Targeting multidrug resistance proteins and C-type natriuretic peptide to optimise cyclic GMP signalling in cardiovascular disease

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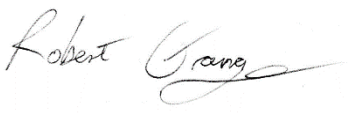

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PUBLISHED ABSTRACTS

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Bubb KJ, Trinder SL, Baliga RS, **Allen RMH**, Patel J, Clapp LH, MacAllister RJ, Hobbs AJ (2015). Natriuretic peptides in the treatment of pulmonary hypertension: PDE2 inhibition augments their therapeutic capacity. *Microcirculation.* 22(7): 542-676

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ABSTRACT

Cyclic-3',5'-guanosine monophosphate (cGMP) is a fundamental intracellular signalling molecule that regulates vascular homeostasis through the tight control of vascular smooth muscle cell (VSMC) reactivity (i.e. vasoconstriction/relaxation) and proliferation. Aberrant VSMC growth and sustained vasoconstriction are hallmarks of cardiovascular disease, exemplified by pulmonary hypertension (PH). Multidrug resistance proteins (MRPs) are membrane bound transporters that facilitate cGMP cellular export thereby representing a potential mechanism that regulates intracellular cGMP-driven signalling. C-type natriuretic peptide (CNP) is an important vasoactive peptide released from the endothelium that maintains vascular homeostasis. CNP binds to natriuretic peptide receptor-B (NPR-B), generating cGMP, and NPR-C, which acts as a clearance receptor removing CNP from the circulation and a signalling pathway regulating vascular function via a cGMP-independent mechanism.

Herein, I investigated two separate hypotheses: that MRPs play an important role in maintaining vascular homeostasis, and that endothelium-derived CNP and its cognate receptor, NPR-C, protects against the development of PH.

The role of MRPs in regulating vascular homeostasis was investigated using organ bath pharmacology, human VSMC (hVSMC) proliferation and measuring mean arterial blood pressure (MABP) in conscious and anaesthetised mice. To investigate the role of endothelium-derived CNP and NPR-C in PH, male and female CNP and NPR-C knockout (KO) mice were used in two experimental models of PH: hypoxia plus Sugen5416 (SU5416) and bleomycin-induced. The severity of PH was measured using right ventricular systolic pressure (RVSP), MABP, right ventricular hypertrophy (RVH) and pulmonary vascular remodelling.

MRP inhibition resulted in concentration-dependent vasorelaxation of mouse aorta *per se* and increased the potency of cGMP-dependent vessel relaxation in response to activation of both particulate and soluble guanylate cyclases (pGC and sGC). MRP inhibition alone also caused concentration-dependent

attenuation of hVSMC proliferation, and enhanced cGMP-mediated attenuation of hVSMC growth via pGC and sGC activation. MRP inhibition *per se* did not decrease MABP in either anaesthetised or telemeterised mice. However, MRP inhibition did dose-dependently enhance reductions in MABP due to pGC activation in anaesthetised mice.

Deletion of endothelial cell-derived CNP (ecCNP KO) in male and female mice did not result in any significant differences in RVSP, RVH or pulmonary vascular remodelling between WT and KO in the hypoxia plus SU5416 model of PH. However, global deletion of NPR-C in both male and female mice caused a significant increase in RVH but not RVSP or vascular remodelling when compared to WT. Both male and female NPR-C KO mice developed significantly increased RVSP compared to WT in the bleomycin-induced model of PH. However, only females exhibited a significant increase in RVH and lung weight in addition to RVSP.

In conclusion, MRP inhibition demonstrates potential therapeutic utility to treat cardiovascular diseases by potentiating the vasodilatory and VSMC anti-proliferative actions of natriuretic peptides and nitric oxide. Endothelial cell-derived CNP is not essential to host protection against PH, whereas its cognate receptor NPR-C demonstrates a cardioprotective capacity. NPR-C attenuates bleomycin-induced PH in both males and females, with a greater effect observed in females. Overall, NPR-C agonism could potentially be used to ameliorate the cardiac and vascular pathology associated with PH.

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ABBREVIATIONS

AC	Adenylyl cyclase
ACh	Acetylcholine
Ang	Angiotensin
ANP	Atrial natriuretic peptide
AVP	Arginine vasopressin
bFGF	Basic fibroblast growth factor
Bleo	Bleomycin
BNP	Brain natriuretic peptide
BHF	British Heart Foundation
BP	Blood pressure
BW	Body weight
CaM	Calmodulin
cAMP	Cyclic adenosine-3',5'- monophosphate
$cANF^{4-23}$	des(Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})-ANP fragment 4-23
cGMP	Cyclic guanosine-3', 5'-monophosphate
CMC	Carboxymethylcellulose sodium
cN	Cyclic nucleotides
CNP	C-type natriuretic peptide
COX	Cyclooxygenase
CVD	Cardiovascular disease
DMSO	Dimethyl sulfoxide
D-NO	Diethylenetriamine/nitric oxide adduct
EC	Endothelial cell
EDHF	Endothelium-derived hyperpolarising factor
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
GC	Guanylyl cyclase
GIRK	G protein-coupled inwardly-rectifying K^+ channel
GS-NO	S-nitroso-L-glutathione
GTP	Guanosine-5'-triphosphate
hCASMC	Human coronary artery smooth muscle cell

hPASMC	Human pulmonary artery smooth muscle cell
HR	Heart rate
Hsp	Heat shock protein
Hyp	Hypoxia
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ILD	Interstitial lung disease
IP_3	Inositol-1, 4, 5-triphosphate
IPF	Idiopathic pulmonary fibrosis
I/R	Ischaemia/reperfusion
Iso	Isoprenaline
KO	Knockout
LPS	Lipopolysaccharide
LW	Lung weight
LV+S	Left ventricle plus septum
MABP	Mean arterial blood pressure
MI	Myocardial infarction
MK	MK571
MLCK	Myosin light chain kinase
MRP	Multidrug resistance protein
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NPR	Natriuretic peptide receptor
OAT	Organic anion transporter
o.p.	Oropharyngeal
PAH	Pulmonary arterial hypertension
PAP	Pulmonary arterial pressure
PB	Probenecid
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PE	Phenylephrine
PF	Pulmonary fibrosis
PGI_2	Prostacyclin

PH	Pulmonary hypertension
PKA	Cyclic adenosine-3',5'-monophosphate-dependent protein kinase
PKG	Cyclic guanosine-3',5'-monophosphate-dependent protein kinase
PLC	Phospholipase-C
p.o.	*Per os* (oral administration)
RNA	Ribonucleic acid
RVH	Right ventricular hypertrophy
RV	Right ventricle
RVSP	Right ventricular systolic pressure
s.c.	Subcutaneous
SNP	Sodium nitroprusside
Sp-NO	Spermine-NONOate
SU5416	Sugen5416
Sug	Sugen5416
TGF	Transforming growth factor
TNF	Tumour necrosis factor
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cell
WHO	World Health Organisation
WT	Wild-type

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INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is defined by dysfunction of the homeostatic mechanisms that regulate the circulatory system (heart and vasculature). As this encompasses a huge multifaceted system, a plethora of diseases are incorporated into the general umbrella term of CVD, including coronary heart disease (CHD) which can lead to a myocardial infarction (MI; “heart attack”), hypertension, stroke, heart failure and atherosclerosis. CVD is the biggest cause of mortality globally; in 2012 31 % of all deaths (~17.5 million people) were due to CVD (WHO, 2015). Within the same year, the most common CVD was CHD, accounting for 7.4 million deaths, followed by stroke (6.7 million deaths); as such, these two disorders give rise to approximately 75 % of the total mortality due to CVD (WHO, 2015).

In the United Kingdom (UK), CVD is the second highest cause of mortality behind cancer (Townsend *et al*., 2015). From 2012 to 2014 CVD dropped from being the highest cause of mortality in the UK; despite this CVD still accounts for over a quarter of all deaths (155,000 people), with similar rates in males (28 %) and females (26 %). Of these, CHD is the biggest single cause of mortality, responsible for 15 % and 10 % of deaths in males and females, respectively (~69,000 deaths in total). Mirroring the global situation, the second most common cause of CVD mortality is stroke, accounting for around 39,000 deaths (6 % and 8 % men and women respectively; Townsend *et al*., 2015). Despite CVD being a substantial cause of mortality in the UK, age-standardised death rate from CVD has continuously decreased since 1961 demonstrating an increasing ability to combat these disorders (Scarborough *et al*., 2011).

In addition to the unacceptably high mortality, the morbidity associated with CVD is substantial. In 2014, CVD accounted for 1.7 million in-patient episodes

in all National Health Service (NHS) hospitals in the UK (Townsend *et al*., 2015). The number of people in the UK living with a CVD is approximately 2.29 million for CHD, 1.18 million for stroke, 9.25 million for hypertension and 493,000 for heart failure. This equates to more than 370 million prescriptions dispensed for CVD in the UK, including anti-platelet, lipid-lowering, anti-hypertensive and heart failure drugs. This equates to a £4.3 billion cost for the treatment and management of CVD (Townsend *et al*., 2015). However, healthcare cost is not the entire financial burden. Cardiovascular disease causes huge losses in productivity in those of working age through a combination of death and illness which greatly impedes UK economics. The Centre for Economics and Business Research (CEBR) published a report in 2014 which states an estimated £11.3 billion total healthcare cost, with a cost from lost work days being £3.9 billion due to mortality and £151.6 million from morbidity. In total, the annual cost of CVD to the UK economy is £15.2 billion, which is predict to increase to £18.7 billion in 2020 (Townsend *et al*., 2015).

In summary, CVD is a massive global health and economic problem, and a major drain of resources to publically-funded healthcare systems. The most disturbing fact is that most CVDs can be prevented by altering lifestyle behaviours, for example smoking (tobacco), alcohol abuse, unhealthy diet and a lack of physical activity. Whilst public health initiatives have helped to curb these precipitating factors, new medicines that target novel pathways responsible for preserving cardiovascular health, or prevent those precipitating CVDs, are a significant unmet medical need.

1.2 Cyclic guanosine-3’,5’-monophosphate (cGMP) signalling

Cardiovascular disease results from an imbalance in many complex and dynamic homeostatic mechanisms. The human circulatory system was originally described by London based physician William Harvey, in 1628, who described the structure of a unidirectional, double circulatory system enforced

by valves, with blood being driven by the heart and transported through arteries, then veins, and returning to the heart (Harvey, 1628). This intricate organ system is regulated by a range of different cells that work together to maintain homeostatic physiology, which in turn are regulated by a multitude of inter- and intra- cellular mechanisms which react to external stimuli leading to a cascade in intracellular signalling, culminating in a specific cellular response. In 1958, Earl Sutherland discovered what is now appreciated as a central player in these homeostatic mechanisms; the intracellular second messenger, cyclic adenosine-3',5'-monophosphate (cAMP; Rall & Sutherland 1958, Sutherland & Rall 1958). As a result of his work on cAMP and the actions of hormones, Earl Sutherland was the solo winner of the Nobel Prize in Physiology or Medicine in 1971 (Nobel Media AB 2014, 2016). Following the identification of cAMP, a sibling molecule, cyclic guanosine-3',5'-monophosphate (cGMP) was synthesised and hypothesised to act in a similar manner to cAMP because of its ability to be enzymatically hydrolysed (Smith *et al*., 1961). Subsequently, cGMP was also found to occur endogenously by a group that isolated and identified the molecule in rabbit urine (Ashman *et al*., 1963). The enzyme responsible for hydrolysing the 3',5'-phosphodiester bond in both cGMP and cAMP was first identified in 1964 (Kuriyama *et al*. 1964; Figure 1). Since then a family of analogous enzymes which hydrolyse these second messengers have been identified and characterised; phosphodiesterases (PDEs; see 1.4.1; Bender & Beavo 2006). Since their discovery over half a century ago, cGMP and cAMP have been at the centre of research that has led to several Nobel Prizes in Physiology or Medicine; furthermore we are still discovering new roles of these fundamental intracellular second messengers.

We now know that spatio-temporal changes in intracellular cGMP concentrations are responsible for controlling a broad range of physiological mechanisms that require a fine-controlled balance between cGMP production and degradation.

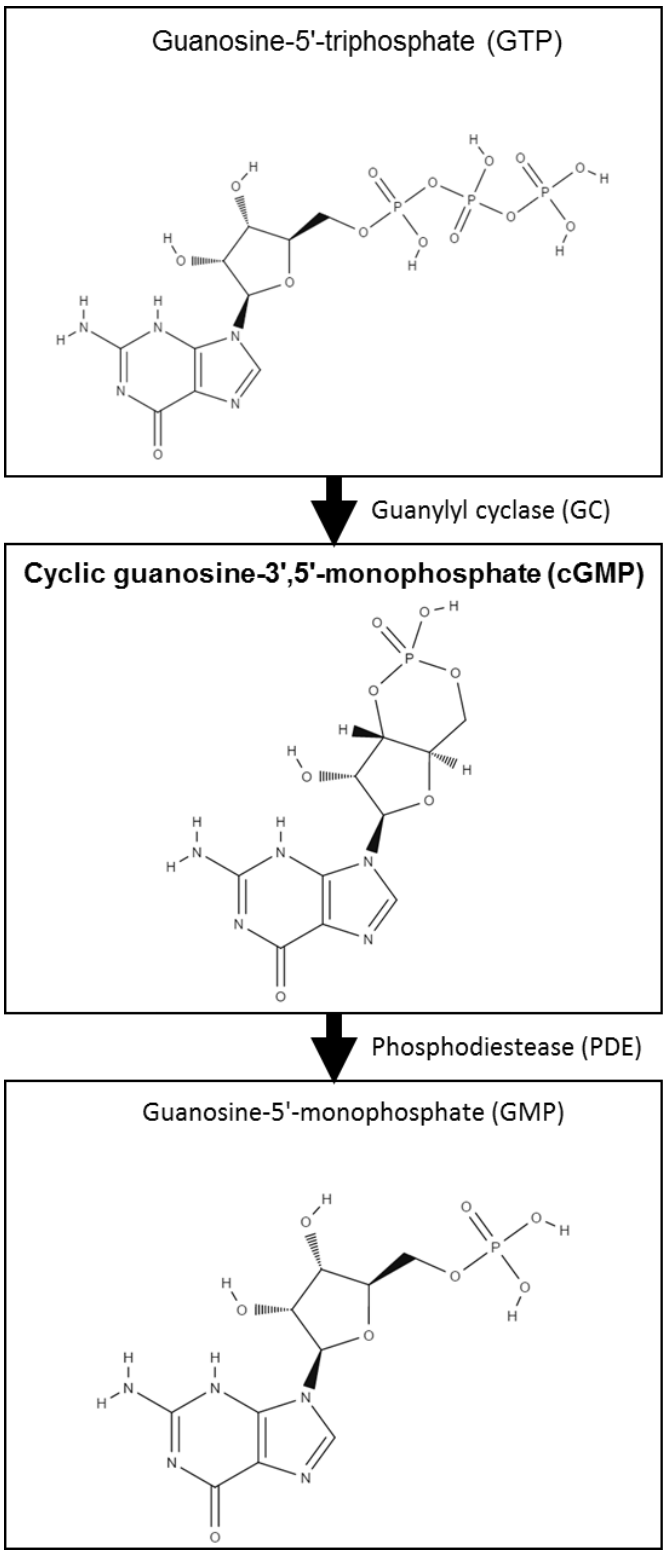

Figure 1. An illustration of the molecular structural changes to cGMP during generation and degradation.

Cyclic guanosine-3',5'-monophosphate (cGMP) is generated from guanosine-5'-triphosphate (GTP) by the enzyme guanylyl cyclase (GC). Cyclic GMP is degraded to guanosine-5'-monophosphate (GMP) by the enzyme phosphodiesterase (PDE).

1.3 Cyclic GMP production

Cyclic guanosine-3',5'-monophosphate is generated from guanosine-5'-triphosphate (GTP) by a family of enzymes termed guanylyl cyclases (GCs; Price *et al*. 1967; Figure 1). GCs are further divided according to structure and membrane localisation; cytosolic or soluble GC (sGC) and membrane-bound or particulate GC (pGC). Although, there is now evidence to suggest that chaperone proteins facilitate the membrane-association of sGC (e.g. heat-shock protein^{90}; Hsp^{90}; Agullo *et al*. 2005,Venema *et al*. 2003).

1.3.1 Soluble guanylyl cyclase (sGC)

Soluble GC is a heterodimeric protein formed of two subunits, α and β (Giuili *et al*. 1992, Giles *et al*. 2003). Two isoforms of each subunit exist, α_1, α_2, β_1 and β_2, with the most commonly studied and physiologically relevant combination being an $\alpha_1\beta_1$ dimer, that is ubiquitously expressed in mammals (Budworth *et al*., 1999). Each subunit is composed of the same distinct domains; an N-terminal H-NOX (haem-nitric oxide and oxygen binding family) domain, a Per/Arnt/Sim (PAS) domain, a amphipathic helix region and a C-terminal catalytic domain (Derbyshire and Marletta, 2012). Although, both α and β monomers have an N-terminal H-NOX domain, only the β subunit coordinates a haem iron (Fe^{2+}) capable of binding nitric oxide (NO; Zhao and Marletta, 1997). NO is the major ligand for sGC and upon binding to the haem domain leads to over a 200-fold increase in cGMP production (Stone & Marletta 1994; Figure 2). Soluble GC, and specifically the β_1 subunit, is essential to life demonstrated by the generation of knockout (KO) mice for this protein which die within 3-4 weeks after birth due to intestinal dysmotility. However, these animals also have catastrophic detrimental cardiovascular characteristics, exemplified by dysfunctional platelet aggregation and aortic vasorelaxation, plus systemic hypertension. Moreover, in sGC KO mice NO donors demonstrate a complete inability to relax pre-contracted isolated vessels and lower blood pressure (BP) *in vivo* compared to wild-type (WT) littermates, illustrating the importance of

sGC to orchestrate the cardiovascular effects of NO (see 1.3.2; Friebe *et al*., 2007). These unfavourable cardiovascular traits in sGC KO mice are mirrored in the human population, in which mutations in the genes that encode the sGC β or α subunits are also linked to the development of cardiovascular disease (Ehret *et al*., 2011). Soluble GC expression exhibits anti -proliferative and -migratory effects on vascular smooth muscle cells (VSMCs). For instance, increased expression of sGC in rats using recombinant adenoviruses, increases cGMP production in aortic smooth muscle cells, and decreases carotid neointima formation, in a balloon-induced vascular injury model compared to controls (Sinnaeve *et al*., 2001).

1.3.2 Nitric oxide (NO)

NO was originally discovered to be an endogenously produced, physiologically important signalling molecule during the hunt to identify the elusive endothelium-derived relaxing factor (EDRF) in the 1980s (Katsuki *et al*. 1977, Ignarro *et al*. 1987, Ignarro *et al*. 1986, Ignarro *et al*. 1982, Furchgott & Zawadzki 1980, Arnold *et al*. 1977). The discovery of NO led to Louis Ignarro, Ferid Murad and Robert Fuchgott being awarded the 1998 Nobel Prize in Physiology or Medicine (Nobel Media AB 2014, 2016). The powerful cardiovascular effects of NO were initially illustrated as a vasorelaxant (Palmer *et al*., 1987) and inhibitor of platelet aggregation (Radomski *et al*., 1987a) and adhesion (Radomski *et al*., 1987b), of leukocyte adhesion (Kubes *et al*., 1991), and anti-proliferative mediator in VSMCs (Garg & Hassid 1989). Subsequent to these impressive early findings further work has substantiated the cardiovascular-protective role of NO/sGC/cGMP signalling.

NO is generated enzymatically by nitric oxide synthase (NOS; Alderton *et al*., 2001). NOS is found in mammals expressed as three different isoforms, neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). These enzymes generate NO through the same process but differ in their activation, cellular localisation and expression. NOS proteins are homodimers, with each monomer containing two different

catalytic regions, an oxygenase and a reductase domain (Crane *et al*., 1998). NO is generated via a two-step, 5 electron oxidation of the amino acid L-arginine. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is oxidised by the reductase domain enabling electrons to be transferred to the cofactors flavin adenine dinucleotide (FAD) then flavin mononucleotide (FMN), present within the same region, and subsequently transported to a haem present in the oxygenase domain (Noble *et al*. 1999; Klatt *et al*. 1996). Binding of calmodulin, from elevated intracellular calcium (Ca^{2+}) concentrations, to the reductase domain increases the flow of electrons to the haem, and represents a key mechanism of activation (Abu-Soud and Stuehr, 1993). The haem binds O_2 and L-arginine, and in addition to the transferred electrons, hydroxylates L-arginine in the first step to produce an intermediate compound, N^G-hydroxy-L-arginine, which is in turn oxidised to L-citrulline and NO. This occurs in the presence of an essential cofactor, tetrahydrobiopterin (BH_4), which maintains enzyme structural integrity, facilitates L-arginine binding, and acts as an electron donor (Nishimura *et al*. 1995; Crane *et al*. 1998; List *et al*. 1997; Alderton *et al*. 2001; Figure 2).

