Validation of phosphodiesterase isozymes as targets for pulmonary hypertension

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Declaration

I, Sarah Louise Trinder confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Pulmonary hypertension (PH) is a debilitating disease with a very poor prognosis. Increasing lung cyclic GMP (cGMP) levels is clinically effective in treating the disease, but since many endogenous pathways regulate cGMP synthesis and metabolism, there exists considerable scope for further optimising cGMP-based therapy. In this thesis I have explored this possibility focusing on natriuretic peptides, vasoactive mediators that stimulate cGMP synthesis via a membrane-bound guanylate cyclase, and phosphodiesterases (PDE) which inactivate cGMP.

Using organ bath pharmacology, I assessed the functional reactivity of systemic (aorta) and pulmonary vessels in response to atrial natriuretic peptide (ANP) and nitric oxide (NO; in the form of an NO-donor) in the absence and presence of isoform-selective PDE inhibitors. This was conducted in tissues from normoxic rats and animals exposed to 2 weeks hypoxia (10% O_2). In addition, I undertook RT-PCR studies to determine if expression of specific PDEs changed in these vessels under a hypoxic environment.

My data suggest that PDE 2 and PDE 5 are the principal PDE isoforms regulating ANP-dependent vasorelaxation in the pulmonary vasculature (from a functional and expressional standpoint) and that inhibition of these PDEs should bring about a selective pulmonary dilatation, particularly under hypoxic conditions. Using a model of hypoxia-induced PH, I demonstrated that the PDE2 inhibitor, BAY 60-7550, prevents the pulmonary hypertension, pulmonary vascular remodelling and right ventricular hypertrophy characteristic of the disease. Moreover, combination of BAY 60-7550 with the neutral endopeptidase inhibitor ecadotril (augments circulating natriuretic peptide levels) produces an additive, if not synergistic, benefit against disease severity.

Finally, I built on published work from our laboratory by investigating the effect of the PDE5 inhibitor sildenafil, in combination with ecadotril, in experimental models of PH and pulmonary fibrosis. Again, my data suggest that targeting PDE5 is also an effective therapeutic approach in these lung disorders.

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5-HT	5-hydroxytryptamine
5-HT_{1B}	5-hydroxytryptamine receptor 1B
5-HT_{2A}	5-hydroxytryptamine receptor 2A
5-HT_{2B}	5-hydroxytryptamine receptor 2B
6MWD	6 minute walking distance
8MM-IBMX	8-methoxymethyl-isobutyl-1-methyl xanthine
aa	Amino acids
AC	Adenylate cyclase
ACh	Acetylcholine
ALK1	Activin-like kinase-1
AMP	Adenosine monophosphate
ANP	Atrial natriuretic peptide
APAH	Associated pulmonary arterial hypertension
ApoE	Apolipoprotein E
$\text{ApoE}^{-/-}$	Apolipoprotein E knock out mouse
ATP	Adenosine triphosphate
BH_4	Tetrahydrobiopterin
BK_{Ca}	Large conductance Ca^{2+}-activated K^+ channels
BMPR2	Bone morphogenetic protein receptor 2
BNP	B-type natriuretic peptide
Ca^{2+}	Calcium
CaM	Calcium calmodulin complex
cAMP	Cyclic 3', 5'-adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic 3', 5'-guanosine monophosphate
CMC	Carboxymethyl cellulose
CNP	C-type natriuretic peptide
COPD	Chronic obstructive pulmonary disease
COX-1	Cyclooxygenase-1
CTEPH	Chronic thromboembolic pulmonary hypertension

Dfen	Dexfenfluramine
ECE-1	Endothelin converting enzyme-1
ECM	Extracellular matrix
ECMO	Extra-corporeal membrane oxygenation
EDHF	Endothelium derived hyperpolarising factor
EDRF	Endothelium derived relaxing factor
EHNA	Eiythro-9-(2-hydroxy-3-nonyl)adenine
eNOS	Endothelial nitric oxide synthase
ERAs	Endothelin receptor antagonists
ET_A	Endothelin receptor A
ET_B	Endothelin receptor B
ET-1	Endothelin-1
FAD	Flavin adenine dinucleotide
FePPIX	Iron protoporphyrin IX
FMN	Flavin mononucleotide
GC	Guanylate cyclase
GPCR	G-protein-coupled receptor
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
HPAH	Heritable pulmonary arterial hypertension
HPV	Hypoxic pulmonary vasoconstriction
IL-1R1$^{-/-}$	interleukin-1 receptor-1 knock out
ILD	Interstitial lung disease
iNOS	Inducible nitric oxide synthase
IP	Prostacyclin receptor
IP_3	Inositol-1,4,5-triphosphate
IPAH	Idiopathic pulmonary arterial hypertension
IPF	Idiopathic pulmonary fibrosis
K^+	Potassium
K_{ATP}	ATP-dependent potassium channels
KCl	Potassium chloride
KO	Knock out
MABP	Mean arterial blood pressure
MCT	Monocrotaline

MCTP	Monocrotaline pyrrole
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mRNA	Messenger ribonucleic acid
mPAP	Mean pulmonary arterial pressure
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NEP	Neutral endopeptidase
NEPi	Neutral endopeptidase inhibitor
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NP	Natriuretic peptide
NPR	Natriuretic peptide receptor
NPR-A	Natriuretic peptide receptor-A
NPR-B	Natriuretic peptide receptor-B
NPR-C	Natriuretic peptide receptor-C
NT pro-BNP	N-terminal pro-B-type natriuretic peptide
PAH	Pulmonary arterial hypertension
PAP	Pulmonary arterial pressure
PASMC	Pulmonary arterial smooth muscle cell
PBS	Phosphate buffered saline
PCH	Pulmonary capillary haemangiomatosis
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDE1	Phosphodiesterase 1
PDE1i	Phosphodiesterase 1 inhibitor
PDE2	Phosphodiesterase 2
PDE2i	Phosphodiesterase 2 inhibitor
PDE3	Phosphodiesterase 3
PDE3i	Phosphodiesterase 3 inhibitor
PDE4	Phosphodiesterase 4
PDE4i	Phosphodiesterase 4 inhibitor
PDE5	Phosphodiesterase 5
PDE5i	Phosphodiesterase 5 inhibitor

PDE6	Phosphodiesterase 6
PDE6i	Phosphodiesterase 6 inhibitor
PDEi	Phosphodiesterase inhibitor
PE	Phenylephrine
PEG	Polyethylene glycol
PF	Pulmonary fibrosis
PFA	Paraformaldehyde
pGC	Particulate guanylate cyclase
PGH_2	Prostaglandin H_2/Prostacyclin
PGI_2	Prostaglandin I_2/Prostacyclin
PGIS	Prostacyclin synthase
PGK	cGMP-dependent protein kinase
PH	Pulmonary hypertension
PKA	Protein kinase-A
PLCβ3	Phospholipase-Cβ3
PPHN	Persistent pulmonary hypertension of the newborn
PVOD	Pulmonary veno-occlusive disease
PVR	Pulmonary vascular resistance
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RV	Right ventricle
RVSP	Right ventricular systolic pressure
S.E.M	Standard error of the mean
SERT	Serotonin transporter
sGC	Soluble guanylate cyclase
SMC	Smooth muscle cell
SOD	Superoxide dismutase
SOC	Store-operated calcium channels
SR	Sarcoplasmic reticulum
SSc	Systemic sclerosis
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
TPH1	Tryptophan hydroxylase 1
TRPC6	Transient receptor potential channel 6

TXA_2	Thromboxane A_2
UCR	Upstream conserved region
VSMC	Vascular smooth muscle cells
WHO	World Health Organisation
WT	Wild type

Publications

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Chapter 1: Introduction

1.1 Pulmonary Hypertension

Pulmonary Hypertension (PH) is clinically defined by a mean resting pulmonary arterial pressure (mPAP) that is persistently elevated i.e. >25 mmHg at rest, pulmonary vascular resistance (PVR) ≥3 Wood units and pulmonary wedge pressure ≤15mmHg (Badesch *et al.*, 2009). The disease is diagnosed via catheterisation of the right ventricle. The pathological characteristics of PH are multifactorial and include, increased pulmonary arterial pressure, vascular remodelling of the pulmonary small arteries, right ventricular hypertrophy and ultimately right ventricular failure (Farber & Loscalzo, 2004). The prevalence of all forms of PH in the UK currently stands at 97 per million population; with a gender bias of 1.8 females to every male (The NHS Information Centre, 2011).

The classification of PH has evolved since the World Health Organization's (WHO) first scheme was proposed in 1973 (Hantano & Strasser, 1975). The first major revision in 1998, divided PH into five categories based on *clinical features* and *pathobiology*, "Evian Classification" (Fishman, 2001). Subsequent revisions in 2003, included changing the term primary pulmonary hypertension to idiopathic pulmonary arterial hypertension (IPAH). The most recent modification was in 2008 at the fourth World Symposium on PH in Dana Point, California; with the "Dana Point" classification (Simonneau *et al.*, 2009). The classification of PH (***Table 1.1***) is currently comprised of five major categories. These groups share pathological and clinical features (Simonneau *et al.*, 2009).

1.1.1 Group 1 – Pulmonary arterial hypertension

Group 1 is comprised of a diverse group of diseases and is termed pulmonary arterial hypertension (PAH); this group can be further sub-divided into six areas. Idiopathic PAH (IPAH), formally primary pulmonary hypertension, is a sporadic disease where

there is not a familial history of PAH or identified risk factor. Heritable PAH (HPAH) consists of patients with a germline mutation and PH in a familial context; of which approximately 80% have mutations in bone morphogenetic protein receptor 2 (BMPR2; Austin & Loyd, 2007). Also, 11-40% of IPAH patients with no family history have BMPR2 mutations (Machado *et al.*, 2006) and so belong to this group. Patients with any identified germline mutation, and familial cases where a genetic mutation has yet or has not been detected, are all included in this sub-group.
Drug and toxin induced PAH, which is characterised into drugs or toxins that have a 'definite' association with PAH, such as appetite suppressants identified in the 1960s (Simonneau *et al.*, 2009), form a further subgroup of PAH.

Associated PAH (APAH) comprises patients with existing diseases such as congenital heart disease, connective tissue disease (e.g. Systemic Sclerosis; SSc), schistosomiasis or human immunodeficiency virus (HIV) infection. SSc associated PAH patients have a worse prognosis than those with IPAH, with a 3 year survival rate of only 48.9% (Fisher *et al.*, 2006). Schistosomiasis is one of the most prevalent chronic infectious diseases in the world with >200 million people infected worldwide, APAH occurs in up to 5% of these patients, therefore it is probably the most prevalent cause of PAH (Fernandes *et al.*, 2011; Lapa *et al.*, 2009). The final two sub-groups of PAH are persistent pulmonary hypertension of the newborn (PPHN) and patients with pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary haemangiomatosis (PCH).

1.1.2 Group 2 – Pulmonary hypertension due to left heart disease

The Dana Point scheme Group 2 is PH resulting from left heart disorders. This was initially reported to represent the most frequent cause of PH (Oudiz, 2007), although this is now thought to be that associated with schistosomiasis infection (Fernandes *et al.*, 2011; Lapa *et al.*, 2009). Group 2 have three primary aetiologies, left heart systolic dysfunction, left heart diastolic dysfunction and left heart valvular disease (Simonneau *et al.*, 2009). All three raise pulmonary arterial pressure, which is

predominantly due to elevated pulmonary venous pressure. Currently there are no drugs approved for treatment of this group of PH patients (Nef *et al.*, 2010).

1.1.3 Group 3 – Pulmonary hypertension due lung disease and/or hypoxia

Group 3 is PH resulting from lung disease and/or hypoxia. This includes chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), chronic exposure to high altitude ('chronic mountain sickness'), sleep-disordered breathing, and obesity hypoventilation syndrome. The prevalence of PH in these conditions remains largely unknown (Nef *et al.*, 2010; Simonneau *et al.*, 2009). However several reports suggest this may occur in a significant number of patients. For example, Ryu and colleagues have reported PH may occur in greater than eighty percent of patients with IPF (Behr & Ryu, 2008). A study of COPD patients revealed 25% of patients had PH (mPAP>25mmHg; Chaouat *et al.*, 2008), whilst others estimate 30-70% of COPD have PH (Seimetz *et al.*, 2011). The estimated prevalence varies greatly and are thought to be due to differences in the definition of PH and methods used to determine pulmonary pressures (Minai *et al.*, 2010). In most cases the severity of PH correlates with the extent of the underlying lung disease (Levinson & Klinger, 2011). This has been widely reported in IPF and emphysema patients and the studies have been able to correlate the severity of the lung disease with mPAP and survival (Lettieri *et al.*, 2006; Mejía *et al.*, 2009; Nathan *et al.*, 2008a; Nathan *et al.*, 2008b; Patel *et al.*, 2007).

1.1.4 Group 4 – Chronic thromboembolic pulmonary hypertension

Group 4 is chronic thromboembolic pulmonary hypertension (CTEPH) whereby obstruction of the pulmonary vascular occurs due to thromboemboli. CTEPH occurs in up to 4% of patients post acute pulmonary embolism (Poli & Miniati, 2011). CTEPH is the only class of PH that can be cured; pulmonary thromboendarterectomy (surgical removal of the thrombus) can be performed in some patients and has a perioperative mortality of less than 10% (Stein *et al.*, 2011).

1.1.5 Group 5 – Pulmonary hypertension with unclear multi-factorial mechanisms

Finally Group 5 consists of patients with PH where the cause is due to aetiologies such as sarcoidosis, thyroid disease and glycogen storage disorders or are multifactorial (Levinson & Klinger, 2011).

Table 1.1 The Dana Point 2008 disease classification of pulmonary hypertension

Table 1.1 The Dana Point 2008 disease classification of pulmonary hypertension.

- **Group 1: Pulmonary Arterial Hypertension (PAH)**
  - 1.1 Idiopathic (IPAH)
  - 1.2 Heritable
    - 1.2.1 BMPR2 mutations (>50%)
    - 1.2.2 ALK1, endoglin
    - 1.2.3 Unknown
  - 1.3 Drug and toxin induced
  - 1.4 Associated (APAH)
    - 1.4.1 Connective tissue diseases
    - 1.4.2 HIV infection
    - 1.4.3 Portal
    - 1.4.4 Congenital heart disease
    - 1.4.5 Schistosomiasis
    - 1.4.6 Chronic hemolytic anaemia
  - 1.5 Persistent pulmonary hypertension of the newborn (PPHN)
  - 1' Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary haemanglomatosis
- **Group 2: PH due to left heart disease**
  - 2.1 Systolic dysfunction
  - 2.2 Diastolic dysfunction
  - 2.3 Valvular disease
- **Group 3: PH due to lung diseases and/or hypoxia**
  - 3.1 Chronic obstructive pulmonary disease (COPD)
  - 3.2 Interstitial lung disease
  - 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern
  - 3.4 Sleep disordered breathing
  - 3.5 Alveolar hypoventilation disorders
  - 3.6 Chronic exposure to high altitude
  - 3.7 Developmental abnormalities
- **Group 4: Chronic thromboembolic pulmonary hypertension (CTEPH)**
- **Group 5: PH with unclear multi-factorial mechanisms**
  - 5.1 Haematologic disorders: myeloproliferative disorders, splenectomy
  - 5.2 Systemic disorders: sarcoidosis, pulmonary Langerhans cell histocytosis
  - 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
  - 5.4 Others: tumoural obstruction, fibrosing mediastinitis, chronic renal failure or dialysis

1.1.6 Diagnosis

Diagnosis of PAH is achieved by pulmonary artery catheterisation, this is because it is able to determine if elevated PAP is due to increased precapillary resistance or postcapillary resistance. Echocardiography is unable to do this but is a useful non-invasive technique to measure PAP and is often used to follow disease progression and response to therapy. Three other tests commonly used to follow disease progression and response to therapy are WHO functional class, 6 minute walking distance (6MWD) and plasma B-type natriuretic peptide (BNP) levels (Badesch *et al.*, 2009).

WHO functional class is assessed by the patient's subjective impression of their ability to tolerate various types of physical activity; there are four levels of impairment. WHO functional class I are patients that deny respiratory problems during normal physical activity and are asymptomatic. Class II patients complain of shortness of breath when completing normal daily activities for example climbing stairs and carrying objects. Patients that suffer from shortness of breath when walking or dressing are class III, whilst class IV is when a patient is short breath at rest (Barst *et al.*, 2004b).

The 6MWD test is a measure of sub-maximal exercise. A normal healthy adult can walk at least 500 metres in 6 minutes on level ground. As PH progresses 6MWD steadily decreases, whereby most patients in WHO functional class IV are unable to walk 150 metres in 6 minutes and often are unable to complete the test without stopping to rest (Levinson & Klinger, 2011).

BNP is a secreted polypeptide produced mainly in the cardiac ventricles of the heart as a result of higher ventricular pressure (wall stretch; (Potter *et al.*, 2006). Plasma BNP levels rise as the right ventricle fails, and therefore BNP represents a biomarker of disease severity. For example, Nagaya and colleagues have reported improved prognosis in patients where BNP plasma levels remain below 180pg/ml, or if with 6 months of therapy BNP levels can be reduced to below 180pg/ml (Nagaya *et al.*, 2000). BNP has a short half-life in blood, which may lead to variability due to processing of patient samples. In contrast N-terminal pro BNP (NT pro-BNP) is far

more stable (Mueller *et al.*, 2004). NT pro-BNP is cleaved from pro-BNP with BNP at a 1:1 ratio, however plasma levels vary due to different *in vivo* half-lives; 120 minutes and 22 minutes, respectively (Kemperman *et al.*, 2004). NT pro-BNP is only cleared by the kidneys, whilst BNP is degraded by a number of routes, including by neutral endopeptidase (NEP), natriuretic peptide receptor C (NPR-C) and to a lesser extent cleared by the kidneys (Leuchte *et al.*, 2007). Furthermore, NT-pro BNP and BNP serum levels correlate with haemodynamics, therefore NT pro-BNP may represent a better biomarker for PH than BNP as it has greater stability and a longer half-life (Leuchte *et al.*, 2007).

1.1.7 Pathology and aetiology

PAH carries a high rate of mortality; in the 1980s it was reported the median survival was 2.8 years, with 1, 3 and 5 year survival rates of 68%, 48% and 34% respectively (D'Alonzo *et al.*, 1991). There has been some recent improvement yet median survival is still only 4.56 years with 1, 3 and 5 year survival rates 81.1%, 61.1% and 47.9% respectively (Kane *et al.*, 2011). This is largely due to the lack of satisfactory treatments, especially when it comes to agents that can reverse vascular wall remodelling and vasodilators that selectively combat vasoconstriction of the pulmonary vasculature over that of the systemic vasculature.

PH is a disease of the small pulmonary arteries, whereby PVR progressively increases due to vasoconstriction, vascular proliferation and remodelling, ultimately leading to right ventricular failure and death (Voelkel *et al.*, 2006). Endothelial dysfunction, therefore vasoconstriction and remodelling of pulmonary vessels are responsible for increases in PVR. However, abnormally alleviated haemodynamics are just one facet of PH; decreased apoptosis and enhanced proliferation of pulmonary smooth muscle cells are also a key aspect of the disease (Chan & Loscalzo, 2008). Another hallmark of the human disease is obstruction of the vascular lumen by the formation of plexiform lesions (Schermuly *et al.*, 2011); which is thought to be due to the disorganised proliferation (apoptotic resistant

clones) of endothelial cells and typically arise in arteries with a diameter of 200 to 400μm (Jeffery & Morrell, 2002).

A pro-proliferative phenotype was recognised by the identification of HPAH patients with loss of function mutations in the BMPR2 gene; a member of the transforming growth factor-beta (TGF-β) family. BMPR2-dependant signalling in particular BMP2 and BMP7 inhibit smooth muscle cell proliferation (Loscalzo, 2001). However BMPR2 mutations are not commonly found in non-FPAH patients and only 15% to 20% of individuals with the BMPR2 mutation develop PAH (Newman *et al.*, 2004). Recently mutations within Smad 4 and 8 genes, whose protein products act downstream of BMPR2, have been identified (Nasim *et al.*, 2011; Shintani *et al.*, 2009). Other genetic mutations also predispose individuals to PAH they include activin-like kinase type-1 (ALK1, another member of the TGF-β family; (Trembath *et al.*, 2001) and transient receptor potential channel 6 (TRPC6) (Yu *et al.*, 2009). Again only a small number of individuals present with these mutations and PAH, therefore it is thought the aetiology is due to multiple hits, involving genetic and environmental factors (Machado *et al.*, 2006).

1.2 Endothelial dysfunction in pulmonary hypertension

Chronic vasoconstriction of the pulmonary arteries is thought to be an early element of PH (Humbert *et al.*, 2004b). Such excessive vasoconstriction is associated with endothelial dysfunction (Jeffery & Morrell, 2002). Endothelial dysfunction leads to overexpression of vasoconstrictors such as endothelin-1 (ET-1) coupled to impaired bioavailability of vasodilators such as nitric oxide (NO) and prostacyclin (prostaglandin I_2; PGI_2), so not only is vascular tone increased but vascular remodelling occurs as a consequence of smooth muscle cell proliferation (Badesch *et al.*, 2009; Barst *et al.*, 2004b; Chan & Loscalzo, 2008; Cool *et al.*, 2005; Galie *et al.*, 2010; Humbert *et al.*, 2008; Jeffery & Morrell, 2002; McLaughlin & McGoon, 2006; Schermuly *et al.*, 2011); ***Figure 1.1***). These changes in endothelial function tip the balance to a more vasoconstrictive, pro-proliferative phenotype.

1.2.1 Nitric oxide and nitric oxide synthase

NO is a colourless gas that was discovered by Joseph Priestley in the early 1770s (Priestley, 1774). Furchgott showed in 1980 that endothelial cells release an endothelium derived relaxing factor (EDRF) in response to acetylcholine (ACh) that relaxed underlying smooth muscle (Furchgott & Zawadzki, 1980). These two discoveries seemed unrelated until in 1987, two groups separately proved that EDRF is NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). The following year it was demonstrated that NO is synthesised from the amino acid L-arginine (Palmer *et al.*, 1988) by a group of enzymes termed nitric oxide synthase (NOS).

The NOS family of proteins is comprised of 3 isoforms; neuronal NOS (nNOS), inducible/inflammatory NOS (iNOS) and endothelial NOS (eNOS) otherwise known as NOS1, NOS2 and NOS3, respectively (Stuehr, 1999). All three isozymes of NOS are homodimeric proteins, with each subunit comprised of two domains, namely the oxygenase and reductase domain. The oxygenase domain contains binding sites for iron protoporphyrin IX (FePPIX, haem group), tetrahydrobiopterin (BH_4) and L-arginine (Stuehr, 1997). Whilst the reductase domain contains binding sites for

flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide phosphate (NADPH; (Stuehr, 1997). NOS is activated by binding a calcium (Ca^{2+}) calmodulin (CaM) complex and both the oxygenase and reductase domains have binding sites for CaM. Both eNOS and nNOS require an increase in resting intracellular Ca^{2+} concentrations in order to bind CaM and so become fully activated, thus making them Ca^{2+} dependent. In contrast iNOS is independent of intracellular Ca^{2+} transients because it has such a high affinity to CaM (Schmidt *et al.*, 1991; Stuehr *et al.*, 1991) it is constitutively bound to the enzyme.

BH_4 appears to have four important roles to ensure optimal eNOS activity. Firstly, BH_4 enables the haem catalytic site to function normally, in its absence the ferric ion of the haem group is reduced to Fe(II) dioxygen complex which results in superoxide formation (Gielis *et al.*, 2011). If BH_4 is present iron-oxy species are formed resulting in L-arginine hydroxylation and hence NO generation (Gielis *et al.*, 2011). Secondly, BH_4 increases the affinity of eNOS to L-arginine (Gorren *et al.*, 1998). Thirdly, it is thought to convert to a BH_3 radical during the first catalytic reaction; thus playing a role in the electron transfer of this reaction (Kotsonis *et al.*, 2000). Finally, BH_4 stabilises dimerisation of the two monomers of eNOS by interacting with amino acids from both molecules (Panda *et al.*, 2002).

1.2.1.1 Nitric oxide, nitric oxide synthase and pulmonary hypertension

Endothelial NOS expression and activation have been shown to be increased in the pulmonary arteries in several animal models of PH, including chronic hypoxia and monocrotaline (MCT; (Fagan *et al.*, 2001; Le Cras *et al.*, 1998; Resta *et al.*, 1997; Zuckerbraun *et al.*, 2010). Furthermore caveolin null mice have chronic upregulation of eNOS activity, resulting in the development of PH (Zhao *et al.*, 2009). This is despite compromised NO bioavailability and bioactivity in PH (Baliga *et al.*, 2012; Zuckerbraun *et al.*, 2011). Increased eNOS expression has been consistently demonstrated in animal models; however this has not been the case in human tissues. There have been conflicting reports of increased eNOS

immunostaining (Xu & Johns, 1995) and decreased eNOS immunostaining (Giaid & Saleh, 1995) in the lungs of patients with pulmonary hypertension. More recently there have been reports of increased eNOS expression in the lungs of children or neonates with PH (Hoehn *et al.*, 2009; Sood *et al.*, 2011).

Increased eNOS expression and activation coupled with decreased NO bioavailability and bioactivity is thought to be due to 'eNOS uncoupling'. In PH eNOS uncoupling has been shown to be due to defective synthesis of BH_4 (Khoo *et al.*, 2005; Nandi *et al.*, 2005); as explained in 1.2.1 BH_4 plays a vital role in eNOS activity. Decreased BH_4 bioavailability results in increased production of reactive oxygen species (ROS; rather than NO) by eNOS and also disruption of the eNOS dimer (Zuckerbraun *et al.*, 2011), thus rendering the eNOS enzyme an ineffective producer of NO. Furthermore the eNOS KO mouse is more susceptible to hypoxia induced pulmonary hypertension than wild type (Baliga *et al.*, 2012; Fagan *et al.*, 1999).

ROS contributes to the pathophysiology of PH via several mechanisms (Wolin *et al.*, 2010). Human studies have suggested there is increased oxidative stress in PH, thus inferring that ROS is increased too (Wong *et al.*, 2012). ROS oxidises the ferrous haem of soluble guanylate cyclase thus rendering it insensitive to NO as explained in 1.2.2 and 1.2.2.1, therefore blocking NO-mediated relaxation (Schrammel *et al.*, 1996). ROS also cause vasoconstriction and have been implicated in pulmonary artery smooth muscle cell (PASMC) proliferation (Sanders & Hoidal, 2007). Furthermore superoxide dismutases (SOD; metabolises superoxide, O_2^-) have been shown to be effective in preventing vascular remodelling in the chronic hypoxia animal model of PH (Hoshikawa *et al.*, 2001a).

1.2.2 Soluble guanylate cyclase

Soluble guanylate cyclase (sGC) is an enzyme that converts guanosine triphosphate (GTP) to cyclic 3', 5'-guanosine monophosphate (cGMP); it is the primary intracellular receptor for NO and mediates the vast majority of physiological effects

of NO including, vasorelaxation, anti-leukocyte and anti-platelet activities (Hobbs & Ignarro, 1996). sGC is a haem-containing protein found in the cytosol of virtually all mammalian cells (Hobbs, 1997). The highest concentrations of sGC can be found in the lung and brain. sGC is comprised of an α (large) and a β (small) subunit (Humbert *et al.*, 1990; Kamisaki *et al.*, 1986). Currently two isoforms for each subunit have been identified and are termed α_1, α_2 and β_1, β_2, respectively. To date only $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers of sGC have been found to be naturally occurring and active (Russwurm *et al.*, 1998).

Each subunit constitutes a haem-binding domain, dimerisation region and a catalytic domain. The haem-binding domain is found in the N-terminal portion of each subunit; the haem moiety gives sGC its sensitivity to NO (Schmidt *et al.*, 2004). Within the C-terminus of each subunit is the catalytic domain. Co-expression of the catalytic domains of both the α and β subunits is required for cyclase activity (Hobbs, 1997). The central region linking the catalytic and haem-binding domains is important in the dimerisation of the α and β subunits.

NO binds to the haem iron of sGC to form a haem-NO complex, which results in the breakage of the histidine-haem bond. This conformational change is transduced to the catalytic domain, the result of which is an up to 200-fold activation of the enzyme (Humbert *et al.*, 1990). sGC becomes insensitive to NO if the Fe^{2+} haem moiety is removed or oxidised and this stimulates the degradation of sGC (Stasch *et al.*, 2006). As discussed in section 1.2.1.1, oxidative stress is increased in patients with PH, therefore oxidation of sGC by ROS is increased.

To produce sGC KO mice, deletion of the β_1 subunit was found to result in a parallel loss of α subunit expression due to proteolytic degradation (Friebe *et al.*, 2007). These β_1 KO mice only live for 3-4 weeks unless given a fibre-free diet, this is due to intestinal dysmotility, suggesting sGC is vital for the maintenance of normal peristalsis of the gut. The β_1 KO mice show a pronounced increase in systemic blood pressure (~30mmHg greater than WT), smooth muscle cell specific β_1 KO mice are also hypertensive (Groneberg *et al.*, 2010); highlighting the importance of sGC in the maintenance of cardiovascular smooth muscle tone *in vivo*. Megakaryocyte and platelet specific β_1 KO mice have prolonged tail-bleeding times

and impaired thrombus formation (Zhang *et al.*, 2011). Therefore sGC also plays a key role in stimulating platelet activation, *in vivo* thrombosis and haemostasis.

Mice deficient in either the α_1 (two strains have been generated) or α_2 sGC subunits have been generated and have normal life expectancy (Buys *et al.*, 2008; Mergia *et al.*, 2006). sGC expression is reduced by over 90% in mice lacking the α_1 subunit compared to WT, due to a corresponding reduction of the β_1 subunit (α_2 KO mice show a minor reduction of the β_1 subunit); therefore the β_1 subunit is unstable without it's dimerising partner (Friebe & Koesling, 2009).

Vasorelaxation by NO donors, sGC activators and ACh in α_1 KO mice is greatly impaired but not obliterated; indicating that the residual sGC containing the α_2 subunit can assume functions in vascular smooth muscle cells (VSMC) but requires far higher concentrations of NO than sGC containing the α_1 subunit (Buys *et al.*, 2008; Mergia *et al.*, 2006). Blood pressure in α_1 KO mice was not raised as in β_1 KO mice, which was surprising. Furthermore there were differing blood pressure phenotypes between the two strains; this appears likely to be due to the different genetic backgrounds of the mice. Gender specific hypertension was reported by Buys *et al.*, male mice (KO: 147mmHg, WT: 125mmHg) were hypertensive whilst females (KO: 115mmHg, WT: 119mmHg) were not (Buys *et al.*, 2008). The males' hypertension was age dependant and only occurred after about 14 weeks of age. They showed that testosterone played a key role; orchidectomised α_1 KO males were not hypertensive and α_1 KO females given testosterone had greatly increased blood pressure (vehicle: 120mmHg, testosterone: 180mmHg). This could be due to females producing EDHF to compensate, as this is the predominant mediator in female mice whilst NO and PGI_2 are predominant in male mice (Scotland *et al.*, 2005). Mergia *et al.* found that their α_1 KO mice had moderately elevated blood pressure (KO: 111mmHg, WT: 104mmHg) regardless of gender (Mergia *et al.*, 2006).

