± 1%; resistance vessels; 3 ± 6% (n=4, NS *versus* normoxic animals, Student's *t*-test). As no significant differences could be seen in the levels of G3PDH, this confirms that equal amounts of total RNA had been used for the amplification of the PDEγ1 and PDEγ2 transcripts.

5.3.4.2 Verify removal of genomic DNA

Contamination of RNA with genomic DNA would generate a PCR product leading to misinterpretation of results. It is therefore important to include a "minus –reverse transcriptase" negative control during the cDNA synthesis. This involved assembling a cDNA synthesis reaction that contained RNA and all other reagents except for the reverse transcriptase. This negative-reverse transcriptase control was then used as a template for PCR in parallel with the other experimental cDNA samples. As no cDNA can be synthesised without the inclusion of reverse transcriptase, the generation of a PCR product from this negative control indicates genomic DNA contamination. If genomic DNA was found in the samples, the results from the parallel RT-PCR with reverse transcriptase were discarded.

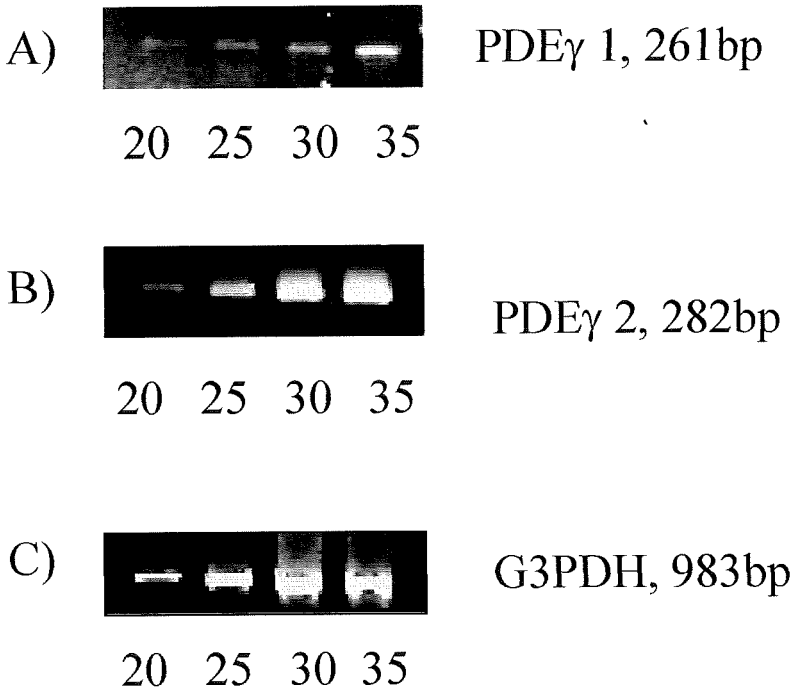


Figure 5.3.1. RT-PCR of the linear amplification of PDEγ1, PDEγ2, and G3PDH transcripts in rat control first branch pulmonary arteries

RT-PCR amplification using specific primers, as described in 2.2.2.4, of: (A) PDEγ1, 261bp; (B) PDEγ2, 282bp; and (C) G3PDH, 983bp from control rat first branch pulmonary arteries. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Each reaction was carried out using 20, 25, 30, and 35 cycles of amplification. Above is a representative result of 3 individual experiments, quantified by densitometry.

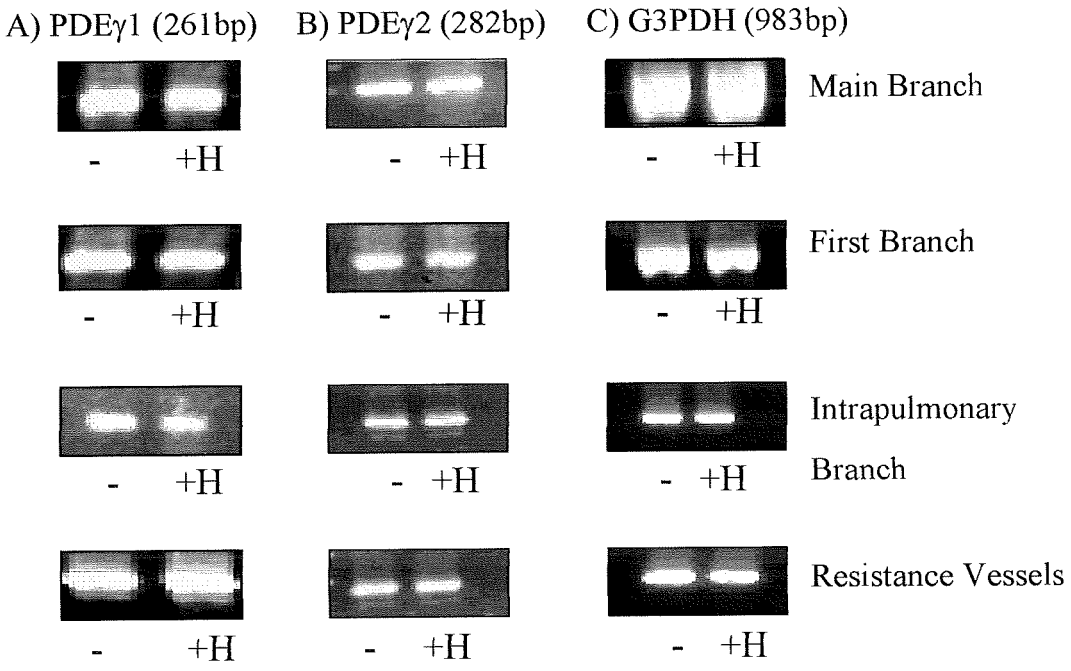


Figure 5.3.2. RT-PCR of PDEγ1, PDEγ2 and G3PDH transcripts from control and hypoxic rat pulmonary arterial branches

RT-PCR with specific primers, as described in 2.2.2.4, for (A) PDEγ1, 261bp; (B) PDEγ2, 282bp; and (C) G3PDH, 983bp, from main, first branch, intrapulmonary, and resistance vessels from Wistar rats maintained under normoxic (-) and chronic hypoxic (+H) conditions. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 4 individual experiments, quantified by densitometry.

5.3.5 Effect of hypoxia on PDEγ1/2-protein levels in rat PA

As changes in protein levels may not correspond with changes in mRNA levels, Western blotting was carried out as in 2.2.3.3 using homogenates from the main, first branch, intrapulmonary and resistance vessels. The anti-PDEγ antibody used was common for both PDEγ1 and PDEγ2 (raised to the C-terminal domain). PDEγ1/2 (M_r=14/18kDa) was expressed in the main pulmonary artery, the first branch pulmonary artery, the intrapulmonary artery and the resistance vessel. The percentage increase in PDEγ1/2 protein expression from basal was 26 ± 10% in the main PA, 97 ± 8% in the first branch PA, 110 ± 9% in the intrapulmonary artery, and 127 ± 9% in the resistance vessels (n=3, P<0.05 *versus* normoxic animals, Student's *t*-test, figure 5.3.3). These results suggest that in each PA studied, PDEγ1/2 protein levels increased with hypoxia. The increase in PDEγ1/2 expression with hypoxia is most marked in the resistance vessels.

5.3.6 Effect of hypoxia on phospho- and total- p42/p44 MAPK protein levels in rat PA

To link PDEγ1/2 expression with increased proliferation through regulation of p42/p44 MAPK, Western blotting was carried out as in 2.2.3.3. Homogenates from the main, first branch, intrapulmonary and resistance vessels and anti-phospho p42/p44 MAPK and total p44 MAPK antibodies were used. Phospho p42/p44 MAPK (M_r=42/44kDa) and total p44 MAPK (M_r=44kDa) were expressed in the main pulmonary artery, the first branch pulmonary artery, the intrapulmonary artery and the resistance vessel. In general it was found that the extent to which p44 MAPK was phosphorylated exceeded that of p42 MAPK in each PA (figure 5.3.4). The extent to which p42/p44 MAPK was phosphorylated increased in the vessels where PDEγ1/2 protein expression was also elevated by chronic hypoxia (figure 5.3.3). The percentage increase in phosphorylated p42/p44 MAPK in each vessel *versus* main branch (100 ± 4%) was 146 ± 9% in the first branch, 154 ± 7% in the intrapulmonary artery, and 184 ± 11% in the resistance vessels (n=3, P<0.05 *versus* main branch vessel, Student's *t*-test, figure 5.3.4A). The most significant increase in the phosphorylation of p42/p44 MAPK between the PA branches from the CH was observed in the resistance vessels, and correlated with the most pronounced increase in PDEγ1/2 expression. The corresponding changes in p44 MAPK protein levels in each vessel *versus* main branch (100 ± 1%) was 1 ± 0.2% in the first

branch, 1 ± 0.05% in the intrapulmonary artery, and 1 ± 1.1% in the resistance vessels (n=3, NS, *versus* main branch vessel, Student's *t*-test, figure 5.3.4B). As the expression of p44 MAPK was shown to be very similar in each vessel, the chronic hypoxic-dependent changes in the phosphorylation state of p42/p44 MAPK is not due to an increase in the expression of the kinase. The largest increase in the phosphorylation of p42/p44 MAPK between the PA branches from the CH was observed in the resistance vessels, and correlated with the most pronounced increase in PDEγ1/2 expression.

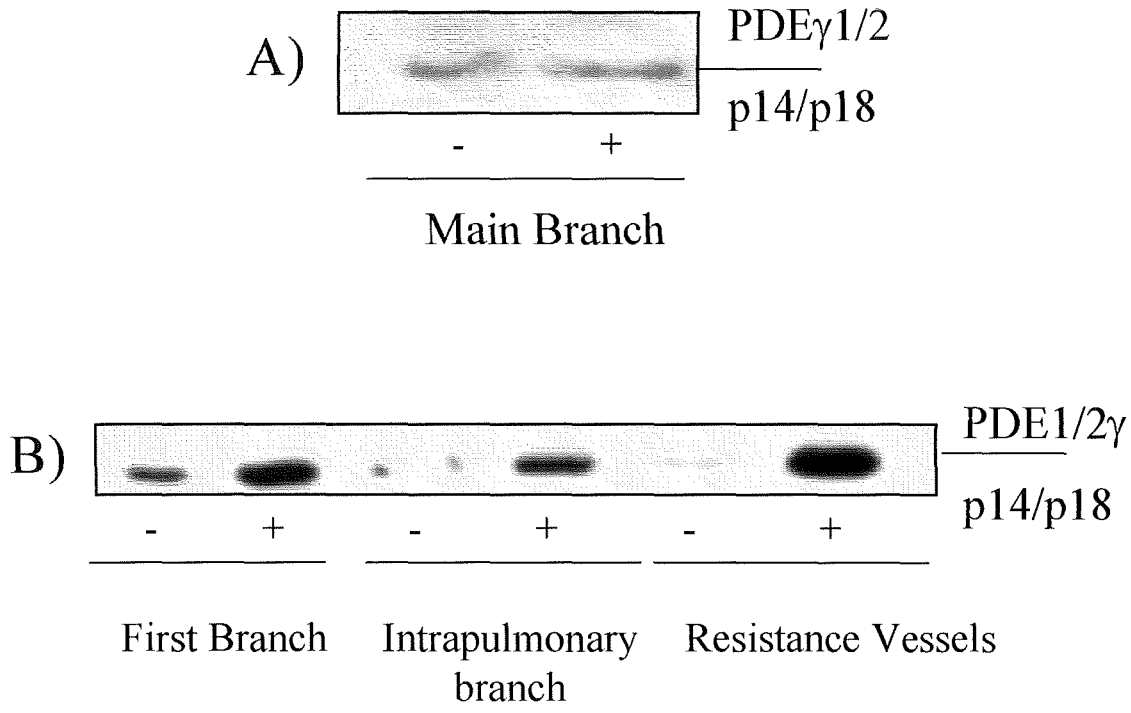


Figure 5.3.3. Western blot analysis of PDEγ1/2 from control and hypoxic rat pulmonary arterial branches

Western blot analysis using an anti-PDEγ1/2 antibody, as described in 2.2.3.3-2.2.3.5, showing the expression level of PDEγ1/2 (p14/p18) in homogenates from main, first branch, intrapulmonary and resistance vessels from rat maintained under normoxic (-) and chronic hypoxic (+H) conditions. 10μg protein/sample were loaded onto SDS-PAGE. Above is a representative result of 3 individual experiments, quantified by densitometry.

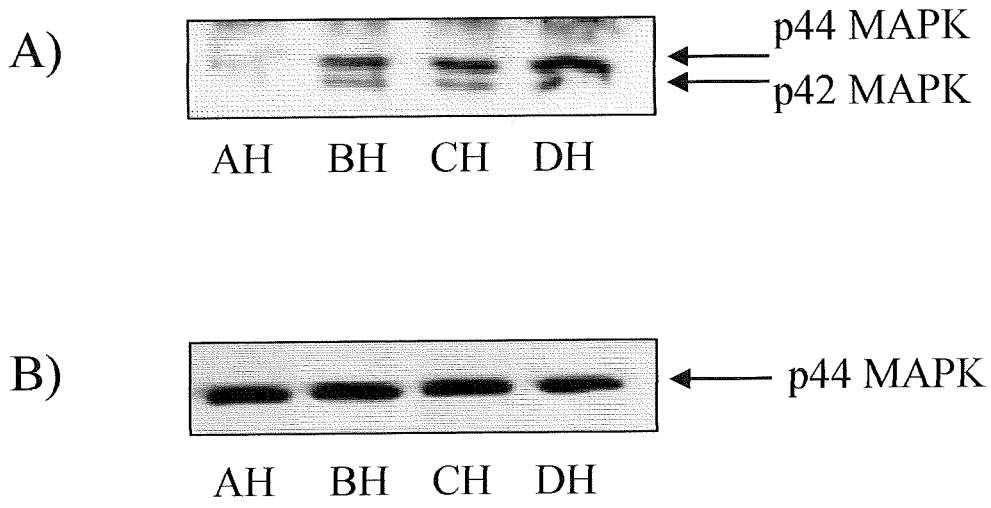


Figure 5.3.4. Western blot analysis of phospho p42/p44 MAPK and total p44 MAPK from hypoxic rat pulmonary arterial branches

Western blot analysis using A) anti-phospho p42/p44 MAPK and B) total p44 MAPK antibodies, as described in 2.2.3.3-2.2.3.5, showing the expression level of A) phospho p42/p44, and B) total p44 in homogenates from main (AH), first branch (BH), intrapulmonary (CH) and resistance vessels (DH) from rat maintained under chronic hypoxic conditions. 10μg protein/sample were loaded onto SDS-PAGE. Above is a representative result of 3 individual experiments, quantified by densitometry.

5.3.7 Development of a model of PHT using cultured hPASMC

PDEγ1 and PDEγ2 expression was investigated in hPASMC exposed to 10% 0_2 for 6 hours, 24 hours, 3 days, 7 days, and 14 days. Initially, PDEγ1 and PDEγ2 were amplified from hPASMC at increasing cycle lengths to show linear amplification. From figure 5.3.5 it can be observed that 25 cycles are the optimal conditions where linear amplification can be seen in PDEγ1, PDEγ2 and G3PDH. 25 cycles were used in all subsequent RT-PCR with hPASMC.

Using hPASMC, parallel RT-PCRs for the housekeeping gene G3PDH were used as internal standards and to verify equal loading of total mRNA. Negative controls were included, where reverse transcriptase was omitted during cDNA synthesis. These reactions were performed to ensure that the RT-PCR products were the result of amplification of the cDNA template, and not due to possible contamination by genomic DNA. If contamination of genomic DNA was found, the corresponding RT-PCR results were discarded.

Figure 5.3.5 show that hPASMC express PDEγ1, PDEγ2 and G3PDH. hPASMC were subjected to 10% O_2 for 6 hours, 24 hours, 3 days, 7 days, and 14 days. As seen from figure 5.3.8 no significant differences were seen in any transcript levels, after 6 hours, 24 hours, 3 days, or 7 days (densitometry not shown). After 14 days of sustained hypoxia no significant difference was observed in PDEγ1, PDEγ2, or G3PDH transcript levels (figure 5.3.9). The changes in PDEγ1, PDEγ2 and G3PDH transcript levels with chronic hypoxia versus normoxia in hPASMC were; PDEγ1, 5 ± 11%, PDEγ2, 8 ± 9%, and G3PDH –2 ± 1% respectively (n=3, NS, *versus* normoxic hPASMC, Student's *t*-test). Data can also be expressed as a ratio of G3PDH. The PDγ1/G3PDH and PDEγ2/G3PDH transcript ratio in normoxic and hypoxic hPASMC respectively were: 1 ± 0.04, 1.07 ± 0.12; and 1 ± 0.04, 1.10 ± 0.15 (n=4, NS, *versus* normoxic hPASMC, Student's *t*-test).

The alignment of PDEγ1 and PDEγ2 from hPASMC with the published mouse sequence and the rat PA sequence (obtained experimentally), all revealed >90% similarity in their nucleotide sequences (figures 5.3.6/5.3.7). Figures 5.3.6/5.3.7 show the open reading frame sequence for both PDEγ1 and PDEγ2.

Although PDEγ1 and PDEγ2 transcript levels were unchanged with chronic hypoxia, protein levels were significantly increased (figure 5.3.9). Western blot analysis, showed that PDEγ1/2 was expressed in hPASMCs. Chronic hypoxic treatment resulted in an increase in PDEγ1/2 protein by 65 ± 7% (n=3, $P<0.05$ *versus* normoxic hPASMC, Student's *t*-test). Therefore these results correlated with the rat model of PHT.

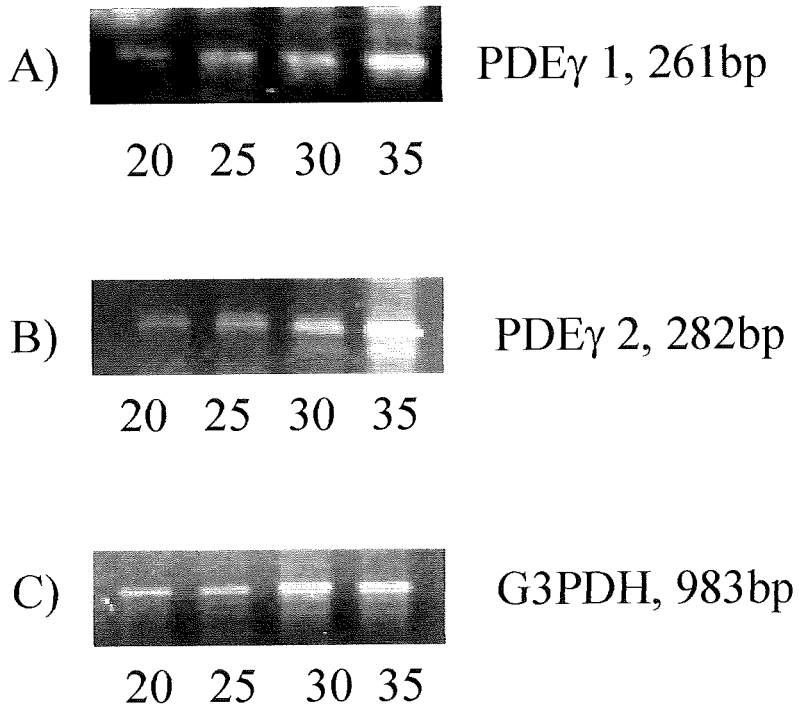


Figure 5.3.5. RT-PCR of the linear amplification of PDEγ1, PDEγ2, and G3PDH transcripts in hPASMC

RT-PCR amplification using specific primers, as described in 2.2.2.4, of: (A) PDEγ1, 261bp; (B) PDEγ2, 282bp; (C) G3PDH, 983bp from control hPASMC. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Each reaction was carried out using 20, 25, 30, and 35 cycles of amplification. Above is a representative result of 3 individual experiments, quantified by densitometry.

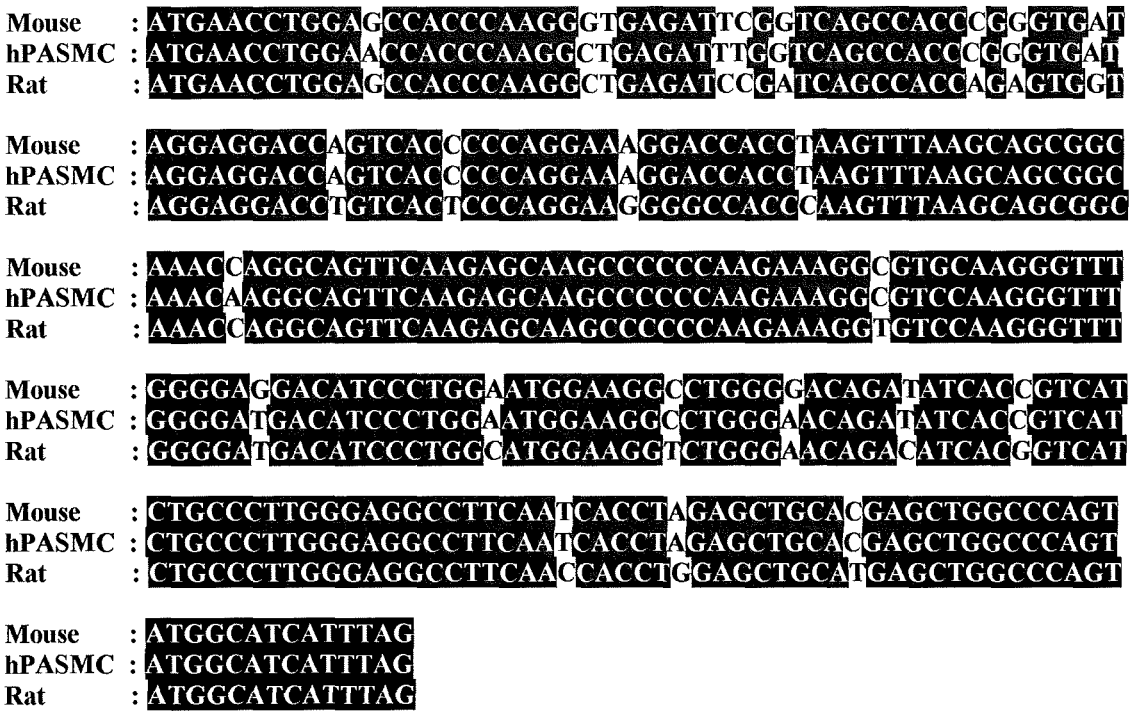


Figure 5.3.6. Rod PDEγ (PDEγ1) ORF cDNA sequence

PDEγ1 open reading frame (ORF) cDNA sequence (264bp) from rat pulmonary arteries and cultured human pulmonary smooth muscle cells aligned with corresponding mouse PDEγ1 to which the primers were designed (using the primers as in 2.2.2.4). The conserved amino acids across all three sequences are shaded black.

```
Mouse   : ATGAGCGACAGCCCTTGCCTGAGTCCTCCAGCACCAAGCCAGGGCCCTAC
Rat     : ATGAGCGACAGCCCTTGCCTGAGTCCTCCAGCACCAAGCCAGGGTCCTAC
hPASMC  : ATGAGCGACAGCCCTTGCCTGAGTCCTCCAGCACCAAGCCAGGGTCCTAC

Mouse   : TACCCCACGCAAAGGGCCCCCCAAGTTCAAGCAGAGGCAGACTCGACAGT
Rat     : TACCCCACGCAAAGGGCCCCCCAAGTTCAAGCAGAGGCAGACGCGACAGT
hPASMC  : TACCCCACGCAAAGGGCCCCCCAAGTTCAAGCAGAGGCAGACGCGACAGT

Mouse   : TCAAGAGCAAGCCTCCCAAGAAAGGGGTGAAAGGGTTTGGAGATGACATC
Rat     : TCAAGAGCAAGCCTCCCAAGAAAGGGGTGAAAGGGTTTGGAGATGACATC
hPASMC  : TCAAGAGCAAGCCTCCCAAGAAAGGGGTGAAAGGGTTTGGAGATGACATC

Mouse   : CCAGGCATGGAGGGGCTAGGAACAGATATCACGGTCATCTGTCCCTGGGA
Rat     : CCAGGCATGGAGGGGCTAGGAACAGATATCACGGTGATCTGCCCCTGGGA
hPASMC  : CCAGGCATGGAGGGGCTAGGAACAGATATCACGGTGATCTGCCCCTGGGA

Mouse   : AGCGTTCAGCCATCTGGAGCTGCATGAGCTTGCTCAGTTCGGGATCATCT
Rat     : AGCATTCAGCCACCTGGAACTGCACGAGCTGGCCCAGTTCGGGATCATCT
hPASMC  : AGCATTCAGCCACCTGGAACTGCACGAGCTGGCTCAGTTCGGGATCATCT

Mouse   : GA
Rat     : GA
hPASMC  : GA
```

Figure 5.3.7. Cone PDEγ (PDEγ2) ORF cDNA sequence

PDEγ2 open reading frame (ORF) cDNA sequence (252bp) from rat pulmonary arteries and cultured human pulmonary smooth muscle cells aligned with corresponding mouse PDEγ2 to which the primers were designed (using the primers as in 2.2.2.4). The conserved amino acids across all three sequences are shaded black.

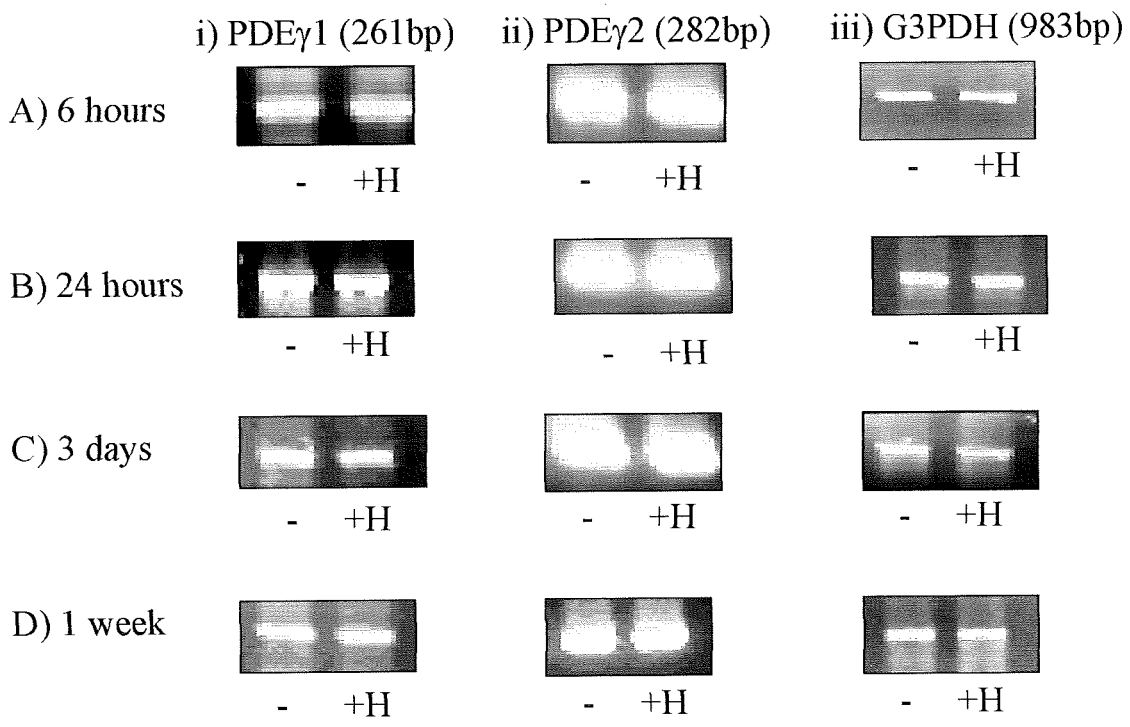


Figure 5.3.8 RT-PCR of PDEγ1, PDEγ2, and G3PDH transcripts in hPASMC exposed to hypoxia (10% O2) or normoxia for 6 hours, 24 hours, 3 days, and 1 week

RT-PCR amplification using specific primers as described in 2.2.2.4. of: i) PDEγ1, 261bp; ii) PDEγ2, 282bp; and iii) G3PDH, 983bp from hPASMC maintained under normoxic (-) and chronic hypoxic (+H) conditions for (A) 6 hours, (B) 24 hours, (C) 3 days, or (D) 1 week. 1μg total RNA/sample was used as a template for cDNA synthesis, of which one fifth was used for each RT-PCR. Above is a representative result of 3 individual experiments, quantified by densitometry.