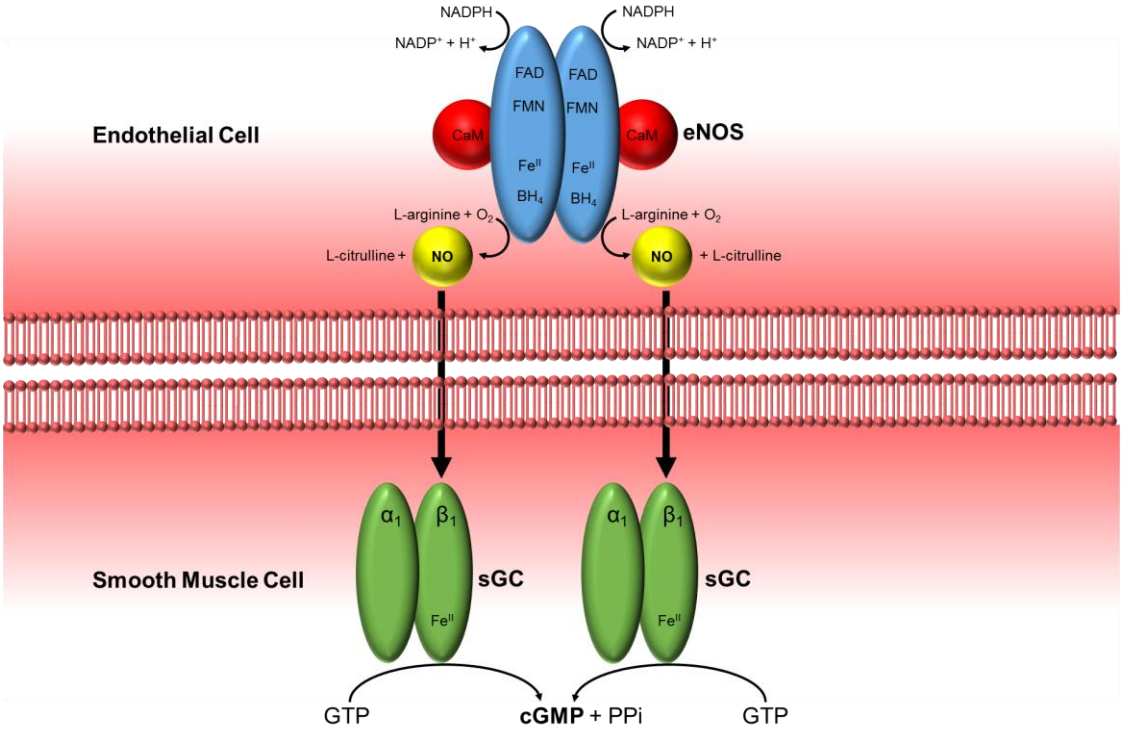

Figure 2. An illustration of the activation of sGC by endothelium-derived NO.

cGMP in vascular smooth muscle cells is generated by sGC, activated by NO, produced by eNOS expressed in vascular endothelial cells.

Cyclic guanosine-3',5'-monophosphate (cGMP), guanosine-5'-triphosphate (GTP), endothelial nitric oxide synthase (eNOS), soluble guanylyl cyclase (sGC), oxygen (O_2), inorganic pyrophosphate (PP_i), nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), calmodulin (CaM), Fe^{2+} (haem).

Endothelial NOS is considered the predominant NOS isoform of the cardiovascular system because of its abundant expression in endothelial cells (ECs) which intuitively enables the enzyme to utilise NO to maintain cardiovascular homeostasis (Pollock and Fiirstermanna, 1993; Pollock *et al*., 1991). In addition to elevated intracellular calcium concentrations, another important cardiovascular dependent-mechanism that can activate eNOS is fluid shear stress. This mechanism occurs through phosphorylation of serine (Ser^{1177}, Ser^{633}), threonine (Thr^{495}) and/or tyrosine (Tyr^{81}, Tyr^{657}) residues present in the eNOS enzyme. Kinases that are known to do this include Akt (activated by oestrogen, vascular endothelial growth factor; VEGF and insulin), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; triggered by bradykinin), and most notably protein kinase-A (PKA; stimulated by fluid shear stress; Boo *et al*., 2002; Fulton *et al*., 1999; McCabe, 2000; Schleicher *et al*., 2009). To illustrate the importance of eNOS in the cardiovascular system, each of the different eNOS KO mice generated to date is hypertensive (Huang *et al*., 1995; Shesely *et al*., 1996; Thoonen *et al*., 2013). Moreover, NO generated specifically from ECs is essential to the maintenance of vascular homeostasis by inhibiting VSMC proliferation (Garg and Hassid, 1989; Nakaki *et al*., 1990; Rudic *et al*., 1998), platelet aggregation (Alheid *et al*., 1987; Busse *et al*., 1987) and leukocyte recruitment (Arndt *et al*., 1993; Zeiher *et al*., 1995). Patients with hypertension demonstrate significant endothelial dysfunction; for example, impaired vascular relaxation to acetylcholine (ACh), which activates eNOS, generating NO (Panza *et al*. 1990). Furthermore, in humans mutations to genes that encode eNOS are associated with cardiovascular disease (Johnson *et al*., 2011; Kathiresan *et al*., 2005; Mitchell *et al*., 2007; Salvi *et al*., 2012). These data demonstrate clearly the importance of EC-derived NO in maintaining vascular function and protection against cardiovascular diseases.

Neuronal NOS is highly expressed throughout the central nervous system (CNS), in the periphery (within non-adrenergic, non-cholinergic [NANC] nerves) as well as skeletal muscle (Nakane *et al*., 1993). Nitric oxide generated by nNOS acts as an important neurotransmitter regulating cognition (memory, learning, neurogenesis/plasticity; Zhou & Zhu 2009) and non-vascular smooth muscle tone (i.e. gastrointestinal tract; Lefebvre, 2002; Tøttrup *et al*., 1991).

Some evidence also points to a direct nNOS-derived NO effect on VSMCs, and thus a cardiovascular role, independent of endothelium-generated NO (Melikian *et al*. 2009). Although, mice rendered genetically-deficient in nNOS don't develop hypertension, in contrast to eNOS KO animals (Melikian *et al*., 2009; Nelson *et al*., 1995). Neuronal NOS KO mice subjected to middle cerebral artery (MCA) occlusion (a model of ischaemic stroke), develop significantly smaller infarct sizes and improved neurological outcome than wild-type (WT) animals, illustrating a pathological neurodegenerative role of nNOS derived NO, alongside it's physiological neuronal signalling function (Huang *et al*., 1994; Lipton *et al*., 1993; Malinski *et al*., 1993). This is in contrast to eNOS KO mice, which develop larger infarct sizes compared to WTs in ischaemic stroke models because of significantly reduced blood flow, lending support to the notion that eNOS is the predominant homeostatic NOS isoform in the cardiovascular system (Huang *et al*., 1996; Lo *et al*., 1996).

Inducible NOS was originally identified in macrophages when exposed to inflammatory stimuli such as bacterial liposaccharide (LPS) or cytokines (Bergmann *et al*., 1992). Inducible NOS isn't basally expressed but can be induced in a variety of cell-types, under a multitude of inflammatory conditions, such as exposure to infectious agents (human immunodeficiency virus; HIV; *helicobacter pylori*; *mycobacterium tuberculosis*), in human autoimmune and chronic inflammatory diseases due to increased production of cytokines (e.g. interleukin-1; IL-1; IL-6), and other conditions including Alzheimer's, Parkinson's, tumours and MI (Kröncke *et al*., 1998). Although, it should be noted that iNOS is constitutively expressed in airway epithelial cells because of continuous exposure to toxins (Guo *et al*., 1995). NO is a free radical, this characteristic enables it to form part of the body's defensive repertoire, where it is utilised to combat infection from microbes and parasites, through its ability to inhibit cellular enzymes, especially those containing a haem domain (Green *et al*., 1990). In addition, NO can decrease the stability of deoxyribonucleic acid (DNA) and proteins, non-specifically, contributing to its anti-microbial affect. However, as a result of this chemistry, excessive production of NO can cause host necrosis and promote tumorigenesis (Kröncke *et al*., 1991; Wink *et al*., 1991). In contrast to nNOS and eNOS, iNOS is constitutively active once

expressed because of its unique ability to bind calmodulin irreversibly at low intracellular calcium concentrations (Cho *et al*., 1992). Constitutively active iNOS results in high-output production of NO and contributes to the life-threatening hypotension found in patients suffering from septic shock (MacMicking *et al*., 1995; Wei *et al*., 1995; Wong and Billiar, 1995).

1.3.3 Particulate guanylyl cyclases (pGCs)

In addition to sGC, cyclic GMP is generated by membrane-bound guanylyl cyclase (pGC). There are seven known pGCs in mammals, GC-A, GC-B, GC-C, GC-D, GC-E, GC-F and GC-G; in humans GC-D and GC-G are pseudogenes and therefore not thought to be expressed or functionally active (Manning *et al*., 2002). GC-C is highly expressed in the intestinal epithelial cells and is activated by the endogenous ligands guanylin and uroguanylin, which regulate colonic epithelial cell proliferation. GC-C is also activated by bacterial heat stable enterotoxins, which causes intestinal epithelial cells causes increased Cl^- secretion in intestinal epithelial cells leading to enhanced intestinal water content and diarrhoea (Potter, 2011). GC-E and GC-F are expressed on rods and cones in the retina and facilitate the conversion of photons into electrical signals allowing light to be perceived (Potter, 2011). However, the most important pGC enzymes in the context of cardiovascular homeostasis and disease are GC-A and GC-B, which are cognate receptors for a family of natriuretic peptides (NPs; see 1.3.4); as such, these pGCs are also known as natriuretic peptide receptor (NPR)-A and NPR-B, respectively. There is a third receptor that binds all three NPs, NPR-C (see 1.3.4.4), but this should not be confused with GC-C since NPR-C does not contain a GC domain and therefore cannot generate cGMP (Fuller *et al.,* 1988).

NPR-A and NPR-B are homodimers composed of an extracellular ligand binding domain, followed by a transmembrane domain, then an intracellular portion consisting of a kinase-like domain (no known kinase activity), followed by a hinge region and finally the GC domain (Garbers & Lowe 1994; Figure 3). In some ways the structures of NPR-A and NPR-B are homologous to sGC due

to their shared GC domain and thus cGMP generating capability (Potter, 2011). Arguably outside the shared GC functionality their structure is quite different (Chinkers and Garbers, 1989; Chinkers *et al*., 1989; Lowe *et al*., 1989; Thorpe and Garbers, 1989). Similar to NPR-A and NPR-B in its extracellular region, NPR-C differs in its intracellular domain which lacks catalytic activity. As a result, this NPR was traditionally thought of as a clearance receptor with no signalling capability; however the receptor is now known to signal as a G-protein coupled receptor (GPCR; Fuller *et al*. 1988; Koh *et al*. 1992; Nussenzveig *et al*. 1990; Figure 3).

1.3.4 Natriuretic Peptides (NPs)

NPR-A and NPR-B are activated by a family of endogenous ligands, the NPs. Each of the three principal mammalian NPs have a very similar structure, sharing a 17 amino acid ring of which 11 amino acids are conserved; the structural differences determine their specific receptor affinities (Potter *et al*. 2006; Figure 4; Table 1). The dissociation constants for each of these ligands binding to NPR-A, -B, and -C were originally published by Bennett *et al*., (1991; Table 1). The ligand selectivity of NPR-A is ANP≥BNP≫CNP, whereas for NPR-B it is CNP≫ANP≥BNP; and finally for NPR-C: ANP≥CNP>BNP (Bennett *et al*. 1991; Koller *et al*. 1991; Suga *et al*. 1992; Table 1). In sum, ANP and BNP are the predominant ligands for NPR-A; CNP is the principal ligand for NPR-B, and NPR-C binds ANP, BNP and CNP almost equally with a slight preference toward ANP.

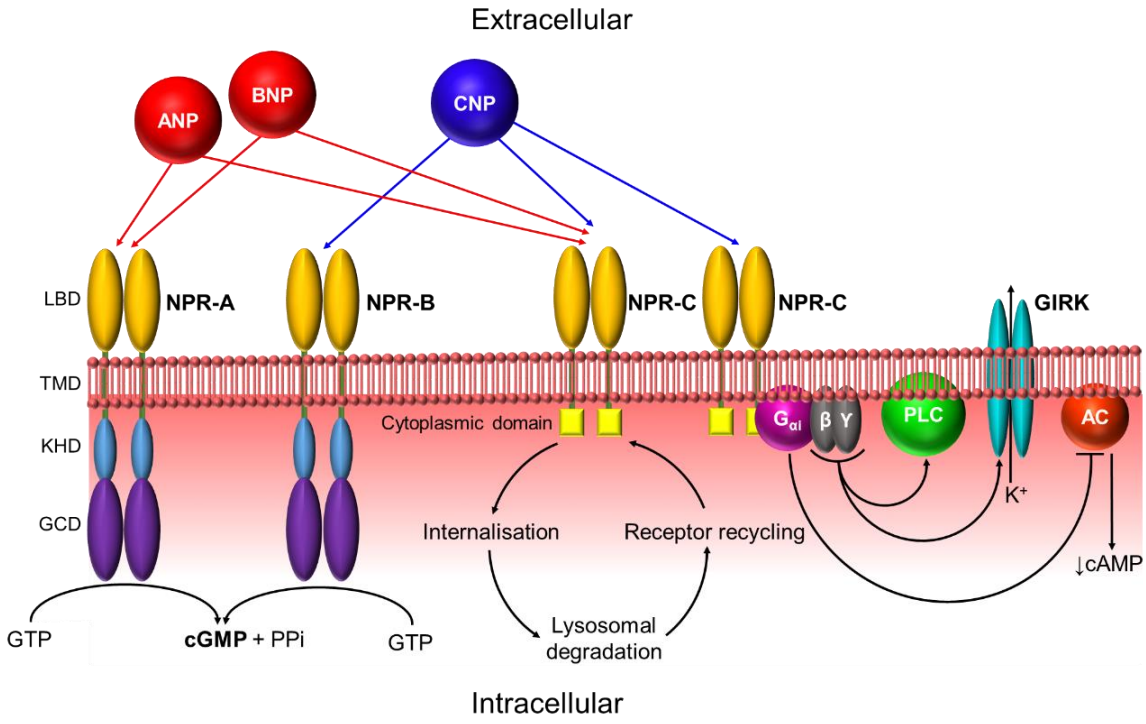

Figure 3. An illustration of natriuretic peptide cellular signalling. Atrial natriuretic peptide (ANP), Brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), natriuretic peptide receptor-A;-B;-C (NPR-A;-B;-C), phospholipase-C (PLC), adenylyl cyclase (AC), cyclic adenosine-3',5'-monophosphate (cAMP), cyclic guanosine-3',5'-monophosphate (cGMP), guanosine-5'-triphosphate (GTP), G protein-coupled inwardly-rectifying potassium channel (GIRK), ligand binding domain (LBD), transmembrane domain (TMD), kinase homology domain (KHD), guanylyl cyclase domain (GCD).

	ANP	BNP	CNP	Summary
NPR-A	1.9 pM	7.3 pM	>500 nM	ANP≥BNP≫CNP
NPR-B	5.4 nM	30 nM	13 pM	CNP≫ANP≥BNP
NPR-C	2.6 pM	13 pM	10.8 pM	ANP≥CNP>BNP

Table 1. A table describing the affinity of each natriuretic peptide for their respective receptors.

Dissociation constants (K_d) of natriuretic peptides for each natriuretic peptide receptor (Bennett *et al*., 1991) and summary of preferential ligand binding (Koller *et al*., 1991; Suga *et al*., 1992a).

1.3.4.1 Atrial Natriuretic Peptide (ANP)

Atrial natriuretic peptide was first identified from atrial myocardial extracts in 1981 by de Bold and colleagues (de Bold *et al*., 1981). Although, a clue as to the physiological effects of ANP was alluded to before the actual discovery; balloon distension of canine atria correlated with urine production (Henry *et al*., 1956). Later, de Bold and colleagues showed that ANP released from atrial myocardial extracts reduced BP through natriuresis and diuresis (Flynn *et al*., 1983). We now know that ANP is first synthesised as a preprohormone, preproANP (151 amino acid peptide) that is cleaved to form proANP (126 amino acid peptide) the predominant form of the peptide, synthesised and stored in atrial cardiomyocyte granules (Hosoda *et al*., 1991; Oikawa *et al*., 1984). The transmembrane cardiac serine protease, corin, is highly expressed on the extracellular surface of atrial cardiomyocytes, and responsible for the conversion of proANP to its physiologically active form ANP (28 amino acid peptide; Yan *et al*. 2000; Figure 4). The physiological relevance of corin was established by generation of the corin deficient mouse that has no detectable endogenous ANP and therefore develops a phenotype dependent on ANP-deficiency; hypertension (enhanced with dietary salt) and increased cardiac hypertrophy compared to WT littermates (Chan *et al*., 2005). Of note, proANP in the kidney can be processed into a longer, related protein, urodilatin (32 amino

acid peptide), and acts as a local paracrine signal increasing natriuresis and diuresis (Forssmann, 2001; Saxenhofer *et al*., 1990). Atrial natriuretic peptide is an integral regulator of cardiovascular homeostasis through a number of different effects. The most obvious of these is in response to increased intravascular volume (hypervolaemia), which stimulates the release of proANP from atrial cardiomyocytes due to wall stretch; ANP is secreted into the circulation, reducing intravascular volume by acting directly on the kidneys, increasing natriuresis and diuresis (de Bold *et al*., 1981). Natriuretic peptide receptor-A is expressed in many tissues including brain, lung, kidney, adrenal, adipose, heart, vascular endothelium and smooth muscle (Goy *et al*., 2001; Lowe *et al*., 1989; Nagase *et al*., 1997; Sabrane *et al*., 2005; Wilcox *et al*., 1991). ANP is active throughout the body, promoting natriuresis and diuresis through a number of different mechanisms. ANP inhibits the release of antidiuretic hormone (ADH; vasopressin) from the hypothalamus, increasing the glomerular filtration rate (GFR) and renal plasma flow (RPF) in the kidney (Samson *et al*., 1987). ANP also modulates the renin-angiotensin aldosterone system (RAAS), specifically by lowering renin and aldosterone secretion from the kidneys and adrenal gland, respectively, thereby reducing production of angiotensin-II (Ang-II; a potent vasoconstrictor) and attenuating sodium re-uptake and water reabsorption (Bianchi *et al*., 1986; Burnett *et al*., 1984; Cogan *et al*., 1986; Fried *et al*., 1986; Kurtz *et al*., 1986; Shi *et al*., 2001, 2003). However, the powerful cardiovascular effects of ANP go beyond this single mechanism (intravascular volume); ANP is a potent vasodilator (Currie *et al*., 1983), inhibitor of: VSMC proliferation (Sharma *et al*., 2002), endothelial permeability (Sabrane *et al*., 2005) and cardiac hypertrophy independent of BP (Knowles *et al*., 2001). Finally, in humans, depression of ANP expression as a consequence of single nucleotide polymorphisms in the *NPPA* gene is associated with the development of hypertension (P. Arora *et al*., 2013; Newton-Cheh *et al*., 2009). Anaritide (Carperitide), a synthetic version of ANP, is prescribed in Japan for the treatment of acute decompensated heart failure (ADHF) and is effective in lowering BP (Morita *et al*., 2012).