There is minimal expression of the α_2 subunit in the vasculature, the α_2 KO mouse as expected showed no significant differences to WT mice with regard to blood pressure and vasorelaxation by agents that activate sGC (Mergia et al., 2006).

1.2.2.1 Soluble guanylate cyclase and pulmonary hypertension

Soluble GC has been shown to be upregulated in the lungs of IPAH patients, as well as in animal models of PH - chronic hypoxic mice and MCT treated rats (Schermuly *et al.*, 2008). However sGC mediated vasodilatation in MCT and chronic hypoxia induced PH in rats is impaired (Crawley *et al.*, 1992; Mam *et al.*, 2010). This is due to impaired bioavailability of NO and oxidation of the ferrous haem of sGC and thus inhibition of its NO-mediated activation (Schrammel *et al.*, 1996). It has been shown that sGC exists within a redox equilibrium, between a reduced (NO sensitive) and oxidised (NO insensitive) state (Stasch *et al.*, 2006). Oxidised sGC has been shown to increase in both human cardiovascular diseases and animal models where ROS is also increased (Stasch *et al.*, 2006). Furthermore, genetic deletion of the α_1-subunit of sGC augments the response to hypoxia-induced PH (Vermeersch *et al.*, 2007).

Soluble GC activators and stimulators have been successfully used to partially reverse hypoxia and MCT induced PH in rats (Weissmann *et al.*, 2009; Schermuly *et al.*, 2008; Dumitrascu *et al.*, 2006) and the sGC stimulator riociguat (BAY 63-2521) is currently in phase III clinical trials for the treatment of PH (Mittendorf *et al.*, 2009; Ghofrani *et al.*, 2010), following a successful evaluation in Phase 2 (Ghofrani et al. 2010).

Figure 1.1 The main impaired signalling pathways implicated in pulmonary hypertension

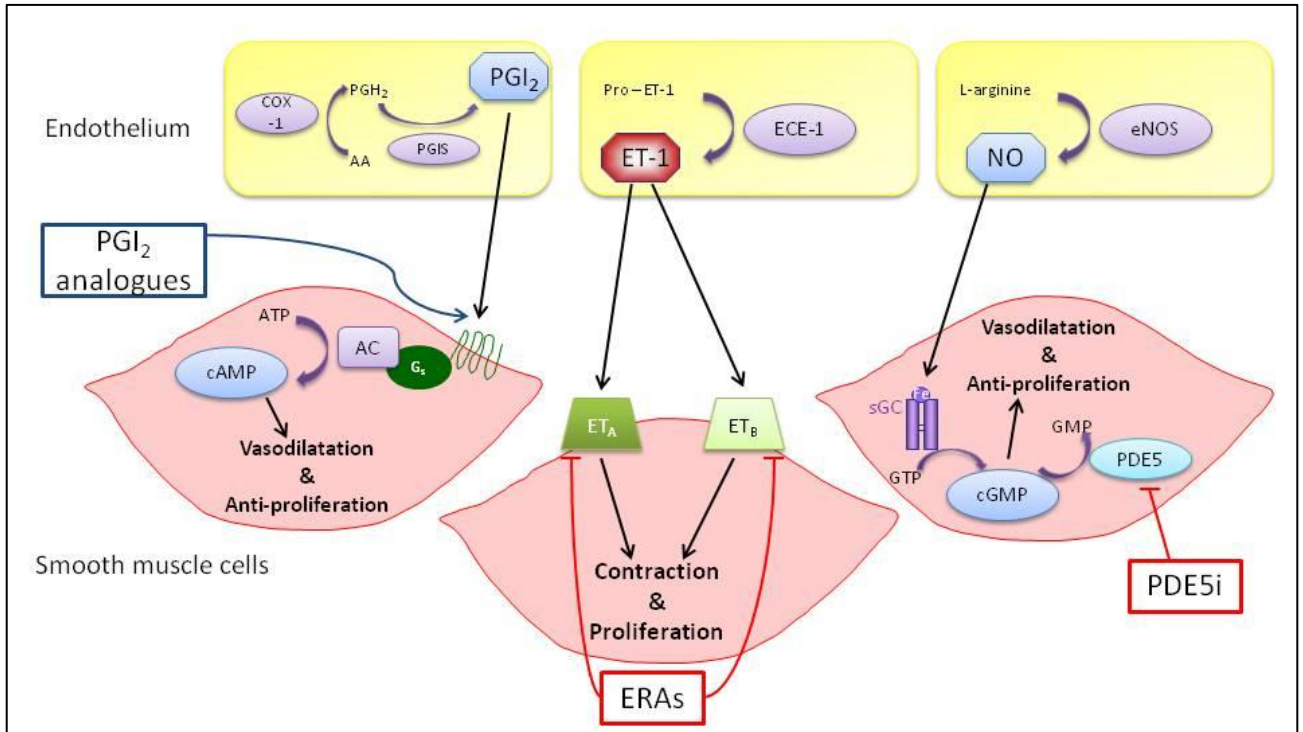


Figure 1.1 Main impaired signalling pathways implicated in pulmonary hypertension. Prostacyclin (PGI_2) analogues activate G_s-coupled IP receptors, once bound activates adenylate cyclase (AC) which produces cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP); resulting in vasodilatation and anti-proliferation of smooth muscle cells (SMCs). Endothelin receptor antagonists (ERAs) block the contractile and proliferative effects of endothelin-1 (ET-1) on SMCs. Phosphodiesterase 5 inhibitors (PDE5i) block the breakdown of cyclic guanosine monophosphate (cGMP) which has vasodilatory and anti-proliferative effects. $ET_{A/B}$, endothelin receptor A/B; ECE-1, endothelin converting enzyme-1; COX-1, cyclooxygenase-1; AA, arachidonic acid; PGH_2, prostaglandin H_2; PGIS, prostacycin synthase; NO, nitric oxide; eNOS, endothelim nitric oxide synthase; sGC soluble guanylate cyclase; GTP, guanosine triphosphate; GMP, guanosine monophosphate.

1.2.3 Cyclic 3',5'-guanosine monophosphate

In 1963 Ashman and colleagues first identified and purified cyclic 3', 5'-guanosine monophosphate (cGMP) from rat urine, they identified the main organic-phosphate containing molecules in urine as cyclic 3', 5'-adenosine monophosphate (cAMP) and cGMP (Ashman DF. *et al.*, 1963). cGMP is a second messenger that is found ubiquitously and mediates a vast number of physiological processes. Some examples of which are cell proliferation, ion channel conductance, apoptosis, cellular mobility and contractility. As mentioned previously cGMP is generated by sGC and particulate guanylate cyclase (pGC; see section 1.3.2) in response to NO and natriuretic peptides (NPs; see section 1.3), respectively (Hobbs, 1997).

This cyclic nucleotide regulates cellular function via three principle mechanisms: 1) cGMP-gated ion channels, 2) cGMP-regulated PDEs (see section 1.5) and 3) cGMP-dependent protein kinase (PGK). cGMP produces many cytoprotective effects in the vasculature including vasodilatation, anti-platelet, anti-leukocyte and inhibition of VSMC proliferation (***Figure 1.2***).

Two PKGs have been identified PKGI and PKGII. PKGI is the main isotype found in the cardiovascular system (Bonnevier *et al.*, 2004). It has two splice variants α and β. PKGI is expressed at high levels in VSMC (PKGIα and PKGIβ) and endothelial cells (PKGIβ), whilst in cardiac myocytes (PKGIα) is expressed at lower levels (Tsai & Kass, 2009). PKGII is not found in the cardiovascular system, it is mainly expressed in the brain, kidney and intestine (Francis *et al.*, 2010).

Figure 1.2 Mechanisms of cGMP in vascular smooth muscle cells

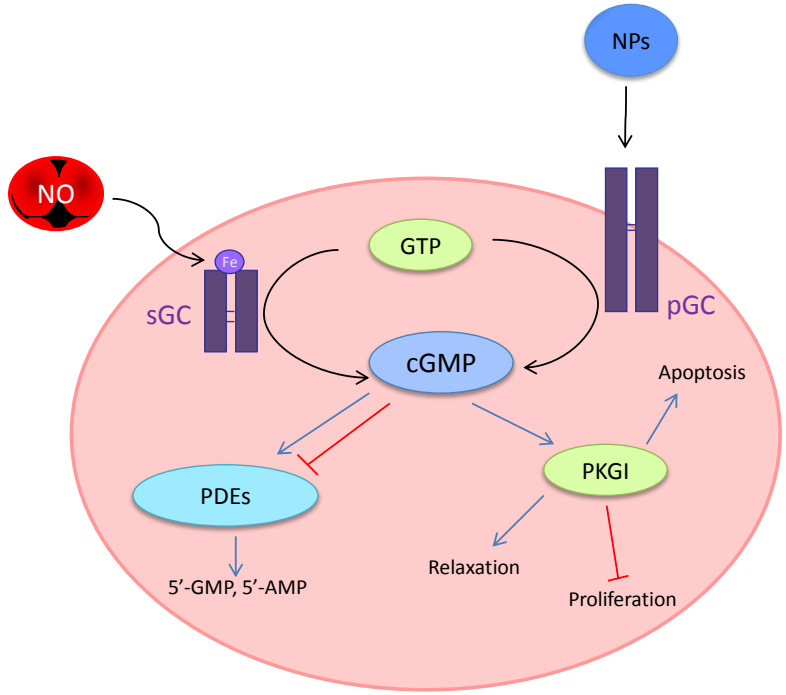


Figure 1.2. Mechanisms of cGMP in vascular smooth muscle cells.
NO, nitric oxide; NPs, natriuretic peptides; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; sGC, soluble guanylate cyclase; pGC, particulate guanylate cyclase; PDEs, phosphodiesterases; PKGI, protein kinase GI; GMP, guanosine monophosphate; AMP, adenosine monophosphate.

The main mechanism of action of PKG in the cardiovascular system is vasodilatation. In VSMC, cGMP activates PKGI which in turn inhibits the release of Ca^{2+} from intracellular stores (Desch *et al.*, 2010; Tertyshnikova *et al.*, 1998), phosphorylates voltage-dependent Ca^{2+} channels ($Ca_v1.2$, L-type Ca^{2+} channels) inhibiting extracellular Ca^{2+} influx (Yang *et al.*, 2007), activates large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) which causes membrane hyperpolarisation and therefore inhibits extracellular Ca^{2+} influx (Fukao *et al.*, 1999), phosphorylates myosin light chain phosphatase (MLCP; (Sauzeau *et al.*, 2000) and decreases the Ca^{2+} sensitivity of the contractile apparatus (Sauzeau *et al.*, 2000); therefore modulating smooth muscle cell relaxation.

1.2.3.1 Cyclic 3', 5'-guanosine monophosphate and pulmonary hypertension

Vascular and lung levels of cGMP are reduced in PH, this can be due to a number of mechanisms; inactivation of GCs, enhanced metabolism of cGMP by PDEs and impaired bioavailability of NO (Archer *et al.*, 1998; Crawley *et al.*, 1992; Steudel *et al.*, 1997; Zhao *et al.*, 1992). PDEs are discussed in depth in section 1.4, NO in section 1.2.1, sGC in section 1.2.2 and pGC in section 1.3.2. Inhibiting PDE5 blocks cGMP hydrolysis, PDE5 inhibitors (PDE5i) are approved therapies for PH and are discussed in section 1.5.5.

1.2.4 Endothelin-1

Endothelin-1 belongs to a family of three peptides, endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). ET-1 is the major cardiovascular isoform of the endothelin family, a 21 amino acid peptide with potent vasoconstrictor activity in most vascular beds (Yanagisawa *et al.*, 1988). The gene product, preproET is cleaved by an endothelin converting enzyme (ECE) into big ET and subsequently to the biologically active ET-1 (Webb, 1991). There are two endothelin receptors both are seven-transmembrane G protein coupled receptors (GPCRs); and are termed

endothelin receptor type A (ET_A) and type B (ET_B). ET_A and/or ET_B activation on smooth muscle cells mediates vasoconstriction, proliferation, and is anti-apoptotic. Conversely activation of ET_B on vascular endothelial cells stimulates the generation of vasodilators, such as NO and PGI_2. Furthermore ET_B on lung endothelial cells acts as a clearance receptor of ET-1 and is accountable for 80% of its clearance, (Shao *et al.*, 2011).

A number of factors stimulate the release of ET-1 from endothelial cells, shear stress, stretch, TGF-β, hypoxia and angiotensin II; all are related to vascular remodelling. Whilst the release of ET-1 is inhibited by NO and PGI_2 (Jeffery & Morrell, 2002). ET-1 has a key developmental and regulatory role, but is one of the most potent and long lasting vasoconstrictors to be discovered (Price & Howard, 2008).

1.2.4.1 Endothelin-1 and pulmonary hypertension

PH patients with various aetiologies have elevated plasma levels of ET-1, as do animals with PH induced by MCT or hypoxia (Jeffery & Morrell, 2002). Moreover, increased levels of circulating ET-1 correlate with increased pulmonary vascular resistance as well as increased mortality in PAH patients (Shao *et al.*, 2011; Rubens *et al.*, 2001). ET_A and ET_B expression is increased in the lungs of PAH patients, although the proportion of ET_A to ET_B remains constant (Davie *et al.*, 2002). Endothelin receptor antagonists (ERAs) are approved therapies for PH, these are discussed later in section 1.5.3.

1.2.5 Prostacyclin

Prostacyclin was discovered by Moncada and colleagues in 1976, it was originally termed PGX and later renamed PGI_2 (Moncada *et al.*, 1976). PGI_2 is synthesised in endothelial cells from arachidonic acid in a two step process. Firstly cyclooxygenase-1 (COX-1) metabolises arachidonic acid into prostaglandin H_2

(PGH_2) and secondly PGH_2 is metabolised by prostacyclin synthase (PGIS) into PGI_2 (Vanhoutte, 2009).

PGI_2 is not only a vasodilator but also inhibits proliferation of vascular smooth muscle cells and decreases platelet aggregation (Wharton *et al.*, 2000). PGI_2 activates the G_s-coupled prostacyclin (IP) receptor, once bound activates adenylate cyclase (AC) which produces cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) and activates protein kinase A (PKA; (Wharton *et al.*, 2000).

1.2.5.1 Prostacyclin and pulmonary hypertension

The expression of IP receptors and PGIS is reduced in pulmonary arteries from PH patients (Tuder *et al.*, 1999). Furthermore it has been shown that the anti-proliferative effects of PGI_2 are mediated via the IP receptor; in hypoxia induced PH, IP receptor KO mice had significantly more medial and vessel wall hypertrophy of peripheral pulmonary arteries than their wild type counterparts (Hoshikawa *et al.*, 2001b). PGI_2 analogues are approved therapies for PH and are discussed in section 1.5.2.

1.2.6 Cyclic 3', 5'-adenosine monophosphate

Cyclic AMP was first discovered by Sutherland and Rall in 1960 (Sutherland & Rall, 1963). cAMP is produced by the membrane-bound enzyme AC from ATP and activates PKA (Wharton *et al.*, 2000). AC is stimulated to produce more cAMP by the activation of numerous GPCRs including β-adrenergic receptors and the IP receptor. As discussed in section 1.2.5 PGI_2 elicits some of its biological effects by increasing intracellular levels of cAMP. The precise mechanisms by which cAMP/PKA elicits vascular relaxation are still unclear (Cogolludo *et al.*, 2007). However it is generally accepted that cAMP induces vascular relaxation by lowering intracellular Ca^{2+} via inhibition of PLCβ and thus the production of inositol-1,4,5-

triphosphate (IP_3; (Barnes & Liu, 1995). IP_3 stimulates the release of Ca^{2+} from the sarcoplasmic reticulum (SR) thus increasing intracellular Ca^{2+}; this in turn leads to increased Ca^{2+} entry to the cell via the activation of store-operated Ca^{2+} channels (SOC) in order to refill SR Ca^{2+} stores (Cogolludo *et al.*, 2007). cAMP, like cGMP is thought to induce smooth muscle hyperpolarisation and thus relaxation by the modulation of K^+ channels. Indeed, cAMP-induced activation of BK_{Ca} and ATP-dependent K^+ channels (K_{ATP}) has been described in PASMCs (Barman *et al.*, 2003). Furthermore, cAMP is also able to regulate vascular tone through Ca^{2+} desensitisation: increased MLCP activity, reducing myosin light chain kinase (MLCK), p42/p44 MAP kinase and Rho kinase activity (Somlyo & Somlyo, 2003).

1.2.7 Serotonin

Serotonin (or 5-hydroxytryptamine; 5-HT) was first associated with PH in the 1960s, when people taking diet pills (appetite suppressants) such as aminorex developed PH (Follath *et al.*, 1971). This observation was confirmed when patients taking other serotonin transporter (SERT) inhibitors, such as dexfenfluramine (Dfen) for more than three months had an increased risk of developing PH compared to non-users (Kramer & Lane, 1998); the 'serotonin hypothesis of PH' was then coined.

5-HT is synthesised in pulmonary artery endothelial cells by the enzyme tryptophan hydroxylase 1 (TPH1) and exerts biological activity via one of several 5-HT receptor subtypes (primarily $5\text{-}HT_{1B}$, $5\text{-}HT_{2A}$ & $5\text{-}HT_{2B}$), and through SERT, to mediate constriction and proliferation of pulmonary artery smooth muscle cells and fibroblasts (MacLean & Dempsie, 2009; Weir *et al.*, 2004).

1.2.7.1 Serotonin and pulmonary hypertension

5-HT production and circulating levels are elevated in PH, as are SERT, $5\text{-}HT_{1B}$ and TPH1 expression in pulmonary artery smooth muscle and endothelial cells from PH patients (MacLean & Dempsie, 2009). Experimentally, inhibition of SERT prevents

serotonin-dependent proliferation in cells, and reduces hypoxic and MCT-induced PH in rodent models (Song *et al.*, 2005; Zhai *et al.*, 2009). Genetically-modified mice have also substantiated a role for 5-HT in the pathogenesis of PH since 5-HT_{1B}, 5-HT_{2B} and SERT KO mice are resistant to the pulmonary vascular remodelling and right ventricular (RV) hypertrophy in response to hypoxia (Eddahibi *et al.*, 2000; Keegan *et al.*, 2001; Launay *et al.*, 2002). Conversely, animals over-expressing SERT exhibit aggravated pathology when exposed to a hypoxic environment (MacLean *et al.*, 2004). There is also evidence to suggest that 5-HT may interact with BMPR2 to markedly increase susceptibility to PH (Long *et al.*, 2006; Willers *et al.*, 2006). The dual 5-HT_{2A} and 5-HT_{2B} antagonist terguride prevents PH in experimental models (Dumitrascu *et al.*, 2011) but was unsuccessful in Phase II clinical trial (Ghofrani *et al.*, 2012).

1.3 Natriuretic peptides

The natriuretic peptide (NP) family consists of three principle members: atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). They all share a common 17 amino acid ring structure (CFGXXXDRIXXXXGLGC, ***Figure 1.3.*** (Barr *et al.*, 1996)), are synthesised as preprohormones and have actions that protect the cardiovascular system, particularly from the effects of salt and volume overload.

1.3.1.1 Atrial natriuretic peptide

ANP was the first NP to be discovered in 1981 (de Bold *et al.*, 1981). It is preferentially synthesised and secreted from the cardiac atria, however under pathological conditions ANP can also be synthesised in the ventricles (Yasue *et al.*, 1994). ANP is stored in granules predominantly in its 126 amino acid proANP form, upon secretion it is rapidly cleaved to the biologically active 28 amino acid peptide form by corin a transmembrane cardiac serine protease (Yan *et al.*, 2000). Biologically active rat and mouse ANP are also 28 amino acids in length. The principle stimulant for ANP release is atrial wall stretch as a result of increased atrial volume (de Bold *et al.*, 1981).

ANP and BNP are described as 'endocrine mediators'. This is because once secreted they perfuse into the coronary sinus thus enabling them to be distributed throughout the body to their target organs. The effects of ANP and BNP are wide ranging and show considerable overlap, but murine knockout models have revealed that ANP and BNP have certain distinct functions (Franco *et al.*, 2004; Mori *et al.*, 2004; Potter *et al.*, 2006; Tamura *et al.*, 2000). ANP knockout (KO) mice exhibit cardiac hypertrophy, vascular remodelling and modest increases in systemic blood pressure (Franco *et al.*, 2004; Mori *et al.*, 2004). These findings suggest ANP is a modulator of vascular remodelling, blood pressure as well as cardiac hypertrophy. In contrast, BNP KO mice exhibit fibrotic lesions in the ventricles; therefore BNP has a role as a regulator of ventricular integrity (Tamura *et al.*, 2000).

Figure 1.3 Amino acid structure of the three natriuretic peptides

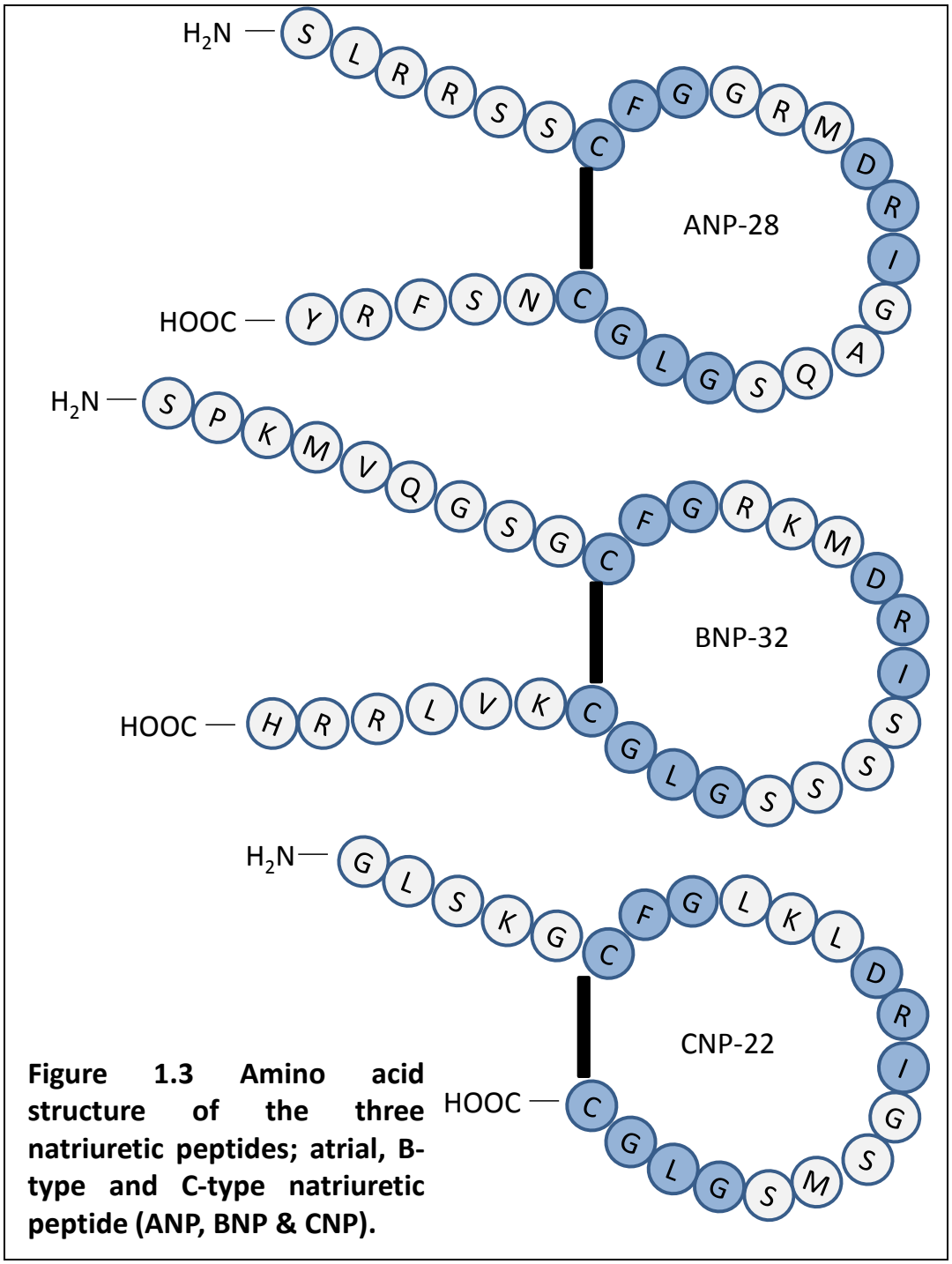


Figure 1.3 Amino acid structure of the three natriuretic peptides; atrial, B-type and C-type natriuretic peptide (ANP, BNP & CNP).

1.3.1.2 B-type natriuretic peptide

BNP is commonly synthesised and secreted from the ventricles, it can however be synthesised in the atria under pathological conditions (Yasue *et al.*, 1994). BNP is stored in granules in the atria with ANP, where-as ventricular production only occurs after stimulation via cardiac wall stretch (Potter *et al.*, 2006). Therefore, BNP release (from the ventricles) is not instantaneous after stimulation akin to ANP, there is a delay whilst it is being transcribed and translated. The biologically active form in humans is 32 amino acids in length; however, rat and mouse BNP is 45 amino acids long. Active human BNP is produced from 108 amino acid pro-BNP (de Bold *et al.*, 1981; Sudoh *et al.*, 1990). Pro-BNP is cleaved by corin and/or furin to produce the active form of BNP and N-terminal pro BNP (NT-proBNP; (Sawada *et al.*, 1997; Yan *et al.*, 2000).

1.3.1.3 C-type natriuretic peptide

CNP was first isolated from porcine brain and identified as a 22 amino acid peptide (Sudoh *et al.*, 1990). CNP is the most conserved NP, CNP-22 and -53 are identical in all mammals. The 103 amino acid pro-CNP is processed by the intracellular endoprotease furin to CNP-53 (Wu *et al.*, 2003). CNP is expressed abundantly in vascular endothelial cells and possibly stored as CNP-53 but released as the 22 amino acid form, as this is the predominant form found in blood (Stingo *et al.*, 1992). Unlike ANP and BNP, CNP acts in a paracrine fashion (rather than endocrine) to regulate local vascular tone and blood flow (Suga *et al.*, 1992b; Villar *et al.*, 2007).

1.3.2 Natriuretic peptide receptors

There are three natriuretic peptide receptors (NPR) termed NPR-A, -B and –C. ANP and BNP are selective agonists for NPR-A, whilst CNP is the sole endogenous ligand for NPR-B (Potter *et al.*, 2006). NPR-A and –B have a similar topology; a

transmembrane protein with an extracellular binding site for NPs, a single short transmembrane domain separating the protein into 2 halves, an intracellular domain, kinase homology and dimerisation regions, and a catalytic GC (Schulz *et al.*, 1989). The GC domain facilitates the conversion of GTP to cGMP at its C-terminal end (Schulz *et al.*, 1989). Hence NPR-A and –B are also termed particulate guanylate cyclase (pGC) or GC-A and –B, respectively.

NPR-C is a truncated NPR, in that the extracellular and transmembrane domains are similar to NPR-A and –B but its intracellular domain is very short and it lacks the GC domain. Instead it possesses an intracellular $G_{i/o}$ G-protein binding domain which inhibits AC activity and activate phospholipase-Cβ3 (PLCβ3; (Madhani *et al.*, 2003). All three NPRs are homodimeric (Potter *et al.*, 2006).

1.3.2.1 Natriuretic peptide receptor-A

NPR-A is widely distributed about the body. It has been implicated in pulmonary and vascular smooth muscle relaxation, inhibition of cardiac hypertrophy and ventricular fibrosis, natriuresis/diuresis, inhibition of the renin/aldosterone system as well as increased endothelial permeability and reduced intravascular volume (de Bold *et al.*, 1981; Kuhn *et al.*, 2002; Oliver *et al.*, 1997; Sabrane *et al.*, 2005; Schmitt *et al.*, 2003). NPR-A KO mice have elevated blood pressure, salt sensitive hypertension and enlarged hypertrophic hearts (Oliver *et al.*, 1997). Such mice highlight the importance of NPR-A activation in the regulation of blood pressure and cardiovascular homeostasis. The rank affinity of NPR-A for NPs is ANP ≥ BNP >> CNP (Suga *et al.*, 1992a).

NPR-A KO mice have an increased susceptibility to hypoxia induced PH (Zhao *et al.*, 1999). Additionally, hypoxia induced PH is attenuated by increased circulating levels of ANP in both transgenic mice that over express ANP (Klinger *et al.*, 1993a) and in rats given a neutral endopeptidase inhibitor (NEPi; NEP hydrolyses ANP) (Baliga *et al.*, 2008; Klinger *et al.*, 1993b).

1.3.2.2 Natriuretic peptide receptor-B

NPR-B is found in chondrocytes, brain, lung, vascular smooth muscle and uterus (Chrisman *et al.*, 1993; Nagase *et al.*, 1997; Schulz *et al.*, 1989). NPR-B is more abundant in the brain than the other NPRs, and activation has been shown to stimulate growth hormone release from anterior pituitary cells in rats (Hartt *et al.*, 1995). NPR-B KO mice suffer from dwarfism, impairment of endochondral ossification and limb bone growth, and the females are infertile (Tamura *et al.*, 2004). The rank affinity of NPR-B for NPs is CNP > ANP ≥ BNP (Suga *et al.*, 1992a).

CNP acting via NPR-B has been shown to be both anti-hypertrophic and anti-fibrotic in the heart (Calvieri *et al.*, 2012). As a compensatory mechanism in the failing heart CNP is produced in an attempt to improve cardiac remodelling and oppose vascular resistance (Kalra *et al.*, 2003). Furthermore NPR-B becomes the predominant NPR in the failing heart (Pagel-Langenickel *et al.*, 2007).

1.3.2.3 Natriuretic peptide receptor-C

NPR-C is known as the 'clearance receptor', because it binds circulating NPs, internalises them and subjects them to lysosomal degradation (Maack *et al.*, 1987). The rank affinity of NPR-C for NPs is ANP ≥ CNP > BNP in both humans and rats (Suga *et al.*, 1992a). The differences in affinity between NPs for NPR-C may play a role in the longer serum half-life of BNP as compared to ANP. However, NPR-C has a positive signalling capacity – not just clearance. It has recently been shown that NPR-C/CNP signalling plays a role in endothelium derived hyperpolarising factor (EDHF) dependent dilatation in rat isolated mesenteric and coronary arteries (Villar *et al.*, 2007). NPR-C KO mice have mild diuresis, low blood volume, and ANP has a longer half-life; this is consistent with NPR-C acting as a clearance receptor (Matsukawa *et al.*, 1999). These transgenic mice also have skeletal bone deformities, perhaps the result of increased CNP levels activating NPR-B (Matsukawa *et al.*, 1999).

1.3.3 Neutral endopeptidase

Neutral endopeptidase is a membrane bound zinc metalloendopeptidase, it is located in numerous tissues and organs including the lung, brain and gut (Thompson & Morice, 1996). NEP degrades ANP, however it has quite broad selectivity and so can also degrade ET-1 (Vijayaraghavan *et al.*, 1990), BNP (Bourne & Kenny, 1990), bradykinin (Klinger *et al.*, 1993b) and substance P (Erdos & Skidgel, 1989). Decreased metabolism of any these compounds could affect both pulmonary and systemic tone.