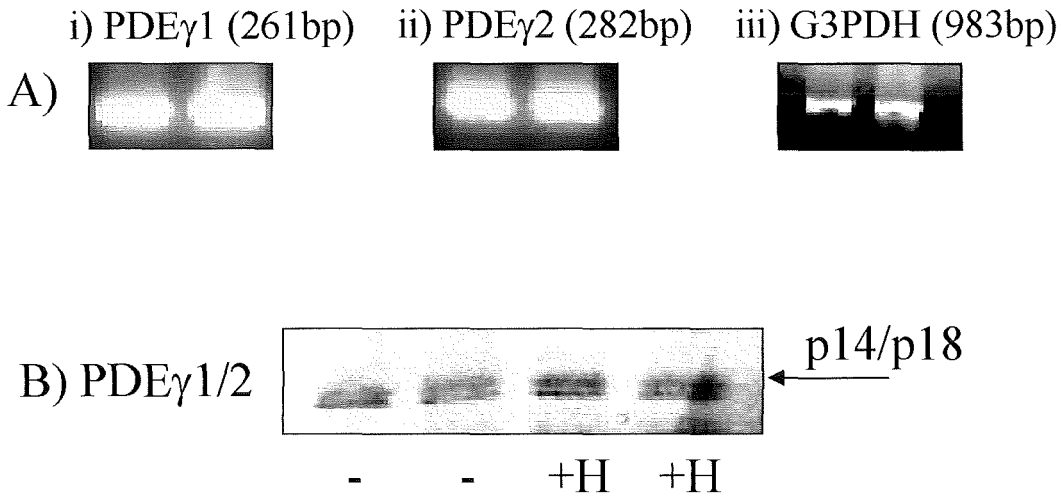


Figure 5.3.9 A) RT-PCR of PDEγ1, PDEγ2 and G3PDH transcript and B) Western blotting of PDEγ1/2 protein from hPASMC exposed to hypoxia (10% O2) or normoxia for 2 weeks

(A) RT-PCR amplification using specific primers, as described in 2.2.2.4, of i) PDEγ1, 261bp; ii) PDEγ2, 282bp; and iii) G3PDH, 983bp, from hPASMC maintained under normoxic (-) and chronic hypoxic (+H) conditions for 2 weeks. 1μg total RNA/sample was used to make cDNA, of which one fifth was used for each RT-PCR. (B) Western blot (2.2.3.5) with anti-PDEγ1/2 antibody showing the expression of PDEγ1/2 in homogenates from hPASMC maintained under normoxic (-) and chronic hypoxic (+H) conditions for 2 weeks. 10μg protein/sample were loaded onto SDS-PAGE. Above is a representative result of 3 individual experiments, quantified by densitometry.

5.3.8 Effect of hypoxia and EGF/PDGF stimulation on phospho- and total-p42/p44 MAPK protein levels hPASMC

To link PDEγ1/2 expression with increased proliferation through regulation of p42/p44 MAPK, Western blotting was carried out as in 2.2.3.3. Homogenates from normoxic and hypoxic hPASMC, and anti-phospho p42/p44 MAPK and total p44 MAPK antibodies were used. Normoxic and hypoxic hPASMC where also stimulated with either 50ng/ml epidermal growth factor (EGF), or 10ng/ml PDGF. Phosphorylated p42/p44 MAPK (M_r=42/44kDa) and total p44 MAPK (M_r=44kDa) protein were both detected in hPASMC.

Normoxic and hypoxic cells were also stimulated with 50ng/ml EGF for 5 min. The % changes in phosphorylated p42/p44 MAPK protein levels *versus* normoxic cells were: hypoxic 54 ± 3%; EGF/normoxic, 102 ± 7%; EGF/hypoxic, 115 ± 9% (n=3, $P<0.05$ *versus* normoxic hPASMC, Student's *t*-test). The corresponding % changes in total p44 MAPK protein levels *versus* normoxic cells were: hypoxic 7 ± 13%; EGF/normoxic, 3 ± 7%; EGF/hypoxic, 5 ± 12% (n=3, NS *versus* normoxic hPASMC, Student's *t*-test).

Normoxic and hypoxic cells were also stimulated with 10ng/ml PDGF for 5 min. The % changes in phosphorylated p42/p44 MAPK protein levels *versus* normoxic cells were: hypoxic 65 ± 12%; PDGF/normoxic, 99 ± 7%; PDGF/hypoxic, 107 ± 11% (n=3, $P<0.05$ *versus* normoxic hPASMC, Student's *t*-test). The corresponding % changes in total p44 MAPK protein levels *versus* normoxic cells were: hypoxic 10 ± 23%; PDGF/normoxic, 12 ± 15%; PDGF/hypoxic, 15 ± 29% (n=3, NS *versus* normoxic hPASMC, Student's *t*-test).

Using monoclonal antibodies raised against total p42/p44 MAPK no significant change in the level of p44 MAPK was detected with hypoxia or on stimulation with the growth factors. This indicates that increased 'basal' p42/p44 MAPK phosphorylation in response to hypoxia and growth factors is not due to an increase in total p44 MAPK. These results show the hypoxic and growth factor dependent stimulation of p42/p44 MAPK in hPASMC. Hypoxia in combination with either EGF, or PDGF did not however appear to act in a synergistic or additive manner to increase p42/p44 MAPK activation.

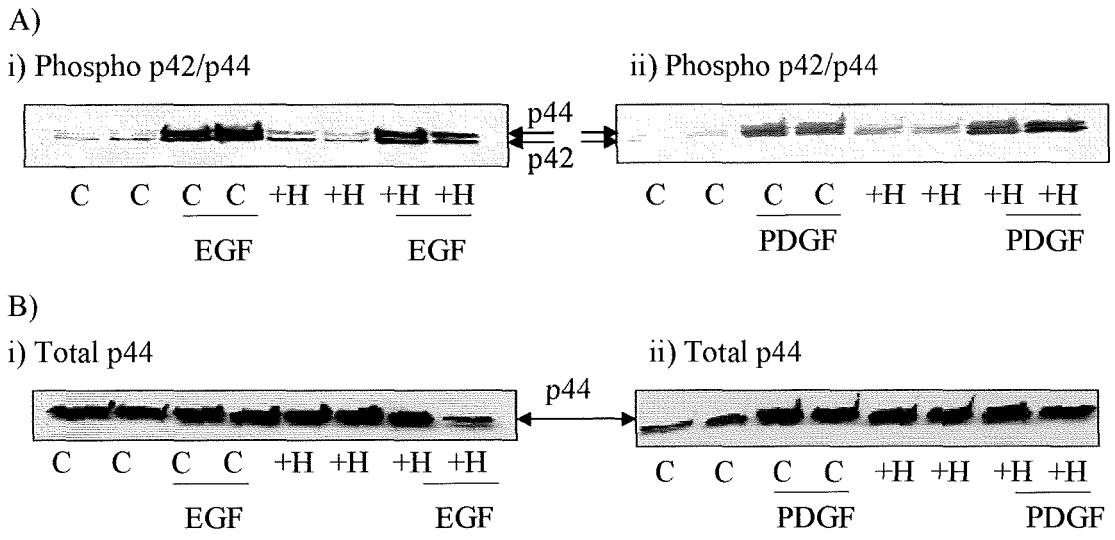


Figure 5.3.10. Western blot analysis of phospho p42/p44 MAPK and total p44 MAPK from control and hPASMC treated with EGF and PDGF

Western blot analysis using A) anti-phospho p42/p44 MAPK and B) total p44 MAPK antibodies, as described in 2.2.3.3-2.2.3.5, showing the expression level of A) phospho p42/p44, and B) total p44 in homogenates from hPASMC maintained under normoxic (-) and chronic hypoxic (+H) conditions treated with 50ng/ml epidermal growth factor (EGF) and 10ng/ml platelet derived growth factor (PDGF) for 5 minutes. 10μg protein/sample were loaded onto SDS-PAGE. Above is a representative result of 3 individual experiments, quantified by densitometry.

5.4 Discussion

The first observation from these experiments is the presence of both PDEγ1 and PDEγ2 mRNA and protein in the main PA, first branch PA, intrapulmonary and resistance vessels of the CH, and in hPASMC (see section 5.3). The presence of PDEγ emphasises a wider role for PDEγ in mammalian cell biology, other than in the phototrasduction cascade in the eye.

The second major finding of this study was that chronic hypoxia induced an increase in the protein expression of PDEγ1/2 above basal in all PAs from the CH studied and in cultured hPASMC (figures 5.3.4 and 5.3.10). The increase in PDEγ1/2 protein with hypoxia did not correlate with increased PDEγ1 and PDEγ2 transcript levels (figures 5.3.3 and 5.3.10). As there was no significant effect on PDEγ transcript levels, it would appear that hypoxia may activate a translation pathway that increases protein synthesis. These results suggest the possibility of post transcriptional or post translational modification to PDEγ1/2 in response to hypoxia.

5.4.1 Possible role of PDEγ in the hypoxic dependent increase in PDE5A

During visual excitation of photoreceptors the removal of the inhibitory action of the PDEγ subunit, triggers PDE6 activation. It was therefore proposed that PDEγ may interact with PDE5 in the lung in a similar inhibitory manner. It was postulated that hypoxia might reduce PDEγ, leading to the observed increase in PDE5 activity and expression in PA from CH (MacLean *et al.*, 1997, see chapter 3.3). Recombinant PDEγ has been shown to modulate PDE5 activity indirectly by preventing its activation by PKA in a concentration-dependent manner (Tate *et al.*, 1998, and Lochhead *et al.*, 1997). Therefore, a decrease in PDEγ in response to hypoxia would increase PKA mediated activation of PDE5, giving rise to the hypoxic-dependent phosphorylation of the enzyme. However, this is not the case, as PDEγ was actually shown to increase in the PA from the CH.

In fact, results from this study suggest that in rat PAs and in hPASMC, changes in PDE5 expression during hypoxia cannot be explained by reduced PDEγ expression. There does not appear to be any consistent correlation between PDE5 expression and

PDEγ expression in PAs from CH or in hypoxia treated hPASMC (sections 3.3 and 5.3). These results do not disprove that PDEγ may interact with PDE5 in these systems. It may be that PDEγ has more of a regulatory than inhibitory role over PDE5. For example, PDEγ could stabilise any increase in PDE5 activity/expression and that the acute PKA-dependent regulation of PDE5 activity is not as significant as the changes in protein expression under hypoxic conditions. This is plausible, as Tsang *et al.* (1996), showed that mice carrying a disruption of the PDEγ gene, ($Pdeg^{tm1}/Pdeg^{tm1}$) rather than increasing PDE6 activity prevented the functioning of the enzyme and elevated cGMP levels. These authors suggested that an interaction between the inhibitory PDEγ subunit and the catalytic subunits of PDE may be critical for the proper action of the enzyme, as well as the correct folding or confirmation of the catalytic sites in photoreceptors.

5.4.2 Possible role of PDEγ1/2 in the remodelling of the pulmonary artery with chronic hypoxia

The increased PDEγ1/2 expression as a result of hypoxia could be related to increased mitogenic signalling. This is proposed as Wan *et al.* (2001), have shown that PDEγ1 regulates both tyrosine kinase and G-protein coupled receptor-dependent stimulation of p42/p44 MAPK. Hence increased PDEγ as a result of hypoxia may potentially improve the efficiency of mitogenic signalling from these receptors, which may subsequently account for the increased proliferation of smooth muscle observed with PHT. The fact that the increase in PDEγ is more profound in PAs (i.e. resistance vessels) that classically show most remodelling in response to hypoxia, provides further evidence for its role in increased proliferation (figure 5.3.4).

A role for the proposed model by Wan *et al.* (2001), appears to be supported by results obtained in both the CH and in hypoxia treated hPASMC. Exposure of both rats and hPASMC to chronic hypoxia resulted in increased PDEγ1/2 expression, which may be responsible for the observed increase in p42/p44 MAPK activation in these vessels. When comparing the vessels within the pulmonary arterial tree of the CH, the greatest increase in both p42/p44 MAPK and PDEγ is observed in the resistance vessels that classically show the most profound remodelling in response to hypoxia. This is a significant finding as p42/p44 MAPK activation plays a key role in regulating cell proliferation, and is therefore likely to play a role in pulmonary vessel remodelling in PHT.

Below is a more detailed explanation of the proposed novel role of PDEγ in regulating p42/p44 MAPK-dependent signalling in HEK293 cells. This is followed by evidence, which may link the model proposed in HEK293 cells, with what may be occurring in the CH, and hypoxia treated hPASMC studied in this chapter.

5.4.2.1 Novel role of PDEγ in p42/p44 MAPK signalling

Wan *et al.*, 2001 provided the first evidence that PDEγ may be a novel intermediate regulating p42/p44 signalling from both receptor tyrosine kinase (RTK) and G-protein coupled receptors (GPCR) in HEK293 cells. These authors initially demonstrated that both the EGF- and $G_{\alpha}i/o$ coupled receptor agonist-dependent activation of p42/p44 MAPK were reduced by the transfection of antisense PDEγ, and conversely increased by the overexpression of recombinant PDEγ. Furthermore, these pathways were shown to require G-protein input through GRK2 (which is activated by Gβγ subunits). PDEγ was shown to be a substrate for GRK2, and their interaction required for its stimulatory effect of p42/p44 MAPK activation.

In addition, PDEγ appeared to interact with dynamin II to regulate p42/p44 MAPK signalling in HEK293 cells (Wan *et al.*, 2001). The interaction with dynamin II suggests PDEγ may have a role in stimulating GTP hydrolysis by dynamin II, promoting endocytosis of receptor signalling complexes resulting in the relocalisation with and activation of p42/p44 MAPK.

5.4.2.2. Evidence for p42/p44 MAPK activation in response to hypoxia

In this study chronic hypoxia (14 days) induced the activation of p42/p44 MAPK in hPASMC. Others have shown that chronic hypoxia induced temporal activation of p42/p44 MAPK. Jin *et al.* (2000), previously investigated the roles of p42/p44 MAPK, JNK, and p38 in hypoxia induced remodelling in PA. This study showed an increase p42/p44 MAPK, JNK and p38 MAPK tyrosine phosphorylation and activities with hypoxia in the main and first branch PA from male Sprague-Dawley rats. JNK activation peaked at day 1, and p42/p44 MAPK and p38 MAPK peaked after 7 days of hypoxia. In addition, both p38 MAPK and p42/p44 MAPK were shown to be activated in fibroblasts derived from the pulmonary arteries, but not from the aorta of CH (Welsh *et al.*, 2001). It was suggested that the fibroblasts from the PA of CH appeared to have

undergone a phenotypic switch, which causes them to exhibit enhanced proliferative responses compared to fibroblasts derived from the PA of normoxic rats.

Activation of MAPK family results in the phosphorylation of several transcription factors such as; early growth resonse-1 (Egr-1), Elk-1, activated transcription factor (ATF)-2 and members of the activator protein-1 (AP-1) family such as *c-jun* and *c-fos*. The activation of downstream effectors of MAPK pathways have been shown to occur in response to hypoxia, and appear to be important for the resultant increased cell growth and proliferation. In fact, *c-fos* gene expression was "turned on" by hypoxia via the p42/p44 MAPK pathway (Muller *et al.*, 1997b; Premkumar *et al.*, 2000). Premkumar *et al.*, 2000, showed that hypoxia activated p42/p44 MAPK and that this was essential fro stimulation of *c-fos* via the *cis* serum response element, a critical immediate early gene involved in regulating mitogenesis. In bovine aortic endothelial cells Lo *et al.*, 2001, outlined a Ca^{2+}-dependent activation of p42/p44 MAPK in response to hypoxia, whereby phospholipase C activated PKCα in association with Raf-1 triggered events leading to the transcription of *Egr-1*. The p42/p44 MAPK pathway is known to phosphorylate hypoxia inducible factor-1α (HIF-1α) and enhance transcriptional activity of HIF-1 (Richard *et al.*, 1999). In endothelial cells it has been documented that hypoxia induces phosphorylation, nuclear translocation and activation p42/p44 MAPK (Minet *et al.*, 2000). Furthermore, HIF-1 was activated in these cells in response to hypoxia. This increase was shown to be dependent on the phosphorylation of the HIF-1α carboxyl-terminal domain by p42 MAPK. These authors concluded that the *temporal* activation of the p42/p44 MAPK pathway appears to be associated with hypoxia-induced pulmonary arterial remodelling.

In addition to the p42/p44 MAPK pathway mediating hypoxic responses, it is also well documented that JNK and p38 MAPK pathways are activated in a low oxygen environment (Bogoyevitch *et al.*, 1996; Seko *et al.*, 1996, Scott *et al.*, 1998, Jin *et al.*, 2000, Das *et al.*, 2001). Of particular interest, it was demonstrated that hypoxic stimulation of PA cells is mediated by activation of the stress-activated protein kinases with particular strong muti-phasic activation of the p38 MAPK pathway (Scott *et al.*, 1998). Due to such studies JNK and p38 MAPK signalling should be looked at in greater detail in the models of hypoxia used in this study, and consequently assess if there may also be a role for PDEγ in these pathways. Variable patterns of activation of ERK, JNK, and p38 MAPK in response to hypoxia have been documented depending

on cell type studied, and the conditions under which the experiments were done. Studies such as those by Jin *et al.* (2000), Das *et al.* (2001), and Scott *et al.* (1998), all show the transient activation of p42/p44 MAPK, JNK, and p38 MAPK in response to hypoxia.

PDEγ1/2 might increase the activation of components involved in p42/p44 MAPK signalling (increase rate of endocytic signalling), or/and reduce MAPK phosphatases to prolonging the *temporal* activation of p42/p44 MAPK (long term adaptation). In fact, MAPK phosphatases (MKPs), which dephosphorylate threonine and tyrosine residues of MAPKs, have previously been shown to be induced in response to hypoxic stimuli. Northern and Western blot analyses verified that MKP-1 mRNA and protein levels were dramatically up regulated by hypoxia in PC12 cells (Seta *et al.*, 2001). Furthermore, Laderoute *et al.* (1999), demonstrated that the transient increase in MAPK activity induced by hypoxia in SiHa cells correlated with both the transcriptional activation of the gene for the MKP family member MKP-1, and the enhanced expression of MKP-1 protein. Together these results suggest that the induction of MKP may be responsible for the *temporal* activation MAPKs in response to hypoxia. Interaction of PDEγ1/2 with MKP may prevent or attenuate its activation, hence indirectly prolonging p42/p44 MAPK. Prolongation of p42/p44 MAPK activation might have a significant effect on gene induction and pulmonary artery remodelling.

It is important to note however the effect of MAPK on MKP is chronic. Therefore, increased p42/p44 MAPK activation by PDEγ will eventually increase MKP. Hence it is essential to make clear that the effects described above could be accounted for by acute inhibition of MKP by PDEγ e.g. direct protein-protein interaction.

5.4.2.3. Evidence for G-protein activation in response to hypoxia

The increased phosphorylation of p42/p44 MAPK in response to hypoxia may be due to increased GPCR or RTK activation. There is evidence for a role of upstream signals of the proposed model in the cellular response to hypoxia.

Primarily it is known that G-proteins can be activated by environmental stimuli known to have a role in cell proliferation. Activation of G proteins have been speculated to be critical in the early responses to hypoxia, and the subsequent modulation of ion channel

activity and cell depolarisation in various cell types (Mironov and Richter, 2000, Kobayashi *et al.*, 1998, Wenzlaff *et al.*, 1998). In particular, $G_{\alpha}i$ has been shown to mediate mitogenic responses as a result of shear stress, mechanical stretch, and reactive oxygen species (Nishida *et al.*, 2000). The mechanism by which $G_{\alpha}i$-coupled receptors activate p42/p44 MAPK has been shown to involve $\beta\gamma$ subunit as well as α-subunit associated coupling (Lopez-Ilasaca *et al.*, 1998). It has been demonstrated that $G_{\alpha}i/o$ is utilized for growth, and subpopulations of smooth muscle cells specifically with $G_{\alpha}i/o$ proliferated in response to hypoxia (Frid *et al.*, 1998). Das *et al.* (2001), demonstrated that pertussis toxin-sensitive G-proteins are essential upstream signalling components of proliferation and activation of MAPK in response to hypoxia in fibroblasts from PA. These authors demonstrated that hypoxia-induced and serum stimulated activation of p42/p44 MAPK, JNK, and the increase in DNA synthesis were, markedly attenuated by pertussis toxin.

Finally, a variety of G-protein receptor agonists are well documented to be elevated with PHT. For example in response to hypoxia there is evidence of $G_{\alpha}i$ protein activation through the NPY1 receptor and 5-$HT_{1D/1B}$ receptor (MacLean *et al.*, 1996). These results together support an upstream role of $G_{\alpha}i$-proteins, in hypoxia induced proliferation through p42/p44 MAPK.

5.4.2.4. Evidence for growth factor and RTK activation in response to hypoxia

Growth factors are also known to play a role in cell replication and division, and several studies suggest they may have a role in the remodelling of PA seen with PHT. PDGF and bFGF have been shown to cause proliferation of rat pulmonary arteries (Rothman *et al.*, 1994; Wang *et al.*, 2000). In fact, the results presented in this study demonstrated that stimulation of hPASMC with growth factors resulted in increase p42/p44 MAPK activation (5.3.10).

In PHT elevations of numerous growth factors and/or their mRNA have been documented, including PDGF-A and PDGF-B (Arcot *et al.*, 1993; Katayose *et al.*, 1993), VEGF (Turder *et al.*, 1995, Christou *et al.*, 1998), TGF-β (Acrot *et al.*, 1993), bFGF (Arcot *et al.*, 1995), IGF-1 (Perkett *et al.*, 1992), and EGF (Gillespie *et al.*, 1989a). In addition, hypoxia caused the upregulation of vascular endothelial growth

factor, and platelet-derived growth factor mRNA with a time course of upregulation that correlated to the activation of p42/p44 MAPK and p38 MAPK (Jin *et al.*, 2000). Finally Xiao *et al.* (1993), also suggested that hypoxia may initiate the secretion of growth factors from endothelial cells, which may be responsible for the proliferation of smooth muscle in PA. This was concluded as hypoxic endothelial cells conditioned medium stimulated proliferation of PASMC, promoting them from G0/D1 phase to S phase and increasing ^{3}H-thymidine incorporation.

It can be seen from figure 5.3.10 that exposing hPASMC to chronic hypoxia increased p42/p44 MAPK activation. This was correlated with a hypoxic-dependent increase in PDEγ1/2 expression. Furthermore, it was investigated whether an increase in p42/p44 MAPK in response to EGF and PDGF may be potentiated in hPASMC previously exposed to chronic hypoxia. However, as seen from figure 5.3.10 hypoxia and growth factors did not appear to have an additive or synergistic activation of p42/p44 MAPK. It may be that the concentrations of both EGF and PDGF used in the study were too high. The maximal p42/p44 MAPK activation may have already been achieved in response to these growth factors alone, therefore any additive effect that hypoxia may have had would not have been seen. An extended concentration response for both EGF and PDGF should be preformed in the presence of hypoxia in hPASMC to test this theory. However, it may also be that the relatively small increase in PDEγ1/2 expression in hPASMC, may not be sufficient to significantly alter the maximal EGF/PDGF–dependent activation of p42/p44 MAPK. The increase in 'basal' p42/p44 MAPK with hypoxia can be correlated with findings in the rat. These results suggest that chronic hypoxia may induce the release of a factor (e.g. growth factor) that can act back on smooth muscle cell receptors to regulate p42/p44 MAPK via a pathway involving PDEγ1/2. This hypothesis may explain how hypoxic-dependent increased expression of PDEγ1/2 may increase an apparent 'basal' p42/p44 MAPK activation.

In conclusion, figure 5.4.1 shows an outline of the proposed model for the role of PDEγ and subsequent p42/p44 MAPK activation in response to hypoxia in rat PA and hPASMC.