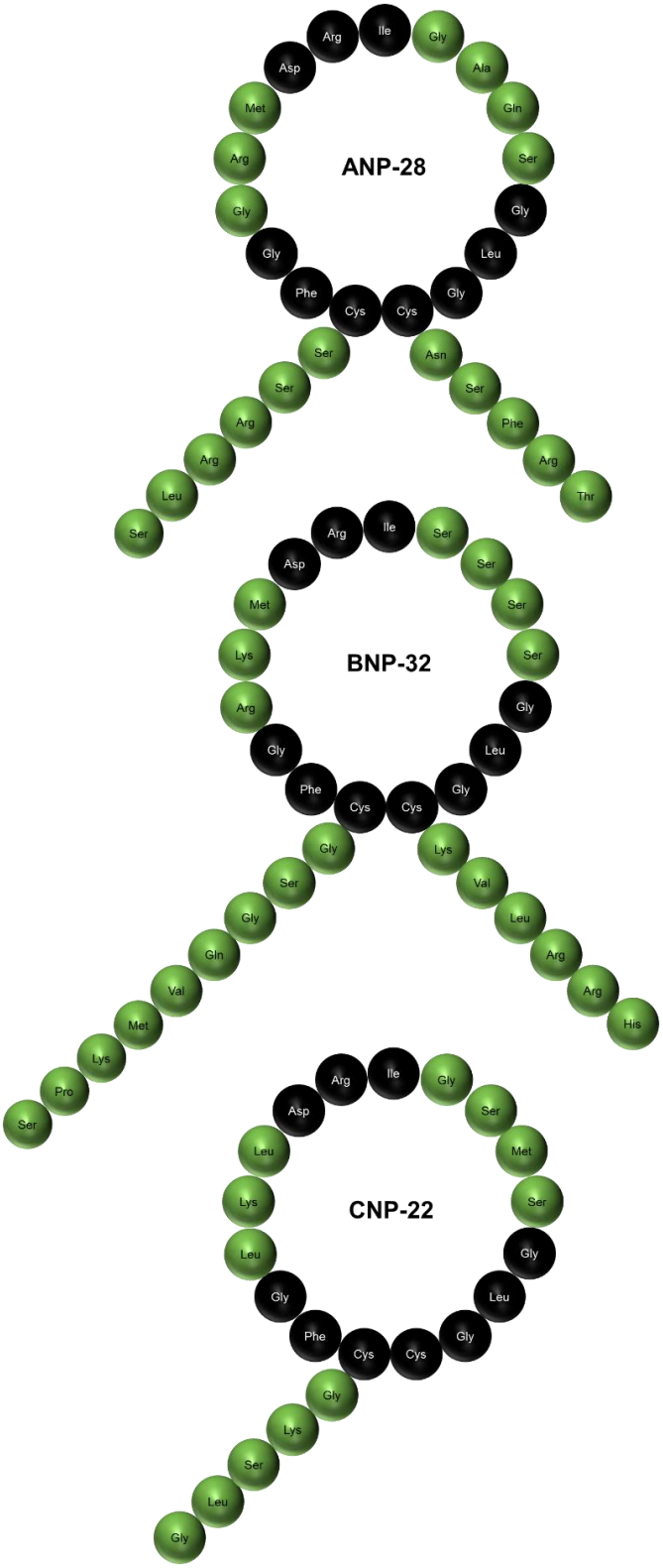

Figure 4. An illustration of the structure of ANP, BNP and CNP.

Atrial natriuretic peptide (ANP), Brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP). Black amino acids are conserved across all three peptides.

1.3.4.2 Brain Natriuretic peptide (BNP)

Brain natriuretic peptide was originally named due to its initial discovery in porcine CNS, however it is now known to be an important cardiac hormone, and the major source of BNP is ventricular cardiomyocytes (Hosoda *et al*., 1991; Mukoyama *et al*., 1991, 1990; Sudoh *et al*., 1988). Mirroring the bioactivation of ANP, preproBNP (134 amino acid peptide) is converted to proBNP (108 amino acid peptide), which is then cleaved to BNP (32 amino acid peptide; Figure 4) by corin (Ichiki *et al*., 2011). Although secreted by ventricular cardiomyocytes, unlike ANP, BNP is not stored in granules, instead BNP gene transcription increases rapidly "on demand," in response to stimuli such as cardiac wall stretch (Grépin *et al*., 1994; Nakagawa *et al*., 1995; Thuerauf *et al*., 1994). Both ANP and BNP are elevated in patients who suffer from cardiovascular diseases; indeed, BNP (and its N-terminal fragment, NT-proBNP) is used as a biomarker, and increased plasma concentrations of this peptide correlates with poor prognosis (Burnett *et al*., 1986; Mark Richards *et al*., 2004). BNP plasma concentrations increase substantially more than ANP, due to differences in their plasma half-life (Mukoyama *et al*., 1991). The half-life of BNP (~20 min; Richards *et al*. 1993) is much longer than ANP (~2 min; Yandle *et al*. 1986; Nakao *et al*. 1986). This is underpinned by differences in degradation and clearance between these two NPs; ANP has a higher affinity for NPR-C and is cleared quicker than BNP (Matsukawa *et al*., 1999) and although both are broken down by neutral endopeptidase 24.11 (neprilysin; NEP), BNP requires prior cleavage by meprin A (a metalloprotease present in the kidney), slowing degradation as a whole (Pankow *et al*., 2007). Both NPs generate cGMP by activating NPR-A but interestingly they have very different biological effects. Mice that have the *NPPA* gene disrupted develop hypertension and cardiac hypertrophy (Melo *et al*., 1999; Newton-Cheh *et al*., 2009; Steinhelper *et al*., 1990); however, disruption of the BNP gene (*NPPB*), does not result in hypertension or left ventricular hypertrophy but rather a significant increase in cardiac fibrosis (Tamura *et al*., 2000) that is due in part to the ability of BNP to promote the degradation of collagen in cardiac fibroblasts (Kapoun *et al*., 2004; Tsuruda *et al*., 2002). Although, *NPPB* over expression in mice reduces blood pressure, however this may be due to cross-activation of the NPR-B receptor

instead of NPR-A from enhanced (10-100 fold) plasma levels of BNP compared to WT littermates (Ogawa *et al*., 1994), this also leads to significant skeletal overgrowth characteristic of CNP/NPR-B signalling (Suda *et al*., 1998; Yasoda *et al*., 1998). However, the cardiovascular effects of BNP being limited to protection against cardiac fibrosis are likely to be underestimated. There is strong evidence supporting a more prominent role of BNP in cardiovascular homeostasis; in humans, mutations in the *NPPB* gene are associated with the development of cardiovascular disease including hypertension (Newton-Cheh *et al*., 2009). Furthermore, nesiritide, a synthetic version of BNP, that is prescribed for the treatment of ADHF, produces significant reductions in blood pressure both in the systemic and pulmonary circulation (Colucci *et al*., 2000; Morita *et al*., 2012). Overall, the evidence demonstrates differential cardiovascular effects of NPR-A activation by BNP compared to ANP; this difference may reflect temporal variations in the production of the two NP ligands depending on the disease.

1.3.4.3 C-type Natriuretic Peptide (CNP)

C-type natriuretic peptide is the third member of the natriuretic peptide family but in contrast to its kin, ANP and BNP, CNP is not a cardiac-derived peptide but rather a prominent EC-derived vascular autocrine/paracrine signalling peptide. First isolated from porcine brain (Sudoh *et al*. 1990), now known to be expressed in the kidney and gastrointestinal tract (Komatsu *et al*., 1991), VSMCs (Casco *et al*., 2002; Kelsall *et al*., 2006; Mendonca *et al*., 2006; Naruko *et al*., 2005, 1996; Suga *et al*., 1998; Woodard *et al*., 2002), fibroblasts (Chrisman and Garbers, 1999; Horio *et al*., 2003), cardiomyocytes (Del Ry *et al*., 2011), brain and CNS (Totsune *et al*., 1994), macrophages (Casco *et al*., 2002), chondrocytes (Hagiwara *et al*., 1994), and ECs (Stingo *et al*., 1992; Suga *et al*., 1998). In contrast to ANP, but similar to BNP, CNP is synthesised on demand. Indeed, under basal conditions CNP levels are low but increase substantially when exposed to pro-inflammatory stimuli such as tumour necrosis factor (TNF; Suga *et al*. 1993) and IL-1β (Suga *et al*. 1993), LPS (Suga *et al*. 1993), and transforming growth factor-β (TGF-β; Suga, Nakao, Itoh, *et al*.

1992), basic fibroblast growth factor (bFGF; Woodard *et al*. 2002), or shear stress (Chun *et al*., 1997). The dogma governing CNP production is similar to the other NPs; CNP is first translated into preproCNP (126 amino acid peptide), which is cleaved to proCNP (103 amino acid peptide), then converted to CNP-53 (53 amino acid peptide) by furin (a ubiquitous proprotein convertase; Wu *et al*. 2004; Thomas 2002). This peptide is further processed by an unknown mechanism, to yield the predominant biologically active form of CNP: CNP-22 (22 amino acid peptide; CNP; Stingo *et al*. 1992; Figure 4).

The cGMP-dependent biological effects of CNP are mediated via activation of NPR-B (Chrisman *et al*., 1993; Drewett *et al*., 1995). NPR-B consists of the same structural domains as NPR-A, possessing an intracellular guanylyl cyclase capable of generating cGMP but differs slightly in its extracellular ligand binding domain eliciting an altered preferred substrate-binding profile (Figure 3; Table 1). Natriuretic peptide receptor-B has a similar tissue expression profile as its ligand. This receptor is expressed in many tissues including bone (Yasoda *et al*., 1998), brain (Herman *et al*., 1996; Langub *et al*., 1995), fibroblasts (Chrisman and Garbers, 1999), kidney (Yoshimoto *et al*., 1996), heart (Del Ry *et al*., 2011; Dickey *et al*., 2007), lung (Bryan *et al*., 2006; Nagase *et al*., 1997), ECs (Del Ry *et al*., 2011) and VSMCs (Abbey and Potter, 2002; Casco *et al*., 2002).

CNP/NPR-B signalling has a number of roles within the cardiovascular system. Firstly, like ANP, CNP is a potent vasodilator of conduit vessels; the ability of the peptide to cause vasodilation via NPR-B has been well characterised using pre-contracted vessels in the absence and presence of the NPR-A/B antagonist HS-142-1, suggesting a prominent role in regulating vascular tone (Drewett *et al*., 1995; Madhani *et al*., 2003; Wennberg *et al*., 1999; Wiley and Davenport, 2001). Further, and similar to NPR-A activation, CNP/NPR-B signalling inhibits VSMC proliferation through the production of cGMP (Doi *et al*., 2001; Furuya *et al*., 1995, 1991; Hutchinson *et al*., 1997). Moreover, CNP/NPR-B signalling is essential for vascular homeostasis by sustaining the integrity of the endothelium; CNP promotes EC proliferation even after injury, thereby preventing the underlying SMC from being exposed to pro-mitogenic factors,

present in the circulation, which may result in pathological VSMC proliferation (Ohno *et al*., 2002; Yamahara *et al*., 2003). From this data one might expect to find clear effects of CNP/NPR-B signalling in reducing BP *in vivo*, however, this data is currently controversial. One of the most definitive methods to investigate the physiological effect(s) of CNP/NPR-B signalling is through the generation of transgenic animals. Both CNP (Komatsu *et al*., 2002) and NPR-B KO (Tamura *et al*., 2004) mice have been generated, and uncovered a striking biological role for CNP/NPR-B signalling; these animals were significantly shorter than WT littermates because of severe bone growth retardation. In both types of transgenic mice, this is due to blunting of endochondral ossification resulting in a reduction in longitudinal vertebra and limb-bone growth, dependent on cGMP generation triggered by CNP/NPR-B activation. The importance of CNP/NPR-B signalling in bone growth is mirrored in humans; loss of function mutations of NPR-B cause a specific type of dwarfism called acromesomelic dysplasia, type Maroteaux (AMDM; Bartels *et al*. 2004). In addition, members of the general population with a single defective allele of NPR-B are on average shorter than others (Olney *et al*., 2006). Surprisingly however, in these studies both CNP and NPR-B KO mice do not have significantly higher BP compared to WT littermates even when given high-salt diet (Komatsu *et al*., 2002; Tamura *et al*., 2004). Indeed, no difference in the cardiovascular system were reported. Although, the characteristic bone growth retardation observed in NPR-B and CNP KO mice may have impeded investigations into the cardiovascular system (Chusho *et al*., 2001; Komatsu *et al*., 2002; Tamura *et al*., 2004).

To overcome this caveat and better investigate the cardiovascular effects of CNP/NPR-B signalling Langenickel *et al*. (2006) generated a transgenic rat expressing a dominant negative NPR-B resulting in “knockdown” of the receptor leading to significantly blunted cGMP generating capacity dependent on NPR-B activation. These rats only develop modest skeletal abnormalities and so can be used to investigate cardiovascular parameters. Again, these NPR-B ‘deficient’ animals did not develop significantly altered haemodynamic capacity measured using telemetry in conscious animals. The rats also exhibited no changes in diuresis or natriuresis, a prominent characteristic of NPR-A activation. However, the animals did develop significant cardiac hypertrophy which was exacerbated

with chronic volume overload, independent of BP, suggesting an important role of NPR-B in cardiac remodelling. This finding has been substantiated by other groups. Using a model of congestive heart failure in mice (trans-aortic banding), Dickey *et al.* (2007) found CNP/NPR-B activity was twice as high compared to ANP/NPR-A activity in failing hearts, and Wang *et al*., (2007), using CNP over-expression in mice, significantly reduced left ventricular hypertrophy developed following ischemia/reperfusion (I/R) injury. Such observations provide further evidence for an essential role of CNP/NPR-B signalling in protection against cardiac hypertrophy. However, despite experimental evidence *in vivo* demonstrating the inability of CNP/NPR-B signalling to reduce blood pressure, one study involving dosing healthy human volunteers with synthetic CNP caused irrefutable reductions in BP, mirrored by significant increases in plasma cGMP concentration, urinary excretion, diuresis and natriuresis, and a reduction in aldosterone secretion (Igaki *et al*., 1998). This effect is also observed in healthy rats, dogs and primates, again, with concomitant increases in cGMP (Aizawa *et al*., 2008; Clavell *et al*., 1993). In the context of cardiovascular disease plasma CNP levels are increased in both heart failure patients (Kalra *et al*., 2003; Tarazón *et al*., 2014; Wright *et al*., 2004) and patients with pulmonary hypertension (Kaiser *et al*., 2015).

In sum, CNP/NPR-B signalling plays an important role in conduit vessel relaxation, inhibiting SMC proliferation, promoting the re-endothelialisation of damaged vessels, protection against cardiac hypertrophy and reducing BP *in vivo*. However, although it is clear CNP infusions significantly reduce blood pressure, deletion of NPR-B does not cause a significant increase in BP, which suggests CNP may be acting through an additional mechanism to regulate blood pressure, possibly within the resistance vasculature and not conduit vessels. This hypothesis is reinforced by HS-142-1, having almost no effect on CNP induced relaxations in isolated rat mesenteric arteries (Chauhan *et al*., 2003).

1.3.4.4 Natriuretic Peptide Receptor-C (NPR-C)

A third member of the natriuretic peptide receptor family, NPR-C, binds all three NPs with similar affinities (Table 1). NPR-C also plays a key role in the regulation of cardiovascular homeostasis but is not a guanylyl cyclase-coupled protein. NPR-C has a similar structure to NPR-A and NPR-B, in that it has a extracellular ligand binding domain that binds all NPs, followed by a transmembrane domain, but it lacks a C-terminal guanylyl cyclase domain and only contains a 37 amino acid long intracellular portion (Fuller *et al*., 1988; Porter *et al*., 1990). NPR-C is expressed throughout the body including the heart, kidney, brain, fibroblasts, adrenal gland, VSMCs, ECs, adipose, lung and platelets (Anand-Srivastava, 2005; Anand-Srivastava *et al*., 1990; Casco *et al*., 2002; Fujishige *et al*., 1998; Fuller *et al*., 1988; Huntley *et al*., 2006; Jeffery *et al*., 2005; Nagase *et al*., 1997; Porter *et al*., 1990; Sun *et al*., 2001; Tsuruda *et al*., 2002; Xing *et al*., 2012). NPR-C was originally thought of as only a clearance receptor internalising and degrading all three NPs with relatively uniform binding affinities. When a NP binds to NPR-C, the peptide is removed from the extracellular space by peptide/receptor internalisation, followed by degradation of the peptide by lysozymes, after which the receptor is recycled back to the membrane (a phenomenon not found to occur with NPR-A and -B receptors; Cohen *et al*., 1996; Fan, 2005; Nussenzveig *et al*., 1990)

The importance of NPR-C as a clearance receptor was made apparent by the generation of NPR-C KO mice which develop significantly elongated spine and limbs, hypotension (in males), and an inability to concentrate urine (Matsukawa *et al*., 1999). The skeletal effects are thought to be due to excessive local CNP concentrations in the vicinity of bone resulting from a deficient clearance mechanism. This hypothesis is supported by the reverse phenotype (dwarfism) observed in both CNP and NPR-B KO mice where endochondral ossification is impaired from reduced CNP/NPR-B signalling (Komatsu *et al*., 2002; Matsukawa *et al*., 1999). Natriuretic peptide receptor-C KO mice generate significantly higher urinary cGMP concentrations suggesting enhanced NPR-A and/or NPR-B activation, and decreased ANP clearance (BNP and CNP were

not reported). However, plasma levels of ANP and BNP (CNP plasma levels were not reported) were not significantly elevated refuting the rationale that reducing the expression of NPR-C leads to significantly higher levels of NPs, in the circulation at least. This led the authors to reasonably infer that NP signalling is enhanced at the local level (D'Souza *et al*., 2004). NPR-C KO mice have a significantly elevated haematocrit, compared to their age matched WT littermates, suggesting NPR-C KO mice have lower intravascular volume. Moreover, NPR-C KO mice have significantly elevated urinary output. Therefore, the absence or reduction in NPR-C, in the kidney specifically, leads to a blunted clearance of ANP (a potent diuretic), increased availability, activation of NPR-A, causing an enhanced GFR, and increased urinary cGMP and urinary output. This hypothesis was supported by water loading NPR-C KO mice which demonstrated an impaired ability to concentrate urine compared to WT littermates, and with the pharmacological use of $cANF^{4-23}$ (a ring-deleted, truncated ANP which acts as an NPR-C partial agonist; Maack *et al*., 1987)

The notion that NPR-C can additionally behave as a direct signalling pathway was first eluded to by Anand-Srivastava & Cantin (1986) who treated cultured rat neonatal atrial and ventricular myocytes with ANP and found it to inhibit, concentration-dependently, inherent adenylyl cyclase activity (lowering cAMP). In addition, adenylyl cyclase activators (e.g. forskolin and isoproterenol) were also inhibited by ANP. This same group determined the mechanism behind this phenomenon by utilising *Pertussis toxin* (PT), a known antagonist of inhibitory guanine nucleotide regulatory proteins ($G_{i/o}$), which abolished the inhibitory effects of ANP on cAMP generation in rat aorta (Anand-Srivastava and Cantin, 1986). The effects of ANP were mimicked using $cANF^{4-23}$ in rat VSMCs, showing specifically that NPR-C agonism leads to decreases in intracellular cAMP without changing intracellular cGMP, ruling out inhibitory effects of cGMP production (Anand-Srivastava *et al*., 1990). Building on this work, using an antibody raised against the intracellular 37 amino acid cytoplasmic domain of NPR-C, the authors were able to block the action of $G_{i/o}$ (Anand-Srivastava *et al*. 1996).

In addition to cANF$^{4-23}$, CNP itself has been found to activate NPR-C signalling. Villar *et al*. (2007), using the NPR-C antagonist M372049, demonstrated that in the resistance vasculature (rat mesentery) vessel relaxations elicited by CNP were dependent on NPR-C activation. In addition, relaxations by ANP and the NO donor Spermine NONOate (Sp-NO) were not affected confirming two independent relaxant mechanisms. This characteristic was specific to the resistance vasculature as M372049 had no effect on CNP, ANP and Sp-NO – induced relaxations in rat aorta. Interestingly, M372049 also blocked the vasoactivity of endothelium-derived hyperpolarising factor (EDHF). Endothelium-derived hyperpolarising factor, as the name suggests, is a mediator(s) released from the endothelium which acts on VSMCs causing vessel relaxation via cell hyperpolarisation (Chen *et al*., 1988). EDHF is the third endothelium-derived factor to be identified after NO and prostacyclin (PGI_2); EDHF has been found to be the predominant mechanism underpinning vessel relaxation in the resistance vasculature (Shimokawa *et al*., 1996). EDHF causes hyperpolarisation by one of two mechanisms, or both; by activating G protein-coupled inwardly rectifying K^+ channels (GIRK) or Na^+/K^+ ATPase (Chauhan *et al*., 2003). Chauhan *et al*., (2003) & Villar *et al*. (2007) found CNP/NPR-C-dependent relaxations of mesenteric resistance arteries were inhibited by barium (Ba^{2+}), a GIRK inhibitor, and M372049, illustrating the importance of endothelial-derived CNP in resistance vessel relaxation, and that CNP acts as an EDHF through NPR-C signalling. Furthermore, Hobbs *et al*. (2004) demonstrated the effectiveness of targeting CNP/NPR-C signalling in the protection against cardiovascular disease; specifically, CNP and cANF$^{4-23}$ acting through NPR-C significantly reduced infarct size and coronary perfusion pressure following I/R injury.

Substantiation of the cardiovascular importance of this novel vascular mechanism (i.e. CNP/NPR-C signalling) came in 2014 with a publication by Moyes *et al*., (2014). The authors generated an endothelial cell specific CNP KO mouse (ecCNP KO), and along with global NPR-C KO mice demonstrated a key function of this pathway in protecting against the development of endothelial dysfunction and hypertension (females only), atherogenesis, and aneurysm (males only). CNP/NPR-C signalling inhibits VSMC proliferation and promotes

the proliferation of ECs explaining, at least in part, why ecCNP KO mice develop atherosclerosis (Khambata *et al*., 2011). Overall, these data suggest that the preferential female cardiovascular protective effect of EDHF described by Scotland *et al*., (2005), is predominantly derived from the release of CNP from the endothelium, to protect against cardiovascular pathogenesis.