1.4 Phosphodiesterases

3',5'-cyclic nucleotide phosphodiesterases (PDEs) are a family of enzymes that hydrolyse cyclic nucleotides and therefore regulate signals conveyed by the second messengers cAMP and cGMP. There are three classes of PDEs I, II and III; mammalian PDEs belong to class I. Twenty-one class I PDE genes have been identified in human, rat and mouse; which encode more than fifty different PDE proteins. The class I PDEs have approximately 270 amino acids (aa) in the C-terminal catalytic domain that are highly conserved (Beavo, 1995). The PDE superfamily is characterised into eleven subfamilies (PDE1 to PDE11), based on structural similarities such as sequence homology, protein domains, substrate specificity, kinetic properties and sensitivity to endogenous regulators and inhibitors (Beavo, 1995).

The protein domains located in the N-terminal catalytic domain give each PDE family their unique characteristics. PDE2, PDE5, PDE6, PDE10 and PDE11 have a protein domain known as a GAF domain and are thus termed GAF-PDEs. GAF domains bind cGMP (some GAF domains have been reported to bind cAMP; (Handa *et al.*, 2008), regulate PDE catalytic domains and thus activity, and facilitate dimerisation of GAF-PDEs (Martinez *et al.*, 2002). When cGMP binds a GAF domain of PDE2 or PDE5 catalytic activity is stimulated via allosteric changes (Martins *et al.*, 1982) or phosphorylation of an adjacent domain (Corbin *et al.*, 2000), respectively. PDE1, PDE3-4 and PDE7-9 do not have GAF domains and so are known as the non-GAF-PDE subfamily. The best characterised PDEs are 1-6 and these are considered in more detail below and in ***Figure 1.4***.

1.4.1 PDE1: 'Calcium/calmodulin-stimulated'

There are three human PDE1 subtypes, PDE1A (535 aa), PDE1B (634 aa) and PDE1C (536 aa). The PDE1 family is unique in that it is Ca^{2+}/calmodulin sensitive and thus Ca^{2+} is required to activate PDE1 isozymes. The PDE1 family can hydrolyse both cAMP and cGMP, but have a greater affinity for cGMP and therefore

preferentially hydrolyse cGMP. PDE1 is localised in the brain, heart, kidney, liver, skeletal muscle as well as vascular and visceral muscle (Wallis *et al.*, 1999). Nuclear PDE1A has been implicated in the regulation of cell proliferation and apoptosis of actively growing vascular smooth muscle cells (Nagel *et al.*, 2006). PDE1 is upregulated in pulmonary artery smooth muscle cells in human IPAH lungs and hypoxia induced PH in mice (Schermuly *et al.*, 2007). Additionally inhibition of PDE1 reverses vascular remodelling in hypoxia induced PH in mice (Schermuly *et al.*, 2007).

1.4.2 PDE2: 'cGMP-stimulated'

There is just one human PDE2 subtype, PDE2A (941 aa). PDE2 is a GAF-PDE and has two GAF domains, GAF-A and GAF-B they have distinct roles in dimerisation and cGMP binding, respectively (Martinez *et al.*, 2002). PDE2 hydrolyses both cAMP and cGMP; it is positively regulated by both cyclic nucleotides, however cGMP is the both the favoured substrate and effector (Erneux *et al.*, 1981). PDE2 is localised in the adrenal cortex, brain, corpus cavernosum, heart, kidney, liver, skeletal muscle and visceral smooth muscle (Wallis *et al.*, 1999). Specific PDE2 inhibitors (PDE2i) have been shown to improve memory in animal models (Boess *et al.*, 2004). PDE2 has been shown to be a regulator of cardiac L-type Ca^{2+} channels in human cardiac myocytes (Fischmeister *et al.*, 2005). In rat cardiac myocytes, PDE2 has been shown to be involved in compartmentalisation of cGMP produced by pGC and sGC; with PDE2 controlling the pGC pool and PDE5 governing the sGC-generated cGMP (Castro *et al.*, 2006). Furthermore, eiythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) a PDE2i was able to reverse hypoxic pulmonary vasoconstriction (HPV) in the perfused rat lung (Haynes *et al.*, 1996).

1.4.3 PDE3: 'cGMP-inhibited'

The two human PDE3 subtypes are PDE3A (1141 aa) and PDE3B (1112 aa). PDE3 has N-terminal hydrophobic membrane association domains and can be either

cytosolic or membrane bound. PDE3 has been shown to be associated with the plasma membrane, sarcoplasmic reticulum, golgi apparatus and nucleus envelope (Omori & Kotera, 2007). PDE3 can hydrolyse both cAMP and cGMP. It has a higher affinity for cGMP but can hydrolyse cAMP 10 times faster than cGMP; therefore cGMP is a competitive inhibitor of cAMP (Zaccolo & Movsesian, 2007). PDE3 is localised in the heart, smooth muscle, adipose tissue and platelets (Wallis *et al.*, 1999). PDE3i have been shown to antagonise platelet aggregation, block oocyte maturation, increase myocardial activity and enhance vascular and airway smooth muscle relaxation (Bender & Beavo, 2006). Intravenous milrinone (PDE3i) is approved for the use in heart failure, but has been shown to increase mortality if used chronically (Packer *et al.*, 1991). PDE3 inhibition potentiates the effect of prostacyclin analogues in the treatment of hypoxia induced PH in rats (Phillips *et al.*, 2005).

1.4.4 PDE4

The PDE4 family is the largest with four human subtypes, PDE4A (647 aa), PDE4B (736 aa), PDE4C (712 aa) and PDE4D (673 aa). They have a unique region known as upstream conserved regions (UCR1 and UCR2), which regulate the catalytic region of PDE4 (Beard *et al.*, 2000). PDE4 exclusively hydrolyses cAMP and is localised in the kidney, lung, mast cells, heart, skeletal muscle as well as vascular and visceral smooth muscle (Wallis *et al.*, 1999). PDE4D KO mice have shown that PDE4D deficiency promotes heart failure (Lehnart *et al.*, 2005). Whilst PDE4B KO mice have shown PDE4B is essential for mounting an inflammatory response to lipopolysaccharide in monocytes and macrophages, and in the production of tumour necrosis factor-α (Jin *et al.*, 2005). Therefore PDE4 inhibitors are being developed with a view to combating inflammatory diseases such as chronic obstructive pulmonary disease (COPD), asthma and arthritis. PDE4 inhibition has been shown to potentiate the effect of prostacyclin analogues in the treatment of hypoxia induced PH in rats (Phillips *et al.*, 2005).

Figure 1.4 Schematic diagram of phosphodiesterases 1-6

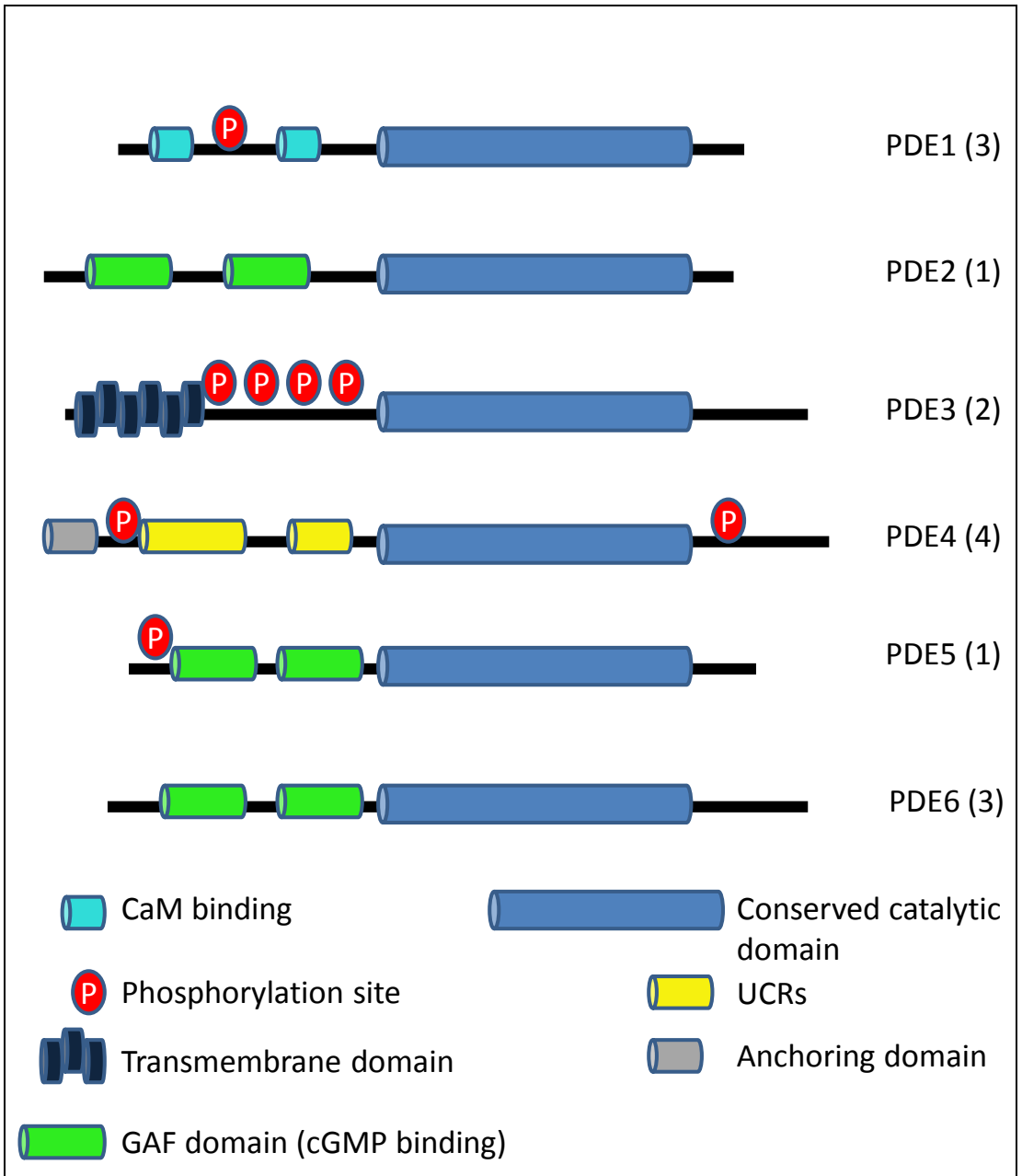


Figure 1.4 Schematic diagram of the structure of phosphodiesterases 1-6. Next to the family name is the number of genes comprising the family. CaM, calmodulin; PDE, phosphodiesterase; GAF, cGMP-activated PDEs; UCRs, upstream conserved regions.

1.4.5 PDE5

The sole human PDE5 is PDE5A (875 aa). PDE5 is a GAF-PDE and has two GAF domains, GAF-A and GAF-B. Unlike PDE2, GAF-A rather than GAF-B is responsible for the allosteric binding of cGMP this promotes phosphorylation, which not only activates the catalytic function but increases cGMP binding affinity (Zoraghi *et al.*, 2005). PDE5 is localised in the corpus cavernosum, platelets, skeletal muscle, vascular and visceral smooth muscle (Wallis *et al.*, 1999). PDE5 regulates vascular smooth muscle relaxation via the regulation of cGMP; particularly in the lung and penis. The PDE5i sildenafil is approved for the treatment of PH and erectile dysfunction. PDE5i in PH are discussed in detail in section 1.5.5.

1.4.6 PDE6

PDE6 is only found in the retina. There are three PDE6 members PDE6A (860 aa), PDE6B (854 aa) and PDE6C (858 aa). Given their localisation in the eye they are often known as photoreceptor PDEs. PDE6 is a GAF-PDE and has two N-terminal GAF domains; GAF-A and GAF-B. GAF-A has a high affinity binding site for cGMP, once bound an allosteric change occurs thus activating PDE6 (Martinez *et al.*, 2008). Little is known about PDE6 due to a paucity of selective inhibitors and KO mice. Sildenafil has been shown to inhibit PDE6, albeit at far greater concentration than required to inhibit PDE5, and so has been suggested to be the source of some visual side effects of sildenafil.

1.4.7 Manipulating cGMP-PDE activity in pulmonary hypertension

A method of increasing cGMP levels is to inhibit the enzyme that hydrolyses it, PDEs. Several PDEs have been implicated in PH, including PDE1 and PDE3, however little is known in comparison to PDE5. For example PDE1i reverses vascular remodelling in hypoxia induced PH in mice (Schermuly *et al.*, 2007). Whilst PDE3i potentiates the effect of prostacyclin analogues in the treatment of

hypoxia induced PH in rats (Phillips *et al.*, 2005). The PDE5i sildenafil is already a licensed therapy for PH, as is tadalafil. Vardenafil a third PDE5i is currently being clinically evaluated in PH. Interestingly sildenafil has been shown to have no effect on RV hypertrophy and vascular remodelling in hypoxia induced PH in NPR-A KO mice and blunted effects on right ventricular systolic pressure (RVSP; (Zhao *et al.*, 2003). Therefore the NP pathway plays a key role in the response to PDE5i in hypoxia induced PH. This phenomenon has been investigated by giving a combination of PDE5i (sildenafil) with a NEPi (ecadotril) to rats subjected to hypoxia (Baliga *et al.*, 2008). This combination treatment is effective in lowering RVSP, RV hypertrophy and vascular remodelling over that of sildenafil or ecadotril alone. Therefore a superior method of treating PH could be to modulate PDE5 and NPs concomitantly. These approaches are discussed in more detail in section 1.5.

1.5 Treatment of Pulmonary Hypertension

Current treatment options for PH include lifestyle modifications (low grade aerobic exercise), conventional treatments (e.g. diuretics and anticoagulants), calcium channel blockers, prostacyclin analogues, endothelin receptor antagonists and phosphodiesterase (PDE) inhibitors (McLaughlin & McGoon, 2006).

There is little data to base exercise recommendations on, but patients are usually advised to partake in low grade aerobic exercise, such as walking in order to prevent deconditioning and worsening of overall function (Humbert *et al.*, 2004c). Diuretics are used to manage oedema as well as volume over load in RV failure, and oxygen supplementation to prevent hypoxemia (a potent pulmonary vasoconstrictor), which has been shown to improve survival in IPAH patients (Fuster *et al.*, 1984). In addition to these, the anti-coagulant warfarin and the cardiac glycoside digoxin are also used as background therapies in PH patients (McLaughlin *et al.*, 2009; Writing Committee *et al.*, 2009). Current frontline therapies are predominately vasodilators that either augment endogenous vasodilator pathways, including NO or PGI_2, or by inhibiting the potent endogenous vasoconstrictor ET-1 (***Figure 1.1***). However these are systemic vasodilators, therefore systemic hypotension can be dose limiting (Galie *et al.*, 2010).

1.5.1 Calcium channel blockers

Calcium (Ca^{2+}) channel blockers can be effective in IPAH patients who respond to an acute challenge during right heart catheterisation and achieve a >20% fall in PAP and no decline in cardiac output (Sitbon *et al.*, 1998). The L-type Ca^{2+} channel blockers commonly used are nifedipine, diltiazem and amlodipine. Unfortunately only a small number (10-15%) of IPAH patients meet the criteria and only half will attain sustained haemodynamic and clinical benefit. Those that do respond have a vastly improved 5-year survival rate to non-responders (94% versus 55%, respectively; (Sitbon *et al.*, 2005).

1.5.2 Prostacyclin analogues

PGI_2 is a ligand of the G_s-coupled IP receptor, once bound it activates AC which produces cAMP and activates PKA. This pathway is anti-proliferative and vasorelaxant and opposes the TXA_2 pathway which cause proliferation and vasoconstriction (Humbert *et al.*, 2004b). PGI_2 and TXA_2 are arachidonic acid metabolites, it is thought in PH there is a balance shift from PGI_2 to TXA_2 (Humbert *et al.*, 2004b); the aim of prostacyclin therapy is to redress the balance back to PGI_2. Prostacyclin therapy has been shown to be effective in patients with PH, however the various analogues available all have their deficiencies (Galie *et al.*, 2010). For example, the route of delivery and the pain associated with this, in the case of intravenous administered epoprostenol, or subcutaneous delivery of treprostinil, or the short half-life of beraprost and iloprost.

Inhaled prostacyclin therapy has been trialled in man to overcome the difficulties described above. Inhaled treprostinil has been shown to be effective in the long-term treatment of adults and short-term treatment of children (Benza *et al.*, 2011; Krishnan *et al.*, 2012). Oral beraprost (TRK-100STP) has been shown to be effective in a 12 week trial of Japanese PAH patients (Kunieda *et al.*, 2009).

1.5.3 Endothelin receptor antagonists

ET-1 is a potent vasoconstrictor as well as mitogenic agent. ET-1 plasma levels are significantly elevated in PAH, and correlates with disease severity (Rubens *et al.*, 2001). There are two cell surface receptors ET_A and ET_B, they are both G-protein-coupled receptors (Shao *et al.*, 2011). On vascular smooth muscle cells activation of both the ET_A or ET_B receptors induces vasoconstriction and proliferation. However, to complicate the story ET_B receptor stimulation on endothelial cells results in NO and PGI_2 release (see section 1.2.4). The ability of the two receptors to mediate disparate downstream functions has directed the targeting of these receptors as therapeutic targets. ERAs such as bosentan (dual ET_A/ET_B), sitaxsentan ($ET_A >> ET_B$) and ambrisentan ($ET_A > ET_B$) have had positive effects on PH patients in

clinical trials (Williamson *et al.*, 2000; Channick *et al.*, 2001; Barst *et al.*, 2004a; Galie *et al.*, 2005a) but elevated hepatic enzymes remain a concern and this class of drugs are teratogenic (McLaughlin & McGoon, 2006).

1.5.4 Inhaled nitric oxide

Inhalation of exogenous NO gas not only decreases PAP but improves oxygenation in numerous forms of PAH, it has been shown to be particularly efficacious in neonates with PPHN (Kinsella *et al.*, 1992). NO is an unstable gas therefore long-term therapy is complicated; there are also concerns with regard to the development of methaemoglobinaemia (NO avidly binds the haem moiety and oxidises it) as well as rebound PH as a consequence of therapy termination (Atz *et al.*, 1996). Nonetheless, inhaled NO has proved a great success in the treatment of PPHN, dramatically reducing the need for extra-corporeal membrane oxygenation (ECMO) and improving survival.

1.5.5 PDE5 inhibitors

PDEs are a family of enzymes that hydrolyse cyclic nucleotides and therefore regulate signals conveyed by the second messengers cAMP and cGMP. There are three classes of PDEs, class I are mammalian PDEs of which there are 11 sub-families (PDE1 to PDE11); see section 1.4. Molecules that block members of this family are collectively known as PDE inhibitors (PDEi); they therefore block the breakdown of cAMP and/or cGMP. PDEi have been a great focus of drug development of recent years, in particular as therapies for cardiovascular disease. This is because they have favourable effects in the vasculature, these include vasodilatation, inhibition of smooth muscle proliferation and prevention of platelet aggregation (Bender & Beavo, 2006). Furthermore, PDE5 expression and activity in the pulmonary vasculature is augmented in PAH (Murray *et al.*, 2002).

PDE5 exclusively metabolises cGMP; PDE5 inhibition reduces both systemic and pulmonary artery pressure in both humans and animals however, there is a greater effect on the pulmonary vasculature thus exhibiting relative selectivity for the pulmonary vasculature in PH, not healthy volunteers (Baliga *et al.*, 2008; Klinger *et al.*, 2006). The PDE5i sildenafil was licensed for the treatment of PH in 2005. Sildenafil has been shown to improve haemodynamic and exercise capacity over several months in patients with IPAH and PH associated with connective tissue disease (Galie *et al.*, 2005b). A similar PDE5i, tadalafil, which has a longer half-life has recently been licensed for the treatment of PAH (Galie *et al.*, 2009). Another PDE5i, vardenafil, has been shown to be effective in the efficacy and safety of vardenafil in the treatment of PAH trial (EVALUATION; (Jing *et al.*, 2011).

1.5.6 Haem-dependent and -independent sGC activators

As described in 1.2.2, signalling through the NO-sGC-cGMP pathway can be compromised in PH by either reduced bioavailability of NO or by the oxidation of sGC thus making it unresponsive to NO and NO donor drugs (Stasch *et al.*, 2006). Two novel classes of compounds have been generated which seem to combat the challenge posed by reduced NO bioavailability or oxidised sGC; namely sGC stimulators and sGC activators.

sGC stimulators are also known as NO-independent, haem-dependent sGC stimulators. This is because they require the sGC haem moiety to be reduced (NO sensitive) in order for sGC to be stimulated and cGMP produced (Stasch & Hobbs, 2009). sGC stimulators can act independently of, or in synergy with, NO (Stasch *et al.*, 2002b; Stasch *et al.*, 2002a). The orally active sGC stimulator riociguat is currently in phase III trials to assess its clinical effectiveness in IPAH and CTEPH (Ghofrani *et al.*, 2010). In earlier clinical trials systemic hypotension and a lack of pulmonary selectivity was observed, this could be limitation of this strategy (Grimminger *et al.*, 2009). Such a shortcoming may have been expected given that sGC stimulators synergise with NO and will augment NO-dependent vasodilation in all vascular beds. The synergy with NO will predominate in the systemic over that

of the pulmonary circulation because in PH bioavailability of NO is impaired in the pulmonary vasculature and not the systemic. Inhalation of riociguat could be an effective route to target the pulmonary vasculature and minimise the side effect of systemic hypotension (Evgenov *et al.*, 2007).

Soluble GC activators are also known as NO- and haem-independent activators. These compounds are able to activate sGC even when the haem moiety is oxidised and therefore insensitive to both NO and sGC stimulators (Stasch *et al.*, 2006). Given the oxidised form of sGC is thought to be prominent in diseased vasculature, sGC activators could prove to be a class of compounds that can specifically target diseased over healthy vessels. These compounds could be an effective method in targeting the 'diseased' pulmonary vasculature over that of the relatively 'healthy' systemic vasculature in PH. Indeed, cinaciguat (BAY 58-2667) has been shown to be effective in experimental pulmonary hypertension (Chester *et al.*, 2011; Dumitrascu *et al.*, 2006).

1.5.7 Combination therapies for pulmonary hypertension

As described earlier, the aetiology of PH is complex and multi-factorial, given current treatments tend to only target one aspect of the disease, combining therapies is a new method of attempting to treat the disease. Treatment using a combination of therapies is not a new idea it has been successfully used in systemic hypertension, rheumatoid arthritis plus many other diseases.

The rational for combining different therapies for the treatment of PH or in fact any disease, is to attack as many pathways as possible that underlie the disease process. ERAs, PDE5i and prostacyclin analogues have all been shown to be effective in lowering pulmonary haemodynamics and improving functional class in PH (Channick *et al.*, 2001; Galie *et al.*, 2005b; Galie *et al.*, 2010). Therefore using two or three of these different therapies together may have additive effects. Furthermore, a combination of these therapies may have synergistic effects, i.e. one therapy facilitating the action of another. The vasodilator activity of prostacyclin analogues

are mostly mediated by cAMP, which is hydrolysed primarily by PDE3 and PDE4 in the lung. PDE3 can also hydrolyse cGMP, for which it has a higher affinity, yet it hydrolyses cAMP 10 times faster; thus making cGMP a competitive inhibitor of PDE3. PDE5i slow the metabolism of cGMP thus making more available, which can increase cAMP levels indirectly by inhibiting PDE3. Therefore, in theory one could further increase cAMP levels by slowing its metabolism if a prostacyclin analogue and a PDE5i were given in combination.

As mentioned in section 1.4.7 a combination of PDE5i (sildenafil) with a NEPi (ecadotril) was given to rats subjected to hypoxia, thus inducing PH (Baliga et al., 2008). This combination treatment is effective in lowering RVSP, RV hypertrophy and vascular remodelling over that of sildenafil or ecadotril alone. Both treatments increase cGMP however in PH the NP system appears to be important because sildenafil is less effective in NPR-A KO mice subjected to hypoxia (Zhao *et al.*, 1999).

Ideally, combination therapy should be designed to increase pulmonary selectivity/efficacy, as well as reverse both structural abnormalities and aberrant haemodynamics. There have been several trials investigating the combination of the following therapies: bosentan and epoprostenol (Humbert *et al.*, 2004a), sildenafil and inhaled iloprost (Ghofrani *et al.*, 2002), sildenafil and epoprostenol (Stiebellehner *et al.*, 2003), bosentan and sildenafil (Hoeper *et al.*, 2004), in PPHN inhaled NO and sildenafil (Stocker *et al.*, 2003). Further large scale studies are required to determine if 'combination therapy' enhances therapeutic outcome.

1.6 Pulmonary Fibrosis

Pulmonary Fibrosis (PF) is an end stage of a group of heterogeneous lung diseases categorised as interstitial lung diseases (ILD). PF is characterised by excessive deposition of extracellular matrix (ECM) proteins in the interstitium (Coward *et al.*, 2010). Alveolar spaces are replaced by large areas of fibrosis and collagen accumulation leading to a loss of tissue architecture thereby preventing efficient gas exchange. It is a progressive disease which eventually leads to respiratory failure and premature death (Coward *et al.*, 2010; Gribbin *et al.*, 2006; Kim *et al.*, 2006). Idiopathic pulmonary fibrosis (IPF) is the most common ILD and has a poor median survival from diagnosis of 3-5 years and an incidence in the UK of 4.6 per 100,000 people, moreover between 1991 and 2003 the incidence increased annually by 11% (Gribbin *et al.*, 2006).

The aetiology of IPF is still largely unknown, however several risk factors have been identified including cigarette smoking (Baumgartner *et al.*, 1997), herpes virus (Tang *et al.*, 2003), hepatitis C virus and Epstein-Barr virus (Borchers *et al.*, 2011). Although the genetic factors which contribute to the development of IPF remain unclear, some 3% of IPF patients have been identified to have the familial form, which suggests that genetic factors may contribute to the risks of developing IPF in certain individuals (Datta *et al.*, 2011). However it is thought the aetiology is likely to be multi-factorial given only a small number of individuals exposed to known risk factors develop IPF.

As the hypotheses on the pathogenesis of IPF have changed or evolved so too has the therapeutic approach. Initially it was thought the lung fibrosis in IPF was as a result of chronic inflammation due to an insult and thus resulting in scarring. Therefore corticosteroids and other anti-inflammatory drugs such as azathioprine have been the standard therapy for IPF. However there appears to be little evidence that these interventions alter the outcome or progression of IPF (Raghu, 2006). In fact it is now questioned if inflammation plays a role in IPF, the evolved view is that IPF is due to abnormal wound healing (Selman *et al.*, 2011). As a result contemporary IPF research has focused on an anti-fibrotic mode of action. There have been some clinical trials using anti-fibrotic drugs however apart from pirfenidone (inhibits the

synthesis of TGF-β and tumour necrosis factor-α; TNF-α) the results so far have been disappointing. So far pirfenidone is the only licensed anti-fibrotic drug for IPF (Richeldi & du Bois, 2011).

Development of PH secondary to ILDs has been shown to occur in up to 38% of scleroderma patients and a third of IPF patients (Lettieri *et al.*, 2006). The presence of PH in patients with advanced IPF has an almost threefold increased risk of death (Lettieri *et al.*, 2006). Such patients fall within the Group III PH classification.

1.6.1 Phosphodiesterase 5 and pulmonary fibrosis

The biological activity of PDE5 in the lung and right ventricle is significantly elevated in the bleomycin mouse model (see section 1.7.4) of PF and PH (Hemnes *et al.*, 2008). Furthermore sildenafil has been shown to attenuate bleomycin induced PF/PH in both mice and rats (Hemnes *et al.*, 2008; Yildirim *et al.*, 2010). However, a clinical evaluation of sildenafil in IPF patients has revealed little benefit (The Idiopathic Pulmonary Fibrosis Clinical Research Network, 2010), suggesting a combination therapeutic approach may be necessary.

1.7 Animal models of pulmonary hypertension

A number of animal models of PH have been developed including insult models, such as the hypoxia Sugen (SU-5416; (Ciuclan *et al.*, 2011; Taraseviciene-Stewart *et al.*, 2001), and bleomycin models (Scotton & Chambers, 2010), as well as genetically engineered predisposition models including the ApoE knockout (Hansmann *et al.*, 2007; Lawrie *et al.*, 2011) or BMPR2 mutants in hypoxia (Burton *et al.*, 2011; Hong *et al.*, 2008; Long *et al.*, 2006; West *et al.*, 2004; West *et al.*, 2008). Two of the most commonly used pre-clinical models for the study of PH are chronic hypoxia and monocrotaline (MCT) injury. Discussed below are these models.

1.7.1 Chronic hypoxia

In the chronic hypoxia model animals are exposed to 10% O_2 for a set length of time to induce PH (i.e. 14 days for rats and 21 days for mice). This model of PH is very predictable and reproducible within age matched animal strains (Stenmark *et al.*, 2006). After two weeks exposure to hypoxia rats develop 'moderate' PH; a doubling of mean pulmonary artery pressure. Structural changes that occur include muscularisation of small arteries in the alveolar wall, increases in the number of cells expressing α-smooth muscle actin (α-SMA) in the walls of previously non-muscularised arterioles and increased thickening of precapillary arteries (Hislop & Reid, 1977; Stenmark *et al.*, 2006). Furthermore RV hypertrophy occurs, however unlike in humans there is little evidence of RV failure (Stenmark *et al.*, 2009). Mice exposed to hypoxia appear to develop less severe PH in comparison to rats; however it has been shown that responses to hypoxia in mice are strain specific (Tada *et al.*, 2008). One of the issues with this model is that hypoxia induced PH in animals is reversible, which is in contrast to human PH (Channick *et al.*, 2001). Additionally this model does not develop plexiform lesions as found in severe forms of human PH and other rat models (Taraseviciene-Stewart *et al.*, 2001; Tuder *et al.*, 1994; Tuder *et al.*, 2001). The minimal vascular remodelling associated with the mouse chronic hypoxia is a short coming that limits the usefulness of knock-out mouse strains to

better understand the distinct roles of a molecule or pathway in the pathogenesis of PH (Taraseviciene-Stewart *et al.*, 2001). See section 1.7.2 for the hypoxia-sugen model which is thought to be better than hypoxia alone as it has a more severe phenotype and develops plexiform lesions. Therefore the chronic hypoxia induced PH model should probably be classed as less severe, and possibly a model of Group 3 PH.

1.7.2 Sugen (SU-5416) and hypoxia

A more recently developed rat model of PH is the Sugen 5416 (SU-5416) and hypoxia model which was developed by Taraseviciene-Stewart and colleagues (Taraseviciene-Stewart et al., 2001). SU-5416 is a vascular endothelial receptor 2 (VEGFR-2) inhibitor. SU-5416 in combination with hypoxia causes the formation of plexiform lesions and the severity of PH progresses even after the rats are removed from hypoxia; in some cases rats die from right heart failure (Taraseviciene-Stewart et al., 2001). Additionally it appears that only the lungs are affected, this is more akin to the human condition.

This model has also been shown to be effective in mice (Ciuclan et al., 2011). The combination of the VEGFR inhibition with chronic hypoxia produced a profoundly exacerbated PH-like pathology in comparison to hypoxia alone. This echoes the rat model. It has been suggested that in endothelial cells VEGF has a pro-survival role, and in severe PH the phenotypically altered pulmonary vessels are due initially to apoptosis, followed by selection of apoptosis resistant, pro-proliferative vascular endothelial cells (Tuder et al., 2007). Therefore VEGFR inhibition drives the initial apoptosis and subsequent selection of endothelial cells that are pro-proliferative and apoptosis resistant, eventually resulting in vascular remodelling.