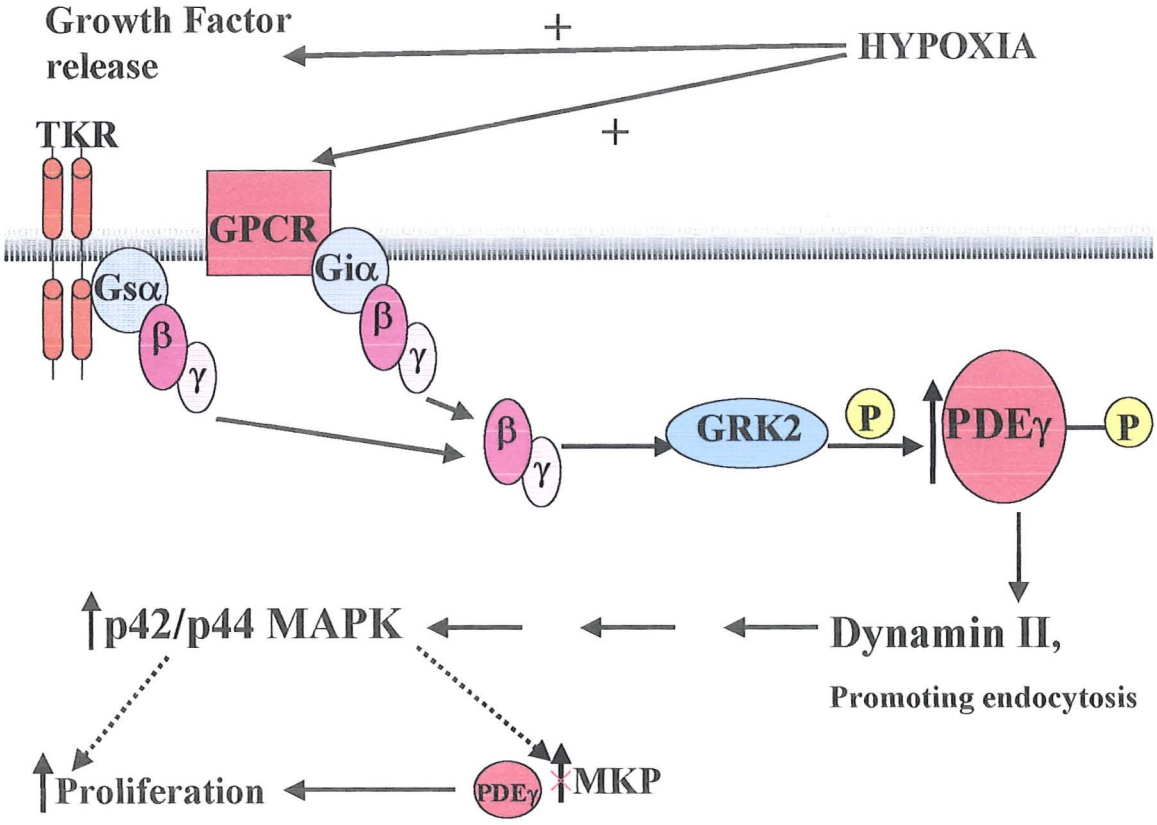


Figure 5.4.1 Proposed role of PDEγ in hypoxia-induced activation of p42/p44 MAPK in hPASMC and PAs from CH

It is proposed in response to hypoxia increased growth factors such as EGF or PDGF and/or GPCR agonists such as 5-HT are released, which act on tyrosine kinase receptors (RTK) or G-protein coupled receptors (GPCR) respectively on the smooth muscle cell surface. It is suggested that this causes the uncoupling of the βγ subunits of G-proteins, which activate G-protein receptor kinase-2 (GRK2). GRK2 may then interact with PDEγ, which stimulates the formation of a complex with dynamin-II. This interaction may be needed for the dynamin-II driven endocytosis of receptor signalling complexes leading to p42/p44 mitogen activated protein kinase (MAPK) activation. Increased p42/p44 MAPK may cause the activation of mitogen activated kinase phosphatases (MKP), which may be responsible for its *temporal* activation (long term adaptation). It is possible PDEγ1/2 may acutely interact with MKPs (possibly directly), prolonging the activation of p42/p44 MAPK. Increased p42/p44 MAPK activity may be responsible for increased cellular growth and proliferation that results in remodelling.

5.4.2.5 Conclusion

These results are merely the beginning of many further investigations required in hPASMC or in the CH, to establish a role for PDEγ in hypoxia induced remodelling. It will be necessary to further elucidate the pathways involved in increased proliferation and remodelling in both these models. It would be advantageous to study the effect of PDEγ on the responsiveness of pulmonary vessels. To address this question further studies will focus on using PDEγ1 knockout and heterozygote mice to assess whether this removal of PDEγ1 would prevent the hypoxic-dependent pulmonary arterial remodelling and reverse MAPK activation.

These experiments did however identify a hypoxic-dependent change in the phenotypic expression of an intermediate protein regulating mitogenic signalling in pulmonary arteries in both the rat and the human. Furthermore these studies may provide evidence for a role of PDEγ in regulation of cellular proliferation through p42/p44 MAPK under hypoxia. This may have a significant effect on the future investigations of arterial remodelling in PHT. The proposal that PDEγ is a novel intermediate involved in p42/p44 MAPK signalling in mammalian cells, opens a new dimension to signal transduction.

CHAPTER 6

GENERAL DISCUSSION

Chapter 6 General Discussion

6.1 General discussion

Both cAMP and cGMP are thought to be involved in maintaining low resistance and tone in the vascular bed of the pulmonary circulation (Murray, 1990; Della Frazia *et al.*, 1997; Koyama *et al.*, 2001). One of the factors in governing signal amplitude in each pathway is the hydrolysis of the cyclic nucleotides by phosphodiesterases (PDEs). Eleven families of PDEs presently exist, differing in tissue distribution, regulatory properties, amino acid sequences, and kinetic characteristics (Beavo *et al.*, 1995, Soderling *et al.*, 1998, Fisher *et al.*, 1998a, Fisher *et al.*, 1998b, Guipponi *et al.*, 1998, Soderling *et al.*, 1999, Fujishige *et al.*, Fawcett *et al.*, 2000, Yuasa *et al.*, 2000). Activity of specific PDE families, in particular PDE3 and PDE5, were reported to be elevated in pulmonary arteries (PA) from chronic hypoxic rats (CH, MacLean *et al.*, 1997). It was hypothesised that altered expression of PDE3 and PDE5 may contribute, in part, to the maintenance of abnormal tone and the remodelling associated with pulmonary hypertension (PHT). The overall aim of this investigation was to study the role of PDE3, and PDE5 and the PDE inhibitory γ-subunit (PDEγ), in response to hypoxia in the pulmonary circulation. Advances in the understanding of the mechanisms of hypoxia-induced signal transduction would prove to be important in finding novel or more effective treatments for PHT, which may even prevent the need for heart-lung transplants.

Chapter 3 of this investigation provides a possible molecular mechanism to explain the previously observed changes in PDE3, and PDE5 activity in the PA branches from CH (MacLean *et al.*, 1997). Using molecular approaches, combined with biochemical techniques, it was concluded that the increased PDE3 activity seen in the main, first and intrapulmonary PA with hypoxia appears to be due to the *de-novo* synthesis of both PDE3A and PDE3B from their respective genes. PDE3A transcript levels and PDE3 activity were also significantly increased in cultured human pulmonary artery smooth muscle cells (hPASMC) maintained under chronic hypoxia. These results were similar to those reported by Wagner *et al.*, 1997, who demonstrated an approximate 3-fold increase in PDE3 transcript in the CH.

Furthermore, a role for the cAMP pathway in regulating PDE3A expression in hPASMC was demonstrated. Roles for both cAMP and protein kinase A (PKA) in the response of the pulmonary circulation to low oxygen was concluded. The enhanced *de-novo* synthesis of PDE3A in response to hypoxia could be mimicked by exposing hPASMC to the membrane permeable analogue of cAMP, 8-Br-cAMP, and prevented by the PKA inhibitor, H8 peptide (chapter 3). It is possible the increased PDE3 expression is directly the consequence of PKA activation *via* increased intracellular cAMP, which may occur in response to the initial hypoxic insult. Equally, increased PKA may increase the *de-novo* synthesis of PDE3 indirectly through the activation of the cAMP response element binding protein (CREB). CREB is a transcription factor that potentially allows cAMP to alter gene expression (reviewed by Shaywitz and Greenberg, 1999; Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989; Montminy, 1997; Roesler, 1999). CREB is phosphorylated in response to hypoxia, providing evidence for this theory (Chida and Voelkel, 1996; Beitner-Johnson and Millhorn, 1998).

Intracellular levels of cAMP are reduced in the main, first branch and intrapulmonary arteries of the CH (MacLean *et al.*, 1996). Together, with the results presented in this study, it can be hypothesised that hypoxia may initially lead to a series of defence and rescue mechanisms to protect PASMC. This may include an initial increase in cAMP and PKA activation, which in turn may lead to the down-regulation of the cAMP pathway. A large increase in cAMP may saturate the PKA substrate phosphorylation sites and initiate cellular processes that inactivate or counter the cyclic nucleotide signalling pathways. Over-stimulation of PKA as a result of chronic hypoxia may lead to the activation of PDE3, and thereby reduce cAMP levels and limit the maximum effect of further stimulation of cAMP. It is important to make clear that the PKA-dependent phosphorylation of PDEs is an acute response. This desensitisation may explain the decrease responsiveness to vasodilators such as isoproterenol and forskolin in promoting smooth muscle relaxation in PA from the CH, and the decrease in cAMP observed in these vessels (Wanstall and O'Donnell, 1992; Jeffery and Wanstall, 1998; Wagner *et al.*, 1997; MacLean *et al.*, 1996). The negative feedback regulation of cAMP has been demonstrated in various other cell types, and is thought to have evolved to prevent excessive accumulation of cyclic nucleotides, and allow for the efficient termination of cyclic nucleotide pathways (Kobayashu *et al.*, 1998, Moon *et al.*, 2002, Corbin *et al.*, 1985, Degerman *et al.*, 1997; Gettys *et al.*, 1987).

Chapter 3 also showed that an increase in the *de-novo* synthesis of PDE5A2 in the conduit PA appears to be responsible for the increase in PDE5 activity previously observed by MacLean *et al.*, 1997. In contrast, the enhanced PDE5 activity seen by these authors in the distal PA of the CH could not be explained by changes in the levels of PDE5A1 or PDE5A2. Hanson *et al.*, 1998, found that increased PDE5 activity in response to PHT correlated with increased phosphorylation of the enzyme. Therefore, it may be that increased phosphorylation may explain the changes in PDE5 activity seen in these vessels of the CH. These results highlight the important point that each branch of the PA tree can respond differently to stimuli, therefore it is essential to know which PA branch is being studied. In the large elastic PA of the CH there is an increase in endogenous tone and a decrease in acetylcholine-induced and sodium nitroprusside-induced vasodilation (MacLean *et al.*, 1995; MacLean *et al.*, 1996; MacLean *et al.*, 1998; Wanstall and O'Donnell, 1992; Oka *et al.*, 2001). However, acetylcholine and sodium nitroprusside-induced relaxation is unchanged in the resistance vessels after hypoxic exposure (Oka, *et al.*, 2001). These authors proposed that the reduced responsiveness of the larger vessels to vasodilators may be due to an increase in PDE5 expression, which can be concluded from this study. The regional differences in the response of the pulmonary circulation to hypoxia is likely to be due to the heterogeneous population of smooth muscle cells present in each PA branch, which means they can express different cytoskeletal and contractile proteins, and potassium channels, and differ in their proliferative and matrix producing abilities (Frid *et al.*, 1997).

In a common mechanism with PDE3 activation, the increased PDE5 expression in the main and first branch PA in response to hypoxia may be due to increased cGMP and the subsequent activation of protein kinase G (PKG). Therefore, a potential mechanism for adaptation to hypoxia at the cellular level could involve the functional regulation of both cyclic nucleotides. Increases in cGMP levels have been shown to activate PDE5, increasing expression, by activating PKG, and by binding to the allosteric sites of PDE5 increasing phosphorylation (Corbin *et al.* 2000; Venkatesh *et al.*, 2001). Elevation of cGMP would cause increase sequestration of cGMP by PDE5, resulting in dampening of the cGMP signal and rendering it unavailable to target protein. The increase in PDE5 expression in response to hypoxia may therefore explain the reduced cGMP levels observed in the PA of CH, and the reduced sensitivity to agents such as nitric oxide in promoting relaxation of pre-contracted pulmonary vessels from the CH and from

patients with PHT (MacLean *et al.*, 1996; MacLean *et al.*, 1997; Shaul *et al.*, 1993). Negative feedback pathways such as these, would suggest that PASMC may not tolerate excessive activation of cyclic nucleotides and protein kinases.

Biochemical 'cross-talk' between the second messengers cAMP and cGMP may also be important during the response of the pulmonary circulation to hypoxia. For example, PDE5 has been shown to be activated not only by PKG, but also by PKA (Burns *et al.*, 1992, Corbin *et al.*, 2000, Murthy *et al.*, 2001; Kotera *et al.*, 1999). Additionally, cAMP response elements have been observed in the 5'-untranslated region and intron of the PDE5A gene (Kotera *et al.*, 1999). These authors demonstrated an increase in cAMP resulted in an increase in PDE5A2 transcript in rat vascular smooth muscle cells (VSMC). Therefore, the activation of PKA, as a result of an acute hypoxic-dependent increase in cAMP, may be responsible either directly or indirectly through CREB, for the increased expression of both PDE3 and PDE5 in the PA of the CH. Furthermore, the increase in PDE5 activity, as a result of hypoxia, would reduce cGMP levels, thereby subsequently dampening its inhibitory action on PDE3, leading to a further increase in PDE3 activity. In fact, the antimitogenic effect of PDE5 inhibitors has been shown to be mediated via PKA (Osinski *et al.*, 2001). These authors suggested than the accumulation of cGMP due to inhibition of PDE5, inhibited PDE3, increasing intracellular levels of cAMP and causing stimulation of PKA. This suggests a very close interaction between cAMP and cGMP mediated effects. During the development of PHT the levels of both cyclic nucleotides may therefore be equally important.

Chapter 4 demonstrated that both the PDE3 inhibitor SKF94836 and the PDE5 inhibitor sildenafil play a functional role in regulating relaxation of PA from control rats, and rats previously exposed to hypoxia. Systemic influences make it difficult to study the direct effects of vasoactive agents on pulmonary vascular smooth muscle tone in the intact animal. Therefore, isolated PA rings were used, so that the vascular smooth muscle vasomotor function could be studied with few compound variables. Both SKF94836 and sildenafil were effective in relaxing precontracted main and first branch PA. The relaxant effect of each PDE inhibitor was dependent on the artery studied and the preconstrictor used to raise tone. Importantly, sildenafil remained potent in the PA from CH, and responses in both the control main and first branch PA were not attenuated by removal of the endothelium. The endothelium-independent action of sildenafil is of advantage in the treatment of PHT, as its potency would not be reduced in patients with

PHT where the endothelium is thought to be damaged (Dinh-Xuan *et al.*, 1991: 1993). Although SKF94836 was still potent in the PA from CH, its actions were endothelium-independent in the main PA, however endothelium-dependent in the first branch PA. These results do not rule out the possibility of SKF94836 as a therapy for PHT, but may merely suggest a fully functional endothelium is required for its optimal effect.

The potencies of each inhibitor were not compromised in well-established PHT (2 weeks hypoxia), despite structural changes such as vascular hypertrophy in the vessels. These results would suggest both SKF94836 and sildenafil may be beneficial in the treatment of PHT. Interestingly, other PDE5 inhibitors such as E-4010 and E-4021 and PDE3 inhibitors such as milrinone, have been shown to cause selective pulmonary vasodilation, and attenuate the increase in pulmonary arterial pressure (PAP), right ventricular hypertrophy, and pulmonary arterial remodelling seen in rat models of PHT (Takahashi *et al.*, 1996, Hanasato *et al.*, 1999; Kato *et al.*, 1998; Baim *et al.*, 1983, Jaski *et al.*, 1985). Sildenafil (100mg) has previously been shown to inhibit the rise in pulmonary arterial pressure in both mice and humans chronically exposed to hypoxia, and in patients with PHT secondary to lung fibrosis (Zhao *et al.*, 2001; Ghofrani *et al.*, 2002). Together results from chapter 3 and chapter 4 offer an explanation to why both PDE3 and PDE5 inhibitors effectively reduce pulmonary vasoconstriction in models of PHT. PDE3 and PDE5 inhibitors act on targets that appear to play significant roles in altering vasoactive responsiveness of the pulmonary circulation. It may be that PDE inhibitors block the vasoconstriction and remodelling associated with PHT by increasing the circulating levels of cyclic nucleotide.

A number of other signalling pathways are thought to be involved in the response of the pulmonary circulation to hypoxia, and may act upon or along side cAMP and cGMP dependent pathways (reviewed by Jeffery and Wanstall, 2001; Archer and Rich 2000). Also, to accommodate changes in their environment cells are known to adjust the pattern of gene expression by regulating of a number of transcription factors (Makarvo, 2000, Baldwin 1996). Results from chapter 3 suggest that NF-κB, the ubiquitous, dimeric transcription factor, may control transcription of PDE5 (NF-κB reviewed by Makarov, 2000, Balwin, 1996; Ghosh *et al.*, 1998). This study showed that the NF-κB inhibitor TLCK reduced the basal expression of PDE5A in hPASMC. The PDE5 gene may have NF-κB binding sites in its promoter region controlling its regulation, or NF-κB may increase PDE5 expression through its known activation of inducible nitric

oxide (iNO, Xie *et al.*, 1993). Activation of iNOS by NF-κB would lead to increased NO production, thereby increasing the levels of intracellular cGMP, which in turn would increase PKG and lead to the subsequent activation PDE5, as proposed above to occur in response to hypoxia. Increased NF-κB activation has been seen in monocrotaline-induced model of PHT, which may provide further evidence for its role in the increase in PDE5 activity and transcript seen in the conduit PA from the CH (Aziz *et al.*, 1997; MacLean *et al.*, 1997). In addition, the catalytic subunit of PKA (PKAc) has been shown to bind IκB proteins, and is associated with the NF-κB/ IκB complex. Active PKAc phosphorylates NF-κB at its PKA consensus site in the Rel domain, and leads to a dramatic increase in transcriptional activity (Blank *et al*, 1992, Verma *et al.*, 1995; Zhong *et al.*, 1997). The proposed increase in PKA in response of the pulmonary circulation to hypoxia may lead to the activation of NF-κB, and thereby be indirectly responsible for the increase in PDE5 transcript observed. Interestingly, PDEs are also thought to have a role in regulating the IκB/NF-κB signalling pathway (Coward *et al.*, 1998; Haddad *et al.*, 2002). PDE5 inhibitors can reduce LPS-mediated NF-κB translocation/activation in epithelial cells, suggesting they may be a novel way to target transcriptional activity implicated in the progression of a number of disorders, such as PHT (Hadded *et al.*, 2002). These findings suggest that NF-κB inhibitors could be a potential new strategy to reduce PDE5 activity indirectly, and could even improve the vasodilator action of PDE5 inhibitors in patients with PHT when taken in combination.

Further signalling pathways activated by cellular stress and hypoxia includes the mitogen activated protein kinase (MAPK) cascades (reviewed by Marshall, 1995 Van Biesen *et al.*, 1995; Serger and Krebs, 1995; Wildmann *et al.*, 1999). The MAPK system provides a route whereby growth factors/hormones can alter transcription and other cellular processes. The role of the MAPK pathway in the hypoxic response of the pulmonary circulation was also studied in chapter 5, in particular concerning its possible regulation by the PDE inhibitory γ-subunit (PDEγ). Wan *et al.*, 2001, have previously shown that PDEγ1 is limiting for both tyrosine kinase and G-protein coupled receptor-dependent stimulation of p42/p44 MAPK. Hence, it was proposed that hypoxia may increase PDEγ and potentially improve the efficiency of p42/p44 MAPK, thereby leading to the remodelling of the PA. Initially, the presence of both PDEγ1 and PDEγ2 mRNA and protein in the main PA, first branch PA, intrapulmonary and resistance

vessels of the CH, and in hPASMC were detected for the first time. Chronic hypoxia induced an increase in the protein levels of PDEγ1/2 in all PA from the CH studied, and in cultured hPASMC. The increase in PDEγ was more profound in the PA (i.e. resistance vessels) that classically show most remodelling in response to hypoxia, which provides evidence for the suggested role of a MAPK/PDEγ interaction in increased proliferation.

The proposed model by Wan *et al.*, 2001, appears to be supported by results obtained and discussed in chapter 5. In both the rat and in hPASMC the increased PDEγ1/2 expression as a result of chronic hypoxia was correlated with an increase in p42/p44 MAPK activation. Furthermore, my results suggested that chronic hypoxia might induce the release of a factor (e.g. growth factor) that can act back on smooth muscle cell receptors to regulate p42/p44 MAPK via a pathway involving PDEγ1/2. This hypothesis may explain how hypoxic-dependent increased expression of PDEγ1/2 may increase 'basal' p42/p44 MAPK activation in the hPASMC. Furthermore, it was suggested that in addition to increasing the activation of components involved in p42/p44 MAPK signalling (increase rate of endocytic signalling), PDEγ1/2 might even interact (direct protein-protein interaction) with and acutely inhibit MAPK phosphatases (MKPs) to prolonging the *temporal* activation of p42/p44 MAPK. This was suggested due to the variable patterns of activation of MAPK pathways in response to hypoxia previously documented (Jin *et al.*, 2000, Das *et al.*, 2001, Scott *et al.*, 1998), and the possible role of MKPs in this transient activation (Alessi *et al.*, 1993; Sun *et al.*, 1993; Ward *et al.*, 1994; Seta *et al.*, 2001; Laderoute *et al.*, 1999). Although a great deal of further research is required surrounding PDEγ, chapter 5 may provide the first evidence for a role of PDEγ in regulating cellular proliferation through p42/p44 MAPK under hypoxia.

It is possible that oxygen deprivation may induce synergism and 'cross-talk' between signalling pathways. For example, the PKA and the MAPK pathways are known to interact at various levels (Wu *et al.*, 1993; Graves *et al.*, 1993). Classically, PKA is known to inhibit vascular smooth muscle cell proliferation by antagonising mitogenic signalling pathways induced by growth factors (reviewed Bornfeldt and Krebs, 1999; Graves *et al.*, 1993). Several studies have shown PKA acts downstream of Ras activation to inhibit the p42/p44 MAPK cascade. It has been suggested that inhibition of p42/p44 MAPK activation in response to cAMP occurs predominantly through the

PKA-dependent phosphorylation of Raf-1, mainly on Ser43, thereby inhibiting its kinase activity (Cook and McCormick, 1993; Wu *et al.*, 1993). It is important to clarify that the PKA-dependent phosphorylation of p42/p44 MAPK, as with PDE, is an acute response. PKA may also inhibit MAPK by inhibition of kinases that activate Raf, for example PKC, although this precise mechanism is still to be defined (Magnuson *et al.*, 1994). In certain conditions, and in certain cell types, cAMP can activate rather than inhibit MAPK pathways, for example through activation of Rap-1 (cAMP activates guanine-nucleotide exchanger for Rap-1), and subsequent activation of B-Raf in PC12 cells (Vossler 1997, York 1998). The overall effect of cAMP appears to be dependent on the relative amount of Rap-1, B-Raf and Raf-1 expressed in the cell at a given time, which may even be altered in response to stimuli. Activation of MAPK pathways, as a direct result of cAMP stimulation, has been shown to result in an increase in DNA synthesis in neonatal, but not adult pulmonary bovine smooth muscle cells (SMC, Guldemeester *et al.*, 1999).

Additionally, in SMCs that express cycloxygenase-2 (COX-2), activation of MAPK can control the activation of PKA. PDGF has been shown to stimulate cAMP synthesis in cultured guinea pig lung smooth muscle cells (Pyne *et al.*, 1997). These authors suggested PDGF stimulates MAPK activation leading to the phosphorylation and activation of phospholipase A_2 ($cPLA_2$), and the subsequent formation of arachidonic acid. In the presence of COX-2, arachidonic acid metabolites such as prostaglandin E_2 (PGE_2) are formed, and released, which stimulate adenylyl cyclase, increase cAMP, and activate PKA (Graves *et al.*, 1996; Pyne *et al.*, 1997). COX-2 mRNA and protein levels have been shown to increase in response to hypoxia in rat lung tissue (Chida and Voelkel, 1996). Together, these results suggest the increased MAPK and COX-2 shown to occur in response to hypoxia in PASMC may contribute to increased PKA activation, and consequently in part to the observed increase in PDE3 and PDE5 activity and transcript. Furthermore, the increased PDEγ associated with hypoxia (chapter 4) could, in part, also play a role in potentiating this pathway, by increasing the activation of components involved in p42/p44 MAPK signalling (increase rate of endocytic signalling) or/and reduce MAPK phosphatases to prolonging the *temporal* activation of p42/p44 MAPK (long term adaptation). This hypothesis may suggest remodelling of the PA occurs primarily in response to hypoxia, which can then lead to the increase tone, by altering the cAMP/cGMP pathways. The exact interaction between these

pathways in the pulmonary circulation in response to hypoxia is still unclear, however worth investigating further.

The MAPK pathway has also recently been shown to regulate specific isoforms of PDEs (Hoffmann *et al.*, 1999; MacKenzie *et al.*, 2000; Baillie *et al.*, 2000). p42 MAPK was shown to have the ability to either increase or decrease cAMP levels dependent upon the pattern of the cAMP-specific PDE (PDE4) isozyme expression. MAPK has been shown to increase cAMP levels in the cell, by phosphorylating PDE4D3 at a single site (Ser579), and inhibiting its activity (Hoffmann *et al.*, 1999). These authors demonstrated this interaction *in vitro* by treating the recombinant enzyme with p42 MAPK, and also in intact COS1 cells transfected to express PDE4D3 and stimulated with EGF. In contrast, the short form of PDE4D1 was shown to be activated when phosphorylated by p42 MAPK (MacKenzie *et al.*, 2000). Baillie *et al.*, 2000, also demonstrated that in common with PDE4D, both PDE4B and PDE4C were shown to be substrates for C-terminal phosphorylation by p42 MAPK at a single serine residue. The short form of PDE4B2 was activated by p42 MAPK phosphorylation, however the long forms of PDE4B and PDE4C isozymes were markedly inhibited (Baillie *et al.*, 2000). It may therefore be possible that MAPK could control the activation of a number of other PDE families in a similar manner. The increase in MAPK in response to hypoxia may be involved in the observed reduction in intracellular levels of cyclic nucleotides by directly increasing the activity of PDEs, for example PDE3.

It is apparent that PHT is a complex multi-factorial process involving many signalling pathways, which may act synergistically and/or interdependently. Undoubtedly, there is no single cause of PHT, and the sensor for hypoxic stimuli that triggers pulmonary vasoconstriction and an increase in tone and proliferation of the PA is still unknown. However, further elucidating the signalling pathways involved in PHT and generally understanding the regulation of processes involved, may lead to the development of a series of treatments that would be beneficial. Due to complexity of the remodelling and the increased tone associated with PHT, drugs with more than one action, or that act on a pathway common to a number of stimuli, would be most successful in its treatment. The results presented in this study establish a rationale for, and demonstrate the potential benefit of, inhibiting both PDE3 and PDE5 activity in the lung as a possible therapy for PHT. Due to their possible cross-talk with the MAPK pathway and transcription factors such as CREB and NF-κB, not only could altering PDE activity

effect the tone of the pulmonary circulation, but also the remodelling of the PA and the transcription of a number of genes. Furthermore, additional studies of the distribution of PDE isoforms in the lung and more precise characterisation will allow the development of more selective drugs targeted to the pulmonary circulation, minimising side effects. For example the detection of different isoforms of PDE5 by Lin *et al.*, 2000 raised the possibility of identifying isoform-specific inhibitors allowing an even more organ-specific enhancement of cGMP-mediated vasodilation. The data presented suggests PDE inhibitors to be used alone or in combination to treat PHT, which is still presently an incurable disease, would appear to be beneficial.

CHAPTER 7

REFERENCES

Chapter 7. References

7.1 References

Abate A, Oberle S, Schroder H. (1998). Lipopolysaccharide-induced expression of cyclooxygenase-2 in mouse macrophages is inhibited by chloromethylketones and a direct inhibitor of NF-κB translocation. *Prostaglandins Other Lipid Mediat.* 56: 277-90.

Abenhaim L, Moride Y, Brenot F, Rich S, Benichou J, Kurz X. (1996). Appetite-suppressant drugs and the risk of primary pulmonary hypertension. International primary pulmonary hypertension study group. *N Eng J Med.* 335: 609-616.

Adachi HS, Yoshitake S, and Saito I. (1994). Cardiohemodynamic profile of E4021, a selective and potent phosphodiesterase type V inhibitor, in anaesthetized pigs. *JPN. J. Pharmacol.* 64:329.