1.3.4.5 Proteolysis of natriuretic peptides (NPs)

In addition to clearance by NPR-C, NPs undergo proteolytic degradation. There are several different proteases that are known to degrade NPs. One of those is neutral endopeptidase-24.11 (NEP; neprilysin; EC 3.4.24.11; enkephalinase; common acute lymphoblastic leukemia antigen; CD10), a type-II integral membrane metallopeptidase (Turner and Tanzawa, 1997). NEP is a membrane-bound zinc-dependent enzyme, which degrades all three NPs but has a preference for ANP over BNP and CNP (Dussaule *et al*., 1993; Kenny *et al*., 1993; Okolicany *et al*., 1992; Watanabe *et al*., 1997). NEP inactivates all three NPs in a similar way; several different peptide bonds are hydrolysed across the ring structure causing the shape to open up, thus inactivating it (Potter, 2015). NEP is expressed throughout the body including kidney, lung, ECs, VSMCs, cardiomyocytes, fibroblasts, neutrophils, brain, and adipose tissue (Erdös and Skidgel, 1989; Graf *et al*., 1995; Kerr and Kenny, 1974a, 1974b; Malroy *et al*., 1978; Shima *et al*., 1988; Standeven *et al*., 2011). This protease does not just hydrolyse NPs but instead has a broad substrate profile, consisting of both vasodilators and vasoconstrictors including angiotensin I, II and III (Ang-I, II, III), endothelin-1 (ET-1), bradykinin (BK), substance P, as well as ANP, BNP and CNP (Emoto and Yanagisawa, 1995; Stephenson and Kenny, 1987; Vijayaraghavan *et al*., 1990). NEP inhibition has been a focus of attention in terms of a novel therapy for cardiovascular disease for many years. NEP inhibition increases circulating ANP concentrations to levels that should be of clinical benefit (Bevan *et al*., 1992; Okolicany *et al*., 1992; Olins *et al*., 1989); however, clinical trials using the NEP inhibitor candoxatril revealed that NEP inhibition does not lower BP, presumably due to simultaneous increases in vasoconstrictors (Bevan *et al*., 1992; Richards *et al*., 1993). To circumvent this

caveat combining NEP inhibition with inhibitors of certain vasoconstrictors (e.g. with an angiotensin receptor blockers in the molecule LCZ696) provides greater efficacy (Mangiafico *et al*., 2013; Vardeny *et al*., 2014). Although NEP inhibition significantly increases plasma ANP concentrations, mice deficient in NEP do not develop skeletal deformities like NPR-C KO mice suggesting NEP dependent degradation of CNP is not as crucial compared to NPR-C dependent clearance (Lu *et al*., 1995).

There are other proteolytic enzymes that degrade NPs such as insulin-degrading enzyme (IDE) and exopeptidase dipeptidyl-peptidase IV (DPP IV/CD26; EC 3.4.14.5). IDE is a zinc metalloprotease found in both cytoplasmic and membrane fractions, and hydrolyses all three NPs with a preference for ANP, suggesting another potential target for increasing circulating NPs for therapeutic gain (Müller *et al*., 1992; Potter, 2015; Volpe, 2014). DPP IV is a cell-surface protease, also present in the plasma in soluble form, is found expressed throughout the cardiovascular system including ECs *(Silva Júnior et al*., 2015). DPP IV has a broad substrate profile but only degrades BNP out of the NPs (Brandt, 2006; Volpe *et al*., 2014),

1.4 Cyclic GMP degradation

1.4.1 Phosphodiesterases (PDEs)

Phosphodiesterases (PDEs) are a superfamily of metallophosphohydrolases that selectively hydrolyse the 3',5'-cyclic phosphate moiety of both cAMP and cGMP generating the inactive forms of these cyclic nucleotides, 5'-AMP and 5'-GMP (Butcher and Sutherland, 1962; Figure 1). PDEs are either membrane-bound or cytosolic enzymes composed of 16 α-helices containing 3 subdomains that define where substrates bind (Conti and Beavo, 2007; Jin *et al*., 1992). In total there are 11 families of PDEs; however, splice variants exist within each family bringing the number of known PDEs to >100 (Bender and Beavo, 2006). The first report of PDE activity was noted in 1961, almost at the same time as

the discovery of cyclic nucleotides themselves, and established by the identification of PDE inhibitors caffeine and theophylline, forms of methylxanthine (Butcher and Sutherland, 1962; Smith *et al*., 1961, 1958). The 11 PDE families can be organised by their substrate specificity; cGMP specific PDEs (PDE5, 6 and 9), cAMP specific PDEs (PDE4, 7 and 8) and dual substrate specificity PDEs (PDE1, 2, 3, 10 and 11; Bender & Beavo 2006; Table 2). This specificity is based on their affinity for each substrate (cGMP and cAMP), and cellular localisation; these characteristics are very important in tightly regulating the local concentration of cAMP and cGMP within the cell. Moreover, compartmentalisation of cyclic nucleotide signalling is crucial in determining the spatial and temporal generation of cGMP (and cAMP) *in vivo* and thus specific physiological effects (K. Arora *et al*., 2013). This phenomenon was first identified 40 years ago in isolated perfused hearts where different contractile responses were observed using two different agonists; prostaglandin E_1 (PGE_1; a prostanoid receptor agonist) and isoproterenol (a β-adrenergic receptor agonist). Both increased intracellular cAMP and activated the same down-stream signalling molecule (i.e. PKA), but isoproterenol activated membrane-bound fractions, compared to PGE_1 that activated cytosolic fractions, explaining the functional differences (Brunton *et al*., 1979; Corbin *et al*., 1977). Ten years later, other studies using PDE inhibitors in guinea pig perfused hearts showed that using isoproterenol to generate intracellular cAMP, and therefore improve contraction, could then be significantly enhanced when combined with 3-isobutyl-1-methylxanthine (IBMX; a non-specific PDE inhibitor) or milrinone (a PDE3 inhibitor) without affecting the activity of known downstream signalling molecules (PKA) despite significant increases in global intracellular cAMP (Rapundalo *et al*., 1989; Weishaar *et al*., 1987). In the context of cGMP generation in ECs, concentrations of cGMP generated by sGC (cytosolic) are smaller and more brief than concentrations of cGMP generated by pGC (membrane-bound), but it is sGC stimulation that is more efficient in relaxing ECs, measured as changes of planar cell surface area, compared to pGC stimulation leading to the conclusion that sGC excitation-relaxation coupling is more efficient (Rivero-Vilches *et al*., 2003). This same cGMP signalling dichotomy (pGC v sGC) has also been observed in cardiac myocytes (Castro *et al*., 2006). Cell signalling compartmentalisation can be observed

using fluorescence resonance energy transfer (FRET) allowing the generation and degradation of intracellular cAMP and cGMP to be visualised in real-time (Honda *et al*., 2001; Ponsioen *et al*., 2004; Stangherlin and Zaccolo, 2012; Zaccolo *et al*., 2000). It is also been possible to observe cGMP signalling coupled with *in vivo* physiological responses using intravital microscopy and transgenic mice expressing FRET based markers for cGMP (Thunemann *et al*., 2014, 2013). Using these techniques scientists are now able to observe that upon generation, cGMP forms “pools” or “clouds” in specific areas of the cell, which is prevented from spreading by the action of PDEs.

There are three cGMP specific PDEs: PDE5, PDE9. PDE6 is known as a photoreceptor PDE because it is widely expressed throughout the mammalian retina and plays an important role in mediating the conversion of a light into a photoresponse (Bender and Beavo, 2006). Until recently little was known about the function of PDE9, despite having a broad tissue expression profile (Bender and Beavo, 2006). Recently the role of PDE9 has been clearly described in hypertrophic heart disease (Lee *et al*., 2015). The authors of this paper showed that PDE9 inhibition protects against pressure-overload stress in heart myocytes and muscle, and reverses pre-established heart disease by increasing cGMP levels, preferentially favouring pGC generated cGMP (since specific sGC generated cGMP had no affect; Lee *et al*., 2015). Arguably the most well-known cGMP-specific PDE is PDE5, which is expressed widely in the body (e.g. VSMC, heart, lung, brain, kidney and platelets; Giordano *et al*., 2001; Kotera *et al*., 2000; Loughney *et al*., 1998; Stacey *et al*., 1998; Yanaka *et al*., 1998). Inhibitors of this enzyme were originally designed to treat hypertension but, during clinical trials, were soon found to produce penile erection in males; these inhibitors, exemplified by sildenafil (Viagra), are now widely prescribed to treat erectile dysfunction. More recently, using vascular reactivity studies, sildenafil has been shown to significantly potentiate the cGMP-dependent vasodilatory effect of ANP in the pulmonary, but not systemic circulation (Baliga *et al*., 2008). In addition, PDE5 expression is significantly increased in the lungs of PH patients and in preclinical models; in accord, clinical evaluation has proven efficacy of sildenafil (and other PDE5 inhibitors) in patients with PH and is now licensed for this indication (Sastry *et al*., 2004; Wharton *et al*., 2005).

cGMP specific	cAMP specific	Dual specificity
PDE5	PDE4	PDE1
PDE6	PDE7	PDE2
PDE9	PDE8	PDE3
		PDE10
		PDE11

Table 2. A table describing cyclic nucleotide degradation specificity of all 11 phosphodiesterases (PDEs).

1.4.1.1 Dual specificity PDEs (PDE2 and PDE3)

PDE2 is expressed widely in mammals including the brain, heart, platelets, ECs, adrenal glomerulosa cells (Bender and Beavo, 2006). Known as a dual substrate enzyme and present in both the membrane and cytosol, PDE2 not only degrades the cAMP and cGMP signal but also acts as an effector protein because the binding of cGMP to an allosteric site stimulates the activity of the enzyme. Through this phenomenon this enzyme mediates both “cross-talk” between the two signalling pathways (cGMP and cAMP), and provides a negative feedback mechanism. Activation of the allosteric site increases the rate at which PDE2 hydrolysis either cAMP or cGMP and therefore its effect is dependent on the local concentration of either cyclic nucleotide at the time. Moreover, in areas of high cGMP concentration, PDE2 will be stimulated and act has a negative feedback mechanism; however, in areas of high concentrations of cAMP, PDE2 can be activated by low concentrations of cGMP (the allosteric site has a ~10-fold higher affinity for cGMP compared to cAMP) to hydrolyse cAMP. An example of this “cross-talk” is from the addition of ANP to adrenal glomerulosa cells which increases the breakdown of cAMP by PDE2 to inhibit aldosterone production (MacFarland *et al*., 1991). PDE2 is also important in the heart as a negative feedback mechanism; PDE5 is a cGMP specific PDE and controls cGMP levels generated in the cytosol of cardiomyocytes, whereas membrane produced cGMP is degraded by PDE2, this is another example of

cGMP signalling compartmentalisation (Fischmeister *et al*., 2006). These phenomena have been demonstrated functionally in a rat disease model of PH, where PDE2 inhibition (BAY 60-7550) potentiates the therapeutic benefit of NPs, NO and the PGI_2 analogue treprostinil, suggesting a new therapeutic target for the treatment of this disease (Bubb *et al*., 2014).

PDE3 is also a membrane-bound and cytosolic, dual substrate, enzyme hydrolysing both cAMP and cGMP (Bender and Beavo, 2006). Unlike PDE2, which is activated by cGMP, PDE3 is inhibited by cGMP and thus another cGMP effector protein (Bender and Beavo, 2006). Although, PDE3 binds to cAMP and cGMP with similar affinities, the concentration at which PDE3 is saturated (V_{max}) when hydrolysing cAMP is 10-fold higher than it is for cGMP, therefore at higher concentrations of cGMP, cAMP degradation is lower (Bender and Beavo, 2006). PDE3 is expressed in platelets, VSMCs, cardiac myocytes and adipose tissue (Shakur *et al*., 2001). The PDE3 inhibitors milrinone, cilostamide, cilostazol, and trequinsin, attenuate platelet aggregation, VSMC proliferation and vasodilation (Maurice and Haslam, 1990; Shakur *et al*., 2001).

1.4.2 Multidrug resistance proteins (MRPs)

In addition to the well-established degradative pathways conveyed by PDEs, efflux of cGMP (and cAMP) has been hypothesised to play a role in dynamically regulating the signalling attributed by both cyclic nucleotide second messengers. Originally, efflux as a mechanism of regulating intracellular cyclic nucleotide concentrations was not deemed important because of its small magnitude compared to degradation by PDEs (Mercapide *et al*., 1999). However, recently there has been an abundance of evidence demonstrating cyclic nucleotide efflux as an important regulator of physiological function.

Cellular efflux of cyclic nucleotides was first observed around the same time as their discovery; hormones stimulating the generation of cAMP led to concomitant increases in extracellular cAMP (Davoren and Sutherland, 1963; Sutherland and Rall, 1958). Indeed, for many years plasma and urinary cAMP

and cGMP concentrations have been used as a routine measure for this phenomenon without knowledge of the mechanisms responsible (Hamet *et al*., 1989). It was not until 1999 that the first multidrug resistance protein (MRP4) capable of transporting nucleotide analogues was identified (Schuetz *et al*., 1999). This was soon followed by identification of MRP5 which has the ability to export cGMP and cAMP (Jedlitschky *et al*., 2000). MRP5 is part of a family of MRPs which are members of the large ABC (ATP-binding cassette) superfamily of energy-dependent, transmembrane transporters, and is also known by the name ABCC5. The subfamily C of the ABC superfamily, in other words the "ABCC" proteins, is composed of 13 separate transporters (Deeley *et al*., 2006; Table 3); these comprise of 10 MRPs, two sulfonylurea receptors (SUR) and the cystic fibrosis transmembrane-conductance regulator (CFTR; Dean *et al*. 2001; Table 3). The general structure of ABC transporters consists of two membrane spanning domains (MSDs) and two intracellular nucleotide binding domains (NBDs; Deeley *et al*. 2006; Figure 5). MRP4, 5, 8 and 9 have the usual ABC transporter structure but MRP1, 2, 3, 6 and 7 have an extra transmembrane domain (three in total; Deeley *et al*. 2006). ABCC family transporters, as a whole, have a hugely diverse substrate profile; this includes chemotherapeutics which, in 1992, was how the first MRP (MRP1) was identified, because of its contribution to multidrug resistance (MDRs) in a human lung cancer cell line (Cole *et al*., 1992; Mirski *et al*., 1987). MRPs have specific properties; they are unidirectional (transport from intracellular to extracellular) that can operate against substantial concentration gradients, ATP-dependent, inhibited by compounds such as probenecid (an organic anion transporter protein inhibitor; OAT) and prostaglandins, and have saturable kinetics (Campbell and Taylor, 1981; Doore *et al*., 1975; Finnegan *et al*., 2011; Rindler *et al*., 1978). Multidrug resistance proteins vary in their ability to export individual substrates but as a family they transport, either on their own or as glucuronide (GSH) or glutathione disulphide (GSSG) conjugates, a huge array of substrates which makes them crucial in the protection against accumulation of cytotoxic xenobiotics (Cole and Deeley, 1998; Deeley *et al*., 2006; Slot *et al*., 2011). MRPs are known to export arachidonic acid derivatives (e.g. PGE_2, PGE_1, leukotriene C_4; LTC_4), steroids and conjugates (e.g. bilirubin glucuronide, glycocholic acid, oestradiol glucuronide), purine nucleotide/nucleoside analogues, pyrimidine nucleoside

analogues, nucleobase analogues, cyclic nucleotides (e.g. cAMP, cGMP), folic acid analogues (e.g. methotrexate), antibiotics, endobiotics, xenobiotics (e.g. paracetamol, morphine, adefovir), tyrosine kinase inhibitors (TKI; e.g. imatinib, nilotinib, erlotinib) and many more substrates (Borst *et al*., 2007; Deeley *et al*., 2006; Slot *et al*., 2011; Sodani *et al*., 2012).

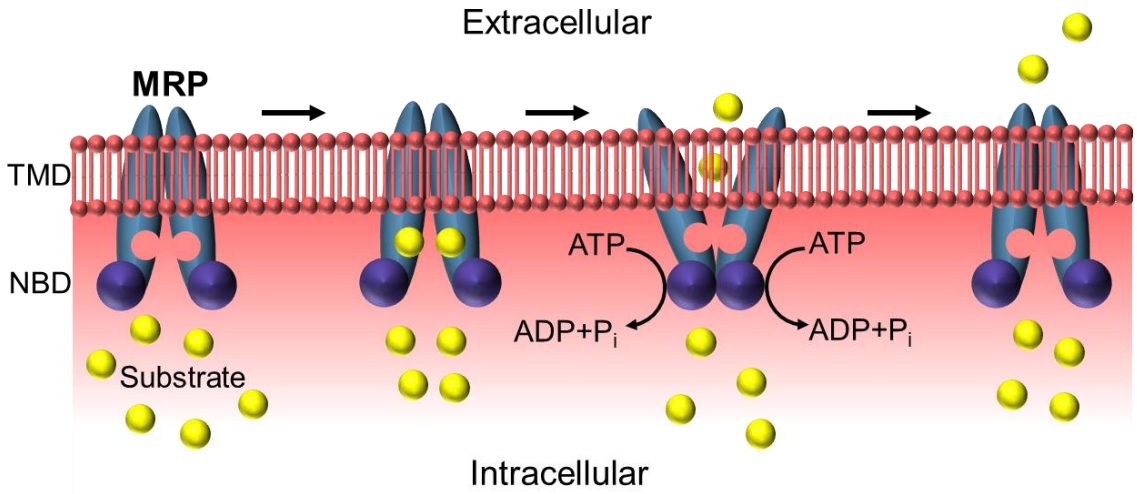

Figure 5. An illustration of multidrug resistance protein substrate efflux.

Multidrug resistance protein (MRP), adenosine-5'-triphosphate (ATP), adenosine-5'-diphosphate (ADP), inorganic phosphate (P_i), transmembrane domain (TMD), nucleotide binding domain (NBD).

Subfamily C of the ABC superfamily	Alternative name	Export cGMP (Yes/No)	Export cAMP (Yes/No)
ABCC1	MRP1	No	No
ABCC2	MRP2	No	No
ABCC3	MRP3	No	No
ABCC4	**MRP4**	**Yes[$]** K_m = 9.69 ± 2.3 µM V_{max} = 2.01 ± 0.34 pmol/mg/min	**Yes[$]** K_m = 44.5 ± 5.8 µM V_{max} = 4.14 ± 0.40 pmol/mg/min
ABCC5	**MRP5**	**Yes[#]** K_m = 2.1 ± 0.2 µM V_{max}/K_m = 2100 µl x mg^{-1} protein x min^{-1}	**Yes[#]** K_m = 379 ± 24 µM; V_{max}/K_m = 90 µl x mg^{-1} protein x min^{-1}
ABCC6	MRP6	No	No
ABCC7	CFTR	No	No
ABCC8	SUR1	No	No
ABCC9	SUR2A/2B	No	No
ABCC10	MRP7	No	No
ABCC11	**MRP8**	**Yes**	**Yes**
ABCC12	MRP9	No	No
ABCC13	MRP10	No	No
ABCG*	**BCRP***	**Yes**	**No**

Table 3. A table describing the members of the ABC superfamily that transport cyclic nucleotides.

A table describing the members of the ABC superfamily that transport cGMP and cAMP (ABCC4/MRP4, ABCC5/MRP5 and ACC11/MRP8), plus ABCG/BCRP. Sulfonylurea receptors SUR, cystic fibrosis conductance regulator (CFTR), multidrug resistance protein (MRP), breast cancer resistance protein (BCRP; Dean and Allikmets, 2001; Dean *et al*., 2001). *BCRP/ABCG is a member of the ABC superfamily but not subfamily C, rather subfamily G. [$]MRP4 K_m value expressed as mean ± SEM (Chen *et al*., 2001). [#]MRP5 K_m value expressed as mean ± standard deviation (SD; Jedlitschky *et al*. 2000).

There are three MRPs that transport both cAMP and cGMP with varying affinities: MRP 4, 5 and 8 (Slot *et al*., 2011; Table 3). The human breast cancer resistance protein (BCRP; ABCG) is also a member of the ABC superfamily but subfamily G (rather than subfamily C); its role in chemotherapy resistance is well characterised but it has only recently been identified to transport cGMP. Little is known about its cGMP-dependent function although it has recently been found expressed in the heart suggesting a possible cardiovascular role (de Wolf *et al*., 2007; Evans *et al*., 2008; Meissner, 2006).