1.7.3 Monocrotaline

Over 40 years ago Kay and colleagues discovered that they could induce PH in rats by giving them ground up *Crotalaria spectabilis* seeds in their food (Kay *et al.*, 1967). MCT is the toxic pyrrolizidine alkaloid present in *Crotalaria spectabilis* that causes PH. MCT is bioactivated in liver microsomes by cytochrome P-450 into monocrotaline pyrrole (MCTP) which causes endothelial injury in the pulmonary vasculature, consequential remodelling of precapillary vessels, and thus PH (Kasahara *et al.*, 1997; Rosenberg & Rabinovitch, 1988). Currently the exact mechanism through which MCT causes PH is unknown. MCT induced PH is progressive, severe and eventually lethal. However MCT has been reported to cause changes in veins, significant liver and kidney damage, as well as myocarditis which affects both the left and right ventricle (Stenmark *et al.*, 2009). Additionally more than 30 agents have been shown to prevent and/or reverse MCT induced PH (Stenmark *et al.*, 2009); suggesting this may not be representative of the human condition and therefore a poor preclinical model for drug development.

A further limitation of the MCT model is the varied results in mice, which limits the opportunities to investigate transgenic mice and therefore the genetic contribution to the pathophysiology of PH. Mice appear to have a degree of resistance to MCT in comparison to rats and do not develop PH to the same degree (Dumitrascu *et al.*, 2008). It is thought that mice metabolise MCT differently to rats and are unable to bioactivate MCT into MCTP (Gomez-Arroyo *et al.*, 2012). To circumvent this problem synthetic MCTP was given via intra venous injection to both mice and rats, which induced acute lung injury in both species, but only severe and progressive PH in rats (Dumitrascu *et al.*, 2008).

1.7.4 Bleomycin

Bleomycin induced pulmonary fibrosis (PF) is a well characterised model, and it has been shown in C57BL/6 mice to also be associated with PH (Ortiz *et al.*, 2002). This matches human idiopathic PF, where PH is a prominent feature (Nathan *et al.*,

2008b). Bleomycin is a glycopeptide antibiotic produced by the bacterium *Streptomyces verticillus* and has been used as an anti-cancer agent (Sleijfer, 2001). However, its usefulness as an anti-neoplastic agent is limited by pulmonary toxicity resulting in fibrosis (Kawai & Akaza, 2003; Sleijfer, 2001). It is thought that bleomycin causes single and double-strand DNA breaks in tumour cells, therefore interrupting the cell cycle. However, this causes the production of superoxide and hydroxide free radicals; overproduction can result in an inflammatory response causing pulmonary toxicity, activation of fibroblasts and subsequent fibrosis (Chaudhary *et al.*, 2006). The mechanism of action by which bleomycin induces PH is not particularly well understood. One hypothesis is that hypoxic vasoconstriction occurs as a result of severe lung damage and in turn induces pulmonary vascular remodelling. A second is the inflammatory environment in the lung caused by bleomycin induces endothelial and smooth muscle cell proliferation in the pulmonary circulation, resulting in PH (Ortiz *et al.*, 2002).

1.7.5 Apolipoprotein E knockout mouse model

Patients with PH have been shown to have reduced messenger ribonucleic acid (mRNA) expression of apolipoprotein E (ApoE) in the pulmonary system, this factor is known to reduce circulating low-density lipoprotein and atherogenesis in the vessel wall (Geraci *et al.*, 2001). When fed a high fat diet (Western or Paigen diet) ApoE knockout ($ApoE^{-/-}$) mice develop increased right ventricular pressures and thus PH (Hansmann *et al.*, 2007; Lawrie *et al.*, 2011). Furthermore the double knock out $ApoE^{-/-}$ and interleukin-1 receptor-1 ($IL\text{-}1R1^{-/-}$) showed a more severe PH phenotype than $ApoE^{-/-}$ alone (Lawrie *et al.*, 2011). It is therefore proposed by the authors that this model could be useful when studying the role of a particular gene in PH. However it could be difficult to separate the effects of each genetic manipulation.

1.7.6 BMPR2 receptor knock out and hypoxia

As described in section 1.2.1 and 1.2.7, BMPR2 mutations play a critical role in the pathogenesis of IPAH and HPAH. However, only 15% to 20% of individuals with the BMPR2 mutation develop PAH (Newman *et al.*, 2004). This suggests that a BMPR2 mutation alone is insufficient for the development of PAH and that multiple environmental or genetic hits are required to trigger the disease.

The mutations in BMPR2 have been reported in coding and non-coding regions of the gene, and collectively appear to lead to reduced cell surface expression of functionally intact BMPR2 protein (Nasim *et al.*, 2012). Considerable efforts utilising a number of transgenic approaches have been applied, seeking to recapitulate the reduced cell surface expression of functional BMPR2 in mice. Mice harbouring dominant negative forms of BMPR2, either a kinase-dead dominant negative form of BMPR2, or clinically relevant functional mutations in the BMPR2 gene develop pulmonary vascular remodelling and hypertension (West *et al.*, 2004; West *et al.*, 2008). In contrast, global silencing of BMPR2 expression by RNA interference did not lead to raised pulmonary arterial resistance, rather mice exhibited a more general vascular pathology, including mucosal haemorrhage (Liu *et al.*, 2007).

The global deletion of BMPR2 leads to an early developmental lethality in mice, whereas heterozygous animals are viable (Beppu *et al.*, 2004). One study has demonstrated $BMPR2^{+/-}$ heterozygous mice develop a mild PH and pulmonary vascular remodelling under basal conditions (Beppu *et al.*, 2004). However, others using the same mouse strain have shown no significant difference (Long *et al.*, 2006). More recently, a number of studies have used conditional knockout approaches targeting deletion of the BMPR2 gene in pulmonary endothelial cells. Investigators found that some, but not all, mice lacking BMPR2 in pulmonary endothelial cells developed RV hypertrophy, and some histopathological features observed in human PAH (Burton *et al.*, 2011; Hong *et al.*, 2008).

The strong genetic link between BMPR2 and the development of heritable forms of PAH is well established. However, the transgenic approaches to recapitulate the

pathophysiology of PAH by modulating the functional expression of BMPR2 in animal models have met with mixed success. Indeed the models highlight the apparent broader role of BMPR2 in the vasculature. Future studies utilising these animal models to explore the environmental triggers that lead to the development of PAH may be well served.

1.8 Hypothesis

PH is a debilitating and deadly disease. There are several approved therapies; however, many of these are vasodilators that do not specifically target the pulmonary vasculature over that of the systemic, therefore systemic hypotension is often dose limiting. A key feature of PH is vascular remodelling and so far there is little evidence that current therapies can reverse said remodelling. Many therapies for PH are either vasodilators or attempt to stem vascular remodelling, not both. Combination therapy could be an effective way forward to the treatment of PH by linking the effects of multiple drugs.

It has been shown that increasing cGMP in PH works in a clinical setting (i.e. inhaled NO, sGC activators and PDE5i). Therefore, in this thesis I have addressed the hypothesis that '***combination of PDE inhibitors with cGMP-elevating agents (i.e. NPs and/or NO) will increase therapeutic efficacy in PH'***.

To address this hypothesis, I have investigated the following specific aims:

1) *In vitro* functional pharmacological assessments of PDE activity, and responsiveness to ANP and NO, in isolated systemic (aorta) and pulmonary vessels
2) To determine the efficacy of PDE5i, PDE2i and NEPi, alone and in combination, in ameliorating PH in *in vivo* models.
3) Investigate PDE isozyme expression in healthy and diseased tissue from animals with experimental PH.

The unique facets of this thesis are the first systematic functional characterisation of PDE1, -2, -3 and -5 on sGC and pGC signalling in pulmonary and systemic vasculature; and the assessment of chronic hypoxia on these systems. In addition, complementary analysis of PDE1, -2, -3 and -5 mRNA expression was performed. I show the first data supporting the functional role of PDE2 (and inhibitors thereof) in lung vascular homeostasis, as well as PDE2 having potential to be a therapeutic target in PH. Furthermore, I demonstrate the beneficial effects of PDE2 are dependent on natriuretic peptide bioactivity and are selective for the pulmonary vasculature. Moreover, the sensitivity of ANP-driven signalling is maintained in

hypoxia whereas NO-based signalling is blunted. Finally, I establish that the combination of PDE5i and NEPi is effective in pre-clinical models of both PH (reversal) and PH associated with IPF.

Chapter 2: Materials and Methods

2.1 Animals

Animals were housed in a specific pathogen-free facility, had access to food and water *ad libitum* and were exposed to a 12 hour light/dark cycle. All animal studies were licensed under the UK Home Office Animals (Scientific Procedures) Act 1986.

2.2 Materials

N-[4-[1-(3-Aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (SPER-NO), 9, 11-dideoxy-11$^{\alpha}$, 9$^{\alpha}$-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619), acetylcholine (ACh), phenylephrine (PE), rat ANP, vinpocetine, milrinone, Krebs Ringer Solution, soda lime and primers were all purchased from Sigma Aldrich Co, Ltd (U.K.). Calcium chloride (1mol/L), potassium chloride (KCl), agarose, carboxymethyl cellulose, polyethylene glycol, silica gel granules with moisture indicator were purchased from VWR International Ltd. (U.K.). BAY 60-7550 was a kind gift of Dr. J.P. Stasch (Bayer, Germany). Sildenafil citrate and ecadotril were obtained from Cogentus (CA, USA). Bleomycin sulphate was purchased from Kyowa Hakko (UK). RNeasy Mini Kit was purchased from QIAGEN (Crawley, UK). qScript cDNA SuperMix kit was purchased from Quanta Biosciences (USA). SYBR Safe gel stain and RNase/DNase free water were purchased from Invitrogen (UK). My Taq Red Mix, Bioline Hyperladder II and loading buffer were purchased from Bioline Reagents Ltd (UK). Mouse monoclonal anti αSMA antibody, biotinylated anti-mouse secondary antibody and ABC-peroxide were purchased from DAKO (UK).

2.3 Functional pharmacological studies

Male Sprague-Dawley rats (200-250g) were sacrificed by cervical dislocation. The pulmonary artery and/or thoracic aorta were carefully excised, cleaned of all connective tissue and cut into segments approximately 3-4mm wide; excess vessel was snap frozen in liquid nitrogen and stored at -80^{o}C for further analysis. The vessel segments were then mounted in 10ml organ baths containing Krebs-bicarbonate buffer (composition mM): Na^{2+} 143; K^{+} 5.9; Ca^{2+} 2.5; Mg^{2+} 1.2; Cl^{-} 128; HCO_3^{-} 25; HPO_4^{2-} 1.2; SO_4^{2-} 1.2; glucose 11 at 37^{o}C and gassed with 95% O_2/5% CO_2. Vessel tension was set at 1g and reset after replacing the Krebs-bicarbonate buffer every 10-15mins during an equilibration period of 1 hour.

After equilibration, the vessels were primed twice with 48mM KCl, 30mins of washing (replacement with fresh Krebs-bicarbonate buffer every 10mins) separated the two concentrations. Following the second KCl-induced contraction the vascular endothelium integrity was tested. A sub-maximal (~80% EC_{80}) concentration of PE (0.1µM) was administered to the bath, once the contraction had stabilised ACh (1µM) was added. If relaxations to ACh were greater than 50% the vessels were deemed endothelium-intact and used for experimentation. If the response to ACh was less than 50% of PE-induced tone, the vessels were discarded. The vessels were washed for another 30mins (as previously described) after which cumulative concentrations of the thromboxane mimetic, U46619 were administered (10nM-0.3µM). A 60min period of washing then ensued to restore basal tone after which the vessels were contracted to approximately the EC_{80} of U46619. Once the U46619-induced contraction had stabilised, cumulative concentration-response curves were constructed to ANP (10pM – 0.3µM) or SPER-NO (1nM - 30µM). The functional activity of PDE1, 2, 3 and 5 against SPER-NO and ANP –induced relaxations were investigated. Concentration-response curves to ANP and SPER-NO were constructed in the absence (control, vehicle [DMSO]) and presence of the following isoform specific PDE inhibitors: PDE1 – vinpocetine, 30µM (Polson & Strada, 2003), PDE2 – BAY 60-5770 (Boess *et al.*, 2004),0.1µM; PDE3 – milrinone, 10µM (Komas *et al.*, 1991), and PDE5 – sildenafil, 3µM (Baliga *et al.*, 2008).

In some studies, Sprague-Dawley rats (160-180g; such that they would be 200-250g by the time of experimentation) were housed in a hypoxic chamber (10% O_2) for two weeks (see 2.5) and functional pharmacological assessment was conducted as described above.

2.4 Reverse transcription polymerase chain reaction

2.4.1 Ribonucleic acid isolation

Thoracic aorta and pulmonary artery were excised from two groups of Sprague-Dawley rats. The first was exposed to normoxia whilst the second was exposed to two weeks of hypoxia to induce pulmonary hypertension (see 2.5). Excess vessel was snap frozen in liquid nitrogen and stored at -80^{oC}.

Total ribonucleic acid (RNA) was isolated from the vessels using a QIAGEN (Crawley, UK) RNeasy Mini Kit following manufacturer's instructions. Frozen vessels were put into liquid nitrogen and thoroughly ground up using a cold pestle and mortar. The tissue powder and liquid nitrogen was poured into a cold RNase-free microcentrifuge tube. The liquid nitrogen was allowed to evaporate, but the tissue powder was not allowed to thaw, then 600µl of RLT buffer was added. The lysate was put into a QIAshedder homogeniser spin column and centrifuged at full speed for 2 minutes. The lysate was centrifuged for 3 minutes at full speed in the collection tube. Supernatant was carefully removed with a pipette and put into a fresh RNase free tube with the equivalent volume of 70% ethanol. 700µl of sample was put into the RNeasy spin column and centrifuged (8000 x g) for 15 seconds, the flow through was discarded and successive aliquots (if sample > 700µl) were centrifuged with the flow through discarded each time. The column was washed with 700µl RW1 buffer and centrifuged (8000 x g) for 15 seconds, the flow through was discarded. Two further washes were performed 500µl RPE buffer was added to the column and centrifuged (8000 x g) for 15 seconds (flow through discarded) then an additional 500µl RPE buffer was added to the column and centrifuged (8000 x g) for 2 minutes. The column was placed into a new 1.5ml collecting tube (supplied in

kit), 30µl RNase free water was added to the column and centrifuged (8000 x g) for 1 minute. The spin column was discarded. The concentration of RNA samples was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). RNA was stored at 25ng/µl in 10µl aliquots at -20^{o}C.

2.4.2 Synthesis of single stranded cDNA

Single stranded complementary deoxyribonucleic acid (cDNA) was synthesised from RNA isolated from normoxic/hypoxic rat thoracic aorta and pulmonary artery (as described in 2.4.1) using qScript cDNA SuperMix kit (Quanta Biosciences, USA). 250ng of RNA (10µl) was combined with 4µl of qScript cDNA SuperMix and 6µl of RNase/DNase free water (Invitrogen, UK) on ice in 0.2ml microtubes. Each reaction was gently vortexed to mix the contents and then briefly centrifuged to ensure all the contents were at the bottom of the tube. The cDNA was amplified using a Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, UK): 5 minutes at 25^{o}C, 30 minutes at 42^{o}C, 5 minutes at 85^{o}C and then held at 4^{o}C.

2.4.3 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed to semi-quantify the expression of PDEs from normoxic/hypoxic rat thoracic aorta and pulmonary artery. 4µl of cDNA (see section 2.4.2 for synthesis method) was combined with 12.5µl Bioline 2x My Taq Red Mix (Bioline Reagents Ltd, UK), 2µl of both the forward and reverse primers (all sequences were designed using BLAST, Apart from β-actin (Duchene *et al.*, 2007), Sigma, UK; ***Table 2.1***) and 4.5µl of RNase/DNase free water on ice in 0.2ml microtubes. Each reaction was gently vortexed to mix the contents and then briefly centrifuged to ensure all the contents were at the bottom of the tube. The reactions were incubated using a Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, UK): 95^{o}C for 30 seconds, 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 67.1^{o}C for 30 seconds and extension at 72^{o}C for 1 minute, followed by 10 minutes final extension at 72^{o}C and then held at 4^{oC}.

The samples were combined with 5µl loading buffer (Bioline Reagents Ltd, UK) and resolved with Bioline Hyperladder II (Bioline Reagents Ltd, UK) on a 2% agarose (VWR, UK) gel made with 1x tris-acetate-ethylenediaminetetraacetic acid (TAE) and 1:10,000 SYBR Safe gel stain (Invitrogen, UK). The gel was run at 110V until the dyefront reached the middle of the gel; approximately 30 minutes. The gel was visualised using an alpha imager (Alpha Innotech, UK).

Table 2.1 Primer sequences and product sizes used for PDE mRNA determination in rat pulmonary artery and aorta

Table 2.1 Primer sequences and product sizes used for PDE mRNA determination in rat pulmonary artery and aorta

PDE		Primer sequence	Product size
PDE1B	forward	5'-TGCTGGAGTCGGATTGCCCGT-3'	384
	reverse	5'-GGAACATCCGCTCCACGAAGATCCC-3'	
PDE1C	forward	5'-CCTTGTCAGTCCCCCTGCCGT-3'	359
	reverse	5'-ACTTAGATCGGGAGAAGCGTGCAA-3'	
PDE2A	forward	5'-CCCAGCCAGAACTCCGAGCA-3'	328
	reverse	5'-GTAGACAGTCTCCACTTTGGGGAGC-3'	
PDE3A	forward	5'-AGGCGCATGGCCTCATTACCG-3'	386
	reverse	5'-GTCAGGGTTGACAGCACTGGGTG-3'	
PDE3B	forward	5'-AGAAAGCGCGGCCGGTTACT-3'	397
	reverse	5'-ACTGGGTTTGCTACTCAACCCCTTG-3'	
PDE5A	forward	GAGAGAGCCATGGCCAAGCA	473
	reverse	TGCTCGCTCCGCTGTATGTA	
β-actin	forward	GAAATCGTGCGTGACATCAAAG	173
	reverse	TGTAGTTTCATGGATGCCACAG	

2.5 Hypoxia-induced pulmonary hypertension – reversal studies

Five groups of male C57/BL6 mice (20-25g) were studied; i) normoxia, ii) hypoxia control receiving daily gavage with vehicle (0.5% w/v carboxymethyl cellulose CMC; VWR, UK) & 0.1% v/v polyethylene glycol (PEG; VWR, UK), iii) hypoxia, daily gavage with vehicle and sildenafil (30mg/kg/day) administered in the drinking water, iv) hypoxia and ecadotril (60mg/kg/day) delivered by gavage and v) hypoxia plus the combined treatment of sildenafil and ecadotril, dosed and delivered as indicated above. The appropriate drug/vehicle treatment was started three weeks after placement of animals into a hypoxic chamber (10% O_2) and lasted for three weeks to induce the development of PH. Thus, this study was designed to evaluate combination therapy on established PH (more clinically relevant), rather than prophylactic treatment.

Hemodynamic measurements of right ventricular systolic pressure (RVSP) and mean arterial blood pressure (MABP) were obtained from the animals after six weeks of hypoxia exposure and relevant drug treatment. The animals were anaesthetised with 1.5% isofluorane and placed supine onto a heating blanket that was thermostatically controlled at 37^{o}C. First the right jugular vein was isolated and a pressure catheter (Millar mouse SPR-671NR pressure catheter with a diameter of 1.4F, Millar Instruments, UK) introduced and advanced into the right ventricle to determine RVSP. Second, MABP was measured by isolating the left common carotid artery and a pressure catheter introduced. Both RVSP and MABP were recorded onto a precalibrated PowerLab system (ADInstruments, Australia). The animal was euthanised by via isofluorane anaesthetic overdose, whole blood collected, heart removed and right and left ventricle weights recorded. Lungs were perfused with 10ml of saline via the right ventricle. The left lung was fixed by inflation with 10% formalin before paraffin embedding and sectioning. The whole blood was centrifuged (220 x g; 2 min), plasma removed and stored at -80^{o}C for further analysis (cGMP, NP levels).

2.5.1 Hypoxia-induced pulmonary hypertension -prophylactic treatment

Five groups of male C57/BL6 mice (20-25g) were studied; i) normoxia, ii) hypoxia control receiving daily gavage with vehicle (0.5% w/v CMC & 0.1% v/v PEG), iii) hypoxia, daily gavage ecadotril (60mg/kg/day) iv) hypoxia and BAY60-7550 (10mg/kg/day) delivered by gavage and v) hypoxia plus the combined treatment of ecadotril and BAY60-7550, dosed and delivered as indicated above. The appropriate drug/vehicle treatment was started immediately before placement of animals into a hypoxic chamber (10% O_2) and lasted for three weeks to induce the development of PH. Thus, this study was designed to evaluate combination therapy (NEPi plus PDE2i) on the development of PH i.e. prophylactic treatment.

Hemodynamic measurements were obtained as described above. As were the collection of blood, heart weights, fixation of lungs. Non-fixed lung tissue and hearts were collected and stored at -80^{o}C for further analysis.

2.6 Bleomycin-induced pulmonary fibrosis and pulmonary hypertension

Male C57BL/6 mice (20-25g) were administered with either bleomycin (1mg/kg in 50µl of saline) or saline via oropharyngeal installation (Lakatos *et al.*, 2006) under light isofluorane-induced anaesthesia. Animals were divided into the following groups: i) saline, ii) bleomycin control receiving daily gavage with vehicle (0.5% w/v CMC & 0.1% v/v PEG), iii) bleomycin, daily gavage with vehicle and sildenafil (30mg/kg/day) administered in the drinking water, iv) bleomycin and ecadotril (60mg/kg/day) delivered by gavage and v) bleomycin plus the combined treatment of sildenafil and ecadotril, dosed and delivered as indicated above. The appropriate drug/vehicle treatment was started 1 hour prior to oropharyngeal installation of bleomycin or saline and continued for 14 days.

RVSP and MABP were obtained as previously described. Whole blood was collected and centrifuged (220 x g; 2 min), plasma removed and stored at -80^{o}C for further analysis (cGMP, NP levels). Lungs were perfused with 10ml saline via the right ventricle; the left lower lobe was then tied off with thread. The trachea was cannulated and lungs insufflated with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at a pressure of 20cm H_2O. The tied off left lower lobe was removed and snap frozen in liquid nitrogen, whilst the heart and remaining inflated lungs were removed and immersed for 4 hours in fresh 4% PFA fixative. The lungs were subsequently transferred into 15% sucrose in PBS, left overnight at 4^{o}C before final transfer to 70% ethanol for analysis of fibrosis.

2.7 Immunohistochemistry

Inflated lungs were fixed in 4% PFA for 4 hours at 4°C and then incubated overnight in 15% sucrose in PBS at 4°C. Tissue was dehydrated in 70% ethanol and embedded in paraffin. Serial sections (4 μm) were used for trichrome blue staining and α smooth muscle actin (αSMA) immunohistochemistry. For the latter, sections were stained with mouse monoclonal anti αSMA antibody (DAKO,UK, 1:1000 dilution), followed by biotinylated anti-mouse secondary antibody. Immunoreactivity was detected using the ABC-peroxide based system (DAKO,UK) following the manufacturer's protocol.

Stained slides were assessed using bright field microscopy by an Axioskop Mot Plus microscope and analysed using KS300 image-analysis software (Carl Zeiss Instruments, Thornwood, NJ). Vessels with a diameter <100μM were counted in each lung section, and defined according to the degree of muscularisation: fully muscularised, partially muscularised and nonmuscularised. Approximately 100 vessels were counted per section (n=6 for each treatment group) and the proportion of vessels in each category was expressed as a percentage of total vessels.

2.8 Data Analysis

For *in vitro* studies, relaxations are expressed as the percentage reversal of U46619-induced tone (mean ± standard error of the mean; SEM of *n* animals). Curves were fitted using non-linear regression and the –log [M] of each drug's half-maximal response (EC_{50}) was used in order to compare potency. Curves were analysed using two-way ANOVA and $P<0.05$ denoted significance.

For *in vivo* studies variation in MABP, RVSP, LV/RV ratio and muscularisation of arteries were analysed by one-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis. Results were expressed as mean ± S.E.M, $P<0.05$ denoted significance. All statistical analysis and curve fitting was undertaken using GraphPad Prism version 5.00 for Windows (GraphPad software, San Diego, U.S.A).

For semi-quantitative analysis of immunoblots, densitometry was performed using ImageJ (National Institutes of Health, USA).

Chapter 3: Results

3.1 Effect of PDE inhibition on the vasorelaxant activity of NO and ANP in normoxic pulmonary and systemic vessels

The purpose of the experiments in section 3.1 was to investigate the functional activity of PDE1, PDE2, PDE3 and PDE5, and their responsiveness to ANP and NO, in isolated systemic (aorta) and pulmonary vessels from healthy (normoxic) rats. Thereby, I attempted to ascertain if a combination of specific isozyme PDE inhibition with ANP or NO could produce selective relaxation of the pulmonary vasculature over that of the systemic vasculature.

Segments (3-4 mm wide) of thoracic aorta and/or pulmonary artery were mounted in organ baths. The vessels were primed twice with 48mM KCl. After which, endothelium integrity was assessed by the ability of ACh (1µM) to induce relaxation in pre-contracted vessels (sub-maximal concentration of PE, 0.1µM; ~EC_{80}). If relaxations to ACh were greater than 50% of PE-induced tone the vessels were deemed endothelium-intact and used for experimentation. The EC_{80} for U46619 in each vessel was ascertained by the addition of cumulative concentrations of the thromboxane mimetic (10nM – 0.3µM). Following the restoration of basal tone the vessels were administered with either a PDEi (see Table 3.1) or vehicle control and contracted to the EC_{80} of U46619. After the U46619-induced contraction had stabilised (20 minutes), cumulative concentration response curves were constructed to ANP (10pM - 0.3µM) or SPER-NO (1nM - 30µM).

SPER-NO (0.1nM - 50µM) and ANP (10pM – 0.5µM) produced concentration dependent relaxations of isolated aortic and pulmonary artery rings.

Table 3.1 IC_{50} and specificity of PDE inhibitors used in organ bath studies

Table 3.1 IC_{50} and specificity of PDE inhibitors used in organ bath studies

Phosphodiesterase isozyme	Inhibitor	IC_{50}	Concentration used	Specificity	Reference
PDE1	Vinpocetine	4.4μM	30μM	PDE7B IC_{50} = 60μM	Polson & Strada, 2003
PDE2	BAY60-5770	5nM	0.1μM	IC_{50} = >250nM for other PDEs	Boess *et al.*, 2004
PDE3	Milrinone	0.45-1μM	10μM	IC_{50} = 17.5 - >300μM for other PDEs	Komas *et al.*, 1991
PDE5	Sildenafil	4nM	3μM	PDE6 IC_{50} = 39nM	Baliga *et al.*, 2008

3.1.1 Phosphodiesterase 1

The potency of SPER-NO in the presence of vinpocetine (PDE1i) in aorta (log EC_{50}, -7.06±0.26 and -7.45±0.13 in the absence and presence of vinpocetine, respectively; P>0.05; ***Figure 3.1***) and pulmonary artery (log EC_{50}, -6.23±0.21 and -6.19±0.14 in the absence and presence of vinpocetine, respectively; P>0.05; ***Figure 3.2***) was not significantly affected compared to control. In accord, the potency of ANP in the presence of vinpocetine in aorta (log EC_{50}, -8.92±0.11 and -8.85±0.12 in the absence and presence of vinpocetine, respectively; P>0.05; ***Figure 3.1***) and pulmonary artery (log EC_{50}, -8.61±0.10 and -8.92±0.12 in the absence and presence of vinpocetine, respectively; P>0.05; ***Figure 3.2***) was also similar to control.

3.1.2 Phosphodiesterase 2

The potency of SPER-NO in the presence of BAY60-7550 (PDE2i) in aorta (log EC_{50}, -7.10±0.08 and -8.59±0.58 in the absence and presence of BAY60-7550, respectively; P<0.05; ***Figure 3.3***) and pulmonary artery (log EC_{50}, -6.13±0.09 and -6.61±0.11 in the absence and presence of BAY60-7550, respectively; P<0.05; ***Figure 3.4***) was significantly increased in comparison to control. Responses to ANP in pulmonary artery in the presence of BAY60-7550 were enhanced (log EC_{50}, -8.58±0.06 and -8.99±0.06 in the absence and presence of BAY60-7550, respectively; P<0.05; ***Figure 3.4***). In contrast however, BAY60-7550 did not alter potency of ANP in aorta (log EC_{50}, -8.76±0.08 and -8.78±0.10 in the absence and presence of BAY60-7550, respectively; ***Figure 3.3***).

Figure 3.1 Effect of the PDE1 inhibitor vinpocetine on ANP and NO-induced relaxation of rat aorta under normoxia

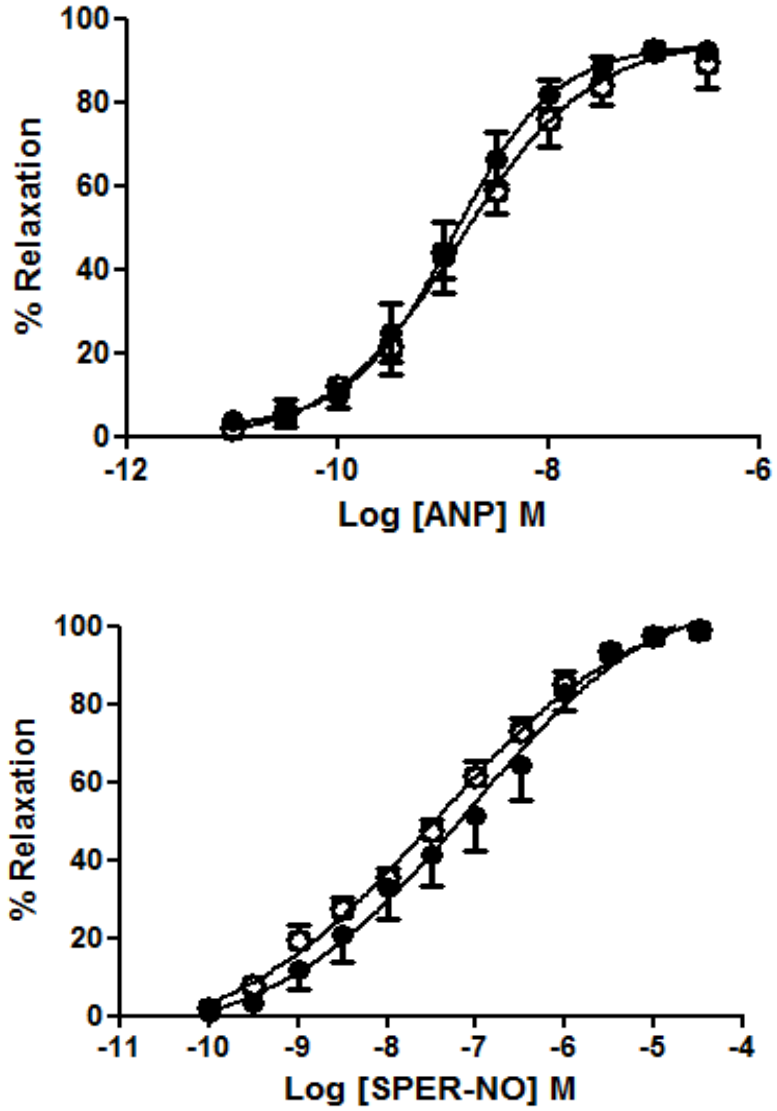


Figure 3.1. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted rat aorta in the absence (closed circles) and presence (open circles) of the PDE1 inhibitor vinpocetine (30µM). n=6.