Adnot S, Raffestin B, Eddahibi S, Braquet P, Chabrier P. (1991) Loss of endothelium-dependent relaxant activity in the pulmonary circulation of rats exposed to chronic hypoxia. *J. Clin Invest.* 87: 155-162.

Ahn HS, Foster M, Cable M, Pitts BJR, Sybertz EJ. (1991). Ca/CaM-stimulated and cGMP-specific phosphodiesterase in vascular and non-vascular tissues. *Adv Exp Med Biol.* 308: 191-197.

Alderton F, Darroch P, Sambi B, McKie A, Ahmed IS, Pyne N, Pyne S. (2001a). G-protein-coupled receptor stimulation of the p42/p44 mitogen-activated protein kinase pathway is attenuated by lipid phosphate phosphatases 1, 1a, and 2 in human embryonic kidney 293 cells. *J Biol Chem.* 276(16):13452-60.

Alderton F, Rakhit S, Kong KC, Palmer T, Sambi B, Pyne S, Pyne NJ. (2001b). Tethering of the platelet-derived growth factor-β receptor to G-protein-coupled receptors. A novel platform for integrative signalling by these receptor classes in mammalian cells. *J Biol Chem.* 276(30):28578-85.

Alessi DR, Smythe C, Keyse SM. (1993). The human CL100 gene encodes a Tyt/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in Xenopus oocyte extracts. *Oncogene*. 8: 2015-2020.

Allen KM, Haworth SG. (1986). Impaired adaptation of pulmonary circulation to extrauterine life in newborn pigs exposed to hypoxia: an ultrastructural study. *J Pathol*. 150(3):205-12.

Anderson GM, Stevenson JM, Cohen DJ. (1987). Steady-state model for plasma free and platelet serotonin in man. *Life Sci*. 41: 1777-1785.

Angel P, and Karin M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta*. 1072: 129-157.

Antezana AM, Antezana G, Aparicio O, Noriega I, Velarde FL, Richalet JP. (1998). Pulmonary hypertension in high-altitude chronic hypoxia: response to nifedipine. *Eur Respir J*. 12(5):1181-5.

Aravind L, Pointing CP. (1997). The GAF domain: an evolutionary link between diverse phototransducing proteins. *Trends Biochem Sci*. 22: 458-459.

Archer SL, Huang JMC, Reeve HL, Hampl V, Tolarova S, Michelakis E, Weir EK. (1996) The differential distribution of electrophysiologically distinct myocytes in conduit and resistance arteries determines their response to nitric oxide and hypoxia. *Circ Res*. 78: 431-442, 1996.

Archer S, Rich S. (2000) Primary pulmonary hypertension: a vascular biology and translational research "Work in progress". *Circulation*. 102(22):2781-91.

Arcot SS, Lipke DW, Gillespie MN, Olson JW. (1993). Alterations of growth factor transcripts in rat lungs during development of monocrotaline-induced pulmonary hypertension. *Biochem Pharmacol*. 46: 1086-1091.

Arcot SS, Fagerland JA, Lipke DW, Gillespie MN, Olson JW. (1995). Basic fibroblast growth factor alterations during development of monocrotaline-induced pulmonary hypertension in rats. *Growth Factors*. 12: 121-130.

Arneklo-Nobin B, Elmer O, Akesson A. (1988). Effect of long-term ketanserin treatment on 5-HT levels, platelet aggregation and peripheral circulation in patients with Raynaud's phenomenon. A double-blind, placebo-controlled cross-over study. *Int Angiol.* 7(1): 19-25.

Aziz SM, Toborek M, Hennig B, Endean E, Lipke DW. (1997). Polyamine regulatory processes and oxidative stress in monocrotaline-treated pulmonary artery endothelial cells. *Cell Biol Int.* 21:801-812.

Badesch DB, Orton EC, Zapp LM, Westcott JY, Hester J, Voelkel NF, Stenmark KR. (1989). Decreased arterial wall prostaglandin production in neonatal calves with severe chronic pulmonary hypertension. *Am J Respir Cell Mol Biol.* 1(6):489-98.

Baeuerle PA, Henkel T. (1994). Function and activation of NF-κB in the immune system. *Annu Rev Immunol.* 12: 141-179.

Baillie GS, MacKenzie SJ, McPhee I, Houslay MD. (2000) Sub-family selective actions in the ability of ERK2 MAP kinase to phosphorylate and regulate the activity of PDE4 cyclic AMP-specific phosphodiesterase. *Brit. J. Pharmacol.* 131: 811-819.

Baim DS, McDowell AV, Cherniles J. (1983). Evaluation of a new bipyridine inotropic agent-milrinone-in patients with severe congestive heart failure. *N Engl J Med.* 309: 748-56, 1983.

Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, and Naylor AM. (1998). Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol.* 159: 2164-2171.

Baldwin AS. (1996). The NF-κB and I-κB proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14: 649-683.

Banner KH, Page CP. (1995). Theophylline and selective phosphodiesterase inhibitors as anti-inflammatory drugs in the treatment of bronchial asthma. *Eur Respir J.* 8(6):996-1000.

Barnard JW, Barman SA, Adkins WK, Longenecker GL, Taylor AE. (1991). Sustained effects of endothelin-1 on rabbit, dog, and rat pulmonary circulations. *Am J Physiol.* 261(2 Pt 2): H479-86.

Barnes, PJ, and Belvisi, MG. (1993). Nitric oxide and lung disease. *Thorax*. 48, 1034-1043.

Barnes PJ and Liu SF. Regulation of pulmonary vascular tone.(1995) *Pharmacol. Rev.* 47: 87-101.

Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, Groves BM, Tapson VF, Bourge RC, Brundage BH, Koerner SK, Langleben D, Keller CA, Murali S, Uretsky BF, Clayton LM, Jobsis MM, Blakburn SD, Shortino D, Crow JW. (1996). A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. *N Engl. J. Med.* 334: 396-301.

Beasley D, Schwartz JH, Brenner BM. (1991). Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J Clin Invest.* 87: 602-608.

Beavo JA. (1995) Cyclic nucleotide phosphodiesterase: functional implications of multiple isoforms. *Physiol Rev.* 75: 725-748.

Beitner-Johnson D, Millhorn DE. (1998). Hypoxia induces phosphorylation of the cyclic AMP response element-binding protein by a novel signalling mechanism. *J Biol Chem.* 273(31):19834-9.

Benotti JR, Grossman W, Braunwald E. (1978). Hemodynamic assessment of amrinone. *N Engl. J. Med.* 299: 1373-1377.

Bentley JK, and Beavo JA. (1992). Regulation and function of cyclic nucleotides. *Current Opinion Cell Biology.* 4: 233-240.

Bergofsky EH. Mechanisms underlying vasomotor regulation of regional pulmonary blood flow in normal and disease states. (1974). *Am. J. Med.* 57: 378-394.

Bergofsky EH, Holtzman S (1967). A study of the mechanisms involved in the pulmonary arterial response to hypoxia. *Circ. Res.* 20: 506-519.

Bevan RD. (1989). Influence of adrenergic innervation on vascular growth and mature characteristics. *Am Rev Respir Dis.* 140: 1478-1482.

Billington CK, Joseph SK, Swan C, Scott MGH, Jobson TM and Hall IP. (1999). Modulation of human airway smooth muscle proliferation by type 3 phosphodiesterase inhibition. *Am J. Physiol.* 276: L412-L419.

Bischoff E, Schneider K. (2001). A conscious-rabbit model to study vardenafil hydrochloride and other agents that influence penile erection. *Int J Impot Res.* 13(4): 230-5.

Bivalacqua TJ, Champion HC, Rajasekaran M, Sikka SC, Kadowitz PJ, Doherty PC, Hellstrom WJ. (1999). Potentiation of erectile response and cAMP accumulation by combination of prostaglandin E1 and rolipram, a selective inhibitor of the type 4 phosphodiesterase (PDE 4). *J Urol.* 162(5): 1848-55.

Black SM, Sanchez LS, Mata-Greenwood E, Bekker JM, Steinhorn RH, Fineman JR. (2001). SGC and PDE5 are elevated in lambs with increased pulmonary blood flow and pulmonary hypertension. *Am J. Physiol. Lung Cell Mol. Physiol.* 281: L1051-L1057.

Blank V, Kourilsky P, and Israel A. (1992). NF-κB and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. *Trends Biochem Sci.* 17: 135-140.

Boerth NJ, Dey NB, Cornwell TL, Lincoln TM. (1997). Cyclic GMP-dependent protein kinase regulates vascular smooth muscle cell phenotype. *J Vasc Res.* 34(4):245-59.

Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH. (1996). Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38 mitogen-activated protein

kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ Res.* 79(2):162-73.

Bolger GB, Erdogan S, Jones RE, Loughney K, Scotland G, Hoffmann R, Wilkinson I, Farrell C, Houslay MD. (1997). Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene. *Biochem J.* 328:539-48.

Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L, Pelicci PG. (1996). Not all Shc's roads lead to Ras. *Trends Biochem Sci.* 21(7):257-61.

Bonisch D, Weber AA, Wittpoth M, Osinski MT, and Schror K. (1998). Antimitogenic effects of trapidil in coronary artery smooth muscle cells by direct activation of protein kinase A. *Mol Pharmacol.* 54: 241-248.

Bonni A, Ginty DD, Dudek H, Greenberg ME. (1995). Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. *Mol Cell Neurosci.* 6(2):168-83.

Boolell M, Allen MJ, Ballard SA, Gepi-Attee S, Muirhead GJ, Naylor AM, Osterloh IH, Gingell C. (1996). Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res.* 8(2):47-52.

Bornfeldt KE, and Krebs EG. (1999). Crosstalk between protein kinase A and growth factor receptor signalling pathways in arterial smooth muscle. *Cell Signal.* 11: 465-477.

Bradford MM. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochemistry.* 72: 248-254.

Brantley DM, Chen C, Muraoka RS, Bushdid PB, Bradberry JL, Kittrell F, Medina D, Matrisian LM, Kerr LD, Yull FE. (2001). Nuclear Factor-κB (NF-κB) regulates proliferation and branching in mouse mammary epithelium. *Mol. Biol. Cell.* 12: 1445-1455.

Brink C, Cerrina C, Labat C, Verley J, Beneveniste J. (1988). The effect of contractile agonists on isolated pulmonary arterial and venous muscle preparations derived from patients with primary pulmonary hypertension. *Am Rev Respir Dis.* 137: A106.

Brown RL. (1992). Functional regions of the inhibitory subunit of retinal rod cGMP phosphodiesterase identified by site-specific mutagenesis and fluorescence spectroscopy. *Biochemistry.* 31(25):5918-25.

Brutsaert D. (1964). Influence of response and of adrenolytic agents on the pulmonary arterial pressor response to hypoxia and catecholamines. *Arch Int Physiol Biochem.* 72: 395-412.

Buchan KW, Magnusson H, Rabe KF, Sumner MJ, Watts IS. (1994). Characterisation of the endothelin receptor mediating contraction of human pulmonary artery using BQ123 and Ro 46-2005. *Eur J Pharmacol.* 260(2-3):221-6.

Buczko W, De Gaetano G, and Garattini S. (1975) Effect of fenfluramine on 5-hydroxytriptamine uptake and release by rat blood platelets. *Br J. Pharmacol.* 53: 563-568.

Budts W, Pokreisz P, Nong Z, Van Pelt N, Gillijns H, Gerard R, Lyons R, Collen D, Bloch KD and Janssens S. Aerosol gene transfer with inducible nitric oxide synthase reduces hypoxic pulmonary hypertension and pulmonary vascular remodelling in rats. *Circulation.* 102: 2880-2885, 2000.

Burns F, Rodger IW, Pyne NJ. (1992). The catalytic subunit of protein kinase A triggers activation of type V cyclic GMP-specific phosphodiesterase from guinea-pig lung. *Biochem J.* 283: 487-491.

Busse R, Mulsch A. (1990). Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS Lett.* 265: 133-136.

Camps M, Nichols A, Gillieron C, Antonsson B, Mudea M, Chabert C, Boschert U, Arkinstall S. (1998) Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. *Science.* 280: 1262-1265.

Carville C, Adnot S, Eddahibi S, Teiger E, Rideau D, and Raffestin B. (1997). Induction of nitric oxide synthase activity in pulmonary arteries from normoxic and chronically hypoxic rats. *Eur Respir J.* 10: 437-445.

Champion HC, Bivalacqua TJ, D'Souza FM, Ortiz LA, Jeter JR, Toyoda K, Heistad DD, Hyman AL, and Kadowitz PJ. (1999). Gene transfer of endothelial nitric oxide synthase to the lung of the mouse. In vivo: effect on agonist-induced and flow mediated vascular responses. *Circ Res.* 84: 1422-32.

Channick RN, Rubin LJ. (2000). Combination therapy for pulmonary hypertension: A glimpse into the future? *Crit.Care Med.* 28:896-897.

Channick RN, Simonneau G, Sitbon O, Robbins IM, Frost A, Tapson VF, Badesch DB, Roux S, Rainisio M, Bodin F, Rubin LJ. (2001) Effects of the dual endothelin-receptor antagonist bosentan in patients with pulmonary hypertension: a randomised placebo-controlled study. *Lancet.* 358(9288):1119-23.

Chen SJ, Chen YF, Meng QC, Durand J, DiCarlo VS, Oparil S (1995). Endothelin-receptor antagonist bosentan prevents and reverse hypoxic pulmonary hypertension in rats. *J. Appl. Physiol.* 79: 2122-2131.

Chen YR, Meyer CF, Tan TH. (1996). Persistent activation of c-jun N –terminal Kinase 1 (JNK1) in gamma radiation induced apoptosis. *J. Biol. Chem.* 271: 631-624

Chiarugi V, Magnelli L, Chiarugi A, Gallo O. (1999). Hypoxia induces pivotal tumour angiogenesis control factors including p53, vascular endothelial growth factor and the NFkappaB-dependent inducible nitric oxide synthase and cyclooxygenase-2. *J Cancer Res Clin Oncol.*125: 525-528.

Chiche JD, Schlutsmeyer SM, Bloch DB, Monte SM, Roberts JD, Filippov G, Janssens SP, Rosenzweig A, Bloch KD. (1998). Adenovirus-mediated gene transfer of cGMP-dependent protein kinase increases the sensitivity of cultured vascular smooth muscle cells to the antiprolferative and pro-apoptotic effects of nitric oxide/cGMP. *J. Biol. Chem.* 273: 34263-34271.

Childa M, Voelkel NF. (1996). Effects of acute and chronic hypoxia on rat lung cyclooxygenase. *Am J Physiol.* 270: L872-L878.

Chini CC, Grande JP, Chini EN, Dousa TP. (1997). Compartmentalization of cAMP signalling in mesangial cells by phosphodiesterase isozymes PDE3 and PDE4. Regulation of superoxidation and mitogenesis. *J Biol Chem.* 272(15):9854-9

Choi YH, Ekholm D, Krall J, Ahmad F, Degerman E, Manganiello VC, Movsesian MA. (2001). Identification of a novel isoform of the cyclic-nucleotide phosphodiesterase PDE3A expressed in vascular smooth-muscle myocytes. *Biochem J.* 353(Pt 1): 41-50.

Choukroun G, Hajjar R, Kyriakis JM, Bonventre JV, Rosenzweig A, Force T. (1998). Role of the stress-activated protein kinases in endothelin-induced cardiomyocyte hypertrophy. *J Clin Invest.* 102(7):1311-20.

Christman BW. (1998). Lipid mediator dysregulation in primary pulmonary hypertension. *Chest.* 114:205S-207S.

Christou H, Yoshida A, Arthur V, Morita T, Kourembanas S. (1998). Increased vascular endothelial growth factor production in the lungs of rats with hypoxia-induced pulmonary hypertension. *Am J Respir Cell Mol Biol.* 18: 768-776.

Chu Y, Solski PA, Khosravi-Far R, Der CJ, and Kelly K. (1996). The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. *J Biol. Chem.* 271: 6497-6501.

Clarke RH, Kueser TJ, Walker MW. (2000). Low-dose nitric oxide therapy for persistent pulmonary hypertension of the newborn: Clinical Inhaled Nitric Oxide Research Group. *N. Eng. J. Med.* 342: 469-474.

Clarke WR, Uezono S, Chambers A, Doepfner P. (1994) The type II phosphodiesterase inhibitor milrinone and type V inhibitor dipyridamole individually and synergistically reduce elevated pulmonary vascular resistance. *Pulm. Pharmacol.* 7: 81-89.

Clayton RA, Nally JE, MacLean MR, Thomson NC, McGrath JC. (1999). Chronic exposure to hypoxia attenuates contractile responses in rat airways in vitro: a possible role for nitric oxide. *Eur. J. Pharmacol.* 385: 29-37.

Clemens MJ, Trayner I, Menaya J. (1992). The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. *J Cell Sci.* 103 (Pt 4):881-7.

Cody RJ, Haas GJ, Binkley PF, Capers Q, Kelley R. (1992). Plasma endothelin correlates with the extent of pulmonary hypertension in patients with chronic congestive heart failure. *Circulation.* 85: 504-504.

Cohen ML, Kronzon I. (1981). Adverse hemodynamic effects of phentolamine in primary pulmonary hypertension. *Ann Intern Med.* 95(5):591-2.

Cohen AH, Hanson K, Morris K, Fouty B, McMurty IF, Clarke W, and Rodman DM. (1996). Inhibition of cyclic 3'-5'-guanosine monophospate-specific phosphodiesterase selectively vasodilates the pulmonary circulation in chronically hypoxic rats. *J. Clin. Invest.* 97: 172-179.

Conrad PW, Rust RT, Han J, Millhorn DE, 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.* 13;274(33):23570-6.

Conti M, Swinnen JV, Tsikalas KE, and Jin SL. (1992). Structure and regulation of the rat high-affinity cyclic AMP phosphodiesterase. A family of closely related enzymes. *Adv. Second Messenger Phosphoprotein Res.* 25: 87-99.

Conti M, Iona S, Cuomo M, Swinnen JV, Odeh J, Svoboda ME. (1995a). Characterization of a hormone-inducible, high affinity adenosine 3'-5'-cyclic monophosphate phosphodiesterase from the rat Sertoli cell. *Biochemistry.* 34(25):7979-87.

Conti M, Nemoz G, Sette C, Vicini E. (1995b). Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endo Rev.* 16: 370-389.

Conti JD, Francis SH, (1999). Cyclic GMP phosphodiesterase-5: target of sildenafil. *J. Biol. Chem*. 274: 13729-13732.

Conti M. (2000). Phosphodiesterase and cyclic nucleotide signalling in endocrine cells. *Mol. Endocrinology*. 14: 1317-1327.

Conti JD, Turko IV, Beasely A, Francis SH. (2000). Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. *Eur. J. Biochem*. 267: 2760-2767.

Conway A, Rakhit S, Pyne S, Pyne NJ. (1999). Platelet-derived-growth-factor stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle: role of pertussis-toxin-sensitive G–proteins, cSrc tyrosine kinase and phosphoinositide 3-kinase sensitive G-protein, c-Src tyrosine kinases and phosphoinositide 3-kinase. *Biochem J*. 337: 171-177.

Cook SJ, McCormick F. (1993). Inhibition by cAMP of Ras-dependent activation of Raf. *Science*. 262(5136): 1069-72.

Corbin JD, Beebe SJ, and Blackmore PF. (1985). cAMP-dependent protein kinase activation lowers hepatocyte cAMP. *J Biol. Chem*. 260: 8731-8735.

Corbin JD, Francis SH. (1999). Cyclic GMP Phosphodiesterase-5: Target of Sildenafil. *J Biol. Chem*. 274. 13729-13732.

Corbin JD, Turko IV, Beasley A, and Francis SH. (2000) Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. *Eur J Biochem*. 267: 2760-2767.

Cornwell TL, Arnold E, Boerth NJ and Lincoln TM. (1994). Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am. J. Physiol*. 267: C1405-C1413.

Cornwell TL, and Lincoln. (1989). Regulation of intracellular calcium levels in cultured vascular smooth muscle cells. Reduction of calcium by aptriopeptin and 8-bromo-cyclic

GMP is mediated by cyclic GMP-dependent protein kinase. *J. Biol. Chem.* 264: 1146-1155.

Cortijo J, Bou J, Belrta J, Cardelus I, Llenas J, Morcillo E, Gristwood (1993). Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with humanbronchus. *Br J. Pharmacol.* 108: 562-568.

Cowan KN, Joones PL, Rabinovitch M. (2000). Elastase and matrix metalloproteinase inhibitors induce regression and tenascin-C antisense prevents progression of vascular disease. *J. Clin. Invest.* 105: 21-34.

Crilley TK, Wanstall JC, Bonnet PA. (1998). Vasorelaxant effects of SCA40 (a phosphodiesterase III inhibitor) in pulmonary vascular preparations in rats. *Clin Exp Pharmacol Physiol.* 25(5): 355-360.

Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MC, Lefkowitz RJ. (1998). Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J. Biol. Chem.* 273: 685-688.

Damke H, Baba T, Warnock DE, Schmid SL. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J Cell Biol.* 127(4):915-34

Das M, Bouchey DM, Moore MJ, Hopkins DC, Nemenoff RA, Stenmark KR. (2001) Hypoxia-induced proliferative response of vascular adventitial fibroblast is dependent on G-protein activation of mitogen activated protein kinases. *J. Biol. Chem.* 276: 15631-15640.

Daub H, Weiss FU, Wallasch C, Ullrich A. (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature. 379(6565):557-60.

Daub H, Wallasch C, Lankenau A, Herrlich A, Ullrich A. (1997). Signal characteristics of G-protein-transactivated EGF receptor. *EMBO J.* 16(23):7032-44.

Defer N, Best-Belpomme M, Hanoune J. (2000). Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. *Am J Physiol Renal Physiol.* 279(3):F400-16.

Degerman E, Belfrage P, Manganiello VC. (1996) cGMP-inhibited phosphodiesterases (PDE3 gene family). *Biochem. Soc Trans.* 24: 1010-1013.

Degerman E, Belfrage P and Manganiello VC. (1997). Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J Biol Chem.* 272: 6823-6826.

Della Fazia MA, Servillo G, Sassone-Corsi P. (1997). Cyclic AMP signalling and cellular proliferation: regulation of CREB and CREM. *FEBS Lett.* 410:22-24.

Delpy E, Coste H, Gouville AC. (1996a). Effects of cyclic GMP elevation on isoprenaline-induced increase in cyclic AMP and relaxation in rat aortic smooth muscle: role of phophodiesterase 3. *Br J. Pharmacol.* 119: 471-480.

Delpy E, le Monnier de Gouville AC. (1996). Cardiovascular effects of a novel, potent and selective phosphodiesterase 5 inhibitor, DMPPO: in vitro and in vivo characterization. Br J. Pharmacol. 118: 1377-1384.

Dempsey EC, Stenmark KR, McMurtry IF, O'Brien RF, Voelkel NF, Badesch DB. (1990). Insulin-like growth factor I and protein kinase C activation stimulate pulmonary artery smooth muscle cell proliferation through separate but synergistic pathways. *J Cell Physiol.* 144(1):159-65.

Dempsey EC, McMurtry IF, O'Brien RF. (1991). Protein kinase C activation allows pulmonary artery smooth muscle cells to proliferate to hypoxia. *Am J Physiol.* 260(2 Pt 1):L136-45

Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fisher SG, Barst RJ, Hodge SE, Knowles. (2000) Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenic protein receptor-II gene. *AM. J. Hum. Genet.* 67: 737-744.

Dent G, Magnussen H, and Rabe KF. (1994). Cyclic nucleotide phosphodiesterase in the human lung. *Lung.* 172: 129-146.

DeRooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bros JL. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*. 396: 474-477.

DiCarlo VS, Chen SJ, Meng QC, Durand J, Yano M, Chen YF, Oparil S. (1995). ETA-receptor antagonist prevents and reverses chronic hypoxia-induced pulmonary hypertension in the rat. *Am J. Physiol.* 269: L690-L697.

Dinh-Xuan AT, Higenbottam TM, Clelland CA, Pepke-Zaba J, Cremona G, Butt AY, Large SR, Wells FC, and Wallwork J. (1991). Impairment of endothelium-dependent pulmonary-artery relaxation in chronic obstructive lung disease. *N Engl J Med.* 324: 1539-1547.

Dinh-Xuan AT, Pepke-Zaba J, Butt AY, Cremona G, Higenbottam TW. (1993) Impairment of pulmonary-artery endothelium-dependent relaxation in chronic obstructive lung disease is not due to dysfunction of endothelial cell membrane receptors nor to L-arginine deficiency. *Br J Pharmacol.* 109(2):587-91.

Docherty CC and MacLean MR (1998). Endothelin-B receptors in rabbit pulmonary resistance arteries: effect of left ventricular dysfunction. *J Pharmacol Exp Ther.* 284: 895-903.

Doggrell SA, and Brown L. Rat models of hypertension cardiac hypertrophy and failure (1998). *Cardiovasc Res.* 39: 89-105.

Doherty AM. Phosphodiesterase 4 inhibitors as novel ant-inflammatory agents. *Cur Opinion Chem. Biol.* 3, 466-473, 1999.

Dukarm RC, Morin III FC, Russell JA, Steinhorn RH, Fishman AP. (1998). Pulmonary and systemic effects of the phosphodiesterase inhibitor dipyridamole in newborn with persistent pulmonary hypertension. *Ped. Res.* 44:831.

Dumas JP, Bardou M, Goirand F, Dumas M. (1999) Hypoxic pulmonary vasoconstriction. *Gen Pharmacol.* 33: 289-297.

Eckhart AD, Zhu ZM, Arendshorst WJ. (1996). Oxygen modulates α_1-adrenergic receptor gene expression by arterial but not venous vascular smooth muscle. *Am J. Physiol.* 40: I11599-I11608.

Eddahibi S, Raffestin B, Braquet P, Chabrier PE, Adnot S. (1991). Pulmonary vascular reactivity to endothelin-1 in normal and chronically pulmonary hypertensive rats. *J Cardiovasc. Pharmacol.* 17: S358-S361.

Eddahibi S, Raffestin B, Pham I, Launay JM, Aegerter P, Sitbon M. (1997). Treatment with 5-HT potentiates development of pulmonary hypertension in chronically hypoxic rats. *Am J Physiol.* 272: H1173-1181.

Eddahibi S, Raffestin B, Le Monnier de Gouville AC, Adnot S. (1998). Effect of DMPPO, a phosphodiesterase type 5 inhibitor, on hypoxic pulmonary hypertension in rats. *Br J Pharmacol.* 125(4): 681-688.

Eddahibi S, Fabre V, Boni C, Martres MP, Raffestin B, Hamon M, Adnot S. (1999) Induction of serotonin transporter by hypoxia in pulmonary vascular smooth muscle cells relationship with the mitogenic action of serotonin. *Circ. Res.*, 84: 329-336

Epinat JC and Gilmore TD. (1999). Diverse agents act at multiple levels to inhibit the Rel/NF-κB signal transduction pathway. *Oncogene*. 18: 6896-6909.