MRP4 is ubiquitously expressed in the mammalian body, found in the adrenal glands, brain, heart, lung, kidney, erythrocytes, platelets, VSMCs and ECs (Mitani *et al*., 2003; Ritter *et al*., 2005; Sager and Ravna, 2009; Slot *et al*., 2011; Tagami *et al*., 2010). MRP4 exports both cGMP and cAMP leading to the rationale that this protein may play a role in cardiovascular homeostasis (Chen *et al*. 2001; Cheepala *et al*. 2011; Wielinga *et al*. 2003; Table 3). In 2008, Sassi *et al*. established for the first time a definitive link between cyclic nucleotide-dependent decreases in VSMC proliferation due to MRP inhibition. The authors of this study, using cultured human coronary smooth muscle cells (hCASMC), found a MRP4 specific silencing ribonucleic acid (siRNA) significantly inhibited proliferation with a concomitant increase in both cAMP and cGMP intracellular/extracellular ratios. This effect was further enhanced upon addition of the non-specific PDE inhibitor IBMX, suggesting MRPs are used as a compensatory cyclic nucleotide “degradative” mechanism in the absence of functional PDE. Interestingly, the authors found that MRP4 expression at the mRNA and protein level was significantly enhanced in proliferating hCASMCs suggesting a possible therapeutic target in pathological VSMC proliferation. Furthermore, using an *in vivo* model, rat carotid artery balloon injury, treatment with an adenoviral vector expressing short-hairpin RNA (shRNA) against rat MRP4 resulted in a significantly reduced intima thickening (i.e. VSMC proliferation). However, despite significant increases in intracellular/extracellular ratios of both cGMP and cAMP, it appeared that this was predominantly a cAMP-dependent mechanism because only inhibition of PKA, and not PKG, re-established VSMC proliferation. The authors of this study concluded that MRP4, in this context, acted as a predominantly cAMP extrusion mechanism (Sassi *et*

al., 2008). These findings led to further studies from this same group in a preclinical model of PH (Hara *et al*., 2011). The authors found that MRP4 expression was significantly increased, in both VSMCs and ECs, at the mRNA and protein level in lungs from patients with PH compared to controls. This finding was mirrored in mice exposed to chronic hypoxia (5 weeks, 10 % O_2) in which MRP4 expression was significantly increased in the pulmonary arteries compared to normoxic control mice. Furthermore, these authors used the MRP inhibitor MK571 and MRP4 KO mice to implicate this MRP isoform in the pathogenesis of PH, and as a potential therapeutic target. Mice administered MK571 or deficient in MRP4 had significantly reduced right ventricular systolic pressures (RVSP), right ventricular hypertrophy (RVH) and muscularised pulmonary vessels, all hallmarks of PH. Interestingly, in lung lysates the activity PKA and PKG were significantly increased in mice treated with MK571, suggesting MRP inhibition was eliciting its therapeutic action by significantly increasing cAMP and cGMP levels. This observation was mirrored in cultured human pulmonary artery smooth muscle cells (hPASMCs), treated with an MRP4 specific siRNA, in which the activity of PKA and PKG were also significantly increased (as shown by significantly elevated intracellular/extracellular ratios of both cAMP and cGMP). Notably, the effect of MRP inhibition was accentuated in the presence of sildenafil, suggesting that in the absence of PDE degradation, MRP activity is significantly elevated, which enhances cGMP concentrations, subsequently increasing cAMP levels, possibly via PDE3 inhibition. Indeed, genetic deletion of MRP4 leads to significantly increased heart weight, cardiomyocyte size, left ventricular wall thickness and interventricular septum with a concomitant increase in cardiac ANP gene expression (Sassi *et al*., 2012). These mice also have extended bleeding times demonstrating that MRP4 promotes platelet aggregation by decreasing intracellular cAMP and cGMP (Borgognone and Pulcinelli, 2012; Decouture *et al*., 2015). Such observations substantiate a cardiovascular homeostatic role for this protein, and cAMP/cGMP efflux.

To further investigate a possible the link between MRP4 and cGMP efflux in the vasculature, Krawutschke *et al*. (2015) used a FRET based system to visualise in real-time the generation of cGMP in primary VSMCs. In this setting, the

authors used MK571 as an “MRP4-specific” inhibitor, which enhanced the magnitude and duration of cGMP generated from increasing concentrations of CNP, ANP and the NO donor S-nitroso-L-glutathione (GS-NO). Although, the authors concluded this was a MRP4 specific phenomenon, MK571 has also been shown to inhibit MRP5 (Reid *et al*., 2003).

In sharp contrast to MRP4, little or nothing is known about the function of MRP5 in the context of cyclic nucleotide transport. MRP5 is not ubiquitously expressed and appears to have a more specific expression profile in brain, heart, fibroblasts, erythrocytes, VSMCs and ECs (Borst *et al*., 2007; Dazert *et al*., 2003; Meissner *et al*., 2007; Mitani *et al*., 2003; Sager, 2004; Slot *et al*., 2011). MRP5 exports both cAMP and cGMP making it a prime candidate, alongside MRP4, for regulating cardiovascular homeostasis; however, the actual role of MRP5 is not fully understood (Cheepala *et al*., 2011; Jedlitschky *et al*., 2000; Reid *et al*., 2003; Slot *et al*., 2011). Sassi *et al*., (2008) consider MRP5 as an endothelial specific protein because minimal expression of this MRP can be found in VSMCs. However, this is in contrast to other evidence showing significant MRP5 expression in VSMCs (Dazert *et al*., 2003; Meissner *et al*., 2007; Mitani *et al*., 2003; Xu *et al*., 2004). MRP5 is highly expressed in throughout the human heart, in cardiac ECs and VSMCs, atrial and ventricular cardiomyocytes, and the endocardium (Dazert *et al*., 2003). Interestingly, MRP5 expression is also increased in the hearts of patients suffering from ischemic (ICM) and dilated (DCM) cardiomyopathy, suggesting MRP5 may be contributing to the pathology and that inhibition of MRP5 might be an attractive target to increase beneficial intracellular cyclic nucleotide concentrations (Dazert *et al*., 2003). Notably, MRP5 KO mice do not have an overt phenotype, and this is the same for the MRP4/MRP5 double KO (Borst *et al*., 2007). This is at odds with the adverse cardiovascular phenotype in MRP4 KO mice, and more in-depth and rigorous cardiovascular investigations need to be performed to discern differences (Sassi *et al*., 2012).

Even less is known about the physiological role of MRP8; it is known to export both cAMP and cGMP, and is found in a variety of tissues including the brain, lung, liver and kidney (Bera *et al*., 2001; Slot *et al*., 2011; Tammur *et al*., 2001;

The Human Protein Atlas, 2008; Yabuuchi *et al*., 2001). However, there is no evidence describing its role in the cardiovascular system. Interestingly, a single nucleotide polymorphism exists in the human gene that codes for MRP8; this mutation is commonly found in East Asian populations and causes dry earwax because of its role in determining the type of earwax (cerumen) produced by the ceruminous apocrine glands through secretion of the aliphatic or aromatic hydrocarbon constituents, although it is not known whether cyclic nucleotides are involved in this phenomenon (Martin *et al*., 2010; Yoshiura *et al*., 2006).

1.5 Pulmonary hypertension (PH)

1.5.1 Classification

One cardiovascular pathology in which modulation of cGMP signalling has proven particularly fruitful in terms of therapeutics, and in understanding the cardio- and vaso- protective mechanisms proffered by cyclic nucleotides, is pulmonary hypertension (PH).

PH is a syndrome *per se*, manifested by a large collection of diseases that mainly affect the cardiorespiratory system. PH is defined by a single diagnostic haemodynamic feature: mean pulmonary artery pressure >25 mm Hg, at rest (PAP_m >25 mm Hg, at rest; Hoeper *et al*., 2013). Patients diagnosed with PH are categorised into a subgroup based on identifiable causes and risk factors, and similar pathological findings, hemodynamic characteristics and treatment management. Group 1: pulmonary arterial hypertension (PAH); Group 2: pulmonary hypertension due to left heart disease; Group 3: pulmonary hypertension due to lung diseases and/or hypoxia; Group 4: chronic thromboembolic pulmonary hypertension (CTEPH); Group 5: pulmonary hypertension with unclear multifactorial mechanisms (Simonneau *et al*. 2013; Figure 6). Group 1 PH or pulmonary arterial hypertension (PAH) is clinically defined by a pulmonary artery wedge pressure (PAWP) ≤15 mm Hg and elevated pulmonary vascular resistance (PVR) >3 wood units (WU), in addition

to PAP_m ≥25 mm Hg, at rest and in the absence of other known causes of PH (Hoeper *et al*. 2013; Figure 6). The Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease Management (REVEAL Registry) conducted in the United States of America (USA) includes, to date, the largest cohort of PH patients (3,515) of any such study, and found the median survival for patients diagnosed with PAH to be seven years (Benza *et al*., 2012; McGoon *et al*., 2013). Although this is a major improvement on the median survival of patients diagnosed with PAH in 1991 (2.8 year), and reflects the substantial improvement in treatment strategies. In addition, PAH demonstrates a significant sex-difference; within the REVEAL study cohort, around 80 % of patients were female (McGoon *et al*., 2013). Even with modern therapies patients suffer from a 15 % mortality rate within the first year of receiving treatment, demonstrating that PAH is still a severe unmet medical need and would benefit from novel effective therapies (Alonzo *et al*., 1991; Benza *et al*., 2012; Thenappan *et al*., 2007).

1. Pulmonary arterial hypertension (PAH)

- **1.1** Idiopathic PAH
- **1.2** Heritable PAH
  - **1.2.1** BMPR2
  - **1.2.2** ALK-1, ENG, SMAD9, CAV1, KCNK3
  - **1.2.3** Unknown
- **1.3** Drug and toxin induced
- **1.4** Associated with:
  - **1.4.1** Connective tissue disease
  - **1.4.2** HIV infection
  - **1.4.3** Portal hypertension
  - **1.4.4** Congenital heart diseases
  - **1.4.5** Schistosomiasis

1' Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis

1''. Persistent pulmonary hypertension of the newborn (PPHN)

2. Pulmonary hypertension due to left heart disease

- **2.1** Left ventricular systolic dysfunction
- **2.2** Left ventricular diastolic dysfunction
- **2.3** Valvular disease
- **2.4** Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies

3. Pulmonary hypertension due to lung diseases and/or hypoxia

- **3.1** Chronic obstructive pulmonary disease
- **3.2** Interstitial lung disease
- **3.3** Other pulmonary diseases with mixed restrictive and obstructive pattern
- **3.4** Sleep-disordered breathing
- **3.5** Alveolar hypoventilation disorders
- **3.6** Chronic exposure to high altitude
- **3.7** Developmental lung diseases

4. Chronic thromboembolic pulmonary hypertension (CTEPH)

5. Pulmonary hypertension with unclear multifactorial mechanisms

- **5.1** Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy
- **5.2** Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
- **5.3** Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
- **5.4** Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

Figure 6. Current classification of pulmonary hypertension.

Bone morphogenic protein receptor type II (BMPR); caveolin-1 (CAV1); endoglin (ENG); human immunodeficiency virus (HIV); pulmonary arterial hypertension (PAH); mothers against decapentaplegic 9 (Smad9); activin-like receptor kinase-1 (ALK1); bone morphogenic protein receptor type 2 (BMPR2); potassium channel super family K member-3 (KCNK3).

1.5.2 Pathogenesis

PAH is a complex and severe pan-vasculopathy with increased PAP resulting from a combination of sustained vasoconstriction, progressive vascular remodelling and thrombosis *in situ,* primarily affecting the pre-capillary arterioles (<100 µm diameter; Archer *et al*., 2010; Intengan and Schiffrin, 2000; Tuder *et al*., 2013). A loss of vascular luminal cross-sectional area restricts flow, increasing pulmonary vascular resistance (PVR), generating a compensatory increase in RVSP; the right ventricle (RV) remodels to sustain high pressures through adaptive RVH, which eventually leads to maladaptive right ventricular hypertrophy (RVH), failure, and ultimately death (Voelkel *et al*., 2012, 2006; Vonk-Noordegraaf *et al*., 2013).

It is thought that endothelial dysfunction or injury initiates PAH pathogenesis; however, the cause(s) of the deficit is yet to be determined (Rabinovitch, 2012). Examples of endothelial dysfunction are chronically impaired production of the vasodilators PGI_2 and NO, and/or a shift to production of vasoconstrictors thromboxane A_2 (TXA_2), ET-1 and 5-hydroxytryptamine (5-HT; Christman 1992; Zakrzewicz *et al*. 2007; Giaid & Saleh 1995; Bauer *et al*. 2002; Behr & Ryu 2008; Yu *et al*. 2004; Hervé *et al*. 1995; Stewart *et al*. 1991; Tuder *et al*. 1999; Rubens *et al*. 2001). In addition, expression and activity of cGMP degrading PDEs (e.g. PDE1 and PDE5) is increased in PAH, further impairing the beneficial effects of cGMP signalling (Baliga *et al*., 2008; Klinger *et al*., 2006; Rabe *et al*., 1994). In addition to effects of vascular tone, endothelial dysfunction/injury promotes proliferation in pulmonary VSMCs leading to the characteristic histopathological features medial hyperplasia and hypertrophy (Pietra *et al*., 2004; Stacher *et al*., 2012). Pulmonary VSMC proliferation is promoted in a number of different ways; the vasoconstrictors ET-1 and 5-HT promote growth (Davie *et al*., 2002; Eddahibi *et al*., 2006), altered expression and function of K^+ and Ca^{2+} channels can lead to membrane depolarisation and an increase in intracellular Ca^{2+} triggering cell-cycle progression and proliferation (Burg *et al*., 2008; Yu *et al*., 2004; Yuan *et al*., 1998). Increased expression of tyrosine kinase receptors (e.g. vascular endothelial growth factor

receptor 2; VEGFR-2) as well as secretion of growth factors (e.g. VEGF, bFGF, TGF-α, PDGF; epidermal growth factor; EGF) act as potent mitogens of fibroblasts, ECs and VSMCs (Archer *et al*., 2010; Benisty *et al*., 2004; Geiger *et al*., 2000; Izikki *et al*., 2009; Kranenburg *et al*., 2005; Tuder *et al*., 2001). During the later stages of the disease, there is an appearance of complex plexiform arteriopathy, commonly known as “plexiform lesions” (Abe *et al*., 2010; Archer *et al*., 2010; Pietra *et al*., 2004). These lesions are a hallmark of PAH and lead to vessel occlusion and obliteration (Tuder *et al*. 2007; Heath & Edwards 1958; Abe *et al*. 2010; Stacher *et al*. 2012; Figure 7). This is illustrated experimentally where inhibition of VEGFR combined with hypoxic conditions causes apoptosis of ECs and gives rise to a pro-survival and anti-apoptotic EC phenotype, leading to the development of the characteristic lesions (Abe *et al*., 2010; Taraseviciene-Stewart *et al*., 2001).

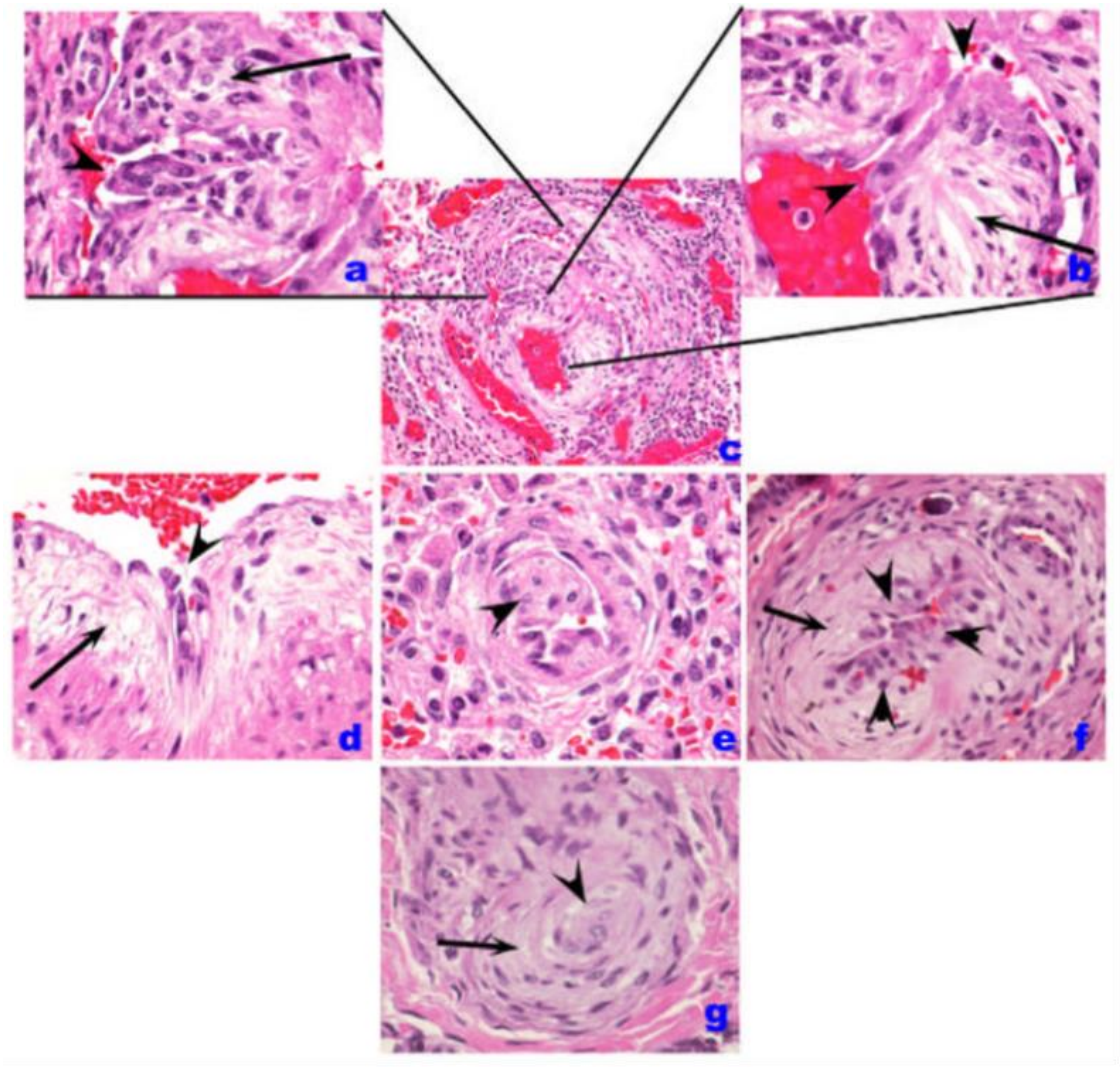

Figure 7. Examples of pulmonary vascular remodelling in PH.

Plexiform lesion (**B**) with high magnification of proliferated endothelial cells (**A**; arrowhead marks a luminal projection, and arrows mark cellular core). Atypical endothelial cell (**B**; arrowheads) with a myxoid component organised as concentric layers of smooth muscle cells (arrow). Abnormal endothelial cells (arrowhead) and the myxoid sub-endothelial layer (arrow; **D**). Intimal projection (arrowhead; **E**). Intimal projection (arrowhead), endothelial cells (arrowheads) in the intima, myxoid subintimal layer (arrow; **F**). Plexiform lesion, endothelial cells (arrowhead) smooth muscle-like cells (arrow; **G**; adapted from R M Tuder *et al*., 2007).

1.5.3 Pharmacotherapy

Until the 1990's PAH was historically considered a vasoconstrictive disease with treatment being predominantly calcium (Ca^{2+}) channel blockers based on retrospective experience and uncontrolled studies (Fuster *et al*., 1984; Rich and Brundage, 1987; Rich, 1988; Rich *et al*., 1992; Rubin, 1985; Sitbon *et al*., 2005; Weir *et al*., 1989). This treatment focused on the symptom of the disease, vasoconstriction, rather than the cause, vascular remodelling (Packer, 1985). Progression arrived when evidence based research developed a synthetic version of PGI_2, epoprostenol (Christman, 1992; Tuder *et al*., 1999). Epoprostenol along with PGI_2 analogues were not only found to improve vascular tone but also reverse vessel remodelling, raising hopes for a treatment that targets the cause of the disease (Akagi *et al*., 2014; Barst *et al*., 1996; Clapp *et al*., 2002; Kuhn *et al*., 2003; Sitbon *et al*., 2002). However, the effectiveness of epoprostenol and PGI_2 analogues in attenuating pulmonary vascular remodelling is controversial. One example is of a single patient treated long-term (18 year) with epoprostenol, which prolonged life (the patient ultimately died of colon cancer) but did not halt the progression of vascular remodelling (Rich *et al*., 2010). Histopathological and morphometric studies have been conducted on explanted lungs of PH patients who received PGI_2 treatment and such individuals showed no difference in percentage thickness of intima, media, or adventitia or in the density of plexiform lesions compared to untreated patients (Achcar *et al*., 2006). This illustrates the need for more effective treatments that target the pulmonary vascular remodelling component of this disease.

In general, there are three main treatment paradigms for PH, these target three fundamental biological pathways that maintain vascular homeostasis; prostacyclin, endothelin, and GC. Examples of these treatments are the prostanoids (e.g. epoprostenol, treprostinil, beraprost, iloprost), which activate prostanoid receptors and promote vasodilation and prevent proliferation, the endothelin receptor (ET_A and ET_B) antagonists (e.g. ambrisentan, bosentan) that inhibit the potent vasoconstrictor and mitogenic effect of ET-1, and those

treatments that potentiate the cGMP pathway (Gali *et al*., 2013).