Figure 3.2 Effect of the PDE1 inhibitor vinpocetine on ANP and NO-induced relaxation of rat pulmonary artery under normoxia

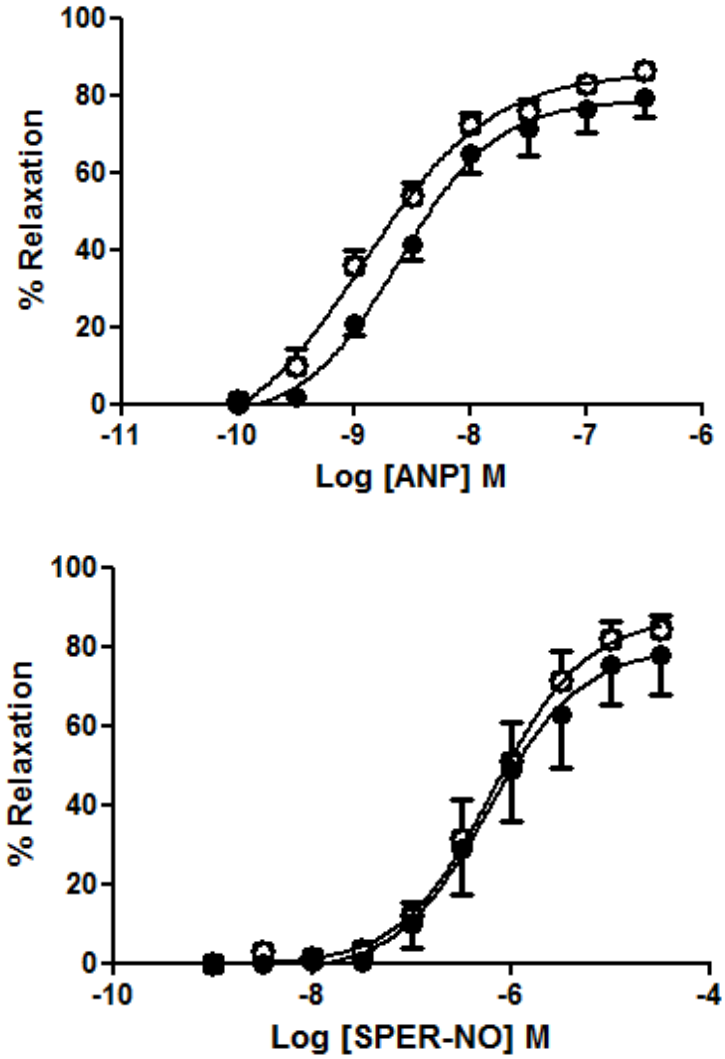


Figure 3.2. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted rat pulmonary artery in the absence (closed circles) and presence (open circles) of the PDE1 inhibitor vinpocetine (30µM). n=6.

Figure 3.3 Effect of the PDE2 inhibitor BAY60-7550 on ANP and NO-induced relaxation of rat aorta under normoxia

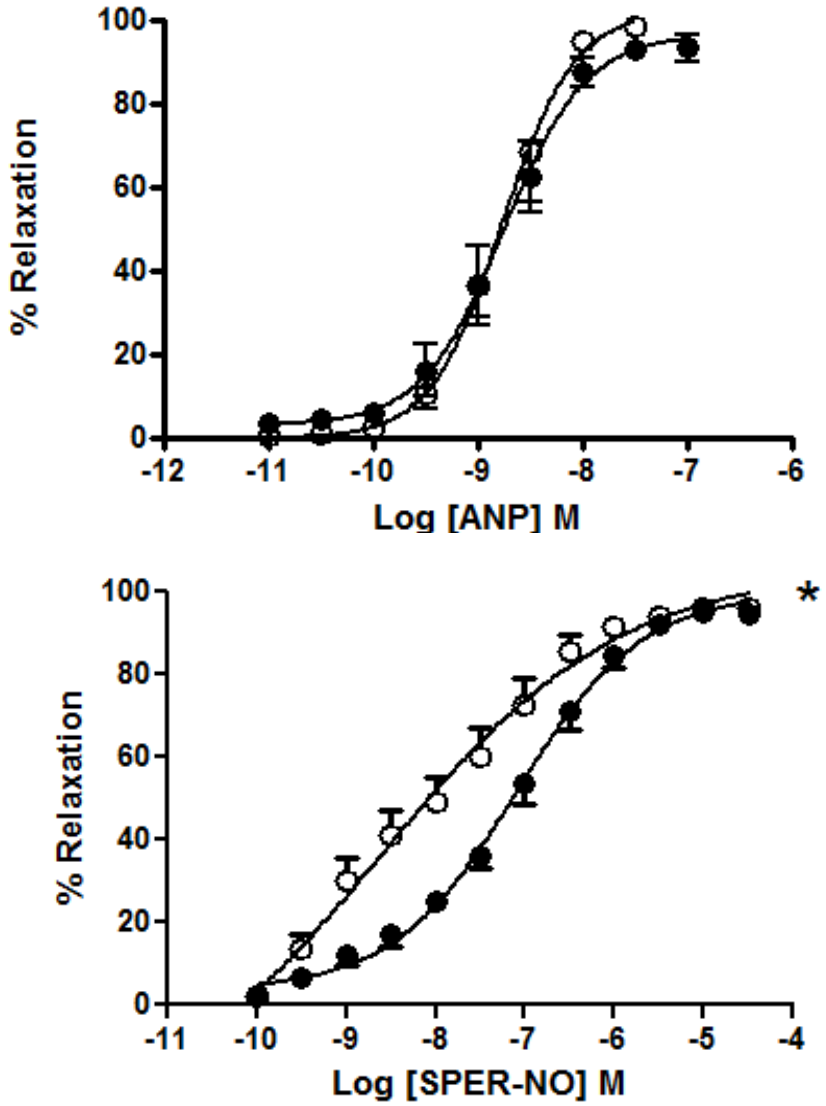


Figure 3.3. Concentration response curves to atrial natriuretic peptide (ANP; upper panel) and spermine NONOate (SPER-NO; lower panel) in U46619 (EC_{80}) pre-contracted rat aorta in the absence (closed circles) and presence (open circles) of the PDE2 inhibitor BAY60-7550 (0.1µM). *P<0.05 across entire curve by 2-way ANOVA; n=6.

Figure 3.4 Effect of the PDE2 inhibitor BAY60-7550 on ANP and NO-induced relaxation of rat pulmonary artery under normoxia

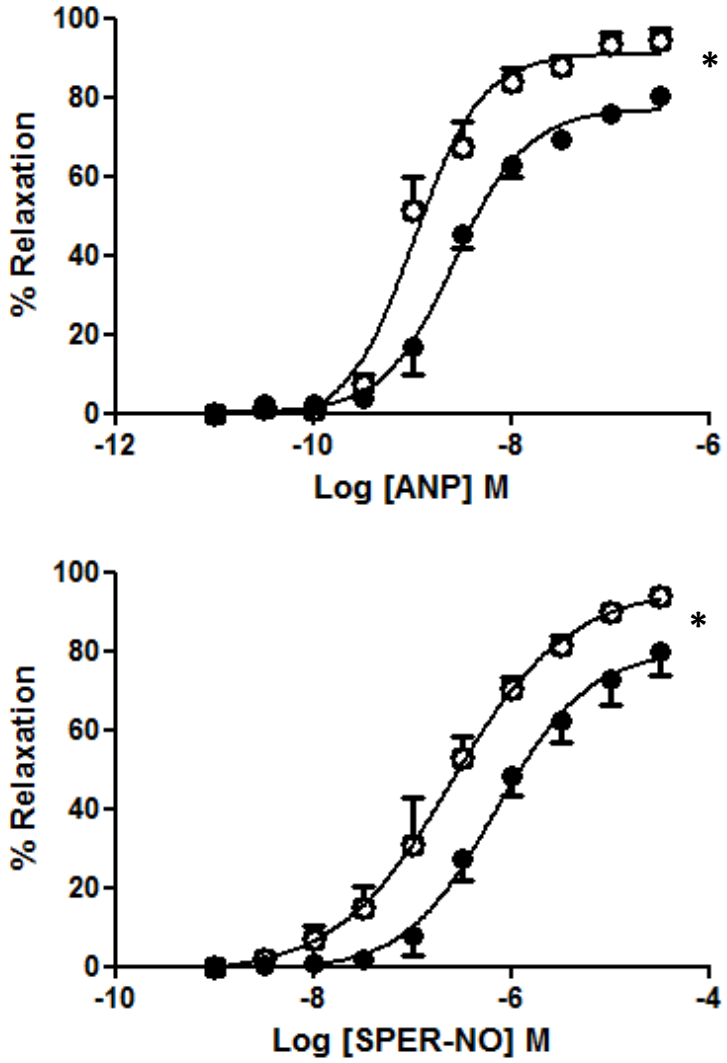


Figure 3.4. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted rat pulmonary artery in the absence (closed circles) and presence (open circles) of the PDE2 inhibitor BAY60-7550 (0.1µM). *P<0.05 across entire curve by 2-way ANOVA; n=6.

3.1.3 Phosphodiesterase 3

The potency of SPER-NO in the presence of milrinone (PDE3i) in aorta (log EC_{50}, -7.31±0.08 and -7.10±0.18 in the absence and presence of milrinone, respectively; P>0.05; ***Figure 3.5***) and pulmonary artery (log EC_{50}, -6.16±0.09 and -6.08±0.13 in the absence and presence of milrinone, respectively; P>0.05; ***Figure 3.6***) was not significantly affected. Likewise, the potency of ANP in the presence of milrinone in aorta (log EC_{50}, -8.88±0.19 and -8.56±0.50 in the absence and presence of milrinone, respectively; P>0.05; ***Figure 3.5***) and pulmonary artery (log EC_{50}, -8.64±0.14 and -8.89±0.11 in the absence and presence of milrinone, respectively; P>0.05; ***Figure 3.6***) was similar to control.

3.1.4 Phosphodiesterase 5

The potency of SPER-NO in the presence of sildenafil (PDE5i) in aorta (log EC_{50}, -6.87±0.37 and -8.66±0.37 in the absence and presence of sildenafil, respectively; ***Figure 3.7***) and pulmonary artery (log EC_{50}, -6.16±0.09 and -6.96±0.09 in the absence and presence of sildenafil, respectively; P<0.05; ***Figure 3.8***) was increased in comparison to control. Responses to ANP in pulmonary artery in the presence of sildenafil were enhanced (log EC_{50}, -8.37±0.05 and -8.98±0.08 in the absence and presence of sildenafil, respectively; P<0.05 ***Figure 3.8***). In contrast however, sildenafil did not alter potency of ANP in aorta (log EC_{50}, -9.08±0.10 and -9.28±0.32 in the absence and presence of sildenafil, respectively; P>0.05; ***Figure 3.7***).

Figure 3.5 Effect of the PDE3 inhibitor milrinone on ANP and NO-induced relaxation of rat aorta under normoxia

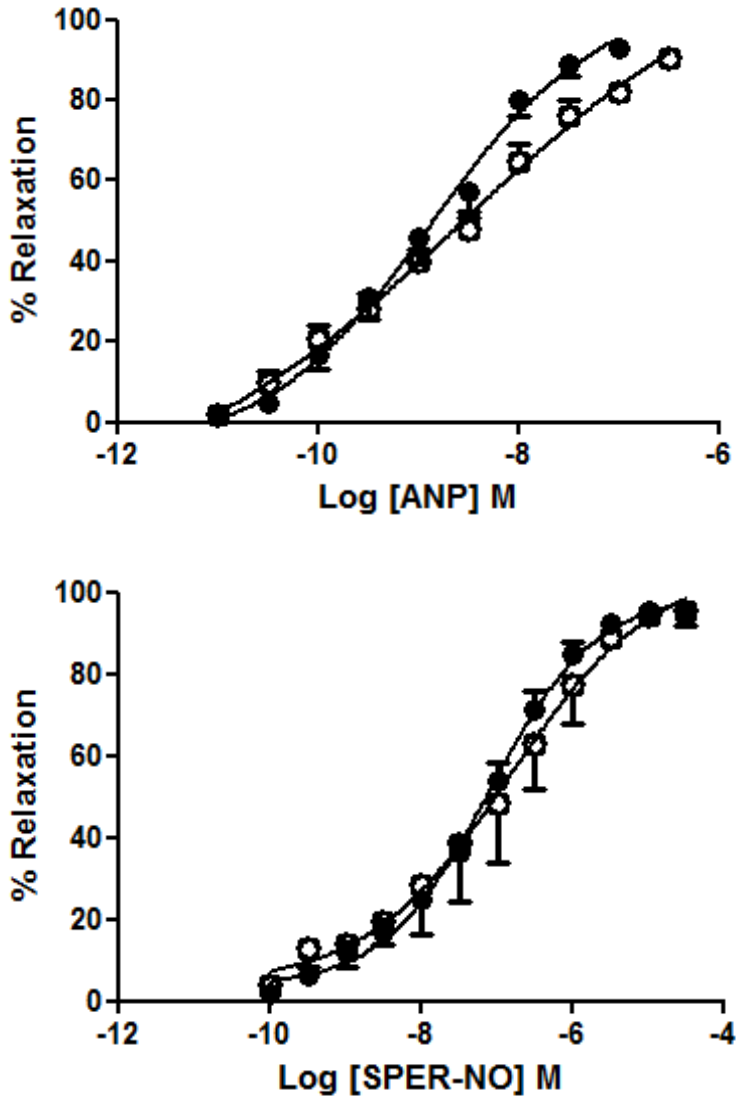


Figure 3.5. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted rat aorta in the absence (closed circles) and presence (open circles) of the PDE3 inhibitor milrinone (10µM). n=6.

Figure 3.6 Effect of the PDE3 inhibitor milrinone on ANP and NO-induced relaxation of rat pulmonary artery under normoxia

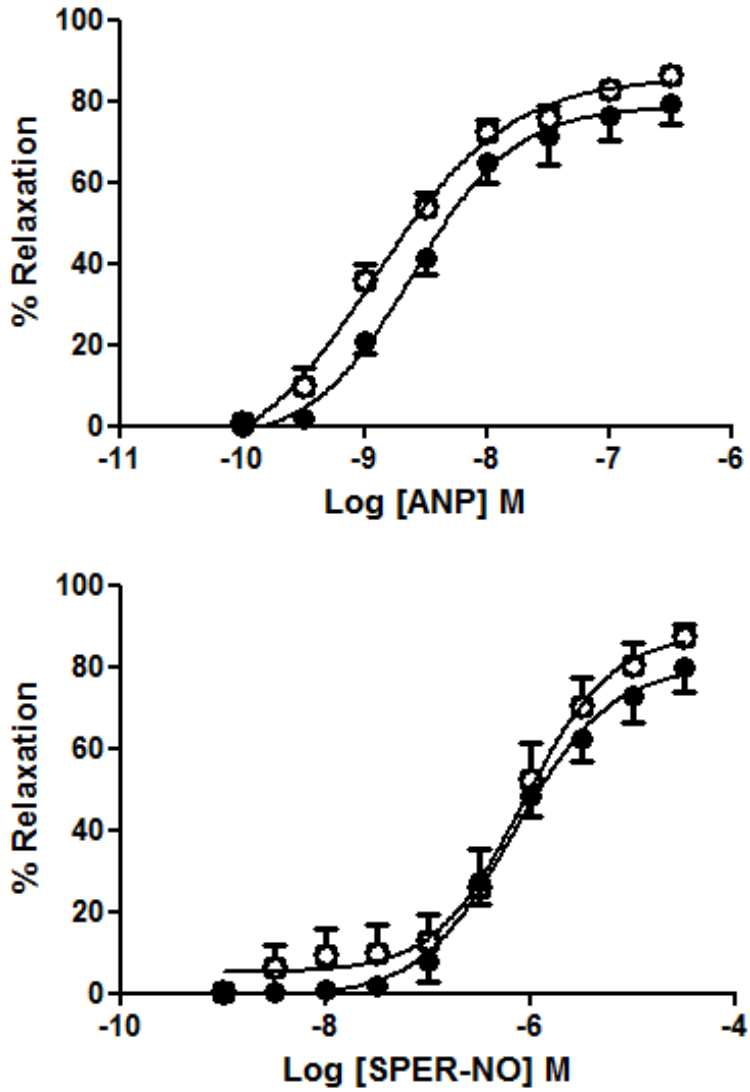


Figure 3.6. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted rat pulmonary artery in the absence (closed circles) and presence (open circles) of the PDE3 inhibitor milrinone (10µM). n=6.

Figure 3.7 Effect of the PDE5 inhibitor sildenafil on ANP and NO-induced relaxation of rat aorta under normoxia

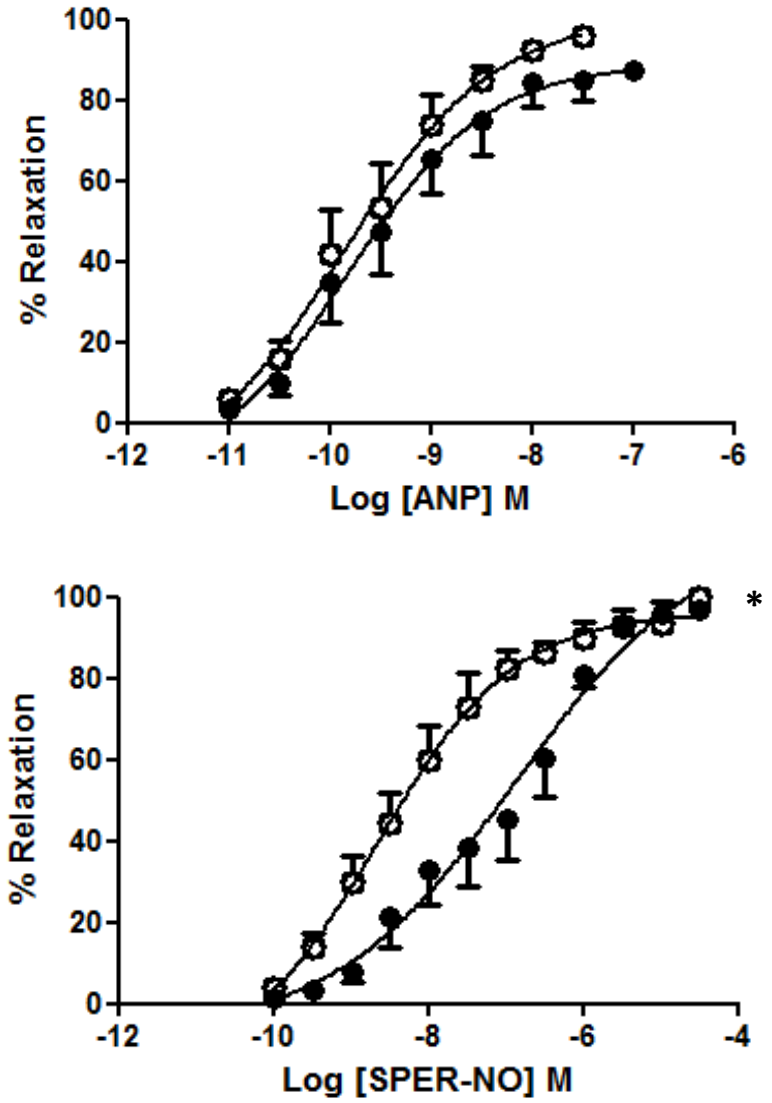


Figure 3.7 Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted rat aorta in the absence (closed circles) and presence (open circles) of the PDE5 inhibitor sildenafil (3µM). *P<0.05 across entire curve by 2-way ANOVA; n=6.

Figure 3.8 Effect of the PDE5 inhibitor sildenafil on ANP and NO-induced relaxation of rat pulmonary artery under normoxia

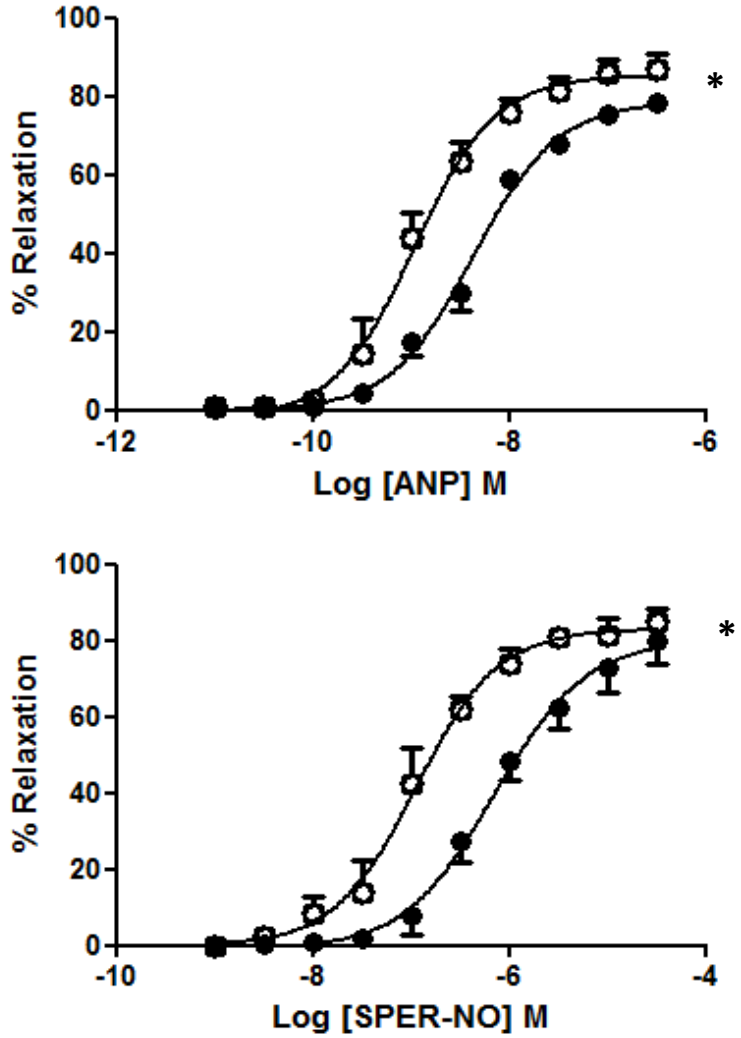


Figure 3.8. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted rat pulmonary artery in the absence (closed circles) and presence (open circles) of the PDE5 inhibitor sildenafil (3µM). *P<0.05 across entire curve by 2-way ANOVA; n=6.

3.1.5 Summary and conclusion

BAY 60-7550 (PDE2i) and sildenafil (PDE5i) were able to augment SPER-NO-induced relaxation in both aorta and pulmonary artery. Interestingly BAY 60-7550 and sildenafil could only augment ANP-induced relaxation in pulmonary artery and not aorta. Therefore the combination of BAY 60-5770 or sildenafil with ANP produced enhanced vasorelaxation specific to the pulmonary vasculature from healthy (normoxic) rats.

Neither vinpocetine (PDE1i) nor milrinone (PDE3i) had any effect on ANP- or NO-induced vasorelaxation in both the pulmonary artery and aorta from healthy (normoxic) rats.

3.2 Effect of phosphodiesterase regulation on the vasorelaxant effects of ANP and NO in hypoxic pulmonary and systemic vessels

The purpose of the experiments in section 3.2 was to investigate the functional activity of PDE1, PDE2, PDE3 and PDE5, and their responsiveness to ANP and NO, in isolated systemic (aorta) and pulmonary vessels from pulmonary hypertensive (hypoxic) rats. Thereby, I attempted to ascertain if a combination of specific isozyme PDE inhibition with ANP or NO could produce selective relaxation of diseased pulmonary vasculature over that of the systemic vasculature.

Sprague-Dawley rats were housed in a hypoxic (10% O_2) environment for 2 weeks to induce pulmonary hypertension. Segments (3-4 mm wide) of thoracic aorta and/or pulmonary artery were mounted in organ baths. The vessels were primed twice with 48mM KCl. After which, endothelium integrity was assessed by the ability of ACh (1µM) to induce relaxation in pre-contracted vessels (sub-maximal concentration of PE, 0.1µM; ~EC_{80}). If relaxations to ACh were greater than 50% of PE-induced tone the vessels were deemed endothelium-intact and used for experimentation. The EC_{80} for U46619 in each vessel was ascertained by the addition of cumulative concentrations of the thromboxane mimetic (10nM – 0.3µM). Following the restoration of basal tone the vessels were administered with either a PDEi (see Table 3.1) or vehicle control and contracted to the EC_{80} of U46619. After the U46619-induced contraction had stabilised (20 minutes), cumulative concentration response curves were constructed to ANP (10pM - 0.3µM) or SPER-NO (1nM - 30µM).

SPER-NO (0.1nM - 50µM) and ANP (10pM – 0.5µM) produced concentration dependent relaxations of isolated aortic and pulmonary artery rings.

3.2.1 Phosphodiesterase 1

The potency of SPER-NO in the presence of vinpocetine in aorta (log EC_{50}, -5.95±0.17 and -6.18±0.12 in the absence and presence of vinpocetine, respectively; P>0.05; ***Figure 3.9***) and pulmonary artery (log EC_{50}, -6.28±0.19 and -6.17±0.21 in the absence and presence of vinpocetine, respectively; P>0.05; ***Figure 3.10***) was not significantly affected. Similarly, the potency of ANP in the presence of vinpocetine in aorta (log EC_{50}, -7.90±0.27 and -7.74±0.20 in the absence and presence of vinpocetine, respectively; P>0.05; ***Figure 3.9***) and pulmonary artery (log EC_{50}, -8.63±0.14 and -8.87±0.15 in the absence and presence of vinpocetine, respectively; P>0.05; ***Figure 3.10***) was not different to control.

3.2.2 Phosphodiesterase 2

The potency of SPER-NO in the presence of BAY60-7550 was unaffected in both aorta (log EC_{50}, -6.19±0.04 and -6.35±0.07 in the absence and presence of BAY60-7550, respectively; P>0.05; ***Figure 3.11***) and pulmonary artery (EC_{50}, -5.96±0.07 and -6.08±0.14 in the absence and presence of BAY60-7550, respectively; P>0.05; ***Figure 3.12***) from rats exposed to 2 weeks hypoxia. The potency of ANP in the presence of BAY60-7550 in pulmonary artery from hypoxic rats (EC_{50}, -8.40±0.17 and -8.70±0.16 in the absence and presence of BAY60-7550, respectively; P<0.05; ***Figure 3.12***) was increased in comparison to control. Whilst the potency of ANP in aorta from hypoxic rats was unaffected by BAY60-7550 (EC_{50}, -7.74±0.26 and -7.51±0.52 in the absence and presence of BAY60-7550, respectively; P>0.05; ***Figure 3.11***).

Figure 3.9 Effect of the PDE1 inhibitor vinpocetine on ANP and NO-induced relaxation of rat aorta under chronic hypoxia

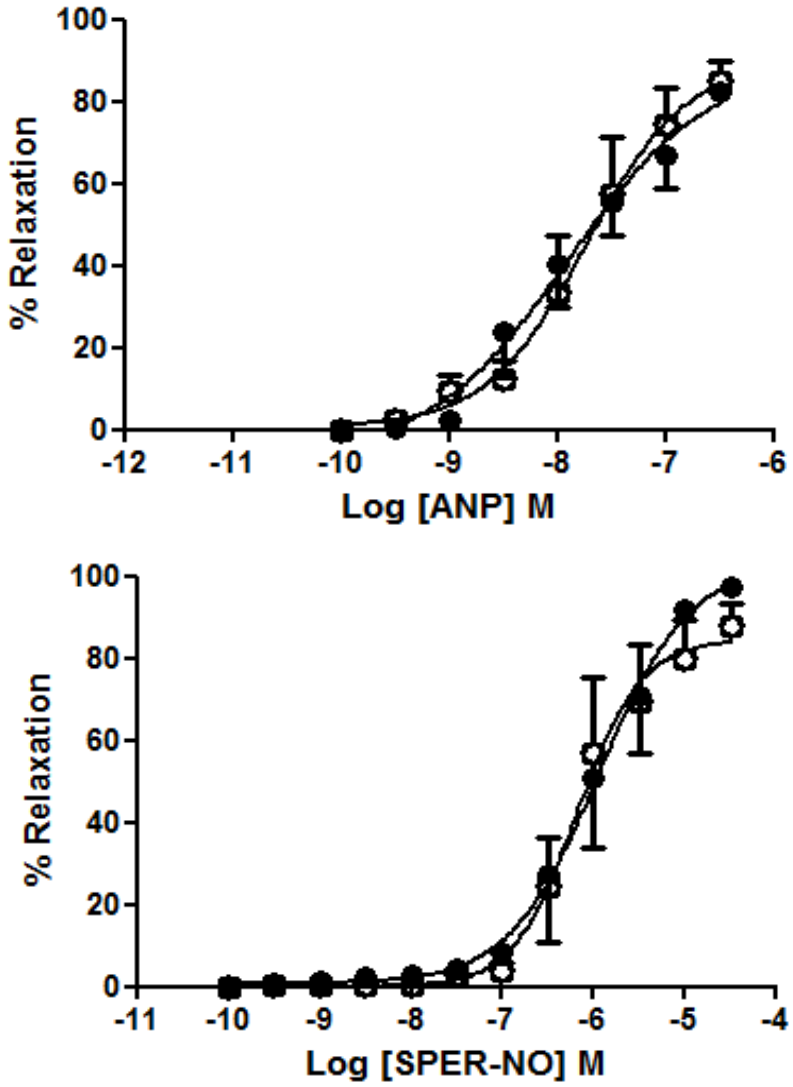
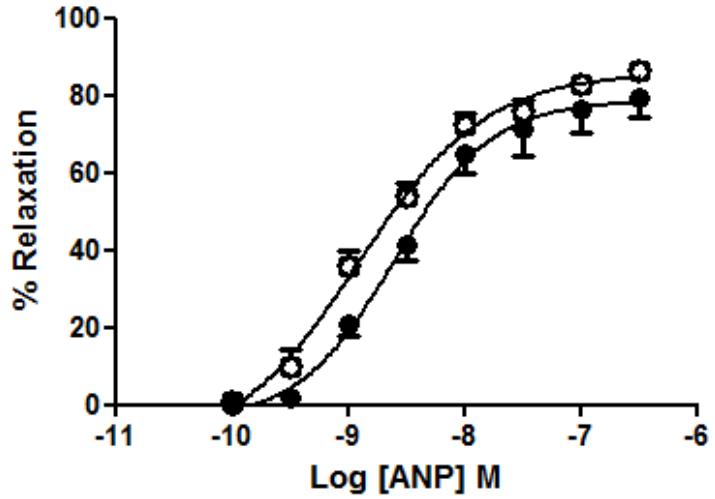


Figure 3.9. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted chronically hypoxic rat aorta in the absence (closed circles) and presence (open circles) of the PDE1 inhibitor vinpocetine (30µM). n=6.