Erhart P. Differential regulation of Raf-1 and B-Raf and Ras-dependent activation of mitogen-activated protein kinase by cyclic AMP in PC12 cells. *Mol. Cell. Biol.* 15: 5524-5530.

Exton JH. (1985) Mechanism involved in α_2-adrenergic phenomena. *Am J Physiol* 248: E633-647.

Faller DV. (1999). Endothelial cell responses to hypoxic stress. *Clin Exp Pharmacol Physiol.* 26(1): 74-84.

Farrukh IS, Gurtner GH, Michael JR. (1987). Pharmacological modification of pulmonary vascular injury: possible role of cAMP. *J Appl Physiol.* 62(1): 47-54.

Fawcett L, Baxendale R, Stacey P, McGrouther C, Harrow I, Soderling S, Hetman J, Beavo JA and Phillips SC. (1999). Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Prac Natl Acad Sci.* 97: 3702-3707.

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

Firth JD, Radcliffe PJ. (1992). Organ distribution of three rat endothelin messenger RNAs and the effect of ischemia on renal gene expression. *J Clin Invest.* 90: 1023-1031.

Fisher DA, Smith JF, Pillar JS, St Denis SH, and Cheng JB. (1998a). Isolation and characterization of PDE8A, a novel human cAMP-specific phosphodiesterase. *Biochem Biophys Res Commun.* 246: 570-577.

Fisher DA, Smith JF, Pillar JS, St Denis SH, and Cheng JB. (1998b). Isolation and characterisation of PDE9A, a novel human cGMP-specific phosphodiesterase. *J Biol Chem.* 273: 1559-15564.

Fishman AP. (1976). Hypoxia on the pulmonary circulation: how and where it acts. *Circ. Res.* 38: 221-231.

Fishman AP. (1985). Pulmonary Circulation. In, Handbook of physiology, The respiratory System. Volume1, Chapter3, pp93-165. Bethesda MD: American Physiological Society.

Fishman AP. (1998) Etiology and pathogenesis of primary pulmonary hypertension: a perspective. *Chest*; 114: 242S-247S.

Forrest IA, Small T, Corris PA. (1999). Effect of nebulized epoprostenol (prostacyclin) on exhaled nitric oxide in patients with pulmonary hypertension due to congenital heart disease and in normal controls. *Clin Sci.* 97(1):99-102.

Fouty B, Komalavilas P, Muramatus M, Cohen A, McMurtry IF, Lincoln TM, Rodman DM. (1998). Protein kinase G is not essential to NO-cGMP modulation of basal tone in rat pulmonary circulation. *Am J Physiol.* 274: H672-H678.

Frame M, Wan KF, Tate R, Vandenabeele P, Pyne NJ. (2001). The gamma subunit of the rod photoreceptor cGMP phosphodiesterase can modulate the proteolysis of two cGMP binding cGMP-specific phosphodiesterases (PDE6 and PDE5) by caspase-3. *Cell Signal.* 13: 735-741.

Francis SH, Thomas MK and Corbin JD. (1980). Cyclic GMP-binding cyclic GMP-specific phosphodiesterase from lung. In "Cyclic Nucleotide Phosphodiesterases: Structure, regulation and drug action". (J. Beavo and MD Houslay, eds), pp. 117-140. Wiley, Chichester, UK.

Francis SH, and Corbin JD. (1990) Purification of cGMP-binding protein phosphodiesterase from rat lung. *Methods Enzymol.* 159: 722-729.

Francis SH, and Corbin JD. (1994). Structure and function of cyclic nucleotide-dependent protein-kinases. *Annu Rev Physiol* 56: 237-227.

Franklin CC, Kraft AS. (1997). Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. *J Biol Chem.* 272(27):16917-23.

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

Frid MG, Aldashev AA, Cabirac GF, Dempsey EC, Stenmark KR. (1998). Hypoxia stimulates proliferation of a unique cell population isolated from the bovine vascular media. *Chest.* 114:28S-29S.

Frostell C, Fratacci MD, Wain JC, Jones R, and Zapol MW. (1991). Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary hypertension. *Circulation* 83: 2038-2047.

Fujishige K, Kotera J, Michibata H, Yuasa K, Takebayashi S, Okumura K, Omori K. (1999). Cloning and characterization of a novel human phophodiesterase of a novel human phosphodiesterase that hydrolyzes both cAMP and cGMP (PDE10A). *J Biol Chem.* 274: 18438-18445.

Fukumoto S, Koyama H, Hosoi M, Yamakawa K, Tanaka S, Morii H, Nishizawa Y. (1999). Distinct role of cAMP and cGMP in the cell cycle control of vascular smooth muscle cells: cGMP delays cell cycle transition through suppression of cyclin D1 and cyclin-dependent kinase 4 activation. *Circ Res.* 85: 985-991.

Fukuroda T, Kobayashi M, Ozaki S, Yano M, Miyauchi T, Onizuka M, Sugishits Y, Goto K, Nishikibe M. (1994). Endothelin receptor subtypes in human versus rabbit pulmonary arteries. *J Appl Physiol.* 76: 1976-1982.

Fuller DV. Endothelial cell response to hypoxic stress. (1999). *Clin Exp Pharmacol Physiol.* 26: 74-84.

Fullerton DA, Agrafojo J, McIntyre RC. (1996). Pulmonary vascular smooth muscle relaxation by cAMP-mediated pathways. J. *Surg. Res.* 61: 444-448

Farrukh IS, Gurtner GH, Michael JR. (1987). Pharmacological modification of pulmonary vascular injury: possible role of cAMP. *J Appl Physiol.* 62(1):47-54.

Furuya M, Takehisa M, Minamitake Y, Kitajima Y, Hayashi Y, Ohnuma N, Ishihara T, Minamino N, Kangawa K and, Matsuo H. (1990). Novel natriuretic peptide, CNP, potently stimulates cyclic GMP production in rat cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 170: 201-208.

Gaine SP, and Rubin LJ. (1998) Primary pulmonary hypertension. *Lancet.* 352: 719-725.

Ganong WF. Review of Medical Physiology. (1995). 17th edition. Chapter 34, pp591-607. Appleton and Lange. 1995.

Garg UC, Hassid A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibits mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest.* 83: 1774-1777.

Geraci MW, Goa B, Shepherd DC, Moore MD, Westcott JY, Fagan KA, Alger LA, Tuder RM, Voelkel NF. (1999). Pulmonary prostacyclin synthase overexpression in

transgenic mice protects against development of hypoxic pulmonary hypertension. *J Clin Invest.* 103: 1509-1515.

Geraci MW, Moore M, Gesell T, Yeager ME, Alger L, Golpon H, GA B, Loyd JE, Tuder RM, Voelkel NF. (2000). Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis. *Circ Res.* 88: 555-562.

Gerondakis S, Grossmann M, Nakamura Y, Pohl T, Grumont R. (1999). Genetic approaches in mice to understand Rel/NF-κB and IκB function: transgenics and knockouts. *Oncogene.* 18(49):6888-95.

Gersony WM. (1973). Persistence of the foetal circulation: a commentary. *J Pediatr.* 82(6):1103-6.

Gettys TW, Blackmore PF, Redmon JB, Beebe SJ, Corbin JD. (1987). Short-term feedback regulation of cAMP by accelerated degradation in rat tissue. *J Biol Chem.* 262: 333-339.

Gettys TW, Vine AJ, Simonds MF, Corbin JD. (1988). Activation of the particulate low Km phosphodiesterase of adipocytes by addition of cAMP-dependent protein kinase. *J Biol Chem.* 263(21):10359-63

Ghofrani HA, Weidemann R, Rose F, Schermuly RT, Olschewski H, Weissmann N, Guther A, Walmrath D, Seeger W, Grimminger F. (2002). Sildenafil for treatment of lung fibrosis and pulmonary hypertension: a randomised controlled trial. *Lancet.* 360: 895-900.

Ghosh E (1998). NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16: 225-260.

Giaid A, Saleh D. (1995). Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med.* 333(4):214-21.

Giais A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, Stewart DJ. (1993). Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med.* 328: 1732-1739.

Gibson A. (2001). Phosphodiesterse 5 inhibitors and nitrergic transmission-from zaprinast to sildenafil. *Eur J Pharamcol.* 411: 1-10.

Gillespie MN, Frederick WB, Altiere RJ, Olson JW, Kimmel EC. (1985). Pulmonary mechanical, ventilatory, and gas exchange abnormalities in rats with monocrotaline-induced pulmonary hypertension. *Exp Lung Res.* 8:191-9.

Gillespie MN, Rippetoe PE, Haven CA, Shiao RT, Orlinska U, Maley BE, Olson JW. (1989a). Polyamines and epidermal growth factor in monocrotaline-induced pulmonary hypertension. *Am Rev Respir Dis.* 140: 1463-1466.

Gillespie PG, Prusti RK, Apel ED, Beavo JA. (1989b) A soluble form of bovine rod photoreceptor phosphodiesterase has a novel 15-kDa subunit. *J. Biol. Chem.* 264: 12187-12193.

Gillespie PG and Beavo JA. (1989). Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M&B 22, 948. *Eur J Pharamcol.* 36, 773-781.

Ginty DD, Bonni A, Greenberg ME. (1994). Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell.* 77(5):713-25.

Goldstein I, Lue TF, Padma-Nathan H, Rosen RC, Steers WD, Wicker PA (1998). Oral sildenafil in the treatment of erectile dysfunction. Sildenafil study group. *New Engl. J. Med.* 338: 1397-1404.

Golpon HA, Geraci MW, Moore MD, Miller HL, Miller GJ, Tuder RM, Voelkel NF. (2000). Hox genes in human lung: altered expression in primary pulmonary hypertension and emphysema. *Am J. Pathol.* 158: 955-66.

Gonzalez GA, and Montminy MR. (1989). Cyclic AMP stimulates somatostatin gene-transcription by phosphorylation of CREB at serine-133. *Cell.* 59: 675-680.

Gonzalez CM, Bervig T, Podlasek C, Huang CF, McKenna KE, McVary KT. (1999). Sildenafil causes a dose- and time- dependent downregulation of phosphodiesterase type 6 expression in the rat retina. *Int J Impot Res.* 11: S9-14.

Gosgnach W, Messika-Zeitoun D, Gonzalez W, Philipe M, Michel J. (2000). Shear stress induces iNOS expression in cultured smooth muscle cells: role of oxidative stress. *Am J Cell Physiol.* 279: C1880-C1888.

Gothie E, Richard DE, Berra E, Pages G, Pouyssegur J. (2000). Identification of alternative spliced variants of human hypoxia-inducible factor-1alpha. *J Biol Chem.* 275(10):6922-7.

Grant PG, Mannarino AF, and Colman RW. (1988). cAMP-mediated phosphorylation of the low Km cAMP phosphodiesterase markedly stimulates its catalytic activity. *Proc. Natl. Acad. Sci.* 85: 9071.

Graves LM, Bornfeldt KE, Raines EW, Potts BC, Macdonald SG, Ross R, Krebs EG. (1993). Protein kinase A antagonises platelet-derived growth factor-induced signalling by mitogen-activated protein kinase in human arterial smooth muscle cells. *Proc Natl Acad Sci.* 90: 10300-10304.

Graves LM, Bornfeldt KE, Argast GM, Krebs EG, Kong X, Tin TA, Lawrence JC Jr. (1995). cAMP- and rapamycin-sensitive regulation of the association of eukaryotic initiation factor 4E and the translational regulator PHAS-I in aortic smooth muscle cells. *Proc Natl Acad Sci.* 92: 7222-7226.

Graves LM, Bornfeldt KE, Sidhu JS, Argast GM, Raines EW, Ross R, Leslie CC, Krebs EG. (1996). Platelet-derived growth factor stimulates protein kinase A through a mitogen-activated protein kinase-dependent pathway in human arterial smooth muscle cells. *J Biol Chem.* 271(1):505-11.

Greenberg B, Rhoden K, Barnes PJ. (1987). Endothelium-dependent relaxation of human pulmonary arteries. *Am J. Physiol.* 252: H434-H438.

Grimshaw MJ, Balkwill FR. Mechanism of hypoxic inhibition of monocyte migration. *Eur. J. Immunol.* 31: 480-489, 2001.

Griscavage JM, Ignarro LJ. (1995). Serine and cysteine proteinase inhibitors prevent nitric oxide production by activated macrophages by interfering with transcription of the inducible NO synthase gene. *Biochem Biophys Res Commun.* 215: 721-929.

Gristwood RW, comer MB, Eden RJ, Taylor EM, Turner JA, Wallduck M, Owen DA (1988). In vivo pharmacological studies with SKF94836, A potent inotrope/vasodilator with a sustained duration of action. *Br J Pharmacol.* 93: 893-901.

Grosser T, Bonisch D, Zucker TP and Schror K. (1995). Iloprost-induced inhibition of proliferation of coronary artery smooth muscle cells is abolished by homologous desensitisation. *Agent Action Suppl.* 45: 58-91.

Gudermann T, Grosse R, Schultz G. (2000). Contribution of receptor/G protein signalling to cell growth and transformation. *Naunyn Schmiedebergs Arch Pharmacol.* 61(4):345-62.

Guipponi M, Scott HS, Kudoh J. (1998). Identification and characterization of a novel cyclic nucleotide phosphodiesterase gene (PDE9A) that maps to 21q22.3: alternative splicing of mRNA transcripts, genomic structure and sequence. *Hum Genet.* 103: 386-392.

Guldemeester A, Stenmark KR, Brough GH, Troy Stevens. (1999). Mechanism regulating cAMP-mediated growth of bovine neonatal pulmonary artery smooth muscle cells. *Am. J. Physiol.* 276: L1010-L1017.

Gutkind JS. (1998). Cell growth control by G-protein-coupled receptors: from signal transduction to signal integration. *Oncogene.* 17(11): 1331-42.

Haddad JJ, Land SC, Tarnow-Mordi WO, Zembala M, Kowalczyk D, Lauterbach R (2002). Immunopharmacological potential of selective phosphodiesterase inhibition. II. Evidence for the involvement of an inhibitory-κB/Nuclear factor-κB-sensitive pathway in alveolar epithelial cells. *J. Pharmacol. Exp. Therp.* 300: 567-577.

Hafner S, Adler HS, Mischak H, Janosch P, Heidecker G, Wolfman A, Pippig S, Lohse M, Ueffing M, Kolch W. (1994). Mechanism of inhibition of Raf-1 by protein kinase A. *Mol Cell Biol.* 14: 6696-6703.

Hai T, Hartman MG. (2001). The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene.* 273(1):1-11.

Hakonarson H, and Gruinstein MM. (1998). Regulation of second messengers associated with airway smooth muscle contraction and relaxation. *Am J Respir Crit Care Med.* 158: S115-22.

Hamad AM, Range SP, Holland E, and Knox AJ. (1997). Regulation of cyclic GMP by soluble and particulate guanylyl cyclases in cultured human airway smooth muscle. *Am J. Physiol.* 273: L807-L813.

Hamad AM, Johnson SR, Knox AJ. (1999a). Antiproliferative effects of NO and ANP in cultured human airway smooth muscle. *Am J Physiol.* 277: L910-L918.

Hamad AM, Range SP, Holland E, and Knox AJ. (1999b). Desensitization of guanylyl cyclases in cultured human airway smooth-muscle cells. *Am. J. Respir.* 20: 1087-1095.

Hameroff SR, Otto CW, Kanel J, Weinstein PR, Blitt CD. (1981). Acute cardiovascular effects of dimethylsulfoxide. *Crit Care Med.* 9(12):855-7.

Hamilton SE, Hurley JB. (1990). A phosphodiesterase inhibitor specific to a subset of bovine retinal cones. *J Biol Chem.* 265(19):11259-64.

Hampl V and Herget J. (2000) Role of nitric oxide in the pathogenesis of chronic pulmonary hypertension. *Physiol Rev.* 80: 1337-1372.

Hanasato N, Oka M, Muramatsu M, Nishino M, Adachi H, and Fukuchi Y. (1999). E-4010, a selective phosphodiesterase 5 inhibitor, attenuates hypoxic pulmonary hypertension in rats. *Am J Physiol.* 227: L225-L232.

Haneda M, Sugimoto T, Kikkawa R. (1999). Mitogen-activated protein kinase phosphatase: a negative regulator of the mitogen-activated protein kinase cascade. *Eur J Pharmacol.* 365(1):1-7.

Hanoune J, Defer N. (2001). Regulation and role of adenylyl cyclase isoforms. *Annu Rev Pharmacol Toxicol.* 41:145-74.

Hanson KA, Burns F, Rybalkin SD, Miller JW, Beavo J, and Clarke WR. (1998a). Developmental changes in lung cGMP phosphodiesterase-5 activity, protein, and message. 158(1): 279-288.

Hanson KA, Ziegler JW, Rybalkin SD, Miller JW, Aman SH, and Clarke WR. (1998b) Chronic pulmonary hypertension increases fetal lung cGMP phosphodiesterase activity. *Am J Physiol.* 275: L931-L941.

Hassoun PM, Thappa V, Landman MJ, Fanburg BL. (1992). Endothelin 1: mitogenic activity on pulmonary artery smooth muscle cells and release from hypoxic endothelial cells. *Proc Soc Exp Biol Med.* 199: 165-170.

Hattoru Y, Nakanishi, Kasai K. (1997). Role of nuclear factor κB in cytokine-induced nitric oxide and tetrahydrobiopterin synthesis in rat neonatal cardiac myocytes. *J. Mol Cell Cardio.* 29: 1585-1592.

Hayashi M, Matsushima K, Ohashi H, Tsunoda H, Murase S, Kawarada Y, Tanaka T. (1998). Molecular cloning and characterisation of human PDE8B, a novel thyroid-specific isozyme of 3', 5'-cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* 250: 751-756.

Hayden MA, Nakayama DK. (1999). Cyclic nucleotides and inducible nitric oxide synthesis in pulmonary artery smooth muscle. *J Surg Res.* 82: 222-227.

Haynes J, Robinson J, Saunders L, Taylor AE, Strada SJ. (1992). Role of cAMP-dependent protein kinase in cAMP-mediated vasodilation. *Am J Physiol.* H511-H516.

Herrlich A, Daub H, Knebel A, Herrlich P, Ullrich A, Schultz G, Gudermann T. (1998). Ligand-independent activation of platelet-derived growth factor receptor is a necessary intermediate in lysophosphatidic, acid-stimulated mitogenic activity in L cells. *Proc Natl Acad Sci.* 95(15):8985-90.

Herve P, Launay JM, Scrobohaci ML, Brenot F, Simonneau G, Petitpretz P, Poubeau P, Cerrina J, Duroux P, Drouet L. (1995). Increase plasma 5-HT in primary pulmonary hypertension. *Am J Med.* 99, 249-254.

Hideyuki A, Saeki T, Mori N, Saito I. (1994). Cardiohemodynamic characteristics of a novel, selective and potent phosphodiesterase V inhibitor, E4021, in the pig. *JPN. J. Pharmacol.* 64:329.

Higashi K, Funkunaga K, Matsui K, Maeyama M, Miyamoto E. (1983). Purification and characterisation of myosin light-chain kinase from porcine myometrium and its phosphorylation and modulation by cyclic AMP-dependent protein kinase. *Biochim. Biophys. Acta.* 747: 232-240.

Hirsch DD, Stork PJ. (1997). Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways in vivo. *J Biol Chem.* 272(7):4568-75.

Hoffmann R, Baillie GS, Mackenzie SJ, Yarwood SJ, Houslay MD. (1999) The MAP kinase ERK2 inhibits the cAMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. *EMBO. J.* 18:893-903.

Hong HJ, Loh SH and Yen MH. (2000). Suppression of the development of hypertension by the inhibitor of inducible nitric oxide synthase. *Br J Pharmacol.* 131:631-637.

Horstman DJ, Frank DU, Rich GF. (1998). Prolonged inhaled NO attenuates hypoxic, but not monocrotaline-induced, pulmonary vascular remodelling in rats. *Anesth Analg.* 86(1):74-81.

Hoshikawa Y, Voelkel NF, Gesell TL, Moore MD, Morris KG, Alger LA, Narumiya S, Geraci MW. (2001). Prostacyclin receptor-dependent modulation of pulmonary vascular remodelling. *Am. J. Respir. Crit. Care. Med.* 164: 314-318.

Houslay MD, and Milligan G. (1997). Tailoring cAMP-signalling responses through isoform multiplicity. *TIBS.* 22: 217-224.

Houslay MD, Sullivan M, Bolger GB. (1998). The multienzyme PDE4 cyclic adenosine monophosphate-specific phosphodiesterase family: Intracellular targeting, regulation and selective inhibition by compounds exerting anti-inflammatory and antidepressant actions. *Advances in Pharmacology.* 44: 225-342.

Hoyer D, Hannon JP, and Martin GR. (2001). Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacol. Biochem. Biohavior*. 72: 1-33.

Huang B, Che D, Zhang W. (1997). Effects of the medium conditioned by endothelial cells under hypoxic condition on the phenotype of porcine pulmonary arterial smooth muscle cells. *J Tongji Med Univ*. 17(4): 209-212.

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.

Hurley JH. (1998). The adenylyl and guanyly cyclase superfamily. *Curr Opin Struct Biol*. 8: 770-777.

Hyman AL, Nandiwada P, Knight DS, Kadowitz PJ. (1981). Pulmonary vasodilator responses to catecholamines and sympathetic nerve stimulation in the cat. Evidence that vascular β_2-adrenoreceptors are innervated. *Circ Res.* 48(3):407-15.

Hyman AL, Kadowitz PJ. (1985). Evidence for existence of postjunctional α_1- and α_2-adrenoceptors in cat pulmonary vascular bed. *Am J Physiol*. 249(4 Pt 2):H891-8.

Hyman AL, Kadowitz PJ. (1986). Enhancement of α- and β-adrenoceptor responses by elevations in vascular tone in pulmonary circulation. *Am J Physiol*. 250(6 Pt 2):H1109-16.

Ichinose F, Hurford WE, Zapol WM. (1995a) Selective pulmonary vasodilation by inhaled nitric oxide (NO) and nebulized zaprinast in awake lambs. *Am J. Respir Crit Care Med*. 151: A730.

Ichinose F, Adrie C, Hurtford WE, Zapol WM. (1995b) Prolonged pulmonary vasodilatory action of inhaled nitric oxide by zaprinast, in awake lambs. *J Appl Physiol*. 78:1288-1295.

Indolfi C, Avvedimento EV, Di Lorenzo E, Esposito G, Rapacciuolo A, Giuliano P, Grieco D, Cavuto L, Stingone AM, Ciullo I, Condorelli G, Chiariello M. (1997). Activation of cAMP-PKA signalling in vivo inhibits smooth muscle cell proliferation induced by vascular injury. *Nat Med*. 3: 775-779.

Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K (1989) The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci.* 86: 2863-2867.

Inoue M, Watanabee K, Mori C. (1994). The effect of bunazosin on monocrotaline-induced pulmonary hypertension in rats. *Acta Paediatr Jpn.* 36: 133-138.

Isaacson TC, Hampl V, Weir EK, Nelson DP, and Archer SL. (1994). Increased endothelium derived NO in hypertensive pulmonary circulation of chronically hypoxic rats. *J Appl Physiol.* 76: 933-940.

Ishihara H, Adachi H, Kodama K, Suzuki S, Takamura T, and Tanaka H. (1998). Pharmacological characterisation of a novel, potent, and selective phosphodiesterase inhibitor, E4010. *Jpn J. Pharmacol.* 76: 175P

Janakidevi K, Fisher MA, Del Vecchio PJ, Tiruppathi C, Figge J, Malik AB. (1992). Endothelin-1 stimulates DNA synthesis and proliferation of pulmonary artery smooth muscle cells. *Am J Physiol.* 263: C1295-C1301.

Jaski BE, Fifer MA, Wright RF. (1985) Positive inotropic and vasodilator actions of milrinone in patients with severe congestive heart failure. *J Clin Invest.* 75: 643-9.

Jeffery TK and Wanstall JC. (1998). Phosphodiesterase III and V inhibitors on pulmonary artery from pulmonary hypertensive rats: differences between early and established PHT. *J. Cardiovascular Pharmacol.* 32: 213-219.

Jeffery TK and Wanstall JC. (2001). Pulmonary vascular remodelling: a target for therapeutic intervention in pulmonary hypertension. *Pharmacol Ther.* 92: 1-20.

Jeon YJ, Yang RH, Pulaski JT (1996). Attenuation of inducible nitric oxide synthase gene expression by Δ9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor-κB/Rel activation. *Mol Pharmacol.* 50: 334-41.

Jeremy JY, Ballard SA, Naylor AM, Miller MAW, Angelini GD. (1997). Effects of sildenafil, a type-5 cGMP phosphodiesterase inhibitor, and papaverine on cyclic GMP and cyclic AMP levels in the rabbit corpus cavernosum in vitri. *Br J. Urol.* 79: 958-963.

Jianming X, Quanfu X, Lirong W. (1991). Effect of hypoxia on the pulmonary β- and α_1-adrenoreceptors in rats. *Chin Med Sci J.* 6: 217-222.

Jin N, Hatton N, Swartz DR, Xia XL, Harrington MA, Larsen SH, Rhoades RA. (2000). Hypoxia activates jun-N-terminal kinase, extracellular signal-regulated protein kinases, and p38 kinase in pulmonary vessels. *Am. J. Respir. Cell. Mol. Biol.* 23: 593-601.

Johnson DE, Georgieff MK. (1989). Pulmonary neuroendocrine cells. Their secretary products and their potential roles in health and chronic lung disease in infancy. *Am Rev Respir Dis.* 140: 1807-1812.

Jones RD, Thompson JS, Morice AH. (1997). The effect of hydrogen peroxide on hypoxia, prostaglandin $F_2\alpha$ and potassium chloride induced contractions in isolated rat pulmonary arteries. *Pulm Pharmacol Ther.* 10(1):37-42

Jourdan KB, Mason NS, Long L, Philips PG, Wilkins MR, Morrell NW. (2001). Characterisation of adenylyl cyclase isoforms in rat peripheral pulmonary arteries. *Am J Physiol Lung Cell Mol Physiol.* 280: L1359-L1369.

Kadowitz PJ, Hyman AL. (1973). Effect of sympathetic nerve stimulation on pulmonary vascular resistance in the dog. *Circ Res.* 32(2):221-7.

Kadowitz PJ, Joiner PD, Hyman AL. (1975). Influence of sympathetic stimulation and vasoactive substances on the canine pulmonary veins. *J Clin Invest.* 56(2):354-65

Kadowitz PJ, Knight DS, Hibbs RG, Ellison JP, Joiner PD, Brody MJ, Hyman AL. (1976). Influence of 5- and 6-hydroxydopamine on adrenergic transmission and nerve terminal morphology in the canine pulmonary vascular bed. *Circ Res.* 39(2):191-9.

Kakkar R, Raju RV, Sharma RK. (1999). Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1). *Cell Mol Life Sci.* 55: 1164-86.

Karamsetty VSNMR, MacLean MR, McCulloch KM, Kane KA, Wadsworth RM. (1996). Hypoxic constrictor response in the isolated pulmonary artery from chronically hypoxic rats. *Resp. Physiol.*105: 85-93.