Cyclic GMP elevating treatments approved for the treatment of PH include the PDE5 inhibitors (sildenafil and tadalafil), inhaled NO (iNO) and the sGC stimulator (Riociguat; Gali *et al*. 2013). Inhaled NO is an extremely effective treatment for persistent pulmonary hypertension of neonates (PPHN) also known as ”blue baby syndrome”, a condition that previously had no non-invasive therapy (Creagh-Brown *et al*., 2009; Macrae *et al*., 2004; Roberts *et al*., 1997, 1992). However, this treatment is very expensive and impractical because NO gas itself is very unstable and stored in large, heavy cylinders (Ichinose *et al*., 2004). Although, the invention of a device that produces NO from the atmosphere, on demand, has been developed and studies show that it can effectively reduce PAP in an acute model of PH in lambs, and thus could provide a low cost, portable alternative iNO treatment for PPHN (Yu *et al*., 2015).

The PDE5 inhibitors sildenafil (Galiè *et al*., 2005), tadalafil (Galiè *et al*., 2009) and vardenafil (Jing *et al*., 2011) are all effective treatments for PH; they improve PAP, exercise capacity, FC and survival. This illustrates the effectiveness of augmenting pulmonary specific NO/sGC/cGMP and NP/NPR/cGMP signalling by PDE5 inhibition, although one caveat is the development of side effects such as flushing, dyspepsia, and diarrhoea. Despite strong evidence in preclinical research, there is currently no evidence demonstrating the effectiveness of PDE5 inhibition in preventing vascular remodelling in PH patients (Galiè *et al*., 2005). Of note, PDE5 inhibitors have been found to also inhibit MRPs, leading to the hypothesis that their therapeutic effectiveness may be, in part, due to synergism through simultaneous prevention of cGMP degradation and export (Jedlitschky *et al*., 2000; Reid *et al*., 2003).

Recently, sGC agonists have been developed for the treatment of PH by increasing cGMP production through augmentation of the NO/sGC pathway. sGC ‘stimulators’ (e.g. riociguat) stimulate the native haem (Fe^{2+}) domain of

sGC and synergise with NO. In contrast to PDE5 inhibition, sGC activation/stimulation lacks pulmonary selectivity which can result in dose-limiting systemic hypotension (Belik, 2009).

1.5.4 Potential role for CNP in pulmonary hypertension (PH)?

Despite the broad cardioprotective role of CNP, maintaining endothelial barrier, function and inhibiting VSMC proliferation and fibrosis (described previously), a function for this peptide in the pulmonary vascular specifically, certainly in the context of PH, remains unclear. Patients with PH have increased plasma concentrations of CNP (Kaiser *et al*., 2015). In addition, rats exposed to chronic hypoxia develop significantly increased plasma levels of CNP, although expression in the lung is decreased (Klinger *et al*., 1998). Itoh *et al*., (2004) investigated the role of CNP in the monocrotaline model of PH and found that CNP infusion increased survival and improved haemodynamics in both prophylactically treated animals and animals with established PH. Most importantly, CNP treatment enhanced expression of markers of proliferation in pulmonary ECs, this was mirrored by a decrease in medial thickness and inflammatory cell infiltrates, and an increase in pulmonary eNOS protein expression. Together these data demonstrate that CNP infusion promotes the maintenance of the EC barrier, a well characterised pathological target of monocrotaline, thus preventing exposure of the underlying VSMCs to mitogens and preventing hyperplasia. In addition, CNP promoted a protective EC phenotype evident by enhancing expression of the NO producing enzyme, eNOS (Itoh *et al*., 2004). This study was followed by a contrasting publication by Casserly *et al*., (2011). This group found using the chronic hypoxia and hypoxia plus SU5416 model (see 1.5.5.3) in rats, that treatment with a continuous infusion of CNP did not attenuate the development of PH. The authors were unable to conclude definitively why CNP infusion was not effective despite its well characterised cardiovascular protective effects; they hypothesised that due to a significant reduction in NPR-B expression measured in the lungs of the hypoxic rats, along with previous evidence showing that pulmonary NPR-C

expression is significantly decreased by hypoxia, that any beneficial effect of increased circulating CNP may have been mitigated (Klinger *et al*., 1994; Sun *et al*., 2000).

CNP potently inhibits leukocyte adhesion and can therefore reduce inflammation associated with pathologic pulmonary vascular remodelling (Moyes *et al*., 2014; Ohno *et al*., 2002; Qian *et al*., 2002). Furthermore, inflammation can precede the deposition of extracellular matrix and fibrosis. This concept has been well characterised in other cardiovascular diseases; CNP infusion reduces cardiac fibrosis (Izumiya *et al*., 2012; Soeki *et al*., 2005), and decreased CNP production is linked with increased left ventricular fibrosis in aged rat hearts (Sangaralingham *et al*., 2011), and CNP infusion attenuates cardiac fibrosis following MI (Soeki *et al*., 2005). In the lung, CNP infusion also increases survival and attenuates pulmonary fibrosis in a mouse model of pulmonary fibrosis (bleomycin-induced; Murakami *et al*., 2004). In this study CNP was able to significantly reduce the number of infiltrating inflammatory cells (macrophages, neutrophils, and lymphocytes) and subsequent secretion of pro-inflammatory cytokines (IL-1β), which prevented the development of pulmonary fibrosis. This work was built on further by Kimura *et al*., (2016) who have shown that CNP over expression in fibroblasts specifically, attenuates the development of pulmonary fibrosis in the bleomycin model through the same process (reduction in inflammation and subsequent fibrosis). In sum, the effectiveness of CNP infusion in treating PH has proven controversial, although CNP is shown to be a potent anti-fibrotic agent and is effective at treating diseases with a substantial fibrotic component. In the context of PH, driven predominantly by vascular remodelling and not fibrosis, it still remains to be understood whether CNP can be used as an effective therapy. However, no matter what the context, the exact mechanism (CNP/NPR-B or CNP/NPR-C) by which CNP is acting to elicit any beneficial action has yet to be delineated.

1.5.5 Animal models of pulmonary hypertension (PH)

Animal models are used to simulate the clinical and pathological features of a disease, with the aim of increasing understanding and ultimately developing novel therapeutic strategies. PH is a multifactorial disease; it has been challenging to simulate the disease accurately and therefore a number of animal models of PH exist, none of which recapitulate all the human manifestations of the disease.

1.5.5.1 Chronic Hypoxia

The link between exposure to either normo- or hypobaric hypoxic conditions and the development of PH was made by studying mountain climbers at high altitude, shedding light into a possible pathophysiological mechanism of PH (Arias-Stella and Saldana, 1963; Houston *et al*., 1987). Pulmonary hypoxia caused by either environmental conditions (e.g. high altitude) or disease (e.g. sleep apnoea) triggers an adaptive pulmonary specific physiological mechanism; the lung attempts to optimise the ventilation/perfusion (V/Q) ratio and gas exchange by diverting blood flow away from poorly ventilated areas (Ward and McMurtry, 2009). O_2 concentrations are detected by mitochondria present in PASMCs, this initiates a signalling cascade, ultimately leading to increased intracellular calcium (Ca^{2+}) concentration and thus vasoconstriction (Aaronson *et al*., 2006; Ward *et al*., 2004; Waypa and Schumacker, 2008). However, in situations where hypoxic conditions are sustained (i.e. chronic hypoxia), for example at high altitude, the enhanced shear stress leads to the release of vasoconstrictors (TXA_2, ET-1, 5-HT), which maintain the high pulmonary vascular tone but also leads to adaptive vascular remodelling (Eddahibi *et al*., 2000; Fike *et al*., 2002; Frid *et al*., 2006; Li *et al*., 1994). In general, this is characterised by PASMC and adventitial fibroblast proliferation causing muscularisation of precapillary resistance vessels with no significant EC proliferation, thus leaving the vessel un-occluded and retaining flow (Rabinovitch *et al*., 1979; Stenmark *et al*., 1987). This response to hypoxia is not universal across species, strains within species and even individuals within

strains; gender and age are factors that influence an individual's response to hypoxic conditions (Dempsey *et al*., 1996; Haworth and Hislop, 2003; Miettinen *et al*., 1981). The most obvious example of species differences is studying animals that do not develop PH at high altitude; for example, the yak, pika, snow pig and llama (Durmowicz *et al*., 1993; Stenmark *et al*., 2006b). Animals that develop PH in hypoxic conditions include humans, mice, rats and neonatal calves; however despite developing PH, they differ in severity and specific pathological features. Moreover, humans and neonatal calves develop severe PH characterised by large increases in PAP_m (can be as high as systemic pressures), significant muscularisation of normally non-muscularised vessels and adventitia thickening, although it's key to note that these features are reversible upon returning to normoxic conditions, contrary to what is observed in patients with PH (Anand *et al*., 1988; Arias-Stella and Saldana, 1963; Stenmark *et al*., 1987). In contrast, rats and mice develop less severe PH characterised by smaller increases in PAP_m and less severe pulmonary vascular remodelling (Estrada and Chesler, 2009; Frank *et al*., 2008; Meyrick and Reid, 1980; Miettinen *et al*., 1981; Tuchscherer *et al*., 2007). In addition, RVH in response to hypoxia is not severe enough to develop to the point of failure, again, in contrast to PH patients where the development of RVH inevitably leads to death (Drexler *et al*., 2008). In addition to differences between animals, chronic hypoxia induces variable pathology between strains within the same species. Different strains of rat, Fischer 344 (F344), Sprague-Dawley (SD), Fawn hooded (FH) and Wistar Kyoto (WKY), respond differently to chronic hypoxia, with some strains demonstrating far greater resistance to others (e.g. FH; Wilkins *et al*., 2015). Although, until recently, the cause of this phenomenon has mostly remained a mystery; except in the case of FH rats who develop PH spontaneously (4 weeks of age) because of an inherent inability to uptake serotonin into platelets, a similar feature observed in PH patients (Cras *et al*., 2000; Hervé *et al*., 1995; Kentera *et al*., 1988). Zhao *et al*., (2015), identified a series of single nucleotide polymorphisms resulting in frameshift mutations in several genes within different strains of rat, which account for their different susceptibility to development of PH. These studies identified a novel pathological mechanism for PH implicating the zinc ion transporter ZIP12, such reduced ZIP12 expression attenuates the development of PH in rats exposed to

chronic hypoxia. Similar stain differences are also seen in mice, driven by a differing gene expression profile within the pulmonary vascular cell (Bull *et al*., 2007; Hoshikawa *et al*., 2003; Tada *et al*., 2013). Despite this, the use of rodents in the chronic hypoxia model for PH research remains the most commonly utilised technique, probably because of its phenotypic reproducibility, practicality and low cost, compared to using larger mammals (Lawrie, 2014).

1.5.5.2 Monocrotaline model

Seeds from the plant *Crotalaria Spectabilis* contain a toxic compound called monocrotaline (MCT) and when ingested causes PH (Kay *et al*., 1967). MCT is a 11-membered macrocyclic pyrrolizidine alkaloid that is subsequently activated to the pyrrole metabolite by oxidases present in the liver, converting it into a reactive bi-functional, cross-linking compound, dehydromonocrotaline (Reid *et al*., 1998; Wilson *et al*., 1992). Production of the reactive pyrrole metabolite is the pathological determining factor. Differences in cytochrome-P450 3A isoform as a result of sex, age and species vary the pathology produced by MCT. The importance of the cytochrome-P450 3A isoform has been demonstrated by specifically inhibiting this enzyme, reducing the severity of MCT-dependent PH. Conversely, induction of this enzyme can increase the severity of pathology (Kasahara *et al*., 1997; Reid *et al*., 1998). The exact mechanism by which MCT causes PH is not completely understood. However, MCT exposure is found to damage the pulmonary arterial endothelial cells (PAECs) causing a disruption of NO signalling and an increase in proliferative and anti-apoptotic factors (Huang *et al*., 2010; Lee *et al*., 2009; Rosenberg and Rabinovitch, 1988). The prominent histopathological feature of this model is medial hypertrophy and not obstructive, EC derived, complex lesion formation (Stenmark *et al*., 2009). The exact mechanism underlying medial hypertrophy is also not known and whether it is directly caused by interactions with ECs or by systemic inflammation triggered by MCT. Therefore, there are several caveats with this model including the limited understanding of the mechanisms underlying vascular remodelling. Firstly, vascular remodelling is not strictly confined to the distal pulmonary arteries as seen in PH patients but is also found in the veins

(Meyrick, 1982; Wilson *et al*., 1989). In addition, other pathological features, not found in PH patients, are associated with MCT exposure, including liver toxicity, occlusion of pulmonary veins, alveolar oedema and alveolar septal cell hyperplasia (Copple, 2003; Dumitrascu *et al*., 2008; Lalich *et al*., 1977). Furthermore and probably most importantly, although MCT treated rats develop significant increases in PAP_m and RVH which eventually leads to heart failure, similar to that of PH patients, MCT acts directly on the heart inducing lymphocytic myocarditis, making RV failure due to PH hard to determine and likely MCT-induced (J. G. Gomez-Arroyo *et al*., 2012).

1.5.5.3 Hypoxia plus SU5416 model

In 2001, Taraseviciene-Stewart *et al*., investigated the role of EC hyper-proliferation that form the characteristic plexiform lesions in an animal model of PAH. VEGF signalling is important for maintaining and differentiating vascular ECs and expression is increased in complex lesions found in PAH patients (Tuder *et al*., 2001, 1994). Increased angiogenesis appears to be a compensatory mechanism in response to hypoxia with enhanced expression of pro-angiogenic substances (e.g. VEGF) attenuating experimental PH, in addition the number of peripheral vessels is commonly reduced in patients with PH, a phenomenon called vascular pruning (McLaughlin and McGoon, 2006; Partovian *et al*., 2000; Pascaud *et al*., 2003). Using the VEGFR-2 inhibitor Sugen5416 (SU5416; Semaxinib), this group found that VEGFR inhibition in normal conditions caused slight pulmonary vascular remodelling, but when combined with hypoxic conditions, resulted in severe, progressive, irreversible PAH, characterised by extensive EC proliferation in the pulmonary arterioles (Taraseviciene-Stewart *et al*., 2001). The authors concluded VEGFR inhibition caused EC apoptosis leading to the rise of an apoptotic-resistant, hyper-proliferating EC phenotype, commonly found in complex lesions of PH patients (Sakao *et al*., 2005; Voelkel *et al*., 2002). This hypothesis was investigated using a caspase inhibitor, preventing EC apoptosis, and attenuating the PH developed in this model (Taraseviciene-Stewart *et al*., 2001). Loss of endothelial barrier integrity through EC apoptosis results in exposing the

underlying PASMCs to circulating growth factors, prompting PASMC proliferation. A significant and defining feature of this model is that in the rats, when returned to normoxic conditions, pulmonary vascular remodelling progresses (unlike in the chronic hypoxia model). Furthermore, this remodelling progresses until complex lesions develop similar to that seen in PH patients, accompanied by severe RVH that eventually leads to failure and death, offering a more representative model of the human disease (Abe *et al*., 2010). Unsurprisingly, upon development of this model this same combination was attempted in mice, largely to allow exploitation of transgenics. Taraseviciene-Stewart *et al*., (2001) found using the same protocol used in rats (a single injection of SU5416) did not produce a PH like pathology (J. Gomez-Arroyo *et al*., 2012). However, subsequently it was found that if the SU5416 was dosed 3 times (once per week) whilst the mice were still exposed to hypoxia, this elicited significant increases in PAP_m, RVH and vascular remodelling with the appearance complex occlusive lesions characteristic of PH, compared to that seen in mice exposed to hypoxia alone (Ciuclan *et al*., 2011). However, slight reversal of RVSP and RVH was observed after returning the animals to normoxia for 2 weeks and the extent of complex lesion were not quantified, begging the question how permanent this pathology is (Ciuclan *et al*., 2011). Nevertheless, the authors of this study did observe significant early EC apoptosis followed by late EC proliferation in the lungs of these mice, evident by significantly enhanced early caspase-3 staining that plateaus over time and is followed by increased EC proliferating cell nuclear antigen (PCNA) staining, demonstrating a shift from EC apoptosis to proliferating phenotype, which is now understood to be the defining feature of PH but this feature has yet to be recapitulated in other mouse models of PH.

1.5.5.4 Bleomycin-induced model of PH

Bleomycin is a chemotherapeutic antibiotic, produced by the bacterium *Streptomyces verticillus* (Adamson, 1976; Umezawa *et al*., 1967). The drug is used in the treatment of lymphoma, squamous cell carcinomas and germ cell tumours, but has the undesirable side effect of producing interstitial lung

disease (ILD), specifically pulmonary fibrosis; this observation led to the rationale that this drug can be used in the research of PH secondary to ILD (Muggia *et al*., 1983). Bleomycin is among a large groups of agents which cause ILD, including: anti-microbial (e.g. amphotericin B), anti-inflammatory (e.g. methotrexate), biological (e.g. rituximab), cardiovascular (e.g. procainamide), chemotherapeutic (e.g. doxorubicin), and miscellaneous (e.g. talc; Schwaiblmair *et al*., 2012). The acute lung injury caused by these agents leads to the development of pneumonia, and the ensuing chronic pulmonary inflammation eventually leads to a fibrotic change that ultimately interferes with gas exchange. Areas of poor gas exchange results in hypoxia and vasoconstriction, thus PH (Seeger *et al*., 2013).

Bleomycin is believed to produce superoxide and hydroxyl free radicals, which disrupt DNA within tumour cells, thereby interrupting the cell-cycle (Claussen and Long, 1999). In the lung, this leads to an inflammatory response, activating fibroblasts, leading to fibrosis (Chaudhary *et al*., 2006; Grande *et al*., 1998). The lungs are particularly susceptible to bleomycin because of relatively low expression of bleomycin hydrolase, an enzyme that inactivates the drug (Sebti *et al*., 1989). Lung instillation (oropharyngeal; o.p.) of bleomycin causes acute lung injury, specifically formed of increased epithelial apoptosis, followed by a characteristic increase in the secretion of pro-inflammatory cytokines (e.g. IL-1β, TNF-α, IL-6, interferon-γ; INF-γ) from macrophages and neutrophils, the crucial first-step in this models fibrotic pathogenesis (Chaudhary *et al*., 2006; Janick-Buckner *et al*., 1989; Mungunsukh *et al*., 2010). Next, significant production of pro-fibrotic markers (TGF-β, fibronectin, procollagen-1) occurs, which peaks at around day 14 (Chaudhary *et al*., 2006; Kolb *et al*., 2001). Of note, TGF-β and myofibroblasts are considered central to the aberrant fibrotic response as they specifically contribute to the exaggerated extracellular matrix deposition and are also found in human IPF (Coward *et al*., 2010; Dhainaut *et al*., 2003; Harari and Caminati, 2010; Hardie *et al*., 2009; Sheppard, 2001; Zhao *et al*., 2002). This extracellular matrix consists mainly of collagen, which forms distinctive histopathological formations; intra-alveolar buds, mural incorporation and obliterative changes (Ebihara *et al*., 2000; Lucey *et al*., 1996; Usuki and Fukuda, 1995). This model of pulmonary fibrosis is highly reproducible and

produces a fibrotic histologic picture similar to human disease, characterised by patchy parenchymal inflammation, epithelial cell injury with reactive hyperplasia, basement membrane damage and interstitial as well as intra-alveolar fibrosis (Epperly *et al*., 2006). However, fibrosis initiated using this model eventually resolves, unlike the human disease, although this process can take many months (Chua *et al*., 2005; Izbicki *et al*., 2002).

1.6 Hypotheses and specific aims

For well over half a century, cGMP has been thoroughly characterised as an integral regulator of cardiovascular homeostasis. My thesis research focused on further optimisation of cGMP for therapeutic gain in cardiovascular disease and comprised two main strands: (1) the role of MRPs in vascular physiology and (2) the cytoprotective function of CNP in pulmonary vascular disease:

(1) There is emerging evidence demonstrating MRPs play a role in regulating vascular homeostasis by modulating cGMP and cAMP levels, and as potential targets for the treatment of cardiovascular disease. However, previous work has focussed on cAMP-dependent effects of MRPs. Therefore, in this thesis I have investigated the hypothesis that ***'MRPs play a pivotal role in regulating local cGMP concentrations and cardiovascular homeostasis'***. To address this question I explored the function of MRPs to potentiate well characterised roles of cGMP in the vasculature; vascular reactivity, VSMC proliferation and BP.

(2) CNP plays an integral role in cardiovascular homeostasis regulating leukocyte adherence, VSMC proliferation, endothelial integrity, BP and fibrosis via both cGMP -dependent and -independent processes. PH is a severe pulmonary vascular disease underlined by vascular dysfunction, which is treated by drugs promoting cGMP signalling, but currently a role for CNP in maintaining pulmonary vascular homeostasis, or the pathogenesis of PH, remains unclear. In addition, the mechanism by which CNP might elicit a beneficial activity has not been elucidated (i.e. CNP/NPR-B or CNP/NPR-C). Thus, in this thesis I have also tested the hypothesis that ***'endogenous endothelial-derived CNP is an innate defence mechanism that protects against PH'***. To achieve this goal, I used an experimental models of PH and mice deficient in endothelial-derived CNP and global NPR-C KO.