Figure 3.10 Effect of the PDE1 inhibitor vinpocetine on ANP and NO-induced relaxation of rat pulmonary artery under chronic hypoxia



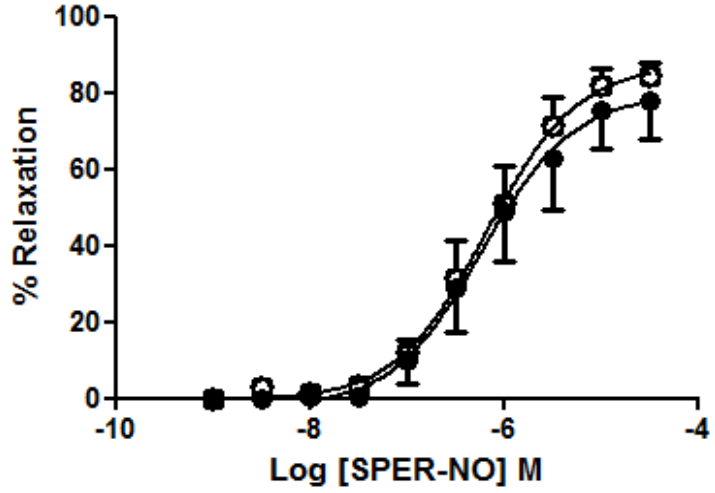


Figure 3.10. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted chronically hypoxic rat pulmonary artery in the absence (closed circles) and presence (open circles) of the PDE1 inhibitor vinpocetine (30µM). n=6.

Figure 3.11 Effect of the PDE2 inhibitor BAY60-7550 on ANP and NO-induced relaxation of rat aorta under chronic hypoxia

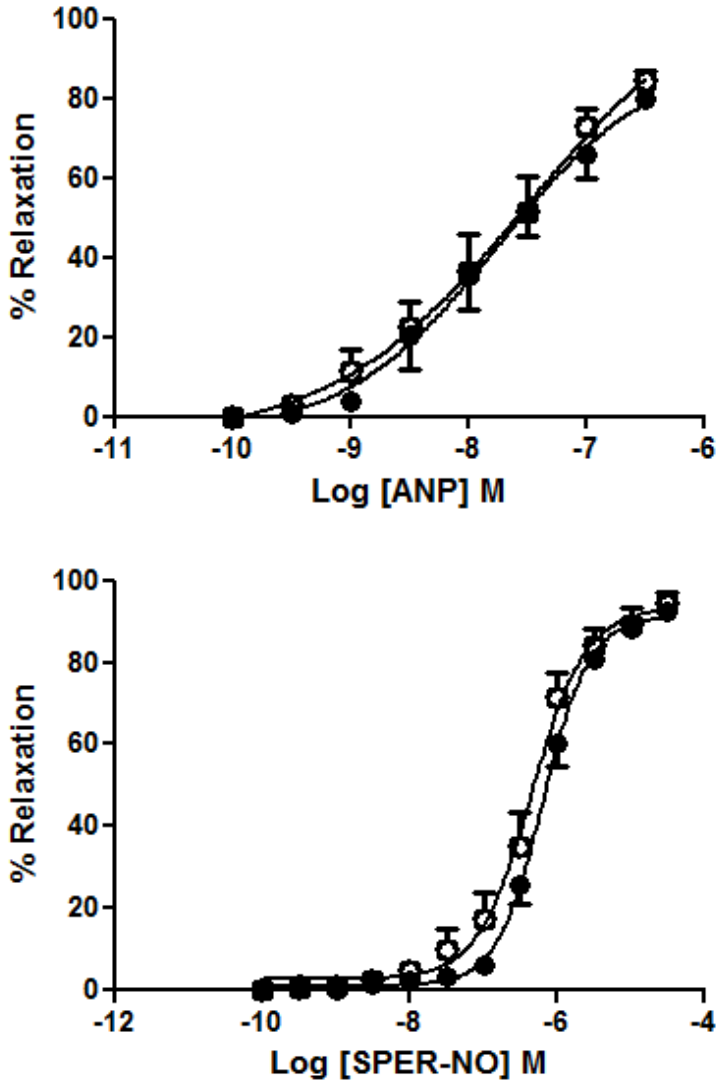


Figure 3.11. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted chronically hypoxic rat aorta in the absence (closed circles) and presence (open circles) of the PDE2 inhibitor BAY60-7550 (0.1µM). n=6.

Figure 3.12 Effect of the PDE2 inhibitor BAY60-7550 on ANP and NO-induced relaxation of rat pulmonary artery under chronic hypoxia

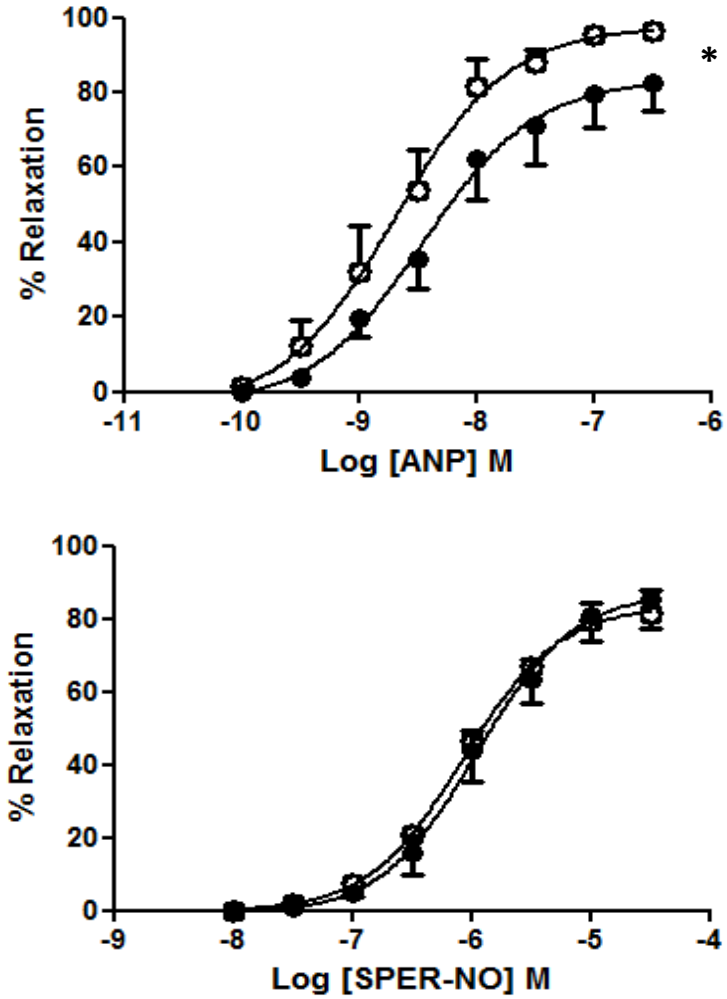


Figure 3.12. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted chronically hypoxic rat pulmonary artery in the absence (closed circles) and presence (open circles) of the PDE2 inhibitor BAY60-7550 (0.1µM). *P<0.05 across entire curve by 2-way ANOVA; n=6.

3.2.3 Phosphodiesterase 3

The potency of SPER-NO in the presence of milrinone in aorta (log EC_{50}, -6.20±0.04 and -6.03±0.08 in the absence and presence of milrinone, respectively; P>0.05; ***Figure 3.13***) and pulmonary artery (log EC_{50}, -6.20±0.11 and -6.13±0.12 in the absence and presence of milrinone, respectively; P>0.05; ***Figure 3.14***) was not significantly affected. In accord, the potency of ANP in the presence of milrinone in aorta (log EC_{50}, -8.33±0.16 and -7.97±0.07 in the absence and presence of milrinone, respectively; P>0.05; ***Figure 3.13***) and pulmonary artery (log EC_{50}, -8.59±0.14 and -8.35±0.12 in the absence and presence of milrinone, respectively; P>0.05; ***Figure 3.14***) was similar to control.

3.2.4 Phosphodiesterase 5

The potency of SPER-NO in the presence of sildenafil in aorta from hypoxic rats (log EC_{50}, -5.56±0.08 and -6.35±0.07 in the absence and presence of sildenafil, respectively; P<0.05; ***Figures 3.15***) was increased in comparison to control. However the potency of SPER-NO in the pulmonary artery was unaffected by sildenafil (log EC_{50}, -5.97±0.08 and -6.24±0.06 in the absence and presence of sildenafil, respectively; P>0.05; ***Figure 3.16***). The potency of ANP in the presence of sildenafil was unaffected in the aorta (log EC_{50}, -7.82±0.25 and -8.12±0.37 in the absence and presence of sildenafil, respectively; P>0.05; ***Figure 3.15***) and was not significantly augmented in the pulmonary artery (log EC_{50}, -8.58±0.16 and -8.80±0.14 in the absence and presence of sildenafil, respectively; P>0.05; ***Figure 3.16***) from hypoxic rats.

Figure 3.13 Effect of the PDE3 inhibitor milrinone on ANP and NO-induced relaxation of rat aorta under chronic hypoxia

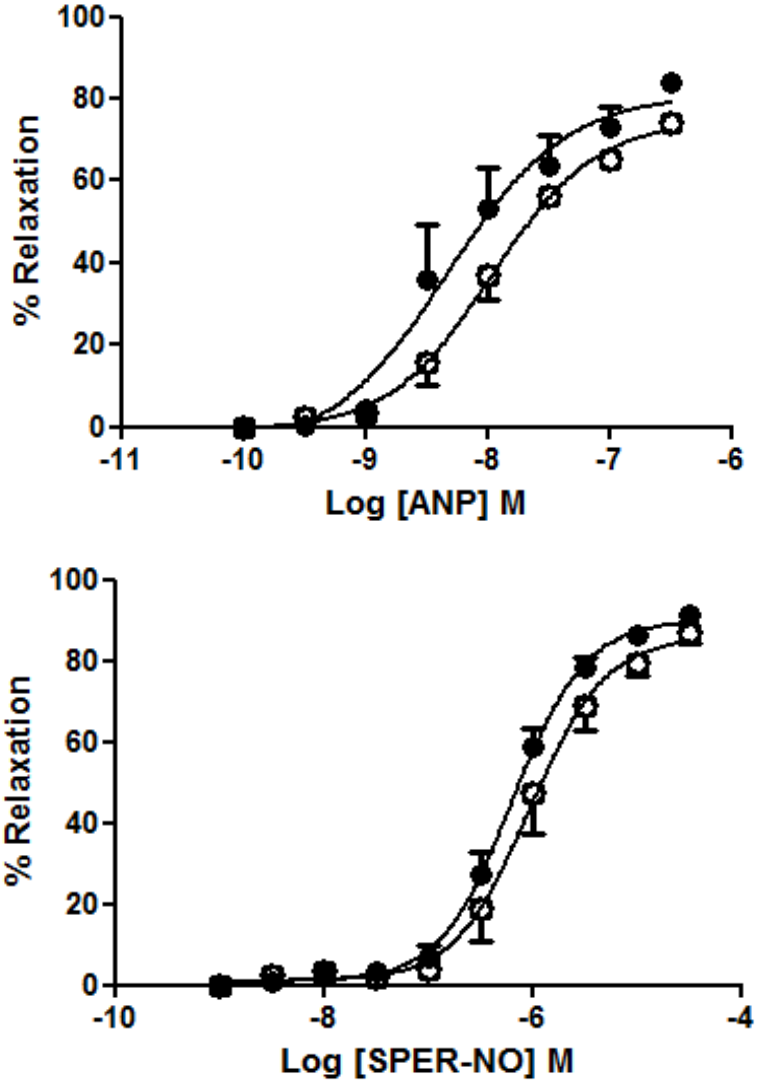


Figure 3.13. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted chronically hypoxic rat aorta in the absence (closed circles) and presence (open circles) of the PDE3 inhibitor milrinone (10µM). n=6.

Figure 3.14 Effect of the PDE3 inhibitor milrinone on ANP and NO-induced relaxation of rat pulmonary artery under chronic hypoxia

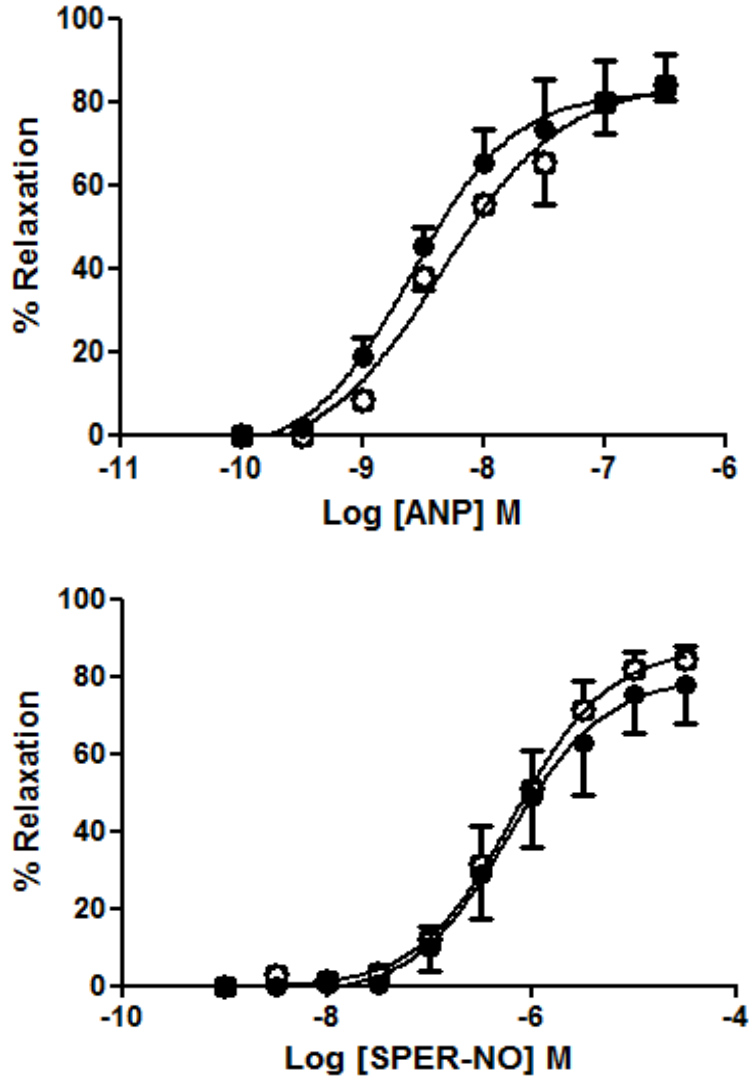


Figure 3.14. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted chronically hypoxic pulmonary artery in the absence (closed circles) and presence (open circles) of the PDE3 inhibitor milrinone (10µM). *P<0.05 across entire curve by 2-way ANOVA; n=6.

Figure 3.15 Effect of the PDE5 inhibitor sildenafil on ANP and NO-induced relaxation of rat aorta under chronic hypoxia

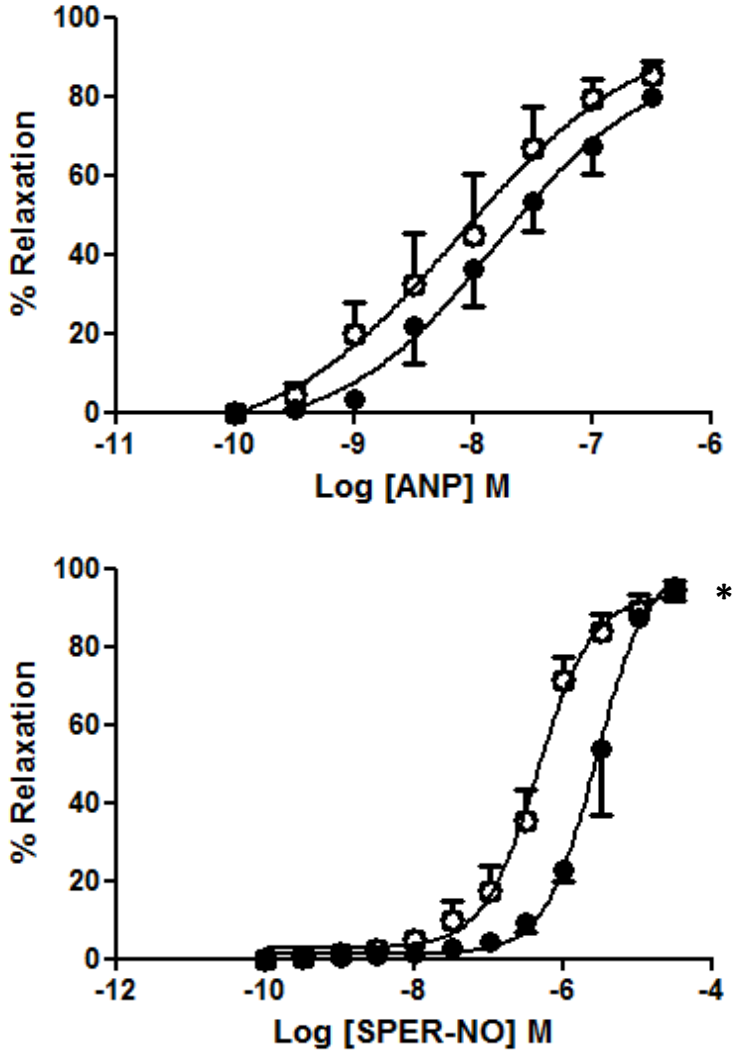


Figure 3.15. Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted chronically hypoxic rat aorta in the absence (closed circles) and presence (open circles) of the PDE5 inhibitor sildenafil (3µM). *P<0.05 across entire curve by 2-way ANOVA; n=6.

Figure 3.16 Effect of the PDE5 inhibitor sildenafil on ANP and NO-induced relaxation of rat pulmonary artery under chronic hypoxia

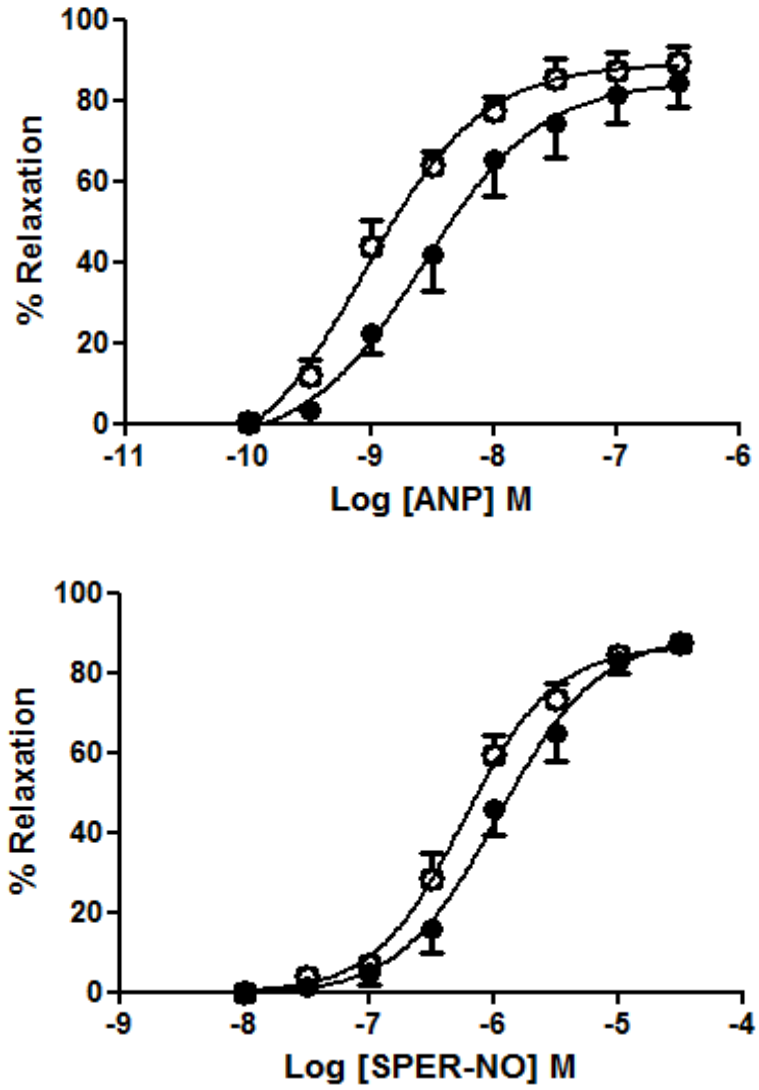


Figure 3.16 Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted chronically hypoxic rat pulmonary artery in the absence (closed circles) and presence (open circles) of the PDE5 inhibitor sildenafil (3µM). n=6.

3.2.5 Summary and conclusion

BAY 60-7550 augmented ANP-induced relaxation in the pulmonary artery but not the aorta from pulmonary hypertensive rats; this is the same response as seen in healthy (normoxic) rats. However, unlike in normoxic animals, BAY 60-7550 was unable to affect the vasodilator activity of SPER-NO in both the pulmonary artery and aorta in pulmonary hypertensive rats. Furthermore sildenafil only augmented the SPER-NO-induced relaxation in the aorta from pulmonary hypertensive rats.

Neither vinpocetine (PDE1i) nor milrinone (PDE3i) had any effect on ANP- or NO-induced vasorelaxation in both the pulmonary artery and aorta from pulmonary hypertensive rats.

3.2.6 Effect of hypoxia on the potency of NO and ANP in the systemic and pulmonary vasculature

Chronic hypoxia significantly attenuated the vasorelaxant effects of both ANP (log EC_{50} -8.43±0.19 and -7.74±0.26 normoxia and chronic hypoxia, respectively, $P<0.05$; ***Figure 3.17***) and SPER-NO (log EC_{50} -7.15±0.08 and -6.11±0.05 normoxia and chronic hypoxia, respectively, $P<0.05$; ***Figure 3.17***) in aorta in comparison to normoxia. In contrast, chronic hypoxia did not significantly affect the vasorelaxant effects of either ANP (log EC_{50} -8.39±0.05 and -8.55±0.18 normoxia and chronic hypoxia, respectively, $P>0.05$; ***Figure 3.18***) or SPER-NO (log EC_{50} -6.11±0.07 and -5.96±0.06 normoxia and chronic hypoxia, respectively, $P>0.05$; ***Figure 3.18***) in pulmonary artery in comparison to normoxia.

Figure 3.17 Effect of hypoxia on ANP and NO-induced relaxation of rat aorta

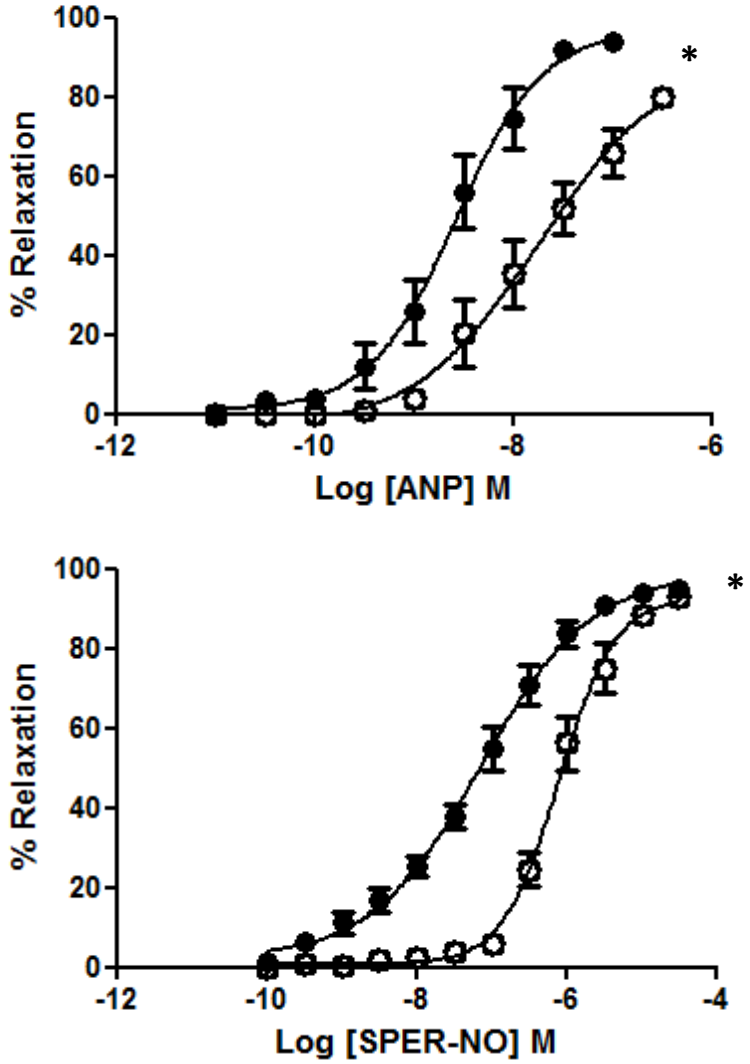


Figure 3.17 Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted normoxic rat aorta (closed circles) and chronically hypoxic aorta (open circles). *P<0.05 across entire curve by 2-way ANOVA; n>10.

Figure 3.18 Effect of hypoxia on ANP and NO-induced relaxation of rat pulmonary artery

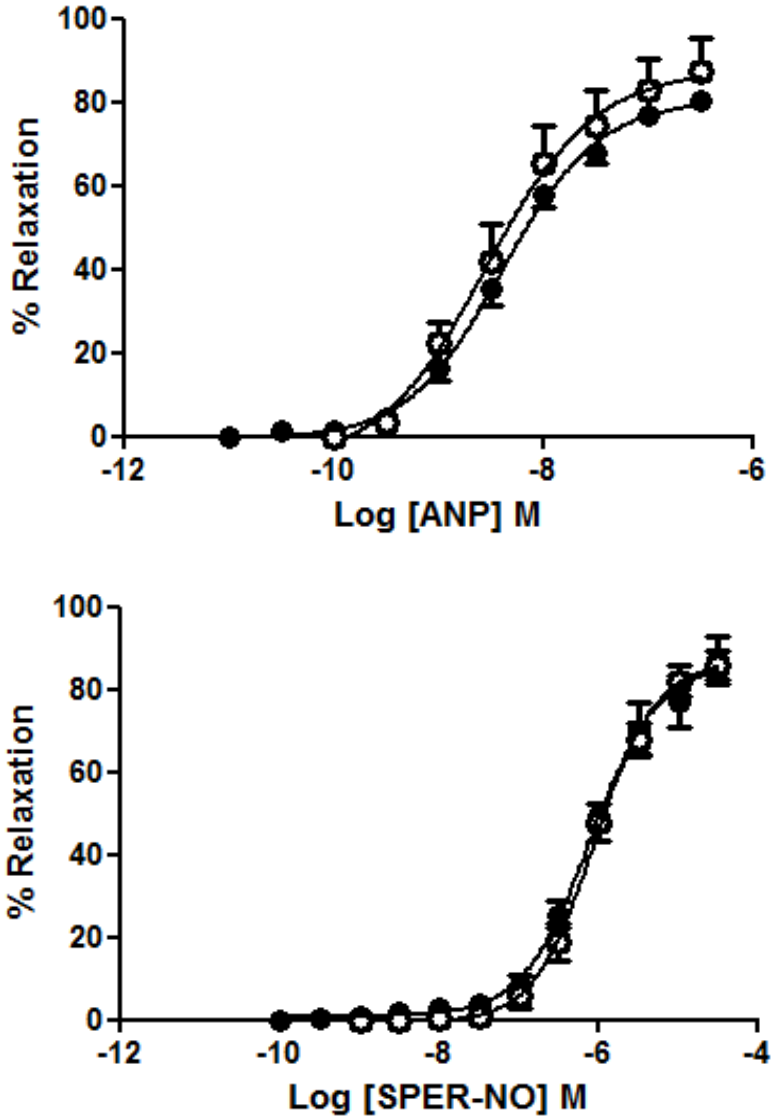


Figure 3.18 Concentration response curves to atrial natriuretic peptide (ANP; upper panels) and spermine NONOate (SPER-NO; lower panels) in U46619 (EC_{80}) pre-contracted normoxic (closed circles) and chronically hypoxic pulmonary artery (open circles). n>10.

3.3 Effect of chronic hypoxia on the expression of phosphodiesterases in the pulmonary and systemic vasculature.

The effect of chronic hypoxia on PDE expression in the pulmonary and systemic vasculature was assessed using RT-PCR. Thoracic aorta and pulmonary artery were excised from two groups of Sprague-Dawley rats. The first was exposed to normoxia whilst the second was exposed to two weeks of hypoxia to induce pulmonary hypertension. Excess vessel was snap frozen in liquid nitrogen and stored at -80^{o}C. RNA was isolated from the vessel samples and used to synthesise cDNA. PCR reactions were performed and the relative expression of PDE1B and 1C, PDE2A, PDE3A and 3B, and PDE5A were assessed.

In rat pulmonary artery chronic hypoxia does not significantly increase the expression of PDE1C, PDE2A, PDE3A and 3B nor PDE5A; that I assessed ($P>0.05$; ***Figure 3.19***). However there does appear to be a trend towards increased expression of PDE1B, PDE2A, PDE3A and PDE3B in pulmonary artery from rats exposed to two weeks hypoxia versus pulmonary artery from normoxic animals (***Figure 3.19***).

PDE expression in rat aorta is not significantly affected by chronic hypoxia ($P>0.05$; ***Figure 3.19***).

Figure 3.19 Effect of hypoxia on the expression PDEs in rat pulmonary artery and aorta

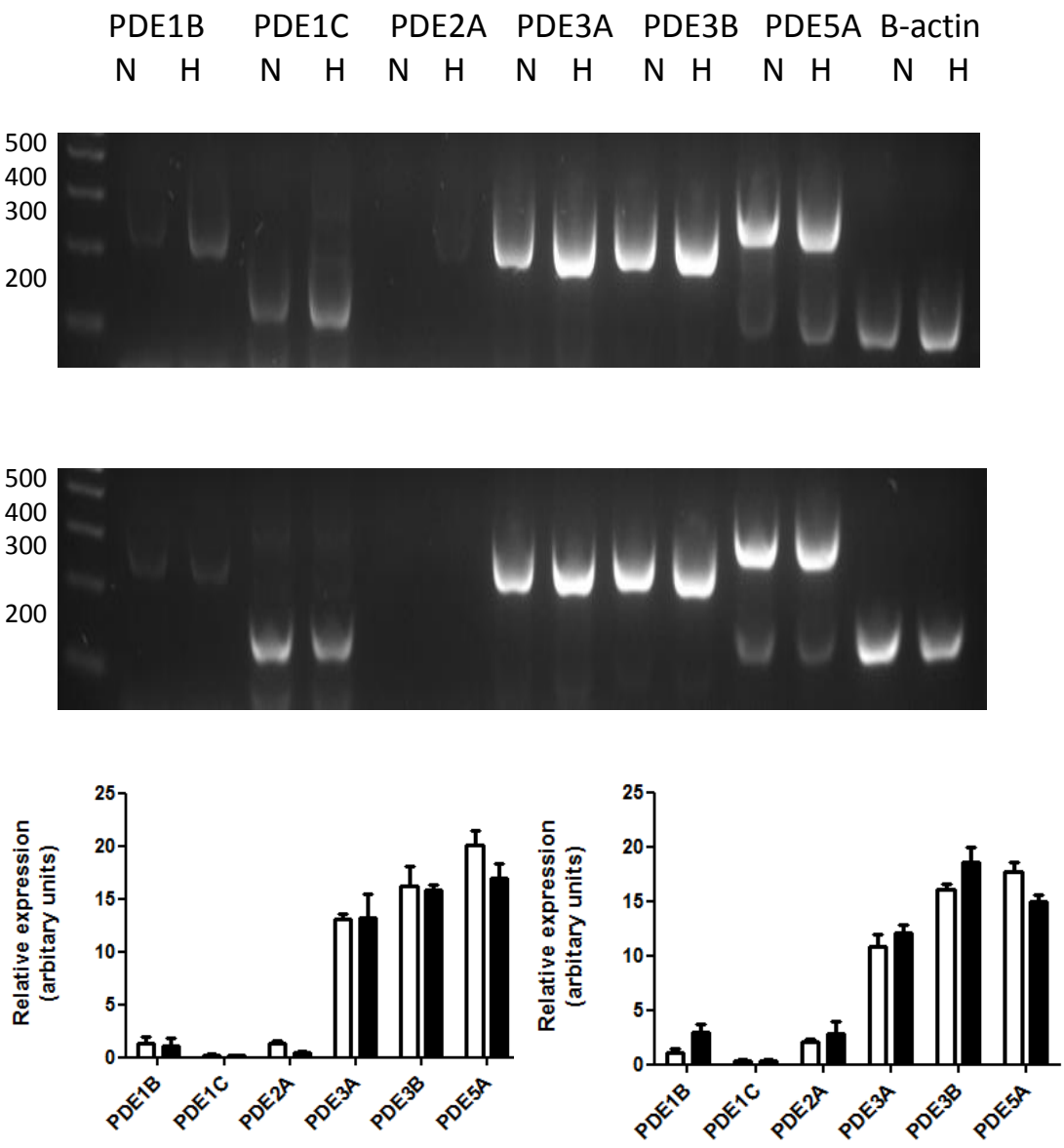


Figure 3.19 Effect of normoxia (N) and chronic hypoxia (H) on the expression of phosphodiesterases (PDEs) in pulmonary artery (upper panel) and aorta (middle panel) with densitometric quantification (lower panel); n=3.