Karin M, Zheng-Gang L, Erbrahim Z. (1997). AP-1 function and regulation. *Curr Opin Cell Biol*. 9: 240-248

Karin M. (1998). New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? *Cell* 93: 487-490.

Karin M, Ben-Neriah Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol*. 18:621-63.

Kariya K, Kawahara Y, Araki S, Fukuzaki H, Takai Y. (1989). Antiproliferative action of cGMP-elevating vasodilators in cultured rabbit aortic smooth muscle cells. *Artherosclerosis*. 80: 143-147.

Karpova AK, Abe MK, Li J, Lui PT, Rhee JM, Kuo WL, Hershenson MB. (1997). MEK1 is required for PDGF-induced ERK activation and DNA synthesis in tracheal monocytes. *Am J Physiol Lung Cell Mol Physiol*. 272: L558-L565.

Kataoka S, Alam R, Dash PK, Yatsu FM. (1997). Inhibition of PDGF-mediated proliferation of vascular smooth muscle cells by calcium antagonists. *Stroke*. 28(2):364-9.

Kato R, Sato J, Nishino T. (1998). Milrinone decreases both pulmonary arterial and venous resistance in the hypoxic dog. *Br J Anaesth*. 81(6): 920-924.

Katayose D, Ohe M, Yamauch K, Shirato K, Fujita H. Shibahara S, Takishima T. (1993). Increased expression of PDGF A- and B-chain genes in rat lungs with hypoxic pulmonary hypertension. *Am. J. Physiol*. 264: L100-L106.

Kauffman RF, Schenck KW, Utterback BG, Crowe VG, and Cohen ML. (1987). *In vitro* vascular relaxation by new inotropic agents: relationship to phosphodiesterase inhibition and cyclic nucleotides. *J Pharmacol Exp. Ther*: 242: 864-872.

Kenan Y, Murata T, Shakur Y, Degerman E, Manganiello VC. (2000). Functions of the N-terminal region of cyclic nucleotide phosphodiesterase 3 (PDE 3) isoforms. *J Biol Chem*. 275(16):12331-8.

Keyes SM. (2000). Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Cur Opinion Cell Biol.* 12: 186-192.

Keyes SM. (1995). An emerging family of dual specificity MAP kinase phosphatases. *Biochemica Biophysica Acta.* 1265, 152-160.

Kilbourne EJ, Nankova BB, Lewis EJ, McMahon A, Osaka H, Sabban DB, Sabban EL. (1992). Regulated expression of the tyrosine hydroxylase gene by membrane depolarisation. Identification of the responsive element and possible second messengers. *J Biol Chem.* 267(11):7563-9.

Kim HJ, Kim KW, Yu BP, Chung HY. (2000). The effect of age on cyclooxygenase-2 gene expression: NF-κB activation and IκBα degradation. *Free Radic Biol Med.* 28(5):683-92.

Kim NN, Huang YH, Moreland RB, Kwak SS, Goldstein I, Traish A. (1999). Cross-regulation of intracellular cGMP and cAMP in cultured human corpus cavernosum smooth muscle cells. *Mol Cell Biol Res Commun.* 4(1): 10-14.

Kinnula VL, Crapo JD, and Raivo KO. (1995). Generation and disposal of reactive oxygen metabolites in the lung. *Lab Invest.* 73: 3-19.

Kirstein M, Rivet-Bastide M, Hatem S, Benardeau A, Mercadier JJ, Fischmeister R. (1995). Nitric oxide regulates the calcium current in isolated human atrial myocytes. *J Clin Invest.* 95(2):794-802.

Klings ES, Farber HW. (2001). Current management of primary pulmonary hypertension. *Drugs.* 61(13):1945-56.

Kloner RA. (2000). Cardiovascular risk and sildenafil. *Am J Cardiol.* 86(2A):57F-61F

Ko FN. (1997). Low-affinity thromboxane receptor mediates proliferation in cultured vascular smooth muscle cells of rats. *Arterioscler Thromb Vasc Biol.* 17(7):1274-82.

Kobayashi S, Beitner-Johnson D, Coforti L, Millhorn DE. (1998). Chronic hypoxia reduces adenosine α-$_{2A}$ receptor-mediated inhibition of calcium current in rat PC12 cells via downregulation protein kinase A. *J. Physiol.* 512: 351-63.

Kodama K, and Adachi H. (1999). Improvement of mortality by long-term E4010 treatment in monocrotaline-induced pulmonary hypertensive rats. *J Pharmacol Exp Ther.* 290: 748-752.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. (1993). Protein kinase Cα activates Raf-1 by direct phosphorylation. *Nature.* 364(6434):249-52.

Kolch W. (2000). Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J.* 351 Pt 2:289-305.

Komas N, Lugnier C, Stoclet JC. (1991). Endothelium-dependent and independent relaxation of the aorta by cyclic nucleotide phosphodiesterase inhibitors. *Br J. Pharmacol.* 104: 495-503.

Kotera J, Fujishige K, Imai Y, Kawai E, Michibata H, Akatsuka H, Yanaka N, Omori K (1999). Genomic origin and transcriptional regulation of two variants of cGMP-binding cGMP-specific phosphodiesterases. *Eur J Biochem.* 262: 866-873.

Koyama H, Bornfeldt KE, Fukumoto S and Nishizawa Y. (2001). Molecular pathways of cyclic nucleotide-induced inhibition of arterial smooth muscle cell proliferation. *J. Cell Physiol.* 186:1-10.

Kronemann N, Nocher WA, Busse R, Schini-Kerth VB. (1999). Growth-inhibitory effect of cyclic GMP- and cAMP-dependent vasodilators on rat vascular smooth muscle cells: effect on cell cycle and cyclin expression. *Br J Pharmacol.* 126: 349-357.

Kwak SP, Hakes DJ, Martell KJ, Dixon JE. (1994). Isolation and characterization of a human dual specificity protein-tyrosine phosphatase gene. *J Biol Chem.* 269(5):3596-604.

Kwasaski H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Houseman DE, and Graybiel AM. (1998). A family of cAMP-binding proteins that directly activate RAP1. *Science* 282: 2275-2279.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*. 369(6476):156-60.

Laderoute KR, Mendonca HL, Calaoagan JM, Knapp M, 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. *J. Biol. Chem.* 274: 12890-12897.

Laemmli UK. (1997). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*. 227, 680-685.

Lane KB, Machado RD, Pauciulo MW Thomson JR, Phillips III JA, Loyd JE, Nichols WC, Trembath RC. (2000). Heterozygous germline mutations in BMPR2, encoding a TGF-β receptor cause familial pulmonary hypertension. *Nature*. 26: 81-84.

Lander HM, Jacovina AT, Davis RJ, Tauras JM (1996). Differential activation of mitogen-activated protein kinase by nitric oxide-related species. *J. Biol. Chem.* 271:19705-19709.

Lander HM, Hajjar DP, Hempstead BL, Mirza UA, Chait BT, Campbell S, and Quilliam LA. (1997). A molecular redox switch on p21ras. Structural basis for the nitric oxide p21ras interaction. *J. Biol Chem*. 272: 4323-4326.

Lang D, Lewis MJ. (1991). Endothelium-deived relaxing factor inhibits the endothlin-1-induced increase in protein kinase C activity in rat aorta. *Br J. Pharmacol.* 104: 139-144.

Lawrence RN, Dunn WR, Wilson VG. (1998). Endothelium-dependent relaxation in response to ethanol in the porcine isolated pulmonary artery. *J Pharm Pharmacol.* 50(8):885-90.

Le Cras TD, Xue C, Rengasemy A, Johns RA. (1996). Chronic hypoxia upregulates endothelial and inducible NO synthase gene and protein expression in rat lung. *Am J Physiol.* 270: L164-170.

Le Cras TD, Tyler RC, Horan MP, Morris KG, McMurty IF, Johns RA, Abman SH. (1998). Effects of chronic hypoxia and altered hemodynamics on endothelial nitric oxide synthase and preproendothelin-1 expression in the adult rat lung. *Chest.* 114(1):35S-36S.

Leach E, Howard P, Barer GR. (1977). Resolution of hypoxic changes in the heart and pulmonary arterioles of rats during intermittent correction of hypoxia. *Clin Sci Mol Med.* 52(2):153-62.

Lee SL, Wang WW, and Fanburg BL. (1997) Association of Tyr phosphorylation of GTPase-activating protein with mitogenic action of serotonin. *Am J. Physiol.* 272: C223-C230.

Levick JR. An Introduction to Cardiovascular Physiology.(1996). 2nd edition, Chapter 13, pp231-254. Butterworth-Heinemann.

Li D, Zhou N, and Johns RA. (1999a) Soluble guanylate cyclase gene expression and localisation in rat lung after exposure to hypoxia. *Am. J. Physiol.*, 277: L841-L847, 1999.

Li F, Che D, Liu S. (1995). Effects of hypoxia on the expression of platelet-derived growth factor (PDGF) gene and PDGF-B chain protein in porcine pulmonary artery endothelial cells. *Chung hua Ping Li Hsueh Tsa Chih.* 24: 139-142.

Li H, Chen S, Chen Y, Meng QC, Durand J, Oparil S, Elton TS. (1994). Enhanced endothelin-1 and endothelin receptor gene expression in chronic hypoxia. *J Appl Physiol.* 77: 1415-1419.

Li L, Yee C, Beavo JA. (1999). CD3- and CD28-dependent induction of PDE7 required for T cell activation. *Science.* 283: 848-851.

Li T, Narhi LO, Wen J, Philo JS, Sitney K, Inoue J, Yamamoto T, Arakawa T. (1998). Interactions between NFκB and its inhibitor IκB: biophysical characterization of a NFκB/IκB-alpha complex. *J Protein Chem.* 17(8):757-63.

Lin YZ, Yao SY, Veach RA, Togerson TR, Hawiger J. (1995). Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J Biol Chem.* 270. 14255-14258.

Lin C, Lau A, Tu R, Lue TF. (2000). Expression of three isoforms of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in human penile cavernosum. *Biochem. Biophys. Res Com.* 268, 628-635.

Lincoln TM, Dey NB, Boerth NJ, Cornwell TL, Soff GA. (1998). Nitric oxide--cyclic GMP pathway regulates vascular smooth muscle cell phenotypic modulation: implications in vascular diseases. *Acta Physiol Scand.* 164(4):507-15.

Lincoln TM, Cornwell TL, and Taylor, AE. (1990). cGMP-dependent protein kinase mediates the reduction of Ca^{2+} by cAMP in vascular smooth muscle. *Am J Physiol.* 258: C399-C407.

Lipkin VM, Udovichenko IP, Bondarenko VA, Yurovskaya AA, Telnykh EV, Skiba NP. (1990). Site-directed mutagenesis of the inhibitory subunit of retinal rod cyclic GMP phosphodiesterase. *Biomed Sci.* 1(3):305-8.

Liu SF, Crawley DE, Evans TE, Barnes, PJ. (1992a). Endothelium-dependent non-adrenergic non-cholinergic neural relaxation in guinea-pig pulmonary artery. J. *Pharmacol. Exp. Ther.* 260:541-548.

Liu SF, Crawley DE, Rohde JAL, Evans TW, Barnes PJ. (1992b). Role of nitric oxide and guanosine 3'-5'-cyclic monophosphate in mediatory nonadrenergic noncholinergic neural relaxation in guinea-pig pulmonary arteries. Br J. Pharmacol. 107: 861-866.

Liu H and Maurice DH. (1998). Expression of cyclic GMP-inhibited phosphodiesterases 3A and 3B (PDE3A and PDE3B) in rat tissues: Differential

subcellular localization and regulated expression by cyclic AMP. *Br J Pharmacol.* 125: 1501-1510.

Liu L, Kwak YT, Bex F, Garcia-Martinez LF, Li XH, Meek K, Lane WS, Gaynor RB. (1998). DNA-dependent protein kinase phosphorylation of IκB-α and IκB-β regulates NF-κB DNA binding properties. *Mol Cell Biol.* 18(7):4221-34.

Liu L, Underwood T, Li Han, Pamukcu R, and Thompson WJ. (2002). Specific cGMP binding by the cGMP binding domains of cGMP-binding cGMP specific phosphodiesterase. *Cellular Signalling.* 14: 45-51.

Liu S, Kuo HP, Sheppard MN, Barnes PJ, Evans TW. (1994). Vagal stimulation induces increased pulmonary vascular permeability in guinea pig. *Am J Respir Crit Care Med.* 149:744-50.

Lo LW, Cheng JJ, Chiu JJ, Wung BS, Liu YC, Wang DL. (2001). Endothelial exposure to hypoxia induces Egr-1 expression involving PKCα-mediated Ras/Raf-1/ERK1/2 pathway. *J Cell Physiol.* 188(3):304-12.

Lockhead A, Nekrasova E, Arshavsky VY, and Pyne NJ. (1997). The regulation of the cGMP-binding cGMP phosphodiesterase by proteins that are immunologically related to γ subunit of the photoreceptor cGMP phosphodiesterase. *J. Biol. Chem.* 272: 18397-18403.

Lopez-Aparicio P, Roscon A, Manganiello VC, Andersson KE, Belfrage P, Degerman E. (1992). Insulin induced phosphorylation and activation of the cGMP-inhibited phosphodiesterase in human platelets. *Biochem. Biophys. Res. Commun.* 186: 517-523.

Lopez-Ilasaca M, Gutkind JS, Wetzker R. (1998). Phosphoinositide 3-kinase γ is a mediator of Gβγ-dependent Jun kinase activation. *J Biol Chem.* 273(5):2505-8.

Loughney K, Hill TR, Florio VA, Uher L, Rosman GJ, Wolda SL, Jones BA, Howard ML, McAllister-Lucas LM, Sonnenburg WK, Francis SH, Corbin JD, Beavo JA, and Ferguson K. (1998). Isolation and characterisation of cDNAs encoding PDE5A, a human cGMP-binding, cGMP-specific 3',5'-cyclic nucleotide phosphodiesterase. *Gene.* 216: 139-47.

Lucas K, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, Waldman SA. (2000). Guanylyl cyclases and signalling by cyclic GMP. *Pharmacol. Rev.* 52: 375-413.

Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, Lefkowitz RJ. (1997) Gβγ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. *J Biol Chem.* 272(7):4637-44.

McAllister-Lucas LM, Sonnenburg WK, Kadlecek A, Seger C, LeTrong H, Colbran JL, Thomas MK, Walsh KA, Francis SH, Corbin JD, and Beavo JA. (1993a). The bovine structure of a bovine lung cGMP-binding, cGMP-specific phosphodiesterase deduced from a cDNA clone. *J. Biol. Chem.* 268: 22863-22873

McAllister-Lucas LM, Sonnenburg WK, Kadlecek A, Seger D, LeTrong H, Colbran JL, Thomas MK, Walsh KA, Francis SH, Corbin JD, Beavo JA. (1993b). The structure of a bovine lung cGMP-binding cGMP-specific phosphodiesterase deduced from a cDNA clone. *J. Biol. Chem.* 268: 22863-22873.

McAllister-Lucas LM, Haik TL, Colbran JL, Sonnenburg WK, Seger D, Beavo JA, Francis SH, and Corbin JD. (1994). Mutagenesis of a lung cGMP-specific phosphodiesterase provides evidence or two distinct sites for allosteric cGMP-binding with an essential aspartic acid at each site. *FASEB J.* 8, A372.

McAuley IW, Kim NN, Min K, Goldstein I, and Traish AM. (2001). Intracavernosal sildenafil facilitates penile erection independent of the nitric oxide pathway. *J. Androl.* 22: 623-628.

McCulloch KM, Docherty CC, MacLean MR. (1998). Endothelin receptors mediating contraction of rat and human pulmonary resistance arteries: effect of chronic hypoxia in the rat. *Br J. Pharmacol.* 123: 1621-1630.

McCulloch KM, Osipenko ON, Gurney AM. (1999) Oxygen-sensing potassium currents in pulmonary artery. *Gen Pharmacol.* 32(4):403-11.

McLaughlin VV, Genthner DE, Panella MM, and Rich S. (1998). Reduction in pulmonary vascular resistance with long-term epoprostenol (prostacyclin) therapy in primary pulmonary hypertension. *N. Engl. J. Med.* 338: 273-277.

McMahon TJ, Ignarro LJ, Kadowitz PJ. (1993). Influence of zaprinast on vascular tone and vasodilator response in the cat pulmonary vascular bed. *J Apl Physiol* 74: 1704-1711.

McMurtry IF, Morris KG, Petrun MD. (1980). Blunted hypoxic vasoconstriction in lungs from short-term high altitude rats. *Am J. Physiol.* 238: H849-H857.

MacDonald KL and Diamond J. (1994). Activation of cAMP-dependent protein kinase in rat aorta by cAMP analogs is not correlated with relaxation. *J. Vasc. Resis.* 31: 121-130.

Machado RD, Pauciulo MW, Thomson JR. Lane KB, Morgan NV, Wheeler L. Philips JA, Newman J, Williams D, Galie N, Manes A, McNeil K, Yacoub M, Mikhail G, Rogers P, Corris P, Humbert M, Donnai D, Martensson G, Tranebjaerg L, Lyod JE, Trebath RC, Nichols WC. (2001). BMPR2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am. J. Hum. Genet.* 68: 92-102.

Machida H, Inoue H, Takagi M, Noto T, Yano K, Kikkawa K. (2002). Sildenafil and T-1032, phosphodiesterase type 5 inhibition showed a different vasorelaxant property in the isolated aorta. *Eur J. Pharmacol.* 440: 45-52.

MacKenzie SJ, Baillie GS, McPhee I, Bolger GB, and Houslay MD. (2000) ERK2 mitogen-activated protein kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterase: The involvement of COOH-terminal docking sites and NH2-terminal UCR region. *J. Biol. Chem.* 275: 16609-16617.

MacLean MR, McCulloch KM, McGrath JC. (1993a), Influence of the endothelium and hypoxia on α_1- and α_2-adrenreceptor agonists in the rabbit isolated pulmonary artery. *Br. J. Pharmacol.* 108: 155-161.

MacLean MR, Smith GC, Templeton AGB (1993b). Adverse reactions associated with sumatriptan. *Lancet.* 341: 1092-1164, 1993.

MacLean MR, Clayton RA, Hillis SW, McIntyre PD, Peacock AJ, Templeton AGB. (1994a). 5HT1-receptor-mediated vasoconstriction constriction in bovine isolated pulmonary arteries: influence of vascular endothelium and tone. *Pulmon. Pharmacol.* 7: 65-72.

MacLean MR, McCulloch KM, and Baird M. Endothelin ETA and ETB receptor-mediated vasoconstriction in rat pulmonary arteries and arterioles. (1994b). *J. Cardio. Pharmacol.* 23: 838-845.

MacLean MR, McCulloch KM, Baird M. (1995) Effects of pulmonary hypertension on vasoconstrictor responses to endothelin-1 and sarafotoxin S6C and on inherent tone in rat pulmonary arteries. *J. Cardiovas. Pharmacol.* 26: 822-830.

MacLean MR, Sweeney G, Baird M, McCulloch KM, Houslay M, and Morecroft I. (1996) 5-hydroxytriptamine receptors mediating vasoconstriction of pulmonary arteries from control and pulmonary hypertensive rats. *Br J Pharmacol.* 119: 917-930.

MacLean MR, Johnson ED, McCulloch KM, Pooley L, Houslay M, and Sweeney G. (1997) Phosphodiesterase isoforms in the pulmonary arterial circulation of the rat: changes in pulmonary hypertension. *J Pharmacol Exper Ther.* 283: 619-624.

MacLean MR, McCulloch KM. (1998a) Influence of applied tension and nitric oxide on responses to endothelins in rat pulmonary resistance arteries. *Br J Pharmacol.* 123: 991-999.

MacLean MR. (1998b) Endothelin-1: a mediator of pulmonary hypertension? *Pulmonary Pharmacol. Therapeutic.* 11: 125-132.

MacLean MR. (1999a) Enothelin and serotonin in pulmonary hypertension. *J Lab. Clin Med.* 134: 105-114.

MacLean MR. (1999b) Pulmonary hypertension anorexigens and 5HT: pharmacological synergism in action? *Trends Pharmacol Sci.* 20: 490-495.

MacLean MR. (1999c) Role of serotonin in the pathogenesis of acute and chronic pulmonary hypertension. Thorax, 54: 161-168.

MacLean MR, Herve P, Eddahibi S, Adnot S. (2000). 5-hydroxytriptamine and the pulmonary circulation: receptors, transporters and relevance to pulmonary arterial hypertension. *Br. J. Pharmacol.* 131: 161-168.

MacPhee CH, Reifsnyder DH, Moore TA, Lerea KM, Beavo JA. (1988) Phosphorylation results in activation of a cAMP phosphodiesterase in human platelets. *J. Biol. Chem.* 263: 10353.

Makarov SS. (2000). NF-κB as a therapeutic target in chronic inflammation: recent advances. *Molecular Medicine Today.* 6: 441-448.

Manganiello VC, Degerman E, Smith CJ, Vasta V, Tornqvist H, and Belfage P. (1992). Mechanisms for activation of the rat adipocyte particulate cyclic-GMP-inhibited cyclic AMP phosphodiesterase and its importance in the antilipolytic action of insulin. *Adv. Second Messenger Phosphoprotein Res.* 25: 147-164.

Manganiello VC, Murata T, Taira M, Belfrage P, Degerman E. (1995a). Diversity in cyclic nucleotide phosphodiesterase enzyme family. *Arch. Biochem. Biophys.* 322: 1-13.

Manganiello VC, Taira M, Degerman E, Belfrage P. (1995b) Type III cGMP-inhibited cyclic nucleotide phophodiesterase (PDE3 Gene Family). *Cellular Signalling.* 7: 445-455.

Mardon K, Merlet P, Syrota A. (1998). Effect of 5-day hypoxia on cardiac adrenergic neurotransmission in rats. *J Appl Physiol.* 85: 890-897.

Mariano AC, Alexandre GM, Silva LC, Romeiro A, Cameron LC, Chen Y, Chase PB, Sorenson MM. (2001). Dimethyl sulphoxide enhances the effects of P(i) in myofibrils and inhibits the activity of rabbit skeletal muscle contractile proteins. *Biochem J.* 358(Pt 3):627-36.

Marinissen MJ, Gutkind JS. (2001). G-protein-coupled receptors and signalling networks: emerging paradigms. *Trends Pharmacol Sci.* 22(7):368-76.

Marshall CJ. (1995). Specificity of receptor tyrosine kinase signalling: transient versus sustained extracellular signal regulated kinase activation. *Cell.* 80, 179-185.

Martin LD, Barnes SD, Wetzel RC. (1992). Acute hypoxia alters eicosanoid production of perfused pulmonary artery endothelial cells in culture. *Prostaglandins*. 42: 371-482.

Mathew R, Zeballos GA ,Tun. H, Gewitz MH. (1995). Role of nitric oxide and endothelin-1 in monocrotaline-induced pulmonary hypertension. *Cardiovascular Res.* 30: 739-746.

Matsuda N, Morita N, Matsuda K, Watanabe M. (1998). Proliferation and differentiation of human osteoblastic cells associated with differential activation of MAP Kinases in response to epidermal growth factor, hypoxia and Mechanical stress *in vitro. Biochem. Biophysic Res Commun*. 249: 350-354.

Mayr B, Montminy M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol*. 2(8):599-609.

Meacci E, Taira M, Moos M Jr, Smith CJ, Movsesian MA, Degerman E, Belfrage P and Manganiello VC. (1992). Molecular cloning and expression of human myocardial cGMP-inhibited cAMP phosphodiesterase. *Proc Natl Acad Sci USA*. 89:3721-3725.

Medina P, Segarra G, Martinez-Leon JB, Vila JM, Aldasoro M, Otero E, Lluch S. (2000). Relaxation induced by cGMP phosphodiesterase inhibitors sildeafil and zaprinast in human vessls. *Ann Thorac Surg*. 70: 1327-31.

Medina P, Segarra G, Torondel B, Chuan P, Domenech C, Vila JM, Lluch S. (2000). Inhibition of neuroeffector transmission in human vas deferens by sildenafil. *Br J Pharmacol.* 131(5):871-4.

Meyrick B and Reid L. (1978). The effect of continued hypoxia on rat pulmonary arterial circulation: an ultrastructural study. *Lab Invest*. 38: 188-200.

Meyrick B, Gamble W, Reid L. (1980). Development of Crotalaria pulmonary hypertension: hemodynamic and structural study. *Am J Physiol*. 239(5):H692-702.

Meyrick B, and Reid L. (1983) Pulmonary hypertension. Anatomical and physiologic correlates. *Clin Chest Med*. 4: 199-217.

Miagkov AV (1998). NF-κB activation provides the potential link between inflammation and hyperplasia in the arthritic joint. *Pro. Natl. Acad. Sci.* 95: 13859-13864.

Michaeli T, Bloom TJ, Martins T, Loughney K, Ferguson K, Riggs M, Rodgers L, Beavo JA, Wigler M. (1993). Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase-deficient Saccharomyces cerevisiae. *J Biol Chem.* 268(17):12925-32.

Michelakis ED, Reeve HL, Huang JM, Tolarova S, Nelson DP, Weir EK, Archer SL. (1997). Potassium channel diversity in vascular smooth muscle cells. *Can. J. Physiol. Pharmacol.* 75: 889-897.

Michie AM, Lobban M, Muller T, Harnett MM, Houslay MD. (1996). Rapid regulation of PDE2 and PDE4 cyclic AMP phosphodiesterase activity following ligation of the T cell antigen receptor on thymocytes: analysis using the selective inhibitors erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and rolipram. *Cell Signal.* 8(2):97-110.

Miller RC, Pelton JT, Huggins JP. (1993). Endothelins--from receptors to medicine. *Trends Pharmacol Sci.* 14(2):54-60.

Milnor WR, Conti RC, Lewis KB, O-Rourke MD. Pulmonary arterial pulse wave velocity and impedance in man. *Cic Res.* 25: 637-649, 1969.

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

Minneman KP. (1988). α_1-Adrenergic receptor subtypes, inositol phosphates and sources of cell Ca^{2+}. *Pharmacol Rev.* 40:87-119.

Mironov SL, Richter DW. (2000). Intracellular signalling pathways modulate K (ATP) channels in inspiratory brainstem neurones and their hypoxic activation: involvement of metabotropic receptors, G-proteins and cytoskeleton. *Brain Res.* 853(1):60-7.

Montminy M. (1997). Transcriptional regulation by cyclic AMP. *Annu Rev Biochem.* 66:807-22.

Moon E, Lee R, Ner R, Wentairs L, Wolda S, Lerner A. (2002). Inhibition of PDE3B Augments PDE4 inhibitor induced apoptosis in a subset of patients with chronic lymphocytic leukemia. *Clinical Cancer Research.* 8: 587-595

Morecroft I, and MacLean MR. (1998). 5-Hydroxytryptamine receptors mediating vasoconstriction and vasodilation in perinatal and adult rabbit small pulmonary arteries. *Br J Pharmacol.* 125: 69-78.

Moreland RB, Goldstein I, Kim, NN, Traish A. Sildenafil Citrate, a selective phosphodiesterase type V inhibitor: research and clinical implications in erectile dysfunction. *Trends in Endocrinology and Metabolism.* 10: 97-104, 1999.