CHAPTER 2

METHOD & MATERIALS

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2.1 Materials & reagents

ANP (Cambridge Bioscience, Cambridge, UK), CNP (Calbiochem, Nottingham, UK), spermine-NONOate (Sp-NO; Sigma Aldrich, Poole, UK), MK571 (MK; Sigma Aldrich, Poole, UK), Iso (Sigma Aldrich, Poole, UK), phenylephrine (PE; Sigma Aldrich, Poole, UK), acetylcholine (ACh; Sigma Aldrich, Poole, UK), were dissolved in deionised, distilled water (ddH_2O). For acute blood pressure studies, sodium nitroprusside (SNP; Sigma Aldrich, Poole, UK), diethylenetriamine/nitric oxide adduct (D-NO; Sigma Aldrich, Poole, UK) and MK571 were dissolved in saline (0.9 %; Baxter, Newbury, UK). For telemetry studies MK571 was dissolved in normal animal drinking water. Probenecid (PB; Sigma Aldrich, Poole, UK) was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Poole, UK) and identical percentage DMSO concentrations added to tissues (0.3 % DMSO). SU5416 (Tocris Bioscience, Bristol, UK) was suspended in carboxymethylcellulose sodium, (CMC; 0.5 %; w/v), NaCl (0.9 %; w/v), polysorbate 80 (Tween 80; 0.4 %; v/v), benzyl alcohol (0.9 %; v/v; all sourced from Sigma Aldrich, Poole, UK).

2.2 Organ bath pharmacology

All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act of 1986 and adhered to ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. Animals were housed in a temperature-controlled environment (~24 °C) with a 12-hour light–dark cycle. Food and water were accessible *ad libitum*.

Male and female mice (C57/BL6; 20-30 g) were euthanised by cervical dislocation. The thoracic aortae were carefully removed, cleaned of connective tissue and cut into three to four ring segments of approximately 4 mm in length. Aortic rings were mounted in 10 ml organ baths containing Krebs-bicarbonate buffer (composition (mM): Na^+ 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; D-Glucose 11) and gassed with carbogen (95 % O_2/ 5 % CO_2; British Oxygen Company; BOC; Guildford, UK). Tension was initially set at 0.3 g and reset at intervals following an equilibration period of approximately 1 h during which time fresh Krebs-bicarbonate buffer was replaced every 15 min. After equilibration, the rings were primed with three separate additions of KCl (48 mM; Sigma Aldrich, Poole, UK), at each addition maximum tension was observed (3 min) before being washed out by the addition of fresh Krebs-bicarbonate buffer at 10 min intervals for a total of 30 min. Cumulative concentrations of PE (1 nM to 3 µM) were then added until a maximum contraction was observed. Another washout period was performed. The vessels were then contracted to 80 % of the tension elicited by the maximum PE concentration (EC_{80}). Once this response had stabilised, a single addition of ACh (1 µM) was added to the bath to assess the integrity of the endothelium. If the contractions to PE were not maintained, or relaxations less than 50 % of the PE-induced tension to ACh were not observed, the tissues were discarded. After another wash period, the vessels were again contracted to 80 % of the maximum tension elicited by PE and then cumulative concentrations of each pharmacological treatment were administered. Pharmacological treatments investigated were ANP (1 pM to 300 nM), ACh (1 nM to 3 µM), MK571 (100 nM to 50 µM), CNP (1 nM to 3 µM), the NO-donor spermine-NONOate (Sp-NO; 1 nM to 30 µM), the β-adrenoceptor agonist Iso (300 pM to 300 nM), and PB (1 µM to 1 mM). In separate experiments, tissues were pre-incubated with sub-threshold concentrations of MK571 (3 µM) or PB (300 µM; determined following construction of concentration-response curves for each compound; Figure 13 & Figure 14) for 10 min prior to cumulative concentrations of either; ACh, ANP, CNP, Sp-NO and Iso being added to pre-contracted vessels.

2.3 Freezing cells

Cells were stored in liquid nitrogen (N_2; BOC) when not being cultured. Cells were aliquoted into cryogenic vials in 90 % bovine serum (BS; Life Technologies Ltd, Paisley, UK) and 10 % DMSO at 0.5 x 10^6 cells/ml. Vials were then slowly frozen in a -80 ºC freezer using a Mr. Frosty™ freezing container (Thermo Fisher Scientific, Leicestershire, UK) for 24 h before being moved and transferred to liquid N_2 for long term storage.

2.4 Thawing cells

Vials of cells were removed from liquid N_2 and immediately placed into a water bath kept at 37 ºC. The lid was unscrewed slightly, to allow pressure equilibration, then re-tightened. Once most of the vial was thawed, pre-warmed cell culture media was added, then the cell suspension pipetted directly into a cell culture flask containing pre-warmed media. The flask was placed in a cell culture incubator over night before the medium was changed the next day to remove all the DMSO.

2.5 Cell proliferation assays

Human coronary artery smooth muscle cells (hCASMC; passage 6-7; Lonza, Basel, Switzerland) were seeded onto 6-well plates at a density of 3 x 10^5 cells/well. Cells were initially grown for 24 h in routine cell culture medium (Smooth Muscle Growth Medium-2; Lonza, Basel, Switzerland) with SmGM™-2 BulletKit containing foetal bovine serum (FBS; 5 %), human epidermal growth factor (hEGF), insulin, human fibroblastic growth factor (hFGF), and gentamicin/amphotericin B (proprietary concentrations). Subsequently, the cells were serum-starved (FBS: 0.5 %; 1:10 dilution of routine cell culture medium) for 48 h to synchronise cell-cycles. Cells were then incubated in medium

containing 5 % serum in the presence of vehicle (sterile ddH_2O) or pharmacological treatment, and cells (stained with trypan blue diluted 1:1 to identify dead cells) counted at 0, 24, 48, 72, 96 h intervals using a haemocytometer. Individual plates were set up with the same treatments, in duplicate wells, one for each time-point. The pharmacological agents investigated were ANP (1 µM), D-NO (10 µM), MK571 (30 nM, 300 nM, 3 µM, 30 µM) and ANP or D-NO combined with MK571 (30 nM).

2.6 Cyclic GMP assay

Intracellular and extracellular cGMP concentrations were measured by enzyme-linked immunosorbent assay (ELISA; GE Healthcare, Hatfield, UK) following 24 h of treatment in the same cells used for the proliferation assays described above. Each well was pre-incubated with the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 µM; Sigma Aldrich, Poole, UK) for 30 min prior to cell and supernatant harvest to prevent breakdown of cGMP during cell processing. Cell pellet (for intracellular cGMP) and supernatant (for extracellular cGMP) were frozen and stored at -80 ^oC until use. The ELISA was conducted as per manufacturer's instructions.

2.7 Acute blood pressure measurement in anaesthetised mice

Male C57/BL6 mice (~25 g) were anaesthetised with 1.5 % isoflurane (Abbott Laboratories Ltd, Queenborough, UK) in O_2 and placed supine on a thermostatically controlled heating blanket (37.0 °C ± 0.5 °C). To measure blood pressure, the left common carotid artery was isolated and a fluid-filled (heparin; 100 U/ml diluted in 0.9 % saline), 0.28 mm internal diameter cannula (Critchley Electrical Products Pty Ltd, Castle Hill, Australia) introduced into the artery. Blood pressure was measured using an in-line P23 XL transducer (Viggo-

Spectramed, USA, California) and PowerLab system (ADInstruments, Castle Hill, Australia), calibrated beforehand, and recorded using LabChart (ADInstruments, Castle Hill, Australia). The jugular vein was cannulated for drug administration. The arterial cannula was flushed once with heparinised saline (heparin; 100 U/ml diluted in 0.9 % saline). After a minimum 10 min equilibration or until continuous stable pressure was observed, mice were given a weight adjusted (1 µl to 1 g of body weight) intravenous bolus injection of accumulative doses of MK571 (0.001, 0.01, 0.1, 1, 3 mg/kg). Since MK571 had little or no effect on blood pressure *per se* (Figure 32), the 3 mg/kg dose was chosen as 'sub-threshold.' Subsequently, blood pressure was measured following intravenous (i.v.) bolus administration of the NO-donor sodium nitroprusside (SNP; 1, 3, 10 µg/kg) or ANP (1, 10, 100 µg/kg) in the absence and presence of MK571 (3 mg/kg; pre-incubated for 30 min).

2.8 Chronic blood pressure measurement in conscious, telemeterised mice

Male C57/BL6 mice (~25 g) were implanted with either a DSI PhysioTel® PA-C10 or HD-X11 telemetry probe (Data Sciences International, Minneapolis, USA). Each probe was cleaned and sterilised according to the manufacturer's guidelines. The probe was soaked in Terg-A-Zyme® (1 % w/v; Sigma Aldrich, Poole, UK) for a maximum of 72 h, rinsed with water, dried and stored until the day of implantation. Prior to insertion the probe was sterilised by soaking in 4 % glutaraldehyde (Sigma Aldrich, Poole, UK) for a maximum of 40 min, then washed with sterile saline and finally re-gelled to ensure the absence of air bubbles in the catheter tip. Mice were sedated with 5 % isoflurane in O_2 and anaesthesia was maintained using 1.5-2 % isoflurane in O_2. The left common carotid artery was exposed, isolated and cleaned of any surrounding tissue. A small incision was made in the carotid and the catheter inserted whilst submerged in saline to ensure no bubble form inside the catheter tip. The tip of the catheter was placed into the aortic arch, securely fastened and the transmitter body placed subcutaneously (s.c.) on the right flank. The incision

was stitched and each animal received post-operative analgesia consisting of 0.3 µg vetergesic (Abbott Laboratories Ltd, Queenborough, UK) in 0.5 ml saline (s.c.). Animals were left to recover for a minimum of 7 days, after which haemodynamic recordings were taken for 64 h over the weekend to minimise noise disturbance. Mean arterial blood pressure (MABP), heart rate (HR) and activity were recorded for 2 min at 15 min intervals using Dataquest Art Acquisition System (Data Sciences International, Minneapolis, USA). Baseline haemodynamic measurements were taken one week before dosing. The body weight of animals was measured at the beginning of the experiment. The mean, normal daily drinking water consumption was calculated by measuring the volume of water consumed per day for three days. At the start of drug administration, drinking water bottles were replaced with bottles containing MK571 at a concentration which entailed each mouse received 25 mg/kg/day (based on a pre-determined mean consumption of 4 ml/mouse/day). A 24 h time period was used for analysis: starting from 1 pm Saturday and ending 1 pm Sunday (12 h light/dark) with dosing commencing 24 h before the start of this time period.

2.9 Experimental models of pulmonary hypertension (PH)

Based on arguments regarding experimental model suitability made in the Introduction, I employed two well-established protocols to evaluate a potential role for CNP in the pathogenesis of PH.

2.9.1 Genotyping of animals

NPR-C KO mice were generously donated to our laboratory by Prof. Oliver Smithies (University of North Carolina, USA; Matsukawa *et al*., 1999). Endothelial-specific CNP KO mice were generated and characterised in our laboratory (Moyes *et al*., 2014). Mouse ear clip samples were digested using DirectPCR lysis reagent (Viagen Biotech, Los Angeles, USA) and 0.3 mg/ml

proteinase K (Scientific Laboratory Supplies Ltd, Benfleet, UK) overnight at 55 ºC. Samples were incubated at 85 ºC for 45 min to denature proteinase K followed by cooling to room temperature. A master mix of the polymerase chain reaction (PCR) mixture composed of: MyTaq™ Red Mix (Bioline, London, UK), primers (Table 4) DNA template (digested mouse ear clip solution) and sterile ddH_2O in a total volume of 25 µl. The PCR conditions for each reaction are described in (Table 5). PCR products were loaded into wells formed from a pre-made 2 % agarose gel (Sigma Aldrich, Poole, UK) containing GelGreen™ (Biotium, Hayward, USA) nucleic acid stain and resolved by gel electrophoresis, then viewed using an AlphaImager (Alpha-Innotech, Kasendorf, Germany). NPR-C KO mice were identified by a product of 413 base pairs (detecting the neomycin cassette used to delete the gene) compared to a 250 base pair product for WT; ecCNP KO mice were identified by the presence of two bands: CNP-floxed (956 base pairs) and Tie^2-Cre (512 base pairs). The WT was identified by a 842 base pair product.

Primer	Primer nucleic acid sequence
Floxed CNP forward	5’-CCCTGTGCTCAGACAGAAATGAG-3’
Floxed CNP reverse	5’-CGCATAACCAGTGAAACAGCATTGC-3’
Tie^2 forward	5’-CCTTTATGCCAAGAGAACTTCCAGGAGG-3’
Tie^2 reverse	5’-TCCTTCCTGACTTCCTTCTGCTCTCTATCC-3’
NPR-C forward	5’-CTTGGATGTAGCGCACTATGTC-3’
NPR-C reverse	5’-CACAAGGACACGGAATACTC-3’
NPR-C NEO	5’-ACGCGTCACCTTAATATGCG-3’

Table 4. A table describing the nucleic acid sequence of the primers used to genotype ecCNP and NPR-C KO and WT mice.

A table describing the nucleic acid sequence of the primers used to genotype endothelial cell-specific C-type natriuretic peptide (ecCNP) and natriuretic peptide receptor-C (NPR-C) knockout (KO), heterozygous and wild-type (WT) mice. Adenine (A), cytosine (C), guanine (G), thymine (T), neomycin (NEO).

PCR Step	Floxed CNP	Tie2	NPR-C
	94°C 10 min	95°C 10 min	95°C 5 min
Denaturation Annealing Polymerisation	x35 cycles 94°C 30 s 60°C 1 min 68°C 2 min	x40 cycles 95°C 30 s 58°C 1 min 72°C 1 min	x35 cycles 93°C 1 min 60°C 1 min 72°C 1 min
Extension	68°C 10 min	72°C 10 min	72°C 10 min

Table 5. A table describing the PCR thermal cycler conditions used for each reaction.

A table describing the polymerase chain reaction (PCR) thermal cycler conditions used to detect each of the three DNA sequences to genotype endothelial cell-specific C-type natriuretic peptide (ecCNP) and natriuretic peptide receptor-C (NPR-C) knockout (KO), heterozygous and wild-type (WT) mice.

2.9.2 Hypoxia plus SU5416 model

The VEGFR-2 inhibitor SU5416 was suspended in CMC (0.5 %; w/v), NaCl (0.9 %; w/v), polysorbate 80 (Tween 80; 0.4 %; v/v), benzyl alcohol (0.9 %; v/v) in ddH_2O, then vigorously vortexed, and sonicated for 6 min to produce a uniform suspension. In optimisation studies, animals (male C57/BL6 mice; ~25 g) were exposed to chronic normobaric hypoxia (10 % O_2) for either 21 or 35 days, or 21 days followed by 14 days of normoxia (21 %; the latter protocol termed ‘Reversal’; Figure 8). Mice were administered SU5416 (20 mg/kg; s.c.) via 3 injections (at weekly intervals). Control mice received vehicle (s.c.) with the same dosing regimen (3 injections, at weekly intervals; days 0, 7 and 14; Figure 8). Control mice were kept at normoxia (21 % O_2) for 21 days and treated with vehicle as described previously. For studies involving transgenic animals (e.g. ecCNP and NPR-C KO), 16 week old, male and female mice received weekly injections of SU5416 (20 mg/kg; s.c.; days 0, 7 and 14) in combination with a 21 day hypoxia (10 % O_2) period (Figure 8). Control mice received vehicle (s.c.;

days 0, 7 and 14) in combination with a 21 day normoxia period (21 % O_2).

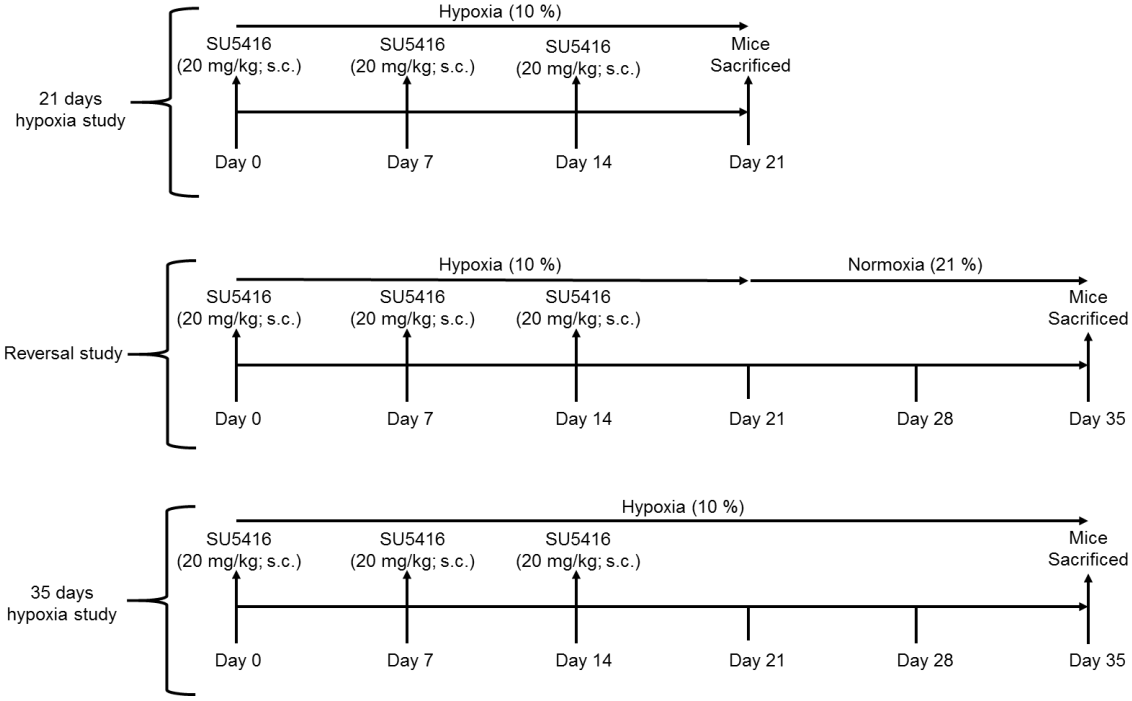


Figure 8. Schematic describing method used for hypoxia plus SU5416 experiments.

For optimisation studies mice were dosed with SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) and exposed to either normoxia (21 % O_2; control) or hypoxia (10 % O_2) for 21 or 35 days, or 21 days hypoxia followed by 14 days normoxia (Reversal). For studies involving male and female endothelium-specific CNP knockout (ecCNP KO) and natriuretic peptide receptor-C knockout (NPR-C KO) mice with corresponding wild-type (WT) littermates involved dosing with SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle and exposure to hypoxia or normoxia for 21 days.

2.9.3 Bleomycin-induced model of pulmonary hypertension (PH)

Male and female WT and NPR-C KO mice received a single oropharyngeal (o.p.) instillation, under light isoflurane anaesthesia, of Bleo-Kyowa® (Bleomycin Sulphate; Bleo; Nippon Kayaku Co. Ltd, Tokyo, Japan) weight adjusted (1 µl to 1 g of body weight), resulting in individual mice receiving 4 mg/kg (4,000 IU or 4 USP) dissolved in saline. Control mice received saline (o.p.) only. The mice were then left in normal housing conditions for 14 days before being euthanised (Figure 9).

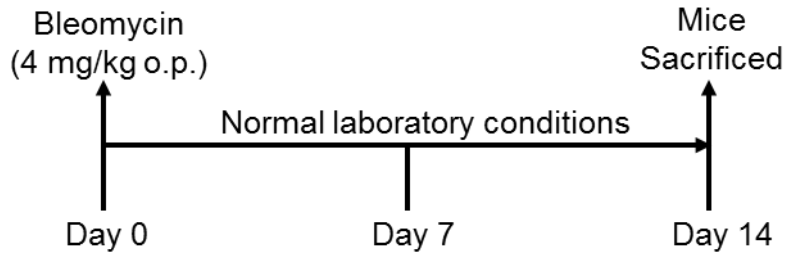


Figure 9. Schematic describing method used for bleomycin-induced pulmonary hypertension model.

Studies involving male and female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates were administered bleomycin (4 mg/kg; o.p.; day 0) followed by 14 days at normal laboratory conditions.

2.9.4 Lung & heart morphology

The whole lung was fixed with 5 % paraformaldehyde (PFA; Sigma Aldrich, Poole, UK) by inflating under constant pressure using a gravity fed apparatus (20 cm of H_2O, 14.7 mm Hg; equivalent to physiological pulmonary pressure). The lungs were then left to fix for 4 h, transferred to a 15 % sucrose (Sigma Aldrich, Poole, UK) solution for 6 h, and permanently stored in 70 % ethanol (Fisher Scientific Ltd, Leicestershire, UK). The left lung was then removed, cut

transversely and the lower section paraffin embedded, sectioned and stained. Embedding, sectioning, mounting and staining were performed by the Barts Cancer Institute (BSI; London, UK) histology department. Mouse lungs from the hypoxia plus SU5416 model of PH were stained for α-smooth muscle actin (α-SMA; Sigma Aldrich, Poole, UK) and used to assess pulmonary vascular remodelling. Pulmonary vascular remodelling was assessed by counting the number of non-muscularised (<25 % total α-SMA staining), partially-muscularised (25 – 75 % α-SMA staining) and fully-muscularised (>75 % α-SMA staining) vessels (Table 21 & Table 22). Every vessel <100 µm in diameter was counted using a microscope (x20 magnification) from a single slice of α-SMA stained lung, for each mouse. Percentage non-muscularised, partially-muscularised and fully-muscularised were then expressed graphically. Mouse lung sections from the bleomycin-induced model of PH were stained with Picrosirius red (PSR; BSI, London, UK) to stain for collagen and representative images of pulmonary fibrosis were taken (Table 23). The heart was removed, the right ventricle dissected under x8 magnification, and weighed to determine RVH (as calculated by right ventricle to left ventricle plus septum ratio; RV/[LV+S]; Fulton's Index).

2.9.5 Right ventricular systolic pressure (RVSP) & mean arterial blood pressure (MABP) measurement

Both RVSP and MABP measurements were taken. The mice were anaesthetised with ~1.5 % isofluorane in O_2 and placed supine on a thermostatically controlled heating blanket (37.0 °C +/- 0.5 °C). RVSP was measured first; the right jugular vein was isolated and a Millar catheter (Millar Instruments; SPR-671, size 1.4F; Houston, USA) introduced into the superior vena cava and then advanced into the right ventricle. The breathing rate was maintained between 100-120 breaths per minute and a 2 min section of stable pressure was recorded. For MABP, the left common carotid artery was isolated and a fluid-filled catheter (described previously) introduced into the artery and a 2 min period of stable pressure recorded. Both MABP and RVSP were

measured using an in-line P23 XL transducer (Viggo-Spectramed, Oxnard, USA) and recorded onto a pre-calibrated PowerLab system (ADInstruments, Castle Hill, Australia). RVSP and MABP were calculated as mean pressure over a 2 min recording.

2.10 Statistics

Statistical analyses were carried out using GraphPad Prism version 5 (GraphPad software, California, USA). When comparing two groups of data a two-tailed, unpaired, Student's t-test was used. Three or more groups of data were compared using one-way ANOVA followed by a Bonferroni multiple comparisons test. Two-way analysis of variance (ANOVA) was used to compare data affected by two factors; organ bath pharmacology (relaxation/contraction & concentration), acute changes in BP (BP & dose), animal models of PH (body weight & time), vascular remodelling (vessel type & number of vessels) and cell proliferation studies (cell growth & time). For organ bath experiments curves were fitted to the data using nonlinear regression and the concentration of each drug, giving a half-maximal response (EC_{50}), was used to compare potency. Exponential growth curves were fitted to cell proliferation data. Statistical significance was achieved when $P<0.05$. Results are expressed as mean ± standard error of the mean (SEM) of n repeats or animals.

CHAPTER 3

RESULTS 1

CHAPTER 3: RESULTS 1

3 Investigation of the role of MRPs in vascular homeostasis

3.1 Effect of MRP inhibition on vascular reactivity

Functional pharmacological studies were performed on mouse thoracic aorta. Concentration-dependent responses to the contractile agent PE were performed to determine vessel viability and an appropriate EC_{80} concentration to pre-contract vessels for the study of vasorelaxant drugs (Figure 10 & Figure 11). All vessels relaxed >50 % to ACh (1 µM) pre-contracted with an EC_{80} concentration of PE, indicating the presence of an intact endothelium (Figure 12). The MRP inhibitors MK571 (100 nM to 50 µM) and PB (1 µM to 1 mM) produced concentration-dependent relaxations of PE-induced tone, with the highest concentrations of each resulting in 100 % relaxation (Figure 13 and Figure 14). Sub-threshold (relaxation <20 %) concentrations of MK571 (3 µM) and PB (300 µM) were chosen to assess the effects of these MRP inhibitors on vasorelaxant responses.

The endothelium-dependent vasorelaxant ACh exerted concentration-dependent relaxations of PE pre-contracted mouse aorta that were not significantly different in the presence of either MK571 (3 µM; Figure 15) or PB (300 µM; Figure 16). The NO-donor, Sp-NO, CNP, ANP and the β-adrenoceptor agonist Iso all induced concentration-dependent relaxations of PE-tone that were sensitive to MRP inhibition. The greatest effect of MRP inhibition was observed on vasorelaxant responses to ANP; MK571 significantly increased ANP potency by almost a half Log (Log EC_{50}: -8.53 ± 0.085 to -9.011 ± 0.12; $P<0.001$; Figure 17). PB also significantly enhanced the vasodilator effect of ANP (Log EC_{50}: -8.51 ±0.091 to -8.64 ± 0.12; $P<0.001$; Figure 18). Responses

to CNP were significantly, albeit modestly, increased by MK571 (Log EC_{50}: -6.84 ± 0.041 to -6.98 ± 0.034; P<0.001; Figure 19) and PB (Log EC_{50}: -6.87 ± 0.14 to -9.011 -6.91 ± 0.098; P<0.05; Figure 20). In addition, PB, but not MK571, significantly enhanced the maximal response to CNP (Emax 88.50 ± 8.94 to 95.97 ± 5.32 % Figure 20). The potency of Sp-NO was marginally, but significantly, enhanced when combined with MK571 (Log EC_{50}: -6.84 ± 0.068 to -6.58 ± 0.028; P<0.01; Figure 21). Conversely, PB reduced the potency of Sp-NO producing a significant rightward shift in the relaxation response curve (Log EC_{50}: -6.36 ± 0.07 to -6.58 ± 0.03; P<0.05; Figure 22). Finally, PB (Log EC_{50} -6.64 ±0.13 to -6.90 ±0.16; P<0.01; Figure 24) and MK571 (Log EC_{50} -6.63 ± 0.14 to -6.97 ± 0.13; P<0.001; Figure 23) significantly increased the potency of Iso.

EFFECT OF PE IN MOUSE AORTIC RINGS

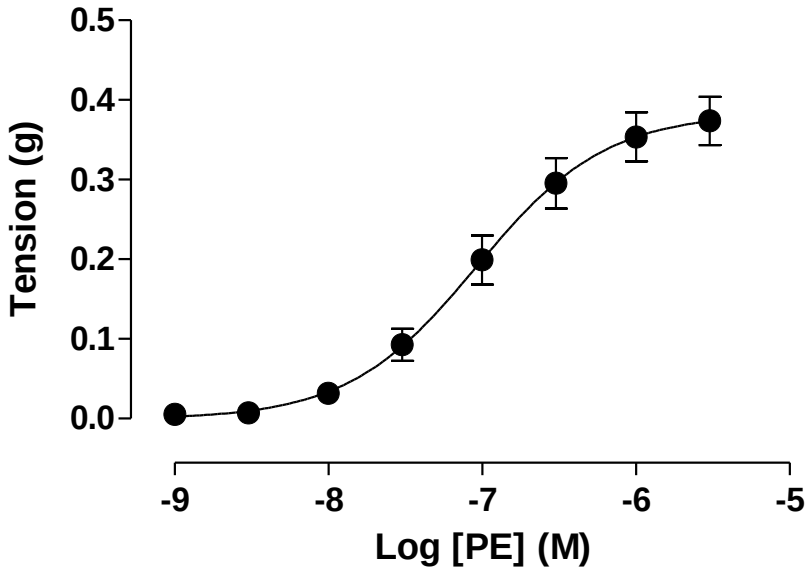


Figure 10. Concentration-response curve to PE in mouse aortic rings.

Contraction is expressed as mean ± SEM increase in absolute tension (g). n=11.

Log EC_{50} (M)	E_{max} (g)
-7.03 ± 0.11	0.38 ± 0.03

Table 6. Log EC_{50} and E_{max} values for PE-induced contraction of mouse aortic rings.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM increase in absolute tension (g). n=11.

EFFECT OF PE IN MOUSE AORTIC RINGS

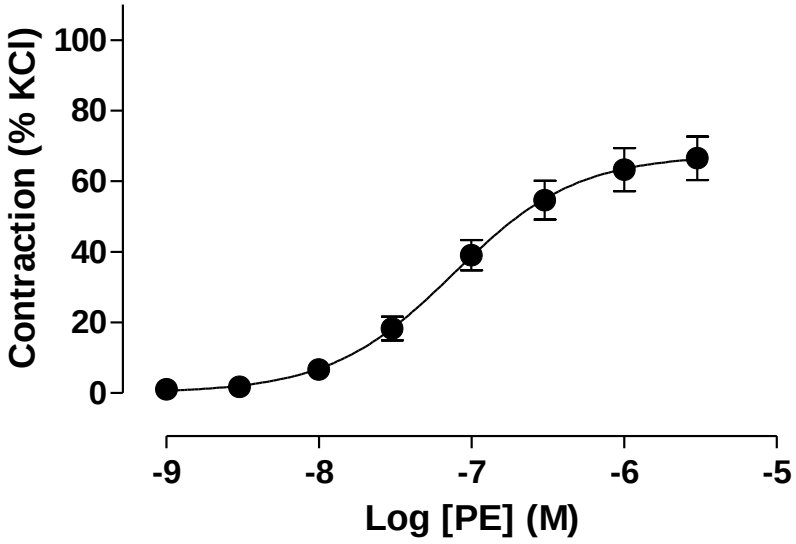


Figure 11. Concentration-response curve to PE in mouse aortic rings.

Contraction is expressed as mean ± SEM percentage of the maximal contraction to K^+ (48 mM). n=7.

Log EC_{50} (M)	E_{max} (%)
-7.12 ± 0.10	67.49 ± 4.24

Table 7. Log EC_{50} and E_{max} values for PE-induced contraction of mouse aortic rings.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage contraction of the maximal increase in tension to K^+ (48 mM). n=7.

EFFECT OF ACh IN MOUSE AORTIC RINGS

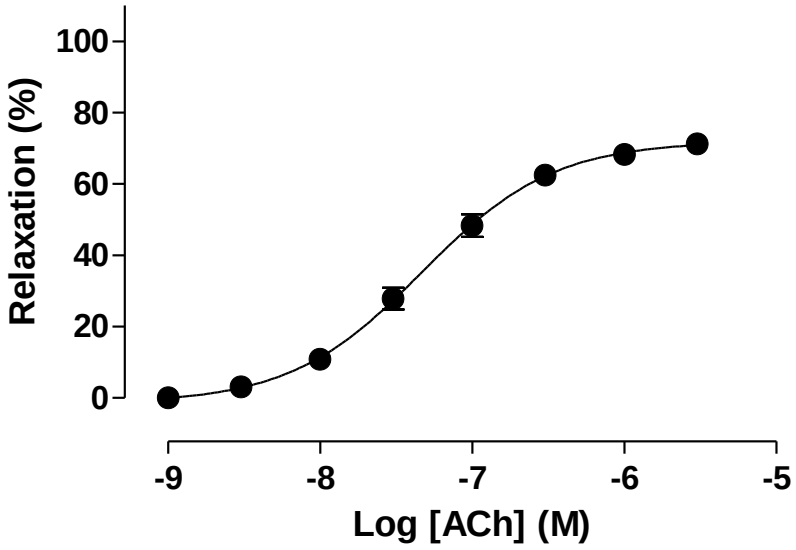


Figure 12. Concentration-response curve to ACh in mouse aortic rings.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone. n=12.

Log EC_{50} (M)	E_{max} (%)
-7.33 ± 0.06	72.02 ± 2.15

Table 8. Log EC_{50} and E_{max} values for ACh-induced relaxation of mouse aortic rings.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone. n=12.

EFFECT OF MK571 IN MOUSE AORTIC RINGS

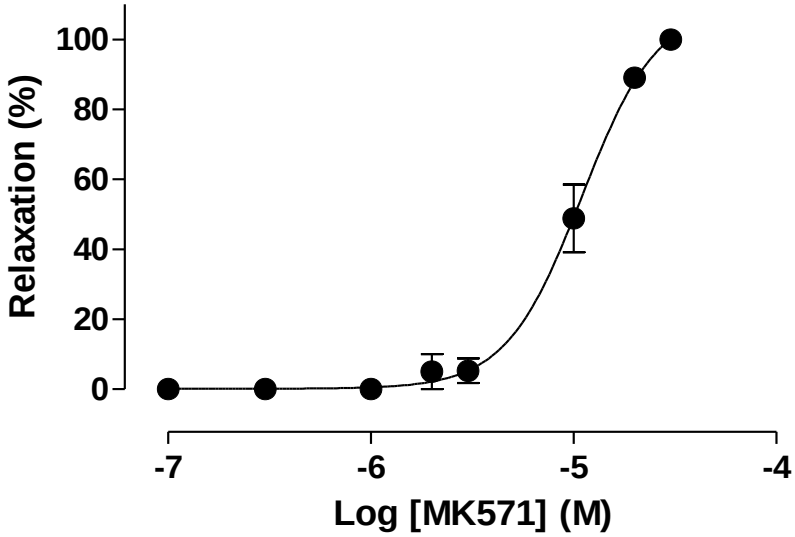


Figure 13. Concentration-response curve to MK571 in mouse aortic rings.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone. n=6.

Log EC_{50} (M)	E_{max} (%)
-4.96 ± 0.05	109.70 ± 10.79

Table 9. Log EC_{50} and E_{max} values for MK571-induced relaxation of mouse aortic rings.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone. n=6.

EFFECT OF PB IN MOUSE AORTIC RINGS

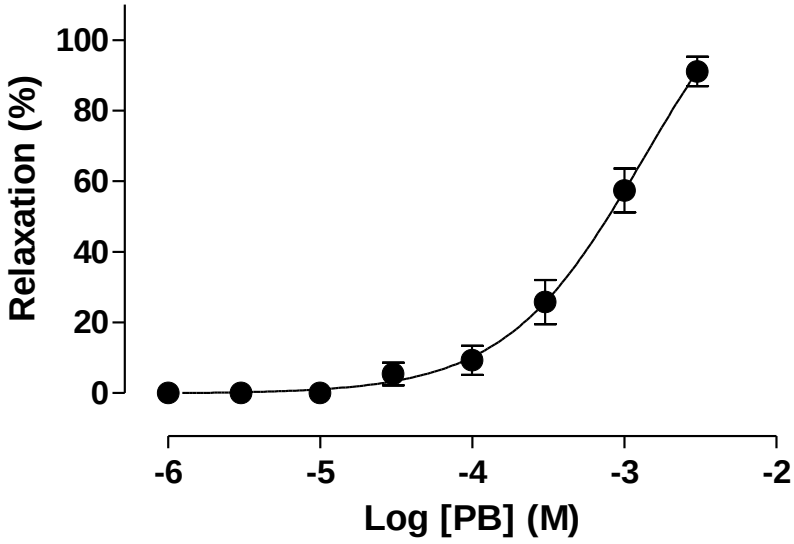


Figure 14. Concentration-response curve to PB in mouse aortic rings.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone. n=4.

Log EC_{50} (M)	E_{max} (%)
-2.87 ± 0.26	133.30 ± 34.63

Table 10. Log EC_{50} and E_{max} values for PB-induced relaxation of mouse aortic rings.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone. n=4.

EFFECT OF ACh IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF MK571

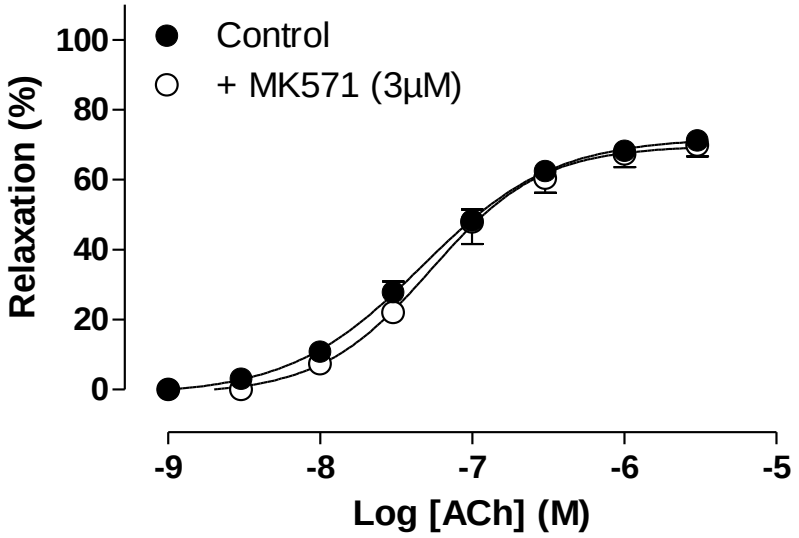


Figure 15. Concentration-response curves to ACh in mouse aortic rings in the absence and presence of MK571.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571. n=6-8.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-7.33 ± 0.055	72.02 ± 2.15
+ MK571 (3 µM)	-7.27 ± 0.072	69.71 ± 2.83

Table 11. Log EC_{50} and E_{max} values for ACh-induced relaxation of mouse aortic rings in the absence and presence of MK571.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571. n=6-8.

EFFECT OF ACh IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF PB

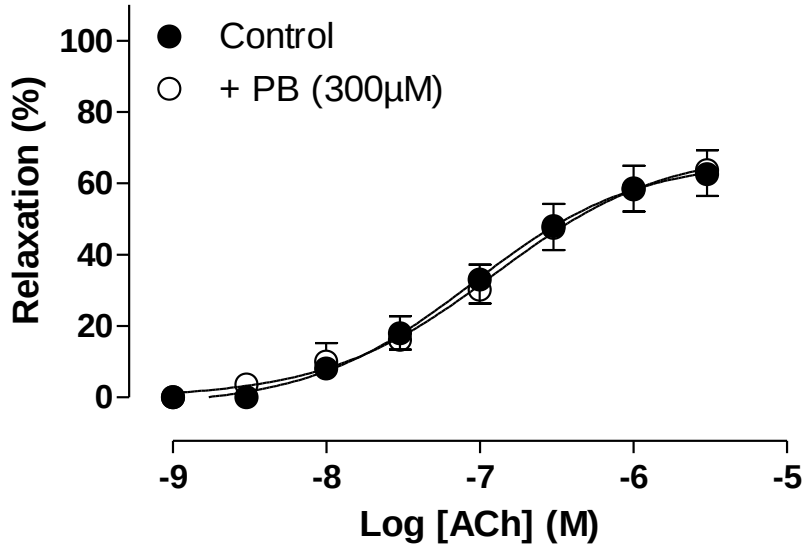


Figure 16. Concentration-response curves to ACh in mouse aortic rings in the absence and presence of PB.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. n=8-12.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-7.05 ± 0.18	66.66 ± 7.86
+ PB (300 µM)	-6.90 ± 0.23	69.36 ± 10.90

Table 12. Log EC_{50} and E_{max} values for ACh-induced relaxations of mouse aortic rings in the absence and presence of PB.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. n=8-12.

EFFECT OF ANP IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF MK571

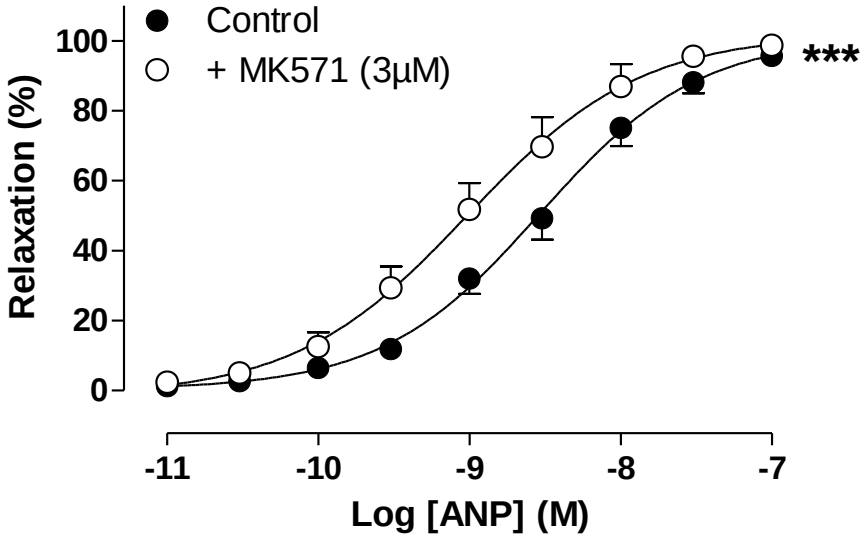


Figure 17. Concentration-response curves to ANP in mouse aortic rings in the absence and presence of MK571.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571. ***P<0.001 v control. n=10-17.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-8.53 ± 0.09	101.00 ± 5.42
+ MK571 (3 µM)	-9.01 ± 0.12	102.00 ± 6.038

Table 13. Log EC_{50} and E_{max} values for ANP-induced relaxation of mouse aortic rings in the absence and presence of MK571.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571. n=10-17.

EFFECT OF ANP IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF PB