3.4 Effect of PDE2 inhibition and ANP augmentation on hypoxia-induced pulmonary hypertension

Control mice exposed to 3 weeks of hypoxia (10% O_2) showed increased RVSP (32.07±1.48mmHg) when compared to normoxic controls (18.97±1.76mmHg; P<0.05; ***Figure 3.20***). The severity of PH (i.e. increased RVSP) following administration of BAY60-7550 (21.66±1.29mmHg) and the combination of BAY60-7550 and ecadotril (29.17±1.97mmHg; each P<0.05; ***Figure 3.20***); was significantly reduced. In contrast ecadotril (26.45±1.91mmHg; P>0.05; ***Figure 3.20***) did not significantly reduce RVSP compared to mice administered vehicle. However, there were no significant changes to MABP across all groups (P>0.05; ***Figure 3.20***). This implies the therapies are selective for the pulmonary circulation.

Exposure to 3 weeks hypoxia resulted in the expected elevation of RV/LV+S ratio, indicative of the development of right ventricular hypertrophy (***Figure 3.21***). The RV/LV+S ratio like RVSP was significantly reduced by BAY60-7550 (0.290±0.006) the BAY60-7550 and ecadotril combination group (0.281±0.007), in comparison to hypoxic controls (0.342±0.019; P<0.05; ***Figure 3.21***). Therefore cardiac hypertrophy was reduced by the combination group and BAY60-7550 alone but not ecadotril.

Exposure to 3 weeks hypoxia resulted in an increase in the percentage of muscularised vessels (53.4±2.4%) when compared to normoxic controls (25.5±3.5%; P<0.05; ***Figure 3.21 and Figure 3.22***). Following the administration of BAY60-7550 (32.0±2.8%) and the combination of BAY60-7550 and ecadotril (35.6±0.8%; each P<0.05; ***Figure 3.21 and Figure 3.22***) hypoxia-induced vessel muscularisation was reduced. Therefore vascular remodelling is reduced by the combination group and BAY60-7550 alone but not ecadotril.

Figure 3.20 Effect of PDE2 inhibition and ANP augmentation on RVSP and MABP in hypoxia-induced pulmonary hypertension

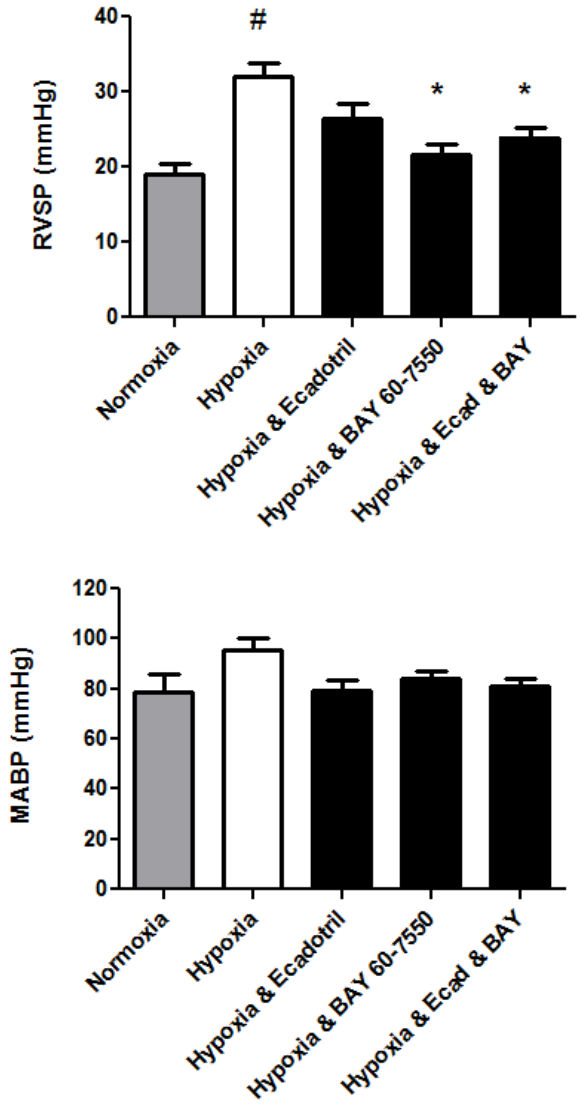


Figure 3.20 Right ventricular systolic pressure (RVSP; upper panel) and mean arterial blood pressure (MABP; lower panel) in normoxic mice (n=8), and mice exposed to 3 weeks hypoxia without treatment (10% O_2; n = 8) or in the presence of ecadotril (60mg/kg/day; weeks 3; n=6), BAY 60-7550 (10mg/kg/day) or BAY60-7550 plus ecadotril (weeks 3; n=8). *P < 0.05 versus hypoxia. #P < 0.05 versus normoxia.

Figure 3.21 Effect of PDE2 inhibition and ANP augmentation on RV hypertrophy and pulmonary vascular remodelling in hypoxia-induced pulmonary hypertension

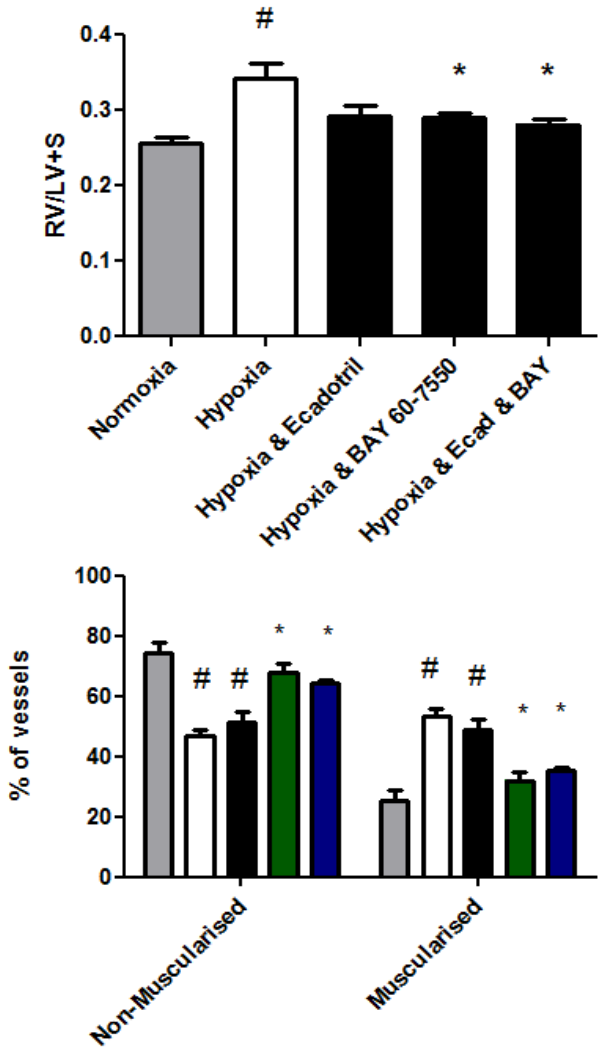


Figure 3.21 Right ventricle:left ventricle plus septum ratio (RV/LV+S; upper panel), non-muscularised and muscularised vessels (lower panel) in the lung of normoxic mice (grey bar; n=8), and mice exposed to 3 weeks hypoxia without treatment (white bar; 10% O_2; n = 8) or in the presence of ecadotril (black bar; 60mg/kg/day; weeks 3; n=6), BAY 60-7550 (green bar; 10mg/kg/day) or BAY60-7550 plus ecadotril (blue bar; weeks 3; n=8). P < 0.05 versus hypoxia. #P < 0.05 versus normoxia.

Figure 3.22 Effect of PDE2 inhibition and ANP augmentation on pulmonary vascular remodelling in hypoxia-induced pulmonary hypertension

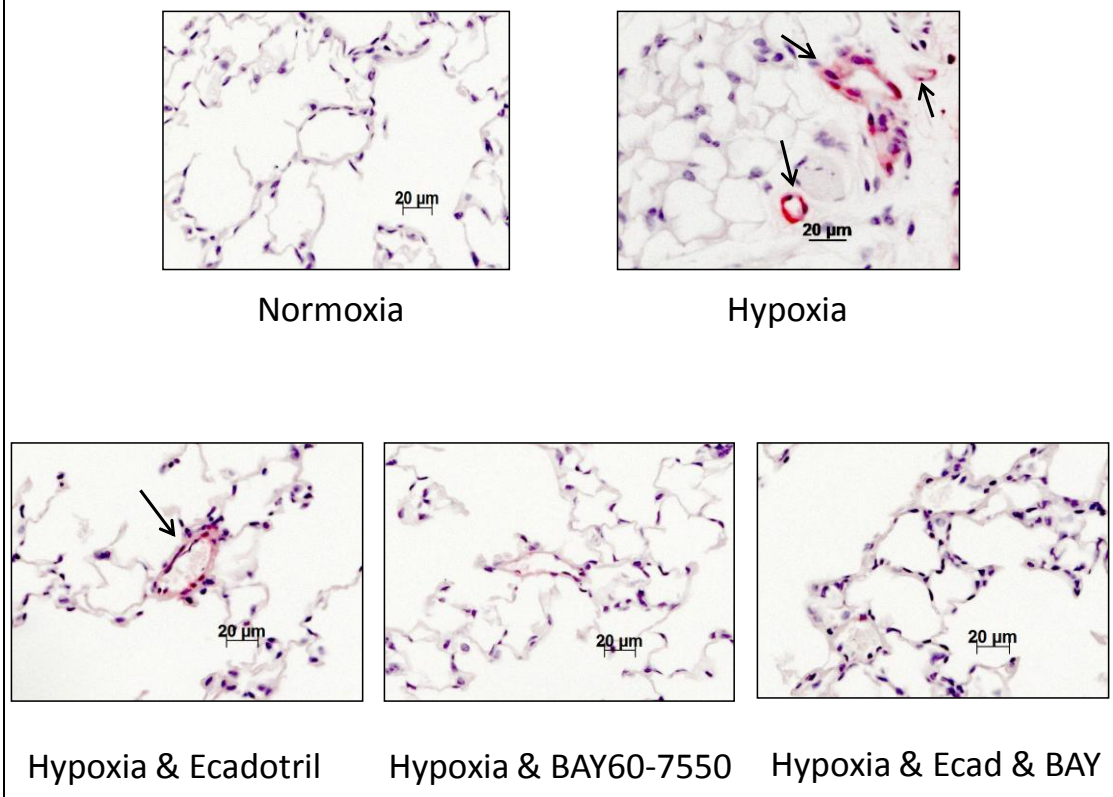


Figure 3.22 Representative light microscope images (20x magnification) of lungs obtained 21 days from initiation of normoxia, hypoxia (10% O_2; weeks 3), hypoxia and ecadotril (60mg/kg/day; weeks 3), hypoxia and BAY60-7550 (10mg/kg/day; weeks 3), hypoxia and ecadotril (Ecad) and BAY60-7550 (BAY) treated mice. Pulmonary arteries were assessed for muscularisation with an anti-alpha smooth muscle actin antibody (indicated by red staining and arrow for overt mucularisation; please refer to section 2.7 for detailed methodology).

3.5 Effect of PDE5 inhibition and ANP augmentation on hypoxia-induced pulmonary hypertension

The effect of 'therapeutic inhibition' (i.e. interventions administered after PH had been established) of PDE5 and augmentation of ANP was assessed in the pre-clinical model of PH in mice – chronic hypoxia. Five groups of male C57/BL6 mice (20-25g) were studied; i) normoxia, ii) hypoxia control receiving daily gavage with vehicle, iii) hypoxia, daily gavage with vehicle and sildenafil (30mg/kg/day) administered in the drinking water, iv) hypoxia and ecadotril (60mg/kg/day) delivered by gavage and v) hypoxia plus the combined treatment of sildenafil and ecadotril, dosed and delivered as indicated above. The appropriate drug/vehicle treatment was started three weeks after placement of animals into a hypoxic chamber (10% O_2) and lasted for three weeks to induce the development of PH (***Figure 3.23***).

Figure 3.23 Experimental design of therapeutic intervention.

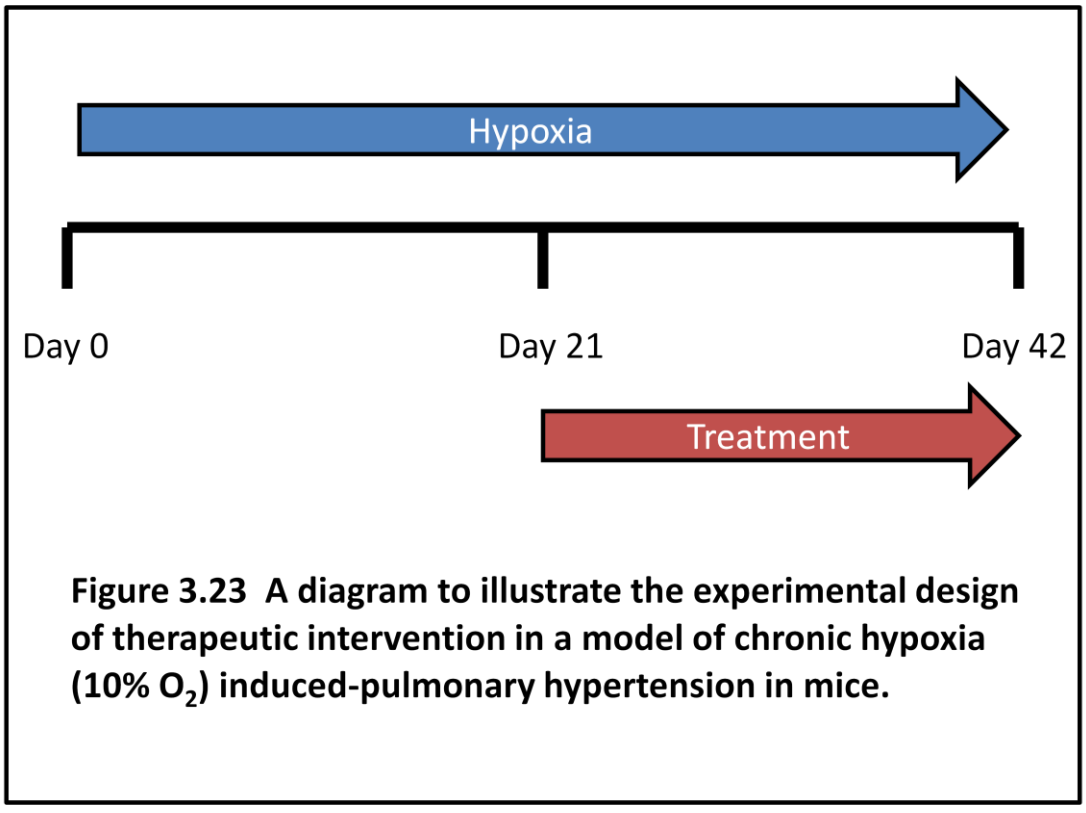


Figure 3.23 A diagram to illustrate the experimental design of therapeutic intervention in a model of chronic hypoxia (10% O_2) induced-pulmonary hypertension in mice.

Control mice exposed to 6 weeks of hypoxia (10% O_2) showed increased RVSP (34.32±3.88mmHg) when compared to normoxic controls (21.57±3.43mmHg; P<0.05; ***Figure 3.24***). The severity of PH (i.e. increased RVSP) following administration of sildenafil (26.32±2.56mmHg), ecadotril (28.03±1.78mmHg) and the combination of sildenafil and ecadotril (29.17±1.97mmHg; each P<0.05; ***Figure 3.24***); from weeks 3-6 was significantly reduced. However, there were no significant changes to MABP across all groups (P>0.05; ***Figure 3.24***). This implies the therapies are selective for the pulmonary circulation.

Exposure to 6 weeks hypoxia resulted in the expected elevation of RV/LV+S ratio, indicative of the development of right ventricular hypertrophy (***Figure 3.25***). The RV/LV+S ratio was only significantly reduced by the sildenafil and ecadotril combination group (0.306±0.014), in comparison to hypoxic controls (0.341±0.033; P<0.05; ***Figure 3.25***). Therefore cardiac hypertrophy was reduced by the combination group but not either drug alone. Total heart weight was not significantly affected by hypoxia or treatment (P>0.05; ***Figure 3.25***).

Figure 3.24 Effect of PDE5 inhibition and ANP augmentation on RVSP and MABP in hypoxia-induced pulmonary hypertension

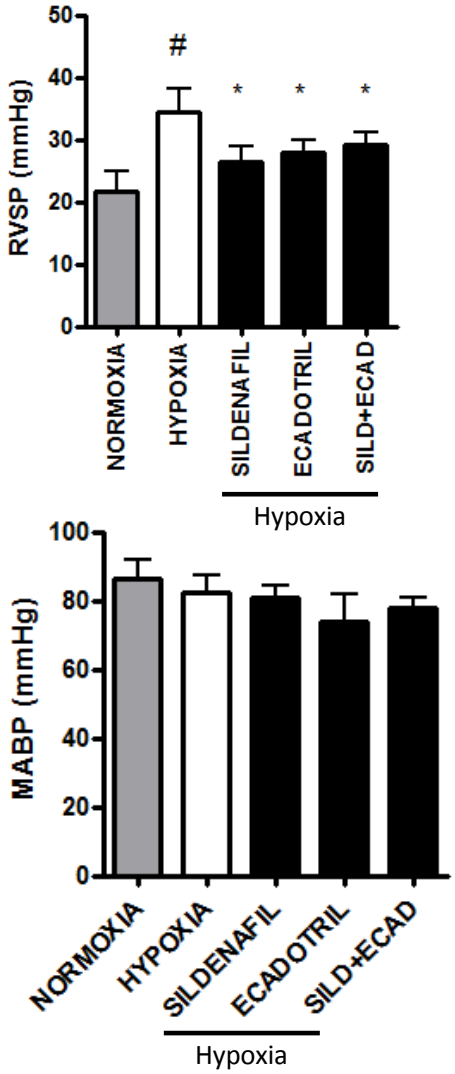


Figure 3.24 Right ventricular systolic pressure (RVSP; upper panel) and mean arterial blood pressure (MABP; lower panel) in normoxic mice (n=6), and mice exposed to 6 weeks hypoxia without treatment (10% O_2; n = 6) or in the presence of sildenafil (30mg/kg/day; weeks 3-6; n=6), ecadotril (60mg/kg/day; weeks 3-6; n=6), or sildenafil plus ecadotril (weeks 3-6; n=8). Data is represented as mean ± S.E.M; *P < 0.05 versus hypoxia. #P < 0.05 versus normoxia.

Figure 3.25 Effect of PDE5 inhibition and ANP augmentation on heart weight and RV hypertrophy in hypoxia-induced pulmonary hypertension

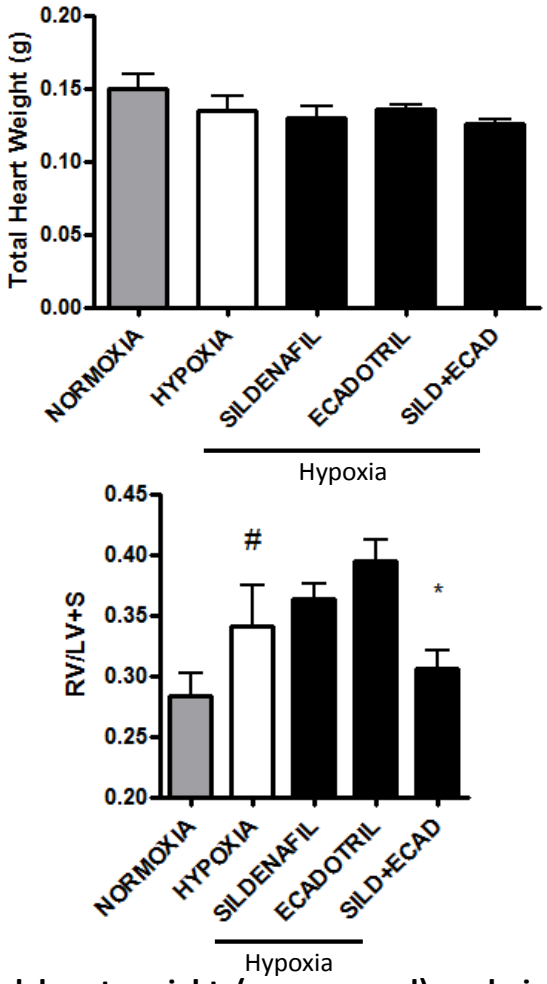


Figure 3.25 Total heart weight (upper panel) and right ventricle:left ventricle plus septum ratio (RV/LV+S; lower panel) in normoxic mice (n=6), and mice exposed to 6 weeks hypoxia without treatment (10% O_2; n = 6) or in the presence of sildenafil (30mg/kg/day; weeks 3-6; n=6), ecadotril (60mg/kg/day; weeks 3-6; n=6), or sildenafil plus ecadotril (weeks 3-6; n=8). Data is represented as mean ± S.E.M; *P < 0.05 versus hypoxia. #P < 0.05 versus normoxia.

3.6 Effect of PDE5 inhibition and ANP augmentation on bleomycin-induced pulmonary hypertension

Interstitial lung diseases, including IPF, are often complicated by the existence of PH; patients with both conditions have a far less favourable outcome (Lettieri *et al.*, 2006; Nathan *et al.*, 2008a; Patel *et al.*, 2007). By adopting a well-validated model of inflammation and lung-injury that is accepted as a model of human lung fibrosis – bleomycin induced lung fibrosis (Scotton & Chambers, 2010), I investigated the effectiveness of the combination treatment, sildenafil plus ecadotril, in a second pre-clinical model of IPF/PH.

Bleomycin challenged mice showed increased RVSP (33.84±2.40mmHg) when compared to saline controls (21.14±4.03mmHg; $P<0.05$; ***Figure 3.26***). Therefore in this model of PF there is accompanying PH. The severity of PH was only significantly reduced following administration of the combination of sildenafil and ecadotril (25.18±1.90mmHg; $P<0.05$; ***Figure 3.26***). Therefore having an additive if not synergistic effect over mono-therapy ($P<0.05$; ***Figure 3.26***). However, there were no significant changes to MABP across all groups ($P>0.05$; ***Figure 3.26***). This implies the therapies are selective for the pulmonary circulation.

Bleomycin challenge resulted in the elevation of RV/LV+S ratio, indicative of the development of right ventricular hypertrophy (***Figure 3.27***). This data supports the haemodynamic that this model of PF has associated PH. The RV/LV+S ratio was significantly reduced by sildenafil (0.250±0.009) or ecadotril (0.240±0.009) treatment as well as the sildenafil and ecadotril combination group (0.239±0.011), in comparison to hypoxic controls (0.312±0.027; $P<0.05$; ***Figure 3.27***). Therefore cardiac hypertrophy was reduced by all treatment groups. Total heart weight was not significantly affected by hypoxia or treatment ($P>0.05$; ***Figure 3.27***).

Figure 3.26 Effect of PDE5 inhibition and ANP augmentation on RVSP and MABP in bleomycin-induced pulmonary hypertension

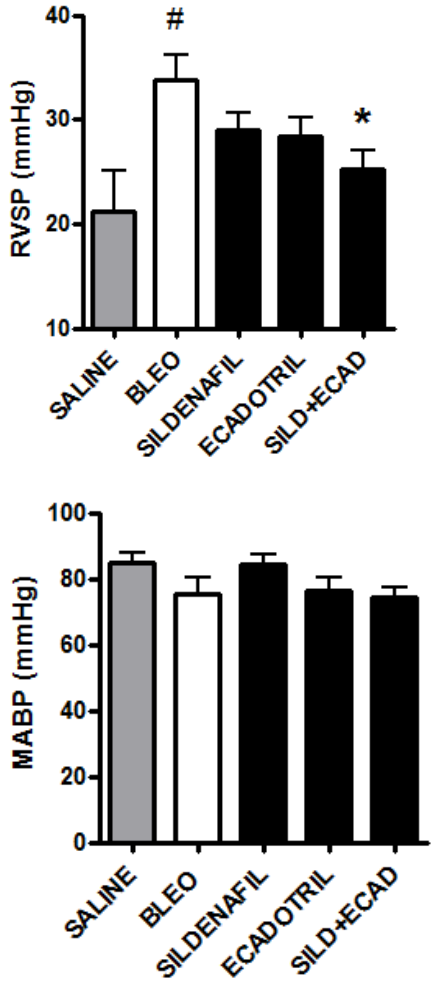


Figure 3.26 Right ventricular systolic pressure (RVSP; upper panel) and mean arterial blood pressure (MABP; lower panel) in saline challenged mice (n=6), and mice challenged with 1mg/kg bleomycin (bleo; n = 6) in the presence of sildenafil (30mg/kg/day; n=9), ecadotril (60mg/kg/day; n=9), or sildenafil plus ecadotril (n=10). *P < 0.05 versus bleomycin. #P < 0.05 versus saline.

Figure 3.27 Effect of PDE5 inhibition and ANP augmentation on heart weight and RV hypertrophy in bleomycin-induced pulmonary hypertension

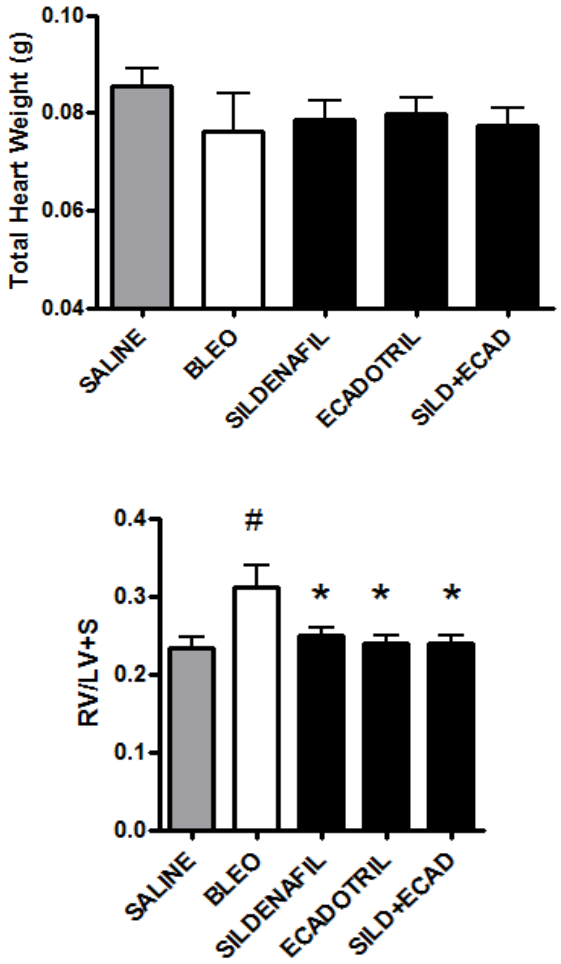


Figure 3.27 Total heart weight (upper panel) and right ventricle:left ventricle plus septum ratio (RV/LV+S; lower panel) in saline challenged mice (n=6), and mice challenged with 1mg/kg bleomycin (bleo; n=6) in the presence of sildenafil (30mg/kg/day; n=9), ecadotril (60mg/kg/day; n=9), or sildenafil plus ecadotril (n=10). *P < 0.05 versus bleomycin. #P < 0.05 versus saline.

Chapter 4: Discussion

4.1 Pulmonary Hypertension

Pulmonary Hypertension is a rare and debilitating disease which has a very poor mortality rate. PH is characterised by increased pulmonary arterial blood pressure, vascular remodelling of the small pulmonary arteries, right ventricular hypertrophy and failure. Whether idiopathic, familial or associated with other diseases (e.g. congenital heart disease, embolism, chronic lung disease), the disorder leads to premature death. This is predominantly due to the paucity of satisfactory treatments, particularly vasodilators that selectively reverse the excessive vasoconstriction observed in the pulmonary vasculature, and inhibitors of vascular wall re-modelling. Despite recent therapeutic advances, including endothelin receptor antagonists and PDE inhibitors, 2-year mortality remains unacceptably high (~15%) and a cure remains elusive. As a consequence, to advance current treatment options, contemporary approaches have focused on developing drug combinations that synergise in the pulmonary vascular bed, improving haemodynamics and reversing structural remodelling.

4.2 Phosphodiesterases and natriuretic peptides in pulmonary hypertension

Natriuretic peptides and NO are involved in the pathology of PH (Klinger *et al.*, 1993b; Klinger *et al.*, 1993a; Zhao *et al.*, 1999). Pharmacological manipulation of either system has therapeutic benefit (e.g. inhaled NO; (Kinsella *et al.*, 1992), sGC activators (Mittendorf *et al.*, 2009), PDEi (Wilkins *et al.*, 2005), but NPs appear to be a better target because they are lung specific (Baliga *et al.*, 2008). Therefore increasing cGMP is a clinically useful approach. However, it is not clear which PDEs are most important in regulating cGMP in the lung, thus providing scope for further optimisation.

Phosphodiesterases are homologous enzymes that facilitate the breakdown of the second messengers cAMP and cGMP. There are 11 distinct PDE families, with each typically consisting of several isoforms and/or splice variants. Molecules blocking the activity of this family of enzymes, collectively known as PDE inhibitors (PDEi), have been a major focus of drug development, particularly for cardiovascular disease (Bender & Beavo, 2006). Indeed, in the vasculature, PDEi exert several favourable effects including vasodilatation, inhibition of smooth muscle proliferation and prevention of platelet aggregation (Bender & Beavo, 2006).