Morice AH, Pepke-Zaba J, Brown MJ, Thomas PS, Higenbottam TW. (1990). Atrial natriuretic peptide in primary pulmonary hypertension. *Eur Respir J.* 3(8):910-3.

Mork A, and Geiser A. (1991). Tosyl-lysyl chloromethylketone inactivation of adenylate cyclases in separate regions of the rat brain. *Arch Int Physiol Biochim Biophys.* 99: 161-164.

Morrison DF, Rider MA, Takemoto DJ. (1987). Modulation of retinal transducin and phosphodiesterase activities by synthetic peptides of the phosphodiesterase γ-subunit. *FEBS Lett.* 222(2):266-70

Mullaney I, Vaughan, DM, MacLean MM. (1998). Endothelin-1 modulation of cAMP in rat pulmonary arteries: effect of chronic hypoxia. *J. Cardio. Physiol.* 31: S112-S114.

Mullaney I, Vaugh DM, MacLean MR. (2000). Regional modulation of cyclic nucleotides by endothelin-1 in rat pulmonary arteries: direct activation of Gi_2-protein in the main pulmonary artery. *Brit. J. Pharmacol.* 129: 1042-1048.

Muller JE, Mittleman A, Maclure M (1997a). Triggering myocardial infarction by sexual activity: low absolute risk and prevention by regular physical exertion. *JAMA.* 275: 1405-1409.

Muller JM, Krauss B, Kaltschmidt C, Baeuerle PA, Rupec RA. (1997b). Hypoxia induces c-fos transcription via a mitogen-activated protein kinase-dependent pathway. *J Biol Chem*. 272(37):23435-9.

Muramatsu M, Tyler RC, Gutkowska J, Klinger JR, Hill NS, Rodman DM, McMurtry IF. (1997). Atrial natriuretic peptide accounts for increased cGMP in hypoxia-induced hypertensive rat lungs. *Am J Physiol*. 272: L1126-1132.

Muraoka RS, Bushdid PB, Brantley DM, Yull FE, and Kerr LD. (2000). Mesenchymal expression of nuclear factor-κB inhibits epithelial growth and branching in the embryonic chick lung. *Dev Biol*. 225: 322-338.

Murga C, Fukuhara S, Gutkind JS. (1999). Novel Molecular Mediators in the Pathway Connecting G-protein-coupled Receptors to MAP Kinase Cascades. *Trends Endocrinol Metab*. 10(4):122-127.

Muroi M, Suzuki T. (1993). Role of protein kinase A in LPS-induced activation of NF-κB proteins of mouse macrophages-like cell line, J774. *Cell Signal*. 5: 289-98

Murray PA, Lodato RF, Michael JR. (1986). Neural antagonists modulate pulmonary vascular pressure-flow plots in conscious dogs. *J. Appl Physiol.* 60: 1900-1907.

Murray TR, Chen L, Marshall BE, Macarak EJ. (1990a). Hypoxic contraction of cultured pulmonary vascular smooth muscle cells. *Am J Resp Cell Mol Biol*. 3: 457-465.

Murray KJ. (1990b). Cyclic AMP and mechanism of vasodilation. *Pharmacol Ther*. 47: 329-345.

Murray KJ, England PJ, Hallam TJ, Maguire J, Moores K., Reeves ML., Simpson AW. Rink TJ. (1991). The effects of siguazodan, a selective phophodiesterase inhibitor, on human platelet function. *Br. J. Pharmacol*. 99: 612-616.

Murtha YM, Allen BM, Orr JA. (1999). The role of protein kinase C in thromboxane A_2-induced pulmonary artery vasoconstriction. *J Biomed Sci*. 6(4):293-5.

Murthy KS. (2001). Activation of phosphodiesterase 5 and inhibition of guanylate cyclase by cGMP-dependent protein kinase in smooth muscle. *Biochem* J. 360: 199-208.

Murthy KS, Zhou H, and Makhlouf GM. (2001). PKA-dependent activation of PDE3A and PDE4 and inhibition of adenylyl cyclase V/VI in smooth muscle. *Am J Physiol Cell Physiol.* 282: C508-C517.

Mustafa SB, and Olson MS. (1998). Expression of nitric oxide synthase in rat kupffer cells is regulated by cAMP. *J Biol Chem.* 272: 5073-5080.

Naccarelli GV, and Golgstein RA. (1989). Electrophysiology of phosphodiesterase inhibitors. *Am J. Cardio* 63: 35A-40A

Nakaki T, Nakayama M, Yamamoto S, Kato R. (1990). α_1-adrenergic stimulation and β_2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol Pharmacol.* 37(1): 30-6

Nakamura T, Houchi H, Minami A, Sakamoto S, Tsuchiya K, Niwa Y, Minakuchi K, Nakaya Y. (2001). Endothelium-dependent relaxation by cilostazol, a phosphodiesterase III inhibitor, on rat thoracic aorta. *Life Sci.* 69: 1079-1715.

Nakazawa H, Hori M, Ozaki H, Karaki H. (1999). Mechanisms underlying the impairment of endothelium-dependent relaxation in the pulmonary artery of monocrotaline-induced pulmonary hypertension rats. *Br. J. Pharmacol.* 128: 1098-1104.

Needham L and Houslay MD. (1988). Tosyl-lysyl chloromethylketone detects conformational changes in the catalytic unit of adenylate cyclase induced by receptor and G-protein stimulation. *Biochem Biophys Res Commun.* 156: 855-859.

Nichols WC, Koller KD, Solis B, Foroud T, Terry VH, Arnold ND, Siemieniak DR, Wheeler L, Philips JA, Newman JH, Conneally PM, Ginsburg D, Lyd JE. (1997). Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31-32. *Nature Gen.* 15: 277-280.

Nicholson CD, Shahid M, Bruin J, Barron E, Spiers I, de Boer J, van Amsterdam RG, Zaagsma J, Kelly JJ, Dent G. (1995). Characterization of ORG 20241, a combined phosphodiesterase IV/III cyclic nucleotide phosphodiesterase inhibitor for asthma. *J Pharmacol Exp Ther*. 274(2):678-87.

Nishida M, Maruyama Y, Tanaka R, Kontani K, Nagao T, Kurose H. (2000). $G_{\alpha}i$ and $G_{\alpha}o$ are target proteins of reactive oxygen species. *Nature*. 408(6811):492-5.

Norton AW, D'Amours MR, Grazio HJ, Herbert TL, Cote RH. (2000). Mechanism of transducin activation of frog rod photoreceptor phosphodiesterase. Allosteric interactions between the inhibitory gamma subunit and the noncatalytic cGMP-binding sites. *J Biol Chem*. 275: 38611-9.

Nunokawa Y, Ishida N, Tanaka S. (1993). Cloning of inducible nitric oxide synthase in rat vascular smooth muscle. *Biochem Biophys Res Commun*. 191: 89-94.

O'Connell MA, Bennett BL, Mercurio F, Manning AM, Mackman N. (1998). Role of IKK1 and IKK2 in lipopolysaccharide signalling in human monocytic cells. *J Biol Chem*. 273(46):30410-4.

Ohnishi M, Oka M, Muramatsu M, Sato K, Kira S, and Fukuchi Y. (1999). E4021, A selective phosphodiesterase 5 inhibitor, potentiates the vasodilator effect of inhaled nitric oxide in isolated perfused rat lungs. *J Cardiovasc. Pharmacol*. 33: 619-624.

Oka M, Hasunuma K, Webb SA, Stelzner TJ, Rodman DM, and McMurtry IF. (1993) EDRF suppresses an unidentified vasoconstrictor mechanism in hypertensive lungs. *Am J. Physiol. Lung*. 264: L587-L597.

Oka M, (2001) Phosphodiesterase 5 inhibition restores impaired ACh relaxation in hypertensive conduit pulmonary arteries. Lung. *Cell. Mol. Pharmacol*. 280: L432-435.

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

Osiniski MT, and Schror K. (2000). Inhibition of platelet-derived growth factor-induced mitogenesis by phosphodiesterase 3 inhibitors. *Biochem Pharmacol.* 60: 381-387.

Osinski MT, Rauch BH, and Schror K. (2001). Antimitogenic actions of organic nitrates are potentiated by sildenafil and mediated via activation of protein kinase A. *Mol Pharmacol.* 59: 1044-1050.

Osipenko ON, Alexander D, MacLean MR, and Gurney AM. (1998). Potassium channels in pulmonary hypertension. *Br J. Pharmacol* 124: 1335-1337

Pahl HL. (1999) Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* 18: 6853-6866.

Palmer D, Maurice DH. (2000). Dual expression and differential regulation of phosphodiesterase 3A and phosphodiesterase 3B in human vascular smooth muscle: Implications for phosphodiesterase 3 inhibition in human cardiovascular tissue. *Mol Pharm.* 58: 247-252.

Palmer LA, Semenza GL, Stoler MH, and Johns RA. (1998) Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. *Am J Physiol Lung Cell Mol Physiol.* 274:L212-L219.

Pan X, Arauz E, Krzanowski JJ, Fritzpatrick DF, and Polson JB. (1994). Synergistic interactions between selective pharmacological inhibitors of phosphodiesterase isozyme families PDE III and PDEIV to attenuate proliferation of rat vascular smooth muscle cells. *Biochem. Pharmacol.* 48: 827-835.

Parker M, Carver JR, Rodeheffer RJ, Ivanhoe RJ, DiBianco R, Zeldis SM, Hendrix GH, Bommer WJ, Elkayam U, Kukin ML, Mallis GI, Sollano JA, Shannon J, Tandon PK, and DeMets DL. (1991). Effect of oral milrinone on mortality in severe chronic heart failure. The PROMISE study research group. *N. Engl J Med.* 325: 1468-1475.

Pasricha PJ, Hassoun PM, Teufel E, Landman MJ, Fanburg BL. (1992). Prostaglandins E1 and E2 stimulate the proliferation of pulmonary artery smooth muscle cells. *Prostaglandins.* 43(1): 5-19.

Peng W, Karwande SV, Hoidal JR, Farrukh IS. (1996). Potassium currents in cultured human pulmonary artery smooth muscle cells. *J. Appl. Physiol.* 80: 1187-1196.

Perkett EA, Badesch DB, Roessler MK. Stenmark KR, Meyrick B. (1992). Insulin-like growth factor I and pulmonary hypertension induced by continuous air embolization in sheep. *Am J Respir Cell Mol Biol.* 6: 82-87.

Perutz MF, Paoli M, Lesk AM. (1999). Fix L, a haemoglobin that acts as an oxygen sensor: signalling mechanism and structural basis of its homology with PAS domains. *Chem Biol.* 6(11):R291-7.

Peterson MB, Huttemeier PC, Zapol WM, Martin EG, Watkins WD. (1982). Thromboxane mediates acute pulmonary hypertension in sheep extracorporeal perfusion. *Am J Physiol.* 243(3):H471-9.

Phillips PG, Long L, Wilkins MR, Morrell NW. (2000). The type 3 phosphodiesterase inhibitor, cilostimide, attenuates acute and chronic hypoxia-induced pulmonary hypertension. *Thorax.* 55 (3): P42, A35.

Piaz VP, Giovannoni MP. (2000). Phosphodiesterase 4 inhibitors, structurally unrelated to Rolipra, as promising agents for the treatment of asthma and other pathologies. *Eur. J. Med. Chem.* 35: 463-480.

Pickering TG, Case DB, Sullivan PA, Laragh JH. (1982). Comparison of antihypertensive and hormonal effects of captopril and propranolol at rest and during exercise. *Am J Cardiol.* 21;49(6):1566-8.

Piene H. (1976). The influence of pulmonary blood flow rate on vascular input impedance and hydraulic power in the sympathetically and noradrenaline stimulated cat lung. *Acta Physiol Scand.* 98(1): 44-53.

Pinsky D, Ox M, Liao H, Morris S, Brett J, Sciacca R, Karakurum M, Van Lookeren Campagne M, Platt J and Norwygrod R. (1993) Restoration of the cAMP second messenger pathway enhances cardiac preservation for transplantation in a heterotrophic rat model. *J. Clin. Invest.* 92: 2994-3002.

Plane F, Garland CJ. (1996). Influence of contractile agonists on the mechanism of endothelium-dependent relaxation in rat isolated mesenteric artery. *Br J Pharmacol.* 119(2):191-3.

Polson JB, and Strada SJ. (1996). Cyclic nucleotide phosphodiesterases and vascular smooth muscle. Chronic hypoxic pulmonary hypertension. Cell biology to pathophysiology. *Annu. Rev. Pharmacol. Toxicol.* 36: 403-427..

Porcelli RJ, Bergofsky EH. (1973). Adrenergic receptors in pulmonary vasoconstrictor responses to gaseous and humoral agents. *J Appl Physiol.* 34: 483-488.

Post JM, Hume JR, Archer SL, Weir EK. (1992). Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. *Am. J. Physiol.* 262: C882-C890.

Premkumar DR, Adhikary G, Overholt JL, Simonson MS, Cherniak NS, Prabhakar NR. (2000). Intracellular pathways linking hypoxia to activation of *c-fos* and *AP-1*. *Adv. Exp. Med. Biol.* 475: 101-109.

Pyne NJ, Burns F. (1993). Lung phosphodiesterase isoenzymes. *Agents Actions Suppl.* 308:191-197.

Pyne NJ, Tolan D, Pyne S. (1997). Bradykinin stimulates cAMP synthesis via mitogen-activated protein kinase-dependent regulation of cytosolic phospholipase A_2 and prostaglandin E_2 release in airway smooth muscle. *Biochem J.* 328 (Pt 2):689-94.

Rabe KF, Tenor H, Dent G, Schudt C, Liebig S, Magnussen H, (1993). Phosphodiesterase isozymes modulating inherent tone in human airways;: identification and characterization. Am J. Physiol. 264: L458-L464.

Rabe KF, Tenor H, Dent G, Schudt C, Nakashima M, and Magnussen H. (1994). Identification of PDE isozymes in human pulmonary artery and effect of selective PDE inhibitors. *Am J Physiol.* 266:L536-L543.

Rabinovitch M, Gamble W, Nadas AS, Meittinen O, Reid L. (1979). Rat pulmonary circulation after chronic hypoxia: haemodynamic and structural features. *Am. J. Physiol.* 236: H818-H827.

Rabinovitch M. (1996). Cell-extracellular matrix interactions in the ductus arteriosus and perinatal pulmonary circulation. *Semin Perinatol.* 20(6):531-41.

Rabinovitch M. (1997). Pulmonary hypertension: updating a mysterious disease. *Cardio. Res.* 34: 268-272.

Raeburn D, Karlsson JA. (1992). Comparison of the effects of isoenzyme-selective phosphodiesterase inhibitors and theophylline on PAF-induced plasma leak in guinea-pig airways in vivo. *Am Rev Respir Dis.* 145: A612.

Rakhit S, Pyne S, Pyne NJ. (2000). The platelet-derived growth factor receptor stimulation of p42/p44 mitogen-activated protein kinase in airway smooth muscle involves a G-protein-mediated tyrosine phosphorylation of Gab1. *Mol Pharmacol.* 58(2):413-20.

Rascon A, Lindgren S, Stavenow L, Belfrage P, Andersson KE, Manganiello VC, and Degerman E. (1992). Purification and properties of the cGMP-inhibited cAMP phosphodiesterase from bovine aortic smooth muscle. *Biochim. Biophys. Acta.* 1134: 149-156.

Raychaudhuri B, Dewik R, Connors MJ (1999). Nitric oxide blocks NF-κB activation in alveolar macrophages. *Am J Respir Cell Mol Biol.* 21: 311-316.

Reeve HL, Archer SL, Weir EK. (1997). Ion channels in the pulmonary vasculature. *Pulmonary Pharmacol and Therapeutics.* 10: 243-252.

Reinhardt RR, Chin E, Zhou J, Taira M, Murata T, Manganiello VC and Bondy CA. (1995). Distinctive anatomical patterns of gene expression for cGMP-inhibited cyclic nucleotide phosphodiesterase. *J Clin Invest.* 95: 1528-1538. .

Resta TC, and Walker BR. (1992). Chronic hypoxia selectively augments endothelium-dependent pulmonary arterial vasodilation. *Am J Physiol Heart Circ Physiol.* 263: L88-L94.

Resta TC, Gonzales RJ, Dail WG, Sanders TC, Walker BR. (1997). Selective upregulation of arterial endothelial nitric oxide synthase in pulmonary hypertension. *Am J Physiol.* 272(2 Pt 2):H806-13.

Rhoades RA, Packer CS, Roepke DA, Jin N, Meiss RA. (1990). Reactive oxygen species alter contractile properties of pulmonary arterial smooth muscle. *Can J Physiol* Pharmacol. 68(12):1581-9.

Rich S, and Kaufmann E. (1991). High dose titration of calcium channel blocking agents for primary pulmonary hypertension: guidelines for short-term drug testing. *J Am Coll Cardiol.* 18: 1323-1327.

Richard DE, Berra E, Gothie E, roux D, Pouyssegur J. (1999) p42/p44 mitogen-activated protein kinase phosphorylate hypoxia-inducible factor 1α (HIF-1α) and enhances the transcriptional activity of HIF-1. *J. Biol. Chem.* 274: 32631-32637.

Rimer S, and Gillis CN. (1993). Site of pulmonary vasodilation by inhaled nitric oxide is due to haemoglobin inactivation. *Circulation.* 88: 2884-2887.

Rimar S, Gillis CN. (1995). Site of pulmonary vasodilation by inhaled nitric oxide in the perfused lung. *J Appl Physiol.* 78(5):1745-9.

Rodeberg DA, Cheet MS, Bass RC, Ardovitz MS Garcia VF. (1995). Nitric Oxide: an overview. *Am J. Surg.* 170: 292-303.

Roesler WJ. (2000). What is a cAMP response unit? *Mol Cell Endocrinol.* 25;162(1-2):1-7.

Rosenberg and Rabinovitch. (1988). Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. *Am. J. Physiol.* 255: H1484-H1491.

Rotella DP, Sun Z, Zhu YH, Krupinski J, Prongrac R, Seiliger L, Normandin D, Macor JE. (2000). N-3-sustituted imidazoquinazoliones: potent and selective PDE5 inhibitors as potential agents for treatment of erectile dysfunction. *J. Med Chem.* 43: 1257-1263, 2000.

Rothman A, Wilner B, Button D, Taylor P. (1994). Immediately-early gene expression in response to hypertrophy and proliferative stimuli in pulmonary arterial smooth muscle cells. *J. Biol Chem.* 269: 6399-6404.

Rothman A, Wolner B, Button D, Taylor. (1994). Immediate early gene expression in response to hypertrophic and proliferative stimuli in pulmonary arterial smooth muscle cells. *J Biol Chem.* 269: 6399-6404.

Rubanyi GM, Polokoff. (1994) Endothelins: Molecular biology, biochemistry, Pharmacology, Physiology and Pathophysiology. *Pharmacol. Rev.* 46: 325-413.

Rubin LJ. (1997). Current concepts: Primary pulmonary hypertension. *New Eng. J. Med.* 336: 111-117.

Rudarakanchana N, Trembath RC, Morrell NW. (2001). New insights into the pathogenesis and treatment of primary pulmonary hypertension. *Thorax.* 56: 888-890.

Rybalkin SD, Rybalkina I, Beavo JA, Bornfeldt KE. (2002). Cyclic nucleotide phosphodiesterase 1C promotes human arterial smooth muscle cell proliferation. *Circ. Res.* 90: 151-157.

Saeki T, Adachi H, Takase Y, Yoshitake S, Souda S, Saito I. (1995) A selective type V phosphodiesterase inhibitor, E4021, dilates porcine large coronary artery. *J. Pharmacol Exp Ther.* 272: 825-831.

Sakou T. (1998). Bone morphogenetic proteins: from basic studies to clinical approaches. *Bone.* 22(6):591-603.

Sampson LJ, Hinton JM, Garland CJ. (2001). Evidence for the expression and function of phosphodiesterase type 5 (PDE-V) in rat resistance arteries. *Br J Pharmacol.* 132:13-17.

Sanchez LS, De La Monte SM, Filippov G, Jones RC, Zapol WM, Bloch KD. (1998). Cyclic-GMP-binding, cyclic-GMP-specific phosphodiesterase (PDE5) gene expression is regulated during rat pulmonary development. *Pediatr Res.* 43: 163-168.

Sasaki S, Kobayashi N, Dambara T, Kira S, Sakai T. (1995), Structural organisation of pulmonary arteries in the rat lung. *Anat Embryol.* 191: 477-489.

Sato K, Oka M, Husunuma K, Ohnishi M, Sato K, Kira S. (1995). Effects of separate and combined ETA and ETB blockade on ET-1-induced constriction in perfused rat lungs. *Am J Physiol.* 269: L668-L672.

Schermuly RT, Ghofrani HA, Enke B, Weissmann N, Grimminger F, Seeder W, Shudt C, Walmrath D. (1999). Low-dose systemic phosphodiesterase inhibitors amplify the pulmonary vasodilatory response to inhaled prostacyclin in experimental pulmonary hypertension. *Am J Respir Crit Care Med.* 160: 1500-1506.

Schermuly RT, Roehl A, Weissmann N, Ghofrani HA, Schudt C, Tenor H, Grimminger F, Seeger W, and Walmrath D (2000). Subthreshold doses of specific phosphodiesterase type 3 and 4 inhibitors enhance the pulmonary vasodilatory response to nebulized prostacyclin with improvement in gas exchange. *J. Pharmacol. Exp. Ther.* 292: 512-520.

Schini-Kerth VB, Boese M, Busse R, Fisslthaler, Mulsch A. (1997). N-α-Tosyl-L-Lysine chloromethylketone prevents expression of iNOS in vascular smooth muscle by blocking activation of NF-κB. *Athero. Thromb. Vasc. Biol.*, 17: 672-679.

Schmidt HH, Lohmann SM, Walter U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim Biophys Acta.* 1178(2):153-75.

Schudt CHR, Tenor H, Wendel A, Rabe K, Loos U, Mallmann P Szamel M, Resch K. (1992). Effect of selective phosphodiesterase (PDE) inhibitors on activation of human macrophages and lymphocytes. *Naunyn Schmiedebergs Archs Pharmacol.* 345: 92.

Schulze-Osthoff K, Ferrari D, Riehemann K, Wesselborg S. (1997). Regulation of NF-κB activation by MAP kinase cascades. *Immunobiology.* 198(1-3):35-49.

Schwartz JH. (2001). The many dimensions of cAMP signalling. *Proc Natl Acad Sci.* 98(24):13482-4.

Schwede F, Maronde E, Genieser HG, and Jastorff B. (2000). Cyclic nucleotide analogs as biochemical tools and prospective drugs. *Pharm. Ther.* 87:199-226.

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

Scott JD, Dell'Acqua ML, Fraser ID, Tavalin SJ, Lester LB. (2000). Coordination of cAMP signalling events through PKA anchoring. *Adv Pharmacol.* 47:175-207.

Seger R, Krebs EG. (1995). The MAPK signalling cascade. *FASEB J.* 9(9):726-35.

Seiler KU, Wassermann O, and Wensky H. (1976). On the role of serotonin in the pathogenesis of pulmonary hypertension induced by anorectic drugs: an experimental study in the isolated perfused rat lung. *Clin Exp. Pharmcol. Physiol.* 3: 323-330.

Seko Y, Tobe K, Ueki K, Kadowaki T, Yazaki Y. (1996). Hypoxia and hypoxia/reoxygenation activate Raf-1. mitogen-activated protein kinase kinase, mitogen-activated protein kinases, S6 kinase in cultured rat cardiac myocytes. *Circ Res.* 78: 82-90.

Semenza GL. (1996) Transcriptional regulation by hypoxia-inducible factor-1: molecular mechanisms of oxygen homeostasis. *Trends Cardiovascular Med.* 6: 151-157.

Semenza GL, Jiang BH, Leung SW, Passantino R, Concorde JP, Maire P and Gillongo A. (1996) Hypoxia response elements in the aldose A, enolase 1, and lactate dehydrogenase A gene contain essential binding sites for hypoxia-inducible factor 1. *J. Biol Chem.* 271: 32529-32537.

Serkkola E, Hurme M. (1993). Activation of NF-κB by cAMP in human myeloid cells. *FEBS Lett.* 334: 327-30.

Seta KA, Kim H, Millhorn DE, Beitner-Johnson D. (2001). Hypoxia-induced regulation of MAPK phosphatase-1 as identified by subtractive suppression hybridisation and cDNA microarray analysis. *J. Biol. Chem.* 276: 44405-44412.

Sette C, Conti M. (1996). Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation. *J Biol Chem*. 271(28):16526-34.

Seybold J, Newton R, Wright L, Finney PA, Suttorp N, Barnes PJ, Adcock IM, and Giembycz MA. (1998). Induction of phosphodiesterase 3B, 4A4, 4D1, 4D2 and 4D3 in Jurkat T cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and Gs coupled receptor antagonists. *J Biol Chem*. 273: 20575-20588.

Sharma RK, and Wang JH. (1984). Calmodulin and Ca2+-dependent phosphorylation and dephosphorylation of the 63-kDa subunit-containing bovine brain calmodulin-stimulated cyclic nucleotide phosphodiesterase isozyme. *J. Biol. Chem*. 259: 9248-9254.

Shaul PW, Muntz KH, DeBeltz D, and Buja LM. (1990). Effects of prolonged hypoxia on adenylate cyclase activity and beta-adrenergic receptors in pulmonary and systemic arteries of the rat. *Circ Res*. 66: 1526-1534.

Shaul PW, Kinane B, Farar MA, Buja LM, and Magness RR. (1991) Prostacyclin production and mediation of adenylyl cyclase activity in the pulmonary artery. Alterations after prolonged hypoxia in the rat. *J. Clin. Invest*. 88: 447-455.

Shaul PW, Lieselotte B, Wells B, and Horning KM. (1993). Acute and prolonged hypoxia attenuate endothelial nitric oxide production in rat pulmonary arteries by different mechanisms. *J. Cardio Pharmacol*. 22: 819-827.

Shaul PW, North AM, Brannon TS, Ujiie K, Wells LB, Nisen PA, Lowenstein CJ, Snyder SH, Star RA. (1995). Prolonged in vivo hypoxia enhances nitric oxide synthase type I and type II gene expression in adult rat lung *Am J Respir Cell Mol Biol*. 13: 167-174.

Shaywitz AJ, and Greenberg ME. (1999). CREB: A stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev. Biochem*. 68: 821-861.

Shen YH, Wang XL, Wilcken DE. (1998). Nitric oxide induces and inhibits apoptosis through different pathways. *FEBS Lett.* 433(1-2):125-31.

Sheng M, McFadden G, Greenberg ME. (1990). Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. *Neuron.* 4(4): 571-82.

Sherman TS, Chen Z, Yuhanna IS, Lau KS, Margraf LR, Shaul PW. (1999). Nitric oxide synthase isoform expression in the developing lung epithelium. *Am J Physiol.* 276(2 Pt 1):L383-90.