It is the cardiovascular consequences of PDE5 inhibition that have been studied most extensively. Blockade of this enzyme, which metabolises cGMP exclusively, lowers systemic and pulmonary artery pressure under physiological conditions in animals and humans (Baliga *et al.*, 2008; Wilkins *et al.*, 2005; Galie *et al.*, 2005b; Klinger *et al.*, 2006; Rubin *et al.*, 2011; Jing *et al.*, 2011; Ghofrani *et al.*, 2002). Moreover, in animal models and patients with PH, PDE5i cause larger reductions in pulmonary than systemic vascular resistance, thereby exhibiting relative selectivity for the pulmonary vasculature (Baliga *et al.*, 2008; Galie *et al.*, 2005b; Klinger *et al.*, 2006; Rubin *et al.*, 2011). This vasoactive profile has culminated in the development and approval of the PDE5i sildenafil as a first-line therapy for PH; the drug elicits an improvement in several indices of disease severity including pulmonary artery pressure, cardiac index, exercise capacity and WHO functional class (Galie *et al.*, 2005b).

However, a significant cohort of PH patients does not respond to sildenafil treatment and in many individuals, indices of disease severity (i.e. 6MWD) return to 'baseline' approx 12 months after initiation of sildenafil therapy. Moreover, in patients who respond well to sildenafil, there is dose-dependent systemic hypotension that limits the beneficial effects of the drug. Thus, there remains considerable scope to optimise interventions targeting cGMP-dependent signalling to improve the treatment of PH.

The mechanism(s) underlying the pulmonary selectivity of PDE5i remain unclear, but may be due, in part, to increased expression and activity of PDE5 in the pulmonary circulation of patients with PH (Wharton *et al.*, 2005). Notwithstanding, the beneficial effects of PDE5i in models of PH are blunted in animals with a genetic deletion of the NPR-A (Zhao *et al.*, 1999). This suggests that the mechanism of pulmonary selectivity of PDE5i depends on the bioactivity of natriuretic peptides (i.e. ANP, BNP or CNP; (Ahluwalia *et al.*, 2004). This view is supported by animal and human data demonstrating that acute infusion of natriuretic peptides in the presence of sildenafil synergistically reduces pulmonary artery pressure (Klinger *et al.*, 2006; Preston *et al.*, 2004). Furthermore, inhibitors of NEPi, an enzyme that degrades natriuretic peptides, significantly reduce the severity of hypoxia-induced PH (Klinger *et al.*, 1993b; Winter *et al.*, 1991). These data highlight the therapeutic potential of manipulating natriuretic peptide bioactivity to reverse the haemodynamic abnormalities associated with PH. This approach additionally holds great promise for attenuating the pulmonary vascular re-modelling that also characterises the disease, since natriuretic peptides inhibit pulmonary vascular smooth muscle proliferation and TGF-β-induced extracellular matrix expression *in vitro*, and prevent structural changes *in vivo* in animal models of PH (Chen *et al.*, 2006; Jin *et al.*, 1991; Klinger *et al.*, 1998; Klinger *et al.*, 1999; Li *et al.*, 2007). Thus, this dual mechanism of action satisfies the well-documented requirement for targeting both haemodynamic and structural aberrations in PH if therapy is to be advanced significantly (Ghofrani *et al.*, 2009; Rhodes *et al.*, 2009).

4.3 In vitro manipulation of phosphodiesterases and cGMP generators in the pulmonary and systemic vasculature

In this study I investigated the effects of PDEi on NO and ANP dependent vasodilatation of the systemic (aorta) and pulmonary (pulmonary artery) vasculature. It has already been shown that ANP dependent vasodilatation can be potentiated by PDE5i (sildenafil) in the pulmonary but not the systemic vasculature (Baliga *et al.*, 2008). Here I show that PDE2i has a similar profile to PDE5i.

In *in vitro* functional pharmacological studies I investigated the effect of PDE1, PDE2, PDE3 and PDE5 on NO and ANP dependent vasodilatation of the systemic (aorta) and pulmonary (pulmonary artery) vasculature. The rationale for these studies was that each of these PDEs hydrolyse cGMP and have either been directly implicated in PH or they are localised in the heart and/or pulmonary vasculature (Beavo, 1995; Bender & Beavo, 2006; Fischmeister *et al.*, 2006).

Only PDE5 and PDE2 inhibitors were able to potentiate the vasodilator effects of NO and/or ANP in normoxic rat vessels; indeed PDE2i and PDE5i produced very similar responses. PDE2 or PDE5 inhibition potentiated the vasodilator effects of NO in both the pulmonary and systemic vasculature, whilst the vasodilator effects of ANP were only potentiated in the pulmonary vessels. These observations suggest that cGMP produced by sGC and pGC in response to NO and NPs, respectively, are independently controlled and do not result in changes in a single cellular pool of cGMP, in the systemic vasculature at least. These results mirror previous findings showing differential effects of sGC and pGC activation on functions within the same cell types (Piggott *et al.*, 2006; Takimoto *et al.*, 2007).

Three PDE2 splice variants have been identified (PDE2A1, PDE2A2 [rat-specific] & PDE2A3) and are expressed in a wide variety of cells and tissues including the heart, platelets and endothelium (Bender & Beavo, 2006). The variants are kinetically indistinguishable, but the 2A2 and 2A3 isoforms have a N-terminal membrane localisation motif that results in a predominantly particulate distribution. This cellular localisation seems key to the functioning of PDE2 in the cardiovascular system. Recent evidence has emerged linking PDE2 and PDE5 in the myocardium

such that compartmentalised cGMP-dependent signalling in response to NO and natriuretic peptides is regulated by PDE5 and PDE2, respectively; this links the membrane-bound natriuretic peptide receptors (i.e. pGCs) with PDE2, and sGC with cytosolic PDE5 (Castro *et al.*, 2006; Fischmeister *et al.*, 2006). This spatially-constrained interaction is thought to play a key role in the development of left ventricular hypertrophy and heart failure (Kass *et al.*, 2007) and it is well-established that PDE2 modulates the activity of the cardiac L-type Ca^{2+} current, opposing the effect of β-agonists (Fischmeister *et al.*, 2005). My findings support the idea of compartmentalisation of intracellular cGMP in vascular smooth muscle cells but also suggest there are differences between the pulmonary and systemic vasculature. Additionally, my data shows for the first time that PDE2 acts in a similar manner to PDE5 when investigating the vasodilator effects of NO and ANP in the pulmonary and systemic vasculature. PDE5 and PDE2 do have some similarities; they hydrolyse cGMP, have GAF domains and are activated by cGMP binding a GAF domain. PDE2 is a 'cGMP-stimulated' PDE which metabolises cAMP and cGMP and, akin to PDE5, possesses tandem GAF activating domains (Aravind & Ponting, 1997) within its N-terminus (Bender & Beavo, 2006) that act as a positive feedback loop to expedite cyclic nucleotide hydrolysis in the presence of cGMP. Therefore cGMP targeted therapies will be suboptimal without blockade of PDE2 and PDE5 because the increase in levels of cGMP will be tempered by increased hydrolysis by these PDEs. Thus, my data suggests PDE2 could be an interesting new target for PH. Furthermore an investigation into the simultaneous inhibition of PDE2 and PDE5 could be an interesting combination for PH.

In the vessels from rats with hypoxia induced PH, neither PDE1i nor PDE3i were able to potentiate NO or ANP dependent vasodilatation in either pulmonary or systemic vessels. PDE2i potentiated ANP dependent vasodilatation in pulmonary artery alone. In contrast PDE5i only potentiated NO dependent vasodilatation in aorta. This data differs from the results in healthy vessels above, and demonstrates how functional activity can differ from healthy to pathological states. PDE2i appears to have 'lost' its ability to potentiate NO dependent vasodilatation (and thus cGMP activity generated by sGC activation) in both the pulmonary and systemic vasculature, whereas PDE5i is unable to affect NO or ANP vasodilatation in the pulmonary vasculature to the same extent it could in healthy vessels. This

loss/dampening of PDE2 and PDE5 inhibition to potentiate vasodilator responses in diseased vessels could be due to greater expression or activity of PDEs. In fact it has been suggested that PDE activity is upregulated in PH (MacLean *et al.*, 1997). My data hints to a trend that PDE expression is increased by exposure to chronic hypoxia but statistical significance was not achieved. Importantly, mRNA levels only represent what is happening at the time the sample was taken, in this case after 14 days exposure to hypoxia. To truly get a sense of the effect of hypoxia on PDE expression, a temporal investigation (e.g. assessments at 0, 7, 14, 21 days of hypoxia) would be superior. A further experimental limitation is that RT-PCR is semi-quantitative; quantitative PCR (qPCR) would provide a more quantitative measure of PDE expression. Furthermore these studies only investigated expression of PDEs, not enzyme activity; although functional pharmacological assessment of the contribution of PDEs to cGMP signalling was performed (as described above).

The inability of PDE2i and PDE5i to potentiate NO-dependent vasodilatation could be due to oxidation of sGC thus rendering it insensitive to NO. Indeed, oxidised sGC has been shown to increase in both human cardiovascular diseases and animal models of cardiovascular disease associated with increased production of reactive oxygen species (ROS) (Stasch *et al.*, 2006). Furthermore sGC activators and stimulators have been successfully used to partially reverse hypoxia and MCT induced PH in rats (Weissmann *et al.*, 2009; Schermuly *et al.*, 2008; Dumitrascu *et al.*, 2006) and the sGC stimulator riociguat (BAY 63-2521) is currently in phase III clinical trials for the treatment of PH (Mittendorf *et al.*, 2009; Ghofrani *et al.*, 2010), following a successful evaluation in Phase 2 (Ghofrani et al. 2010). Given PDE2i in combination with ANP enables selective vasodilatation of the pulmonary vasculature over the systemic vasculature in diseased vessels, PDE2 could be a better target in PH than PDE5.

PDE2 is able to hydrolyse cGMP and cAMP, both of these cyclic nucleotides have been implicated in PH and increasing their intracellular concentration appears to be an effective method of alleviating PH (Dony *et al.*, 2008; Wilkins *et al.*, 2005). Additionally there is interplay between PDE2 and PDE3; PDE2 is stimulated by cGMP whilst cGMP is a competitive inhibitor of cAMP hydrolysis by PDE3. Inhibition of PDE2 increases cGMP concentration which can inhibit PDE3 and

therefore the breakdown of cAMP (Surapisitchat *et al.*, 2007). Therefore increasing the endogenous protection afforded by not just NO and NPs, but prostacyclin too. It has already been shown that PDE3i (hydrolyses cAMP) potentiates the therapeutic effects of prostacyclin analogues in rats with hypoxia induced PH (Phillips *et al.*, 2005). However even though inhibition of PDE3 (milrinone) is approved in the use of heart failure, chronic use has been shown to increase mortality (Packer *et al.*, 1991), reducing enthusiasm for this approach. Furthermore, augmenting NO/NP signalling will stimulate PDE2 (and PDE5) activity and therefore be self inhibiting. Thus, making PDE2i a good strategy to augment cGMP (and cAMP) levels effectively.

PDE 1 and PDE 3 (and splice-variants thereof) have been implicated in pulmonary vascular homeostasis and PH (Bender & Beavo, 2006). These enzymes hydrolyse cGMP and cAMP, although the PDE1A/1B splice variants have a higher affinity for cGMP (Bender & Beavo, 2006). PDE1A and PDE1C expression and activity are up-regulated in animal models of PH and in tissues from patients with the disease (Evgenov *et al.*, 2006; Murray *et al.*, 2007; Schermuly *et al.*, 2007). Moreover, the selective PDE1i 8-methoxymethyl-isobutyl-1-methyl xanthine (8MM-IBMX) reduces proliferation of human vascular smooth muscle cells (Rybalkin *et al.*, 2002) and reverses the haemodynamic and morphological aberrations associated with MCT and hypoxia-induced PH (Schermuly *et al.*, 2007). PDE 3A/3B expression and activity is also enhanced in PH (Murray *et al.*, 2002), and the presence of this 'cGMP-inhibited' PDE might underlie the synergistic cytoprotective activity of NO and prostacyclin in PH, and explain the benefit of co-administration of therapies promoting these pathways concomitantly (i.e. sildenafil and iloprost; Wilkens *et al.*, 2001). Indeed, a dual PDE3/4 inhibitor reverses monocrotaline-induced PH and synergises with iloprost (Dony *et al.*, 2008; Schermuly *et al.*, 2004). Despite this potential, the increased mortality associated with the use of PDE3 inhibitors in (left) heart failure (Amsallem *et al.*, 2005) has limited the therapeutic interest for this approach in PH. Increased expression of PDE1 and PDE3 has been demonstrated in PH (MacLean *et al.*, 1997; Schermuly *et al.*, 2007). Also, inhibition of PDE1 (Schermuly *et al.*, 2007) or PDE3 (Dony *et al.*, 2008; Phillips *et al.*, 2005) has been shown to be effective in alleviating hypoxia induced PH. However, my data intimates that functionally PDE1 and PDE3 are not such key targets, given their

inhibition was unable to potentiate the vasodilator effects of NO and ANP. The beneficial effects of PDE1i and PDE3i (as described in previous published work) maybe due to lack of selectivity of the PDEi used, and crossover inhibition of PDE2 and/or PDE5. Alternatively, PDE1i and/or PDE3i could be more efficacious in inhibiting vascular remodelling or RV hypertrophy, rather than the acute haemodynamic changes I have explored in this thesis. In addition, PDE1 and PDE3 are able to hydrolyse both cAMP and cGMP, but PDE3 hydrolyses cAMP 10 times faster than cGMP; therefore cGMP is a competitive inhibitor of cAMP degradation by this PDE isozyme (Zaccolo & Movsesian, 2007). Given both ANP and NO drive cGMP production PDE3 may already be inhibited by cGMP, entailing that PDE3i are relatively ineffective. Certainly, my data demonstrates the functional effects of PDE2 and PDE5 are more important at least in terms of haemodynamics and therefore possibly better targets for PH.

4.4 PDE inhibition and ANP augmentation in animal models of pulmonary hypertension

Our laboratory have recently exploited a 'natriuretic peptide-centric' approach to evaluate the therapeutic potential of a PDE5i/NEPi combination in PH (Baliga *et al.*, 2008). This work demonstrated that (a) PDE5i augment the vasorelaxant activity of natriuretic peptides selectively in the pulmonary, but not systemic, vasculature; (b) combination therapy is superior to either drug alone in hypoxia-induced PH; and (c) the PDE5i/NEPi combination does not significantly lower systemic blood pressure. These observations suggest that in PH release of natriuretic peptides represents a cytoprotective mechanism that mitigates pathogenesis. By selectively inhibiting PDE isozymes (in this instance PDE5) that (patho)physiologically regulate natriuretic peptide signalling, it is possible to target the pulmonary vasculature and prevent disease progression. In accord, I predicted that by combining NEPi with selective PDEi, or combinations of PDEi, it will be possible to harness further the beneficial effects of natriuretic peptides, thereby optimising pulmonary selectivity and efficacy, and advancing the treatment of PH; investigating this hypothesis represents the central aim of this thesis.

The strategy of elevating cGMP, either by enhancing natriuretic peptide bioactivity or augmenting NO-dependent signalling (e.g. NO inhalation, direct sGC stimulation), is clinically effective in PH (Rhodes *et al.*, 2009). This gives rise to the possibility of targeting alternate cGMP-hydrolysing PDE isozymes to achieve the goal of increasing cGMP in the pulmonary vasculature, thereby reversing disease progression.

4.4.1 PDE2 inhibition and ANP augmentation in hypoxia-induced pulmonary hypertension

My data shows that PDE2 plays a role in the haemodynamic and morphological changes associated with PH, and that PDE2i may be a novel therapeutic approach for this disease. My data shows that BAY60-7550 (PDE2i) alone, as well as BAY60-

7550 and ecadotril in combination could both equally reduce the severity of established hypoxia induced PH (i.e. increased RVSP). Both treatments also reduced right ventricular hypertrophy.

In response to increased resistance, the pulmonary circulation remodels to accommodate the higher pressure and maintain oxygenation. Induction of PH with hypoxia also caused a dramatic increase in the number of muscularised small pulmonary arteries. Herein the re-modelling of the pulmonary circulation was significantly reduced by monotherapy and with dual therapy. This provides further evidence that augmenting natriuretic peptide bioactivity exerts a multi-faceted beneficial effect on many haemodynamic aspects of pathogenesis in PH. Indeed, our lab has recently published evidence that natriuretic peptides differentially regulate endothelial and smooth muscle cell proliferation in the vasculature, suggesting they have the ideal bioactive profile to concomitantly offset endothelial dysfunction/damage and prevent smooth muscle hypertrophy (Khambata *et al.*, 2011). Undoubtedly, if these natriuretic peptide-driven salutary actions translate to the clinical arena, combination treatment should significantly improve outcome. Furthermore there was no significant effect on MABP, indicating systemic hypotension should not be an issue with this combination treatment.

Changes in PDE2 activity in hypoxia-induced PH resemble those of PDE5, intimating that it should be possible to effect a pulmonary-selective dilatation by inhibiting PDE2. As has been published for PDE5 (Baliga *et al.*, 2008), this preferential activity in the pulmonary vasculature appears to be associated with changes in the bioactivity of natriuretic peptides, which would fit well with the membrane association of PDE2 (i.e. co-localisation with natriuretic peptide receptors). Indeed, PDE2 activity may be critical to the development and treatment of PH as a result of the complementary cytoprotective roles of NO, natriuretic peptides and prostacyclin; excessive PDE2 activity would be predicted to curtail the synergism between these cGMP- and cAMP- generating pathways and exacerbate pathology (since it metabolises both cyclic nucleotides), whereas PDE2i would be expected to exert a beneficial effect, as has been reported for the relatively selective PDE2i (EHNA) in acute HPV (Haynes *et al.*, 1996).

4.4.2 PDE5 inhibition and ANP augmentation in hypoxia-induced pulmonary hypertension

Further *in vivo* investigations I undertook were to build on the work by colleagues who demonstrated that PDE5i (sildenafil) and NEPi (ecadotril) combination therapy was effective in hypoxia induced PH, and had an additive effect over monotherapy (Baliga *et al.*, 2008). This previous investigation involved the treatment being given prophylactically; a more clinically relevant experiment would be to induce PH and see if the combination treatment was effective against established PH. My data shows that sildenafil or ecadotril alone, as well as sildenafil and ecadotril in combination could all equally reduce the severity of established hypoxia induced PH (i.e. increased RVSP). Only combination treatment reduced right ventricular hypertrophy. These findings are consistent with studies investigating the effect of PDE5i (sildenafil) in NPR-A KO mice with hypoxia induced PH, that the NPR-A pathway makes a greater contribution to the effect of sildenafil on RV hypertrophy than RVSP (Klinger *et al.*, 1993a; Klinger *et al.*, 1999; Zhao *et al.*, 1999).

This data further emphasises the importance of the combination therapy and the additive if not synergistic effects it elicits on PH. It is reassuring that the combination therapy continues to be effective against both haemodynamic and hypertrophic aspects of PH. This is important as one of the main criticisms of current therapies is their lack of ability to stem or even reverse hypertrophy/remodelling. Given that PH patients eventually succumb to RV failure, it is pleasing to see the combination therapy's anti-hypertrophic affects. Furthermore there was no significant effect on MABP, indicating systemic hypotension should not be an issue with this combination treatment.

4.4.3 PDE5 inhibition and ANP augmentation in bleomycin-induced pulmonary hypertension

Idiopathic pulmonary fibrosis is a progressive fibro-proliferative disorder with a poor prognosis; this is largely the result of a complex and undefined aetiology, and a lack of therapeutic options (Bjoraker *et al.*, 1998; Eickelberg & Laurent, 2010). Interstitial lung diseases, including IPF, are often complicated by the existence of PH; patients with both conditions have a far less favourable outcome (Lettieri *et al.*, 2006; Nathan *et al.*, 2008a; Patel *et al.*, 2007). Our laboratory reported recently that combination of a PDE5i (i.e. sildenafil), that sustains cGMP signalling, and a NEPi (i.e. ecadotril) that slows the breakdown of natriuretic peptides in the circulation, resulted in a significant alleviation of disease severity in hypoxia-induced PH (Baliga *et al.*, 2008). This precedent, coupled to the well-defined anti-fibrotic actions of natriuretic peptides (Das *et al.*, 2010; Nishikimi *et al.*, 2011; Tamura *et al.*, 2000) in cardiac and renal fibrosis, provided the rationale to explore the potential of manipulating natriuretic peptide bioactivity in IPF. Data presented herein suggest that this approach may offer a substantial pharmacodynamic benefit in IPF and associated PH since it combats both the fibro-proliferative and haemodynamic aberrations characteristic of the disease.

Adopting a well-validated model of inflammation and lung-injury that is accepted as a model of human lung fibrosis (Scotton & Chambers, 2010), I demonstrate that focusing efforts to maximize cGMP-dependent signalling using, PDE5i and NEPi, offers a potent means of preventing fibrosis and the accompanying PH. However, caution should be engaged when interpreting these results given a prophylactic treatment regime was employed. Such an approach could have interfered with the inflammation phase immediately post bleomycin in this model, and not necessarily just acted in an anti-fibrotic fashion.

Administration of bleomycin caused the expected increase in lung fibrosis and significantly elevated RVSP; this was accompanied by marked RV hypertrophy. Neither of the components of the combination therapy, sildenafil nor ecadotril, significantly reduced RVSP at the doses employed. However, this provided the ideal background to reveal a clear synergy between the two drugs when administered

together; this cooperative activity mirrored that reported in models of hypoxia-induced PH and patients with the disease (Baliga *et al.*, 2008; Klinger *et al.*, 2006; Preston *et al.*, 2004; Zhao *et al.*, 1999). A salutary effect on the right heart was also evident. Here, sildenafil and ecadotril elicited a marked reduction in RV hypertrophy. Again, an additive benefit of using both drugs together was observed. Interestingly, the effects of the combination therapy were greater (individually and in concert) in the structural changes in the right heart compared to the haemodynamic dysfunction. This disparity suggests that augmentation of cGMP and natriuretic peptide function has direct effects to slow or prevent cardiac hypertrophy, rather than simply secondary to reducing pressure in the pulmonary circulation. This is perhaps not surprising, since all three of the principal members of the natriuretic peptide family exert potent anti-hypertrophic effects in the heart (Klinger *et al.*, 2002; Knowles *et al.*, 2001; Oliver *et al.*, 1997; Tamura *et al.*, 2000). This is a welcome finding since the right heart is often neglected in the consideration of novel therapies for IPF and PH, and since right ventricular failure is often the ultimate cause of death, treatment modalities that directly preserve right heart structure and function are likely to provide a valuable addition to the therapeutic repertoire. A further key facet of this PDE5i plus NEPi combination is that it appears to target the lung, as MABP was not significantly different with either sildenafil or ecadotril, alone or in combination. This selectivity is advantageous in IPF and PH since it avoids the issue of systemic hypotension whilst being able to maximize cGMP signalling in the pulmonary circulation. Indeed, the beneficial pharmacodynamic profile of PDE5i plus NEPi holds promise in the treatment of many forms of PH, since it appears that the combination therapy is effective in models of PH with contrasting aetiologies. This bodes well for porting this dual therapy to other forms of the disease with distinct pathogenesis.

4.5 General summary

I have advanced current knowledge by executing the first systematic functional characterisation of PDE1, -2, -3 and -5 on sGC and pGC signalling in pulmonary and systemic vasculature, and assessed the effect hypoxia has on these systems. In addition, complementary analysis of PDE1, -2, -3 and -5 mRNA expression was performed. I have shown the first data verifying the functional role of PDE2 (and inhibitors thereof) in lung vascular homeostasis, as well as PDE2 having potential to be a therapeutic target in PH. Furthermore, I have established that the beneficial effects of PDE2 are dependent on natriuretic peptide bioactivity and are selective for the pulmonary vasculature. Moreover, the sensitivity of ANP-driven signalling is maintained in hypoxia whereas NO-based signalling is blunted. Finally, I have demonstrated that the combination of PDE5i and NEPi is effective in pre-clinical models of both PH (reversal) and PH associated with IPF.

In summary, a combination therapeutic strategy targeted to enhance natriuretic peptide activity (NEPi) and prevent cGMP metabolism (PDE5i) is a potent and effective combination that prevents and reverses the accompanying PH in bleomycin-induced lung inflammation and injury. Therefore, this NEPi/PDE5i drug combination might be a novel therapeutic approach in PH, which currently has little or no disease-modifying therapy, and consequently significant associated morbidity and mortality. Since both components of the combination are licensed drugs, efficacy in PH patients can be evaluated rapidly and inexpensively; this approach therefore holds promise to improve outcome and lower healthcare costs in this patient cohort

To conclude, these data suggest PDE5i or PDE2i and modulation of the NPR-A pathway could be a method to selectively dilate the pulmonary vasculature over the systemic vasculature. In fact inhibition of PDE2 could be a better target than PDE5 in PH. Therefore *in vivo* investigations into the effect of PDE2i and NEPi, PDE2i and PDE5i or even a combination of all three is warranted. Combination therapy is an attractive method of treatment because reduced doses of each drug can often be given. Not only does this often result in lower incidence of side effects but also gives scope to increase dosage and/or add further drugs if necessary.

Figure 4.1 How PDEs regulate the actions of ANP and NO in the pulmonary circulation

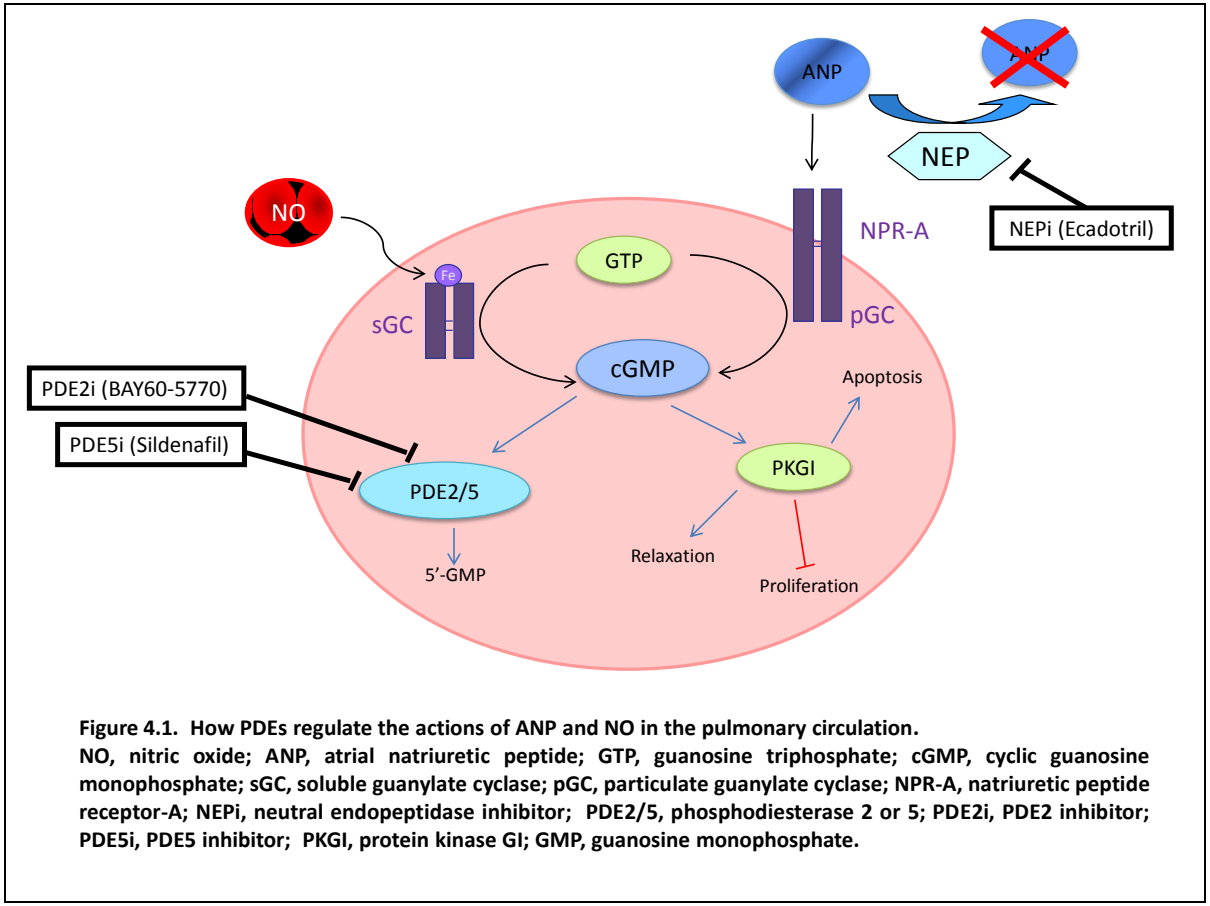


Figure 4.1. How PDEs regulate the actions of ANP and NO in the pulmonary circulation.
NO, nitric oxide; ANP, atrial natriuretic peptide; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; sGC, soluble guanylate cyclase; pGC, particulate guanylate cyclase; NPR-A, natriuretic peptide receptor-A; NEPi, neutral endopeptidase inhibitor; PDE2/5, phosphodiesterase 2 or 5; PDE2i, PDE2 inhibitor; PDE5i, PDE5 inhibitor; PKGI, protein kinase GI; GMP, guanosine monophosphate.

Chapter 5: Further Studies

5.1 Further investigation of PDE2 inhibition

In my *in vivo* hypoxia-induced PH study I used BAY60-7550 (PDE2i) at a concentration of 10mg/kg/day (Boess *et al.*, 2004). At this concentration RVSP, RV hypertrophy and pulmonary vessel muscularisation was returned to baseline. This did not allow any 'window' to see if the combination treatment was of further benefit to mono-therapy. Therefore further investigations should firstly conduct a concentration response, suggested concentrations would be 1, 3 and 10mg/kg/day with and without edcadotril (NEPi; 60mg/kg/day; (Baliga *et al.*, 2008).

In this thesis I have used prophylactic treatment in the chronic hypoxia mouse model of PH. A more clinically relevant treatment regime would be therapeutic dosing i.e. induce PH and then see if the treatment is effective. Given the mild nature of the PH induced by hypoxia alone, an improved model of PH would be the hypoxia-sugen model (Ciuclan *et al.*, 2011).

PDE2 is a 'cGMP-stimulated' PDE which metabolises cAMP and cGMP and, akin to PDE5, possesses tandem GAF activating domains (Aravind & Ponting, 1997) within its N-terminus (Bender & Beavo, 2006) that act as a positive feedback loop to expedite cyclic nucleotide hydrolysis in the presence of cGMP. Therefore cGMP targeted therapies will be suboptimal without blockade of PDE2 and PDE5 because the increase in levels of cGMP will be tempered by increased hydrolysis by these PDEs. Therefore an investigation into the simultaneous inhibition of PDE2 and PDE5 could be an interesting combination for PH.

5.2 Clinical evaluation of PDE5 inhibition and ANP augmentation in PH

Both sildenafil (PDE5i) and racecadotril (NEPi) are already licensed drugs. Therefore there are real translational opportunities with this combination treatment. Currently the annual cost of treating PH patients in the UK is approximately £45,000 (National Institute for Health and Clinical Excellence). However, this combination therapy could be a very affordable treatment strategy given racecadotril (NEPi) is already a generic medicine and sildenafil has just come off patent in the UK.

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