Shimojo T, Hiroe M, Ishiyama S, Ito H, Nishikawa T, Marumo F. (1999). Nitric oxide induces apoptotic death of cardiomyocytes via a cyclic-GMP-dependent pathway. *Exp Cell Res.* 247(1):38-47.

Shirotani M, Yui Y, Hattori R, Kawai C. (1991). U-61431F, a stable prostacyclin analogue, inhibits the proliferation of bovine vascular smooth muscle cells with little antiproliferative effect on endothelial cells. *Prostaglandins.* 41(2):97-110.

Siebenlist U, Franzoso G, Brown K. (1994). Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol.* 405-55.

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

Skiba NP, Artemyev NO, Hamm HE. (1995). The carboxyl terminus of the gamma-subunit of rod cGMP phosphodiesterase contains distinct sites of interaction with the enzyme catalytic subunits and the α-subunit of transducin. *J Biol Chem.* 270(22):13210-5.

Slepak VZ, Artemyev NO, Zhu Y, Dumke CL, Sabacan L, Sondek J, Hamm HE, Bownds MD, Arshavsky VY. (1995). An effector site that stimulates G-protein GTPase in photoreceptors. *J Biol Chem.* 270(24):14319-24.

Soderling SH, Bayuga SJ, and Beavo JA. (1998a). Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. *Proc Natl Acad Sci.* 95: 8991-8996, 1998.

Soderling SH, Bayuga SJ, and Beavo JA. (1998b). Identification and characterisation of a novel family of cyclic nucleotide phosphodiesterases. *J. Biol Chem.* 273: 15553-15558.

Sodering SH, Bayuga SJ, and Beavo JA. (1999). Isolation and characterisation of a dual-substrate phosphodiesterase gene family: PDE10A. *Proc Natl Acad Sci.* 96: 7071-7076,

Sodering SH, and Beavo JA. (2000). Regulation of cAMP and cGMP signalling: new phosphodiesterases and new functions. *Curr. Opin. Cell Biol.* 12: 174-179.

Soff GA, Cornwell TL, Cundiff DL, Gately S, Lincoln TM. (1997). Smooth muscle cell expression of type I cyclic GMP-dependent protein kinase is suppressed by continuous exposure to nitrovasodilators, theophylline, cyclic GMP, and cyclic AMP. *J Clin Invest.* 100(10):2580-7.

Souness JE, Hassall GA, Parrott DP. (1992). Inhibition of pig aortic smooth muscle cell DNA synthesis by selective type III and type IV cyclic AMP phosphodiesterase inhibitors. *Biochem Pharmacol.* 44: 857-866.

Southgate K and Newby AC. (1990). Serum-induced proliferation of rabbit aortic smooth muscle cells from the contractile state is inhibited by 8-Br-cAMP but not 8-Br-cGMP. *Atherosclerosis.* 82: 113-123.

Spence S, Rena G, Sweeney G, Houslay MD. (1995). Induction of Ca2+/calmodulin-stimulated cyclic AMP phosphodiesterase (PDE1) activity in Chinese hamster ovary cells (CHO) by phorbol 12-myristate 13-acetate and by the selective overexpression of protein kinase C isoforms. *Biochem J.* 310:975-82

Steif CG, Uckert S, Becker AJ, Truss MC, and Jonas U. (1998). The effect of the specific phosphodiesterase PDE inhibitors on human and rabbit cavernous tissue *in vitro* and *in vivo*. *J Urol.* 159: 1390-1395.

Stelzner TJ, O'Brien RF, Yanagisawa M, Sakurai T, Sato K, Webb S, Zamora M, McMurtry IF, Fisher JH. (1992). Increased lung endothelin-1 production in rats with idiopathic pulmonary hypertension. *Am J Physiol.* 262: L614-20.

Stewart DJ, Levy RD, Ceracek P, Langleben D. (1991). Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease. *Ann Intern Med.* 114: 464-468.

Stewart AG, Sheedy W, Thompson JS, Morice AH. (1992). Effects of SCH 34826, a neutral endopeptidase inhibitor, on hypoxic pulmonary vascular remodelling. *Pulm Pharmacol.* 5(2):111-4.

Strasser RH, Ihl-Vahl R, Marquetant R. (1992). Molecular biology of adrenergic receptors. *J Hypertens.* 10(6):501-6.

Stryer L. Visual transduction. *J. Biol. Chem.* 266: 10711-10714, 1991.

Sumner MJ, Humphrey PP. (1990). Sumatriptan (GR43175) inhibits cyclic-AMP accumulation in dog isolated saphenous vein. *Br J Pharmacol.* 99(2):219-20.

Sun H, Charles CH, Lau LF, Tonks NK. (1993). MKP-1 (3CH134), an immediate early gene product is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell.* 75: 487-493.

Sundeep SS. (1999). α_1-Adrenergic hypothesis for pulmonary hypertension. *CHEST.* 115:1708-1719.

Suzuki H, and Twarog BM. (1982). Membrane properties of smooth muscle cells in pulmonary hypertensive rats. *Am J. Physiol.* 242: H907-H915.

Sweeney G, Templeton A, Clayton RA. Baird M, Sheridan S, Johnston ED, and MacLean MR. (1995). Contractile response to sumatriptan in isolated bovine pulmonary artery rings: relationship to tone and cyclic nucleotide levels. *Br. J. Pharmacol.* 26: 751-760.

Swinnen JV, Joseph DR and Conti M. (1989). The mRNA encoding a high-affinity cAMP phosphodiesterase is regulated by hormones and cAMP. *Proc. Natl. Acad. Sci.* 86: 8197-8201.

Taira M, Hocjman SC, Calvo JC, Taira M, Belfrage P and Manganiello VC. (1993). Molecular cloning of the rat adipocyte hormone-sensitive cyclic GMP-inhibited cyclic nucleotide phosphodiesterase. *J Biol Chem.* 268: 18573-18579.

Takahashi T, Kanda T, Inoue M, Suzuki T, Kobayashi I, Kodama K, Nagai R. A (1996) selective type V phosphodiesterase inhibitor, E4021, protects the development of right ventricular overload and medial thickening of pulmonary arteries in a rat model of pulmonary hypertension. *Life Sci.* 59: PL371-PL377.

Takahashi T, Kanda T, Sumino H, Inoue M, Sato K, Sakamaki T, Kobyashi I, Iwamoto A, and Nagai R. (1998). Type V phosphodiesterase inhibition modulates endogenous immunoreactivities of endothelin-1 and endothelial nitric oxide synthase in pulmonary arteries in rats with monocrotaline-induced pulmonary hypertension. *Res Exp Med.* 197: 319-328.

Takei K, Mundigl O, Daniell L, De Camilli P. (1996). The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. *J Cell Biol.* 133(6):1237-50.

Takizawa T, Hara Y, Saito T, Masuda Y, Nakaya H. (1996). α_1-Adrenoceptor stimulation partially inhibits ATP-sensitive K^+ current in guinea pig ventricular cells: attenuation of the action potential shortening induced by hypoxia and K^+ channel openers. *J Cardiovasc Pharmacol.* 28(6): 799-808.

Tamm M, Bihl M, Eickelberg O, Stulz P, Perruchoud AP, and Roth M. (1998). Hypoxia-induced interleukin-6 and interleukin-8 production is mediated by platelet-activating factor and platelet-derived growth factor in primary human lung cells. *Am J Respir Cell Mol Biol.* 19: 653-661.

Tate R, Lochhead A, Brzeski H, Arshavsky VY, and Pyne NJ. (1998). The γ subunit of the rod photoreceptor cGMP-binding cGMP-specific PDE is expressed in lung. *Cell Biochem Biophys.* 29: 133-144.

Tate RJ, Arshavsky VY, Pyne NJ. (2001). The identification of the inhibitory γ subunit of the type 6 retinal cyclic GMP phosphodiesterase in non-retinal tissues: Differential processing of mRNA transcripts. *Genomics.* 79: 582-586.

Taylor CT, Furuta GT, Synnestvedt K, Colgan SP. (2000). Phosphorylation-dependent targeting of cAMP response element binding protein to the ubiquitin/proteasome pathway in hypoxia. *Proc Natl Acad Sci.* 97(22):12091-6.

Tenor H, Hatzelmann A, Church MK, Schudt C, Shute JK. (1996). Effects of theophylline and rolipram on leukotriene C4 (LTC4) synthesis and chemotaxis of human eosinophils from normal and atopic subjects. *Br J Pharmacol.* 118(7):1727-35.

Terragno NA and Terragno A. (1977). Role of naturally occurring vasoactive principles in hypertension. State of the art. *Mayo Clin Proc.* 52(7):449-58.

Thanos D, Maniatis T. (1995) NF-κB. A lesson in family values. *Cell.* 80: 529-532.

Thebaud B, Saizou C, Farnoux C, Hartman JF, Mercier JC. (1999). Dypiridamole, a cGMP phosphodiesterase inhibitor, transiently improves the response to inhaled nitric oxide in two newborns with congenital diaphragmatic hernia. *Intensive Care Med.* 25(3): 300-303.

Thomas MK, Francis SH, and Corbin JD. (1990). Substrate- and kinase-directed regulation of phosphorylation of a cGMP-binding phosphodiesterase by cGMP. *J Biol Chem.* 265: 14964-1470.

Thompson WJ, and Appleman MM. (1979). Assay of cyclic nucleotide phosphodiesterase and resolution of multiple forms of the enzyme. *Adv. Cyclic Nucl. Res.* 10:69-92.

Thompson WJ. (1991). Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function. *Pharmacol Ther.* 51:13-33.

Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JS, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, Nichols

WC. (2000). Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-β family. *J Med Genet*. 37(10):741-5.

Torphy TJ, Zhou HL, and Cieslinski LB. Stimulation of beta adrenoreceptors in a human monocyte cell line (U937) up-regulates cyclic AMP-specific phosphodiesterase activity. *J. Pharmacol. Exp. Ther*. 263: 1195-1205, 1992.

Torphy TJ, Undem BJ, Cieslinski LB, Luttmann MA, Reeves ML, Hay DWP. Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle. *J. Pharmacol. Exp. Ther*. 265: 1231-1223, 1993.

Towbin H, Staehelin T, Gordon J. Electrophoresis Transfer of proteins from polyacylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Nat Acad Sci*. 76: 4350-4354, 1979.

Traverse JH, Chen YJ, Du R, Bache RJ. (2000). Cyclic nucleotide phosphodiesterase type 5 activity limits blood flow to hypoperfused myocardium during exercise. *Circulation*. 102(24): 29997-3002.

Troncy E, Francoeur M, Blaise G. (1997). Inhaled nitric oxide: clinical applications, indications, and toxicology. *Can J. Anaesth*. 44: 973-988.

Tsang SH, Gouras P, Yamashita CK, Kjeldbye H, Fisher J, Faber DB, Goff SP (1996) Retinal degeneration in mice lacking the γ subunit of the rod cGMP phosphodiesterase. *Science*. 272: 1026-1029.

Tuder RM, Flook BE, Voelkel NF. (1995). Increased gene expression for VEGF and VEGF receptors KDR/Flt in lungs exposed to acute and chronic hypoxia. Modulation of gene expression by nitric oxide. *J Clin Invest*. 95: 1798-1807.

Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D, Voelkel NF. (1999). Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med*. 159(6):1925-32.

Turko IV, Francis SH, Corbin JD. (1998). Binding of cGMP to both allosteric sites of cGMP-binding cGMP-specific phosphodiesterase (PDE5) is required for its phosphorylation. *Biochem J.* 329: 505-510.

Turko IV, Ballard SA, Francis SH, Corbin JD. (1999). Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. *Mol. Pharmacol.* 56: 124-130.

Udovichenko IP, Cunnick J, Gonzalez K, Takemoto DJ. (1994). Functional effect of phosphorylation of the photoreceptor phosphodiesterase inhibitory subunit by protein kinase C. *J Biol Chem.* 269(13):9850-6.

Underwood DC, Bochnowicz S, Osborn RR, Louden CS, Hart TK, Ohlstein EH, Hay DWP (1998). Chronic hypoxia-induced cardiopulmonary changes in three rat strains: inhibition by the ET receptor antagonist SB217242. *J. Cardiovasc. Pharm.* 31: S453-S455.

Urestsky BF, Jessup M, Konstam MA (1990). Multicenter trial of oral enoximone in patients with moderate to moderately severe congestive heart failure. Lack of benefit compared to placebo. *Circulation.* 82: 774-80.

Van Biesen T, Hawes BE, Luttrell D, Kreuger KM, Touhara K, Profiri E, Sakaue M, Luttrell LM, and Lefkowitz RJ. (1995) Receptor tyrosine kinase and Gβγ mediated MAPK activation by a common signalling pathway. *Nature*. 376, 781-784.

Van Der Zypp A, Retchtman M, Majewski H. (2000). The role of cyclic nucleotides and calcium in the relaxation produced by amrinone in rat aorta. *Gene Pharmacol.* 34: 245-253.

Vender RL. (1994). Chronic hypoxic pulmonary hypertension, cell biology to pathophysiology. *Chest.* 106: 236-243.

Venkatesh K, Gopal, Sharron H, Francis and Corbin J. (2001) Allosteric sites of phosphodiesterase-5 (PDE5). A potential role in negative feedback regulation of cGMP signalling in corpus cavernous. *Eur. J. Biochem.* 268: 3304-3312.

Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev.* 9(22):2723-35.

Veyssier-Belot C and Cacoub P. (1999) Role of endothelial and smooth muscle cells in the physiopathology and treatment of pulmonary hypertension.. *Cardiovascular Res.* 44: 274-282.

Vieira AV, Lamaze C, Schmid SL. (1996). Control of EGF receptor signalling by clathrin-mediated endocytosis. *Science.* 274(5295):2086-9.

Voelkel NF, Hegstrand L, Reeves JT, McMurty IF, Molinoff PB. (1981). Effects of hypoxia on density of β-adrenergic receptors. *J Appl. Physiol.* 50, 363-366.

Voelkel NF. (1986). Mechanisms of hypoxic pulmonary vasoconstriction. *Am Rev Respir Dis.* 133(6):1186-95.

Voelkel NF, Hoeper M, Maloney J, Tuder RM. (1996) Vascular endothelial growth factor in pulmonary hypertension. *Ann N Y Acad Sci.* 796: 186-193.

Voelkel NF, Tuder RM, Weir EK. (1997). Pathophysiology of primary pulmonary hypertension: from physiology to molecular mechanisms. Rubin LK and Rich S (Eds), Primary Pulmonary Hypertension (p83-129). New York: Marcel Dekker Inc.

Voelkel NF. (1997). Appetite suppressants and pulmonary hypertension. *Thorax.* 52:S63-7.

Voelkel NF, Tuder RM. (2000). Hypoxia-induced pulmonary vascular remodelling: a model for what human disease?. *J Clin Invest.* 106: 733-738.

Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ. (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell.* 89(1):73-82.

Wagner RS, Smith CJ, Taylor AM, Rhoades RA. (1997). Phosphodiesterase inhibition improves agonist-induced relaxation of hypertensive pulmonary arteries. *J Pharmacol and Exp Ther.* 282 (3): 1650-1657.

Wan K-F, Sambi B, Frame M, Tate R, and Pyne NJ. (2001) The inhibitory gamma subunit of the type 6 retinal cyclic guanosine monophosphate phosphodiesterase is a novel intermediate regulating p42/p44 mitogen-activated protein kinase signalling in human embryonic kidney 293 cells. *J Biol Chem.* 276: 37802-37808.

Wang CY, Aronson I, Takuma S, Homma S, Nak Y, Alshafie T, Brovkovych V, Malinski T, Oz MC, Pinsky DJ. (2000). cAMP pulse during preservation inhibits the late development of cardiac isograft and allograft vasculopathy. *Circ Res.* 86: 982-988.

Wang HL, Kilfeather SA, Martin GR, and Page CP. (2000). Effects of tetrandrine on growth factor-induced DNA synthesis and proliferative response of rat pulmonary artery smooth muscle cells. *Pulmonary Pharmacol Ther.* 13: 53-60.

Wang Y, Rose PM, Webb ML, and Dunn MJ. (1994). Endothelin stimulates mitogen-activated protein kinase cascade through either ETA or ETB. *Am J. Physiol.* 267: c1130-C1135.

Wanstall JC, and O'Donnell SR. (1992). Responses to vasodilator drugs on the pulmonary artery preparations from pulmonary hypertensive rats. *Br. J. Pharmacol.* 105: 152-158.

Wanstall JC, Hughes IE, O'Donnell SR. (1992). Reduced relaxant potency of nitroprusside on pulmonary artery preparations taken from rats during the development of hypoxic pulmonary hypertension. *Br J Pharmacol.* 107(2):407-13.

Wanstall JC, Hughes IE, O'Donnell SR. (1995). Evidence that nitric oxide from the endothelium attenuates inherent tone in isolated pulmonary arteries from rats with hypoxic pulmonary hypertension. *Br J, Pharmacol.* 114: 109-114.

Wanstall JC. (1996). The pulmonary vasodilator properties of potassium channel opening drugs. *Gen Pharmacol.* 27(4):599-605.

Wanstall JC, Jeffery TK. (1998). Recognition and management of pulmonary hypertension. *Drugs*. 56(6):989-1007.

Ward Y, Gupta S, Jensen P, Wartmann M, Davis RJ, Kelly K. (1994). Control of MAP kinase activation by the mitogen induced threonine/tyrosine phosphatase PAC-1. *Nature*. 367: 651-654.

Wattanapitayakul SK, Bauer JA. (2001). Oxidative pathways in cardiovascular disease. Roles, mechanism, and therapeutic implications. *Pharmacol. and Therap*. 89: 187-206.

Weimann J, Ullrich R, Hromi J, Fujino Y, Clark MWH, Bloch KD, Zapol WM. (2000). Sildenafil is a pulmonary vasodilator in awake lambs with acute pulmonary hypertension. *Anaesthesiology*. 92:1702-1712.

Weinberger B, Heck DE, Laskin DL, Laskin JD. (1999). Nitric oxide in the lung: therapeutic and cellular mechanisms of action. *Pharmacol Ther*. 84(3):401-11.

Weinberger B, Weiss K, Heck DE, Laskin DL, Laskin JD. (2001). Pharmacological therapy of persistent pulmonary hypertension of the newborn. *Pharmacol. Ther*. 89: 67-79.

Weir KE, Reeve HL, Johnson G, Michelakis ED, Nelson DP, Archer SL. (1998) A role for potassium channels in smooth muscle cells and platelets in the etiology of primary pulmonary hypertension. *Chest*. 114: 200S-204S.

Welsh DJ, Peacock AJ, MacLean M. Harnett M. (2001). Chronic hypoxia induces constitutive p38 mitogen-activated protein kinase activity that correlates with enhanced cellular proliferation in fibroblasts from rat pulmonary but not systemic arteries. *Am J. Respir. Crit Care Med*. 164: 282-289.

Wenzlaff H, Stein B, Teschemacher H. (1998). Diminution of contractile response by kappa-opioid receptor agonists in isolated rat ventricular cardiomyocytes is mediated via a pertussis toxin-sensitive G-protein. *Naunyn Schmiedebergs Arch Pharmacol*. 358(3):360-6.

Wharton J, Davie N, Upton PD, Yacoub MH, Polak JM, Morrell NW. (2000). Prostacyclin analogues differentially inhibit growth of distal and proximal human pulmonary artery smooth muscle cells. *Circulation*. 102(25): 3130-6.

Widmann C, Gibson S, Jarpe MB, Johnson GL. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev.* 79(1):143-80.

Wissink S, van Heerde EC, Vand der Burg B, van der Saag PT. (1998). A dual mechanism mediates repression of NF-κB activity by glucocorticoids. *Mol Endocrinol.* 12(3):355-63.

Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW. (1993). Inhibition of the EGF-activated MAP kinase signalling pathway by adenosine 3',5'-monophosphate. *Science* 262: 1065-1069.

Wu KK. (1995). Inducible cyclooxygenase and nitric oxide synthase. *Adv Pharmacol.* 33: 179-207.

Wyatt TA, Naftilan AJ, Francis SH, Corbin JD. (1998). ANF elicits phosphorylation of the cGMP phosphodiesterase in vascular smooth muscle cells. *Am J Physiol.* 274:H448-55.

Xiao F. (1993). [The effect of hypoxic endothelial cells conditioned medium on the growth of pulmonary artery smooth muscle cells]. *Zhonghua Bing Li Xue Za Zhi.* 22(3):166-8.

Xie Q, Whisnant R, Nathan C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *J Exp Med.* 177: 1779-1784.

Xu LX, Tanaka Y, Bonderenko VA, Matsuura I, Matsumoto H, Yamazaki A, Hayashi F. (1998). Phosphorylation of the gamma subunit of the retinal photoreceptor cGMP phosphodiesterase by the cAMP-dependent protein kinase and its effect on the gamma subunit interaction with other proteins. *Biochemistry*. 37(17):6205-13.

Xue C, Appavoo R, Le Cras TD, Koberna PA, Dailey GC, Johns RA. (1994). Distribution of NOS in normoxic vs. hypoxic rat lung: upregulation of NOS by chronic hypoxia. *Am J Physiol.* 267, L667-L678.

Xue C, and Johns RA. (1996). Upregulation of nitric oxide synthase correlates temporally with onset of pulmonary vascular remodelling in the hypoxic rat. *Hypertension.* 28: 743-753.

Yafitz S and Hurley JB. (1994). Transduction mechanisms of vertebrate and invertebrate photoreceptors. *J Biol Chem.* 269: 4329-14332.

Yamamoto KK, Gonzalez GA, Biggs WH, and Montminy MR. (1988). Phosphorylation-induced binding and transcription efficacy of nuclear factor CREB. *Nature.* 334: 494-498.

Yamamoto K, Arakawa T, Ueda N (1995). Transcription roles of nuclear factor κB and nuclear factor-interleukin 6 in the tumour necrosis-α-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem.* 270: 31315-31320.

Yamazaki T, Komuro I, Zou Y, Yazaki Y. (1999). Hypertrophic responses of cardiomyocytes induced by endothelin-1 through the protein kinase C-dependent but Src and Ras-independent pathways. *Hypertens Res.* 22(2):113-9.

Yamboliev IA, Hruby A, Gerthoffer WT. (1998). Endothelin-1 activates MAP kinases and c-Jun in pulmonary artery smooth muscle. *Pulm Pharmacol Ther.* 11(2-3):205-8.

Yanigasawa M, Kurihara H, Kimura S, Masaki T, Kobayashi M, Mitsui Y. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature.* 332: 411-415.

Yan SF, Lu J, Zou YS, Soh-Won J, Cohen DM, Buttrick PM, Cooper DR, Steinberg SF, Mackman N, Pinsky DJ, Stern DM. (1999). Hypoxia-associated induction of early growth response-1 gene expression. *J Biol Chem.* 274(21):15030-40.

Yang X, Sheares KK, Davie N, Upton PD, Taylor GW, Horsley J, Wharton J, Morrell NW. (2002). Hypoxic induction of cox-2 regulates proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Cell Mol Biol.* 27(6):688-96.

York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJ. (1998). Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature*. 392(6676):622-6.

Yu SM, Cheng ZJ, Kuo SC. (1995). Endothelium-dependent relaxation of rat aorta by butein, novel cyclic AMP-specific phosphodiesterase inhibitor. *Eur J. Pharmacol.* 280: 69-77.

Yu SM, Hung MM, and Lin CC. (1997). cGMP-elevating agents suppress proliferation of vascular smooth muscle cells by inhibiting the activation of epidermal growth factor signalling pathway. *Circulation*. 95: 1269-1277.

Yuan XL, Goldman W, Tod M. (1993). Hypoxia reduces potassium currents in cultured rat pulmonary but not mesenteric arterial myocytes. *Am. J. Physiol.* 264: L116-L123.

Yuasa K, Kotera J, Fujishige K, Michibata H, Sasaki T, Omori K. (2000). Isolation and characterization of two novel phosphodiesterase PDE11A variants showing unique structure and tissue specific expression. *J. Biol. Chem*. 275:31469-31479.

Yuasa K, Kanoh Y, Okumura K, Omori K. (2001) Genomic organization of the human phophodiesterase PDE11A gene. Evolutionary relateness with other PDEs containing GAF domains. *Eur. J. Biochem*. 268: 168-178.

Zamora MA, Dempsey EC, Walchak SJ, Stelzner TJ. (1993). BQ123, an ETA receptor antagonist inhibits endothelin-1-mediated proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Cell Mol Biol.* 9: 429-433.

Zamora MA, Stelzner TJ, Webb S, Panos RJ, Ruff LJ, Demsey EC. (1996). Overexpression of endothelin-1 and enhanced growth of pulmonary artery smooth muscle cells from fawn-hooded rats. *Am J Physiol.* 270: L101-109.

Zapol WM, Rimer S, Gillis N, Marletta M, and Bosken CH. (1994) Nitric oxide and the lung. *Am J. Respir. Crit. Care Med.* 149: 1375-1380.

Zenner E, (1999). No cause for alarm over retinal side-effects of sildenafil. *The Lancet.* 353 (9150): 340-314.

Zhao L, Long L, Morrell NW. (1999). NPR-A-deficient mice show increased susceptibility to hypoxia-induced pulmonary hypertension. *Circulation.* 99: 605-607.

Zhao L, Mason NW, Morrell NW, Kojonazarov B, Sadykov A, Maripov A, Mirrakhimov MM, Aldashev A, Wilkins MR. (2001). Sildenafil inhibits hypoxia-induced pulmonary hypertension. *Circulation.* 104: 424-428.

Zhang H, Chen X, Teng X, Snead C, Catravas JD. (1998). Molecular cloning and analysis of the rat inducible nitric oxide synthase gene promoter in aortic smooth muscle cells. *Biochem. Pharmacol.* 55: 1873-1880.

Zhong H, SuYang H, Erdument-Bromage H, Tempst P, and Ghosh S. (1997). The transcriptional activity of NF-κB is regulated by the IκB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell.* 89:413-424.

Ziegler JW, Ivy DD, Fox JJ. Kinsella JP, Clarke WR and Abman SH. (1995) Dipyridamole, a cGMP phosphodiesterase inhibitor, causes pulmonary vasodilation in the ovine fetus. *Am J. Physiol.* 269:H473-479.

Ziegler JW, Ivy DD, Wiggins JW, Kinsella JP, Clarke WR, Abman SH (1998). Effects of dipyridamole and inhaled nitric oxide in pediatric patients with pulmonary hypertension. *Am. J. Resp. Crit. Care Med.* 158:1388-1393.

Zifa E, Fillion G. (1992). 5-Hydroxytryptamine receptors. *Pharmacol Rev.* 44(3):401-58

Zucker TP, Bonisch D, Hasse A, Grosser T, Weber AA, and Schror K. (1998). Tolerance development to antimitogenic actions of prostacyclin but not prostaglandin E1 in coronary artery smooth muscle cells. *Eur J. Pharmacol.* 345: 213-220.

Zuckerman BD, Orton EC, Stenmark KR, Trapp JA, Murphy JR, Coffeen PR, Reeves JT. (1991). Alteration of the pulsatile load in the high-altitude calf model of pulmonary hypertension. *J Appl Physiol.* 70(2):859-68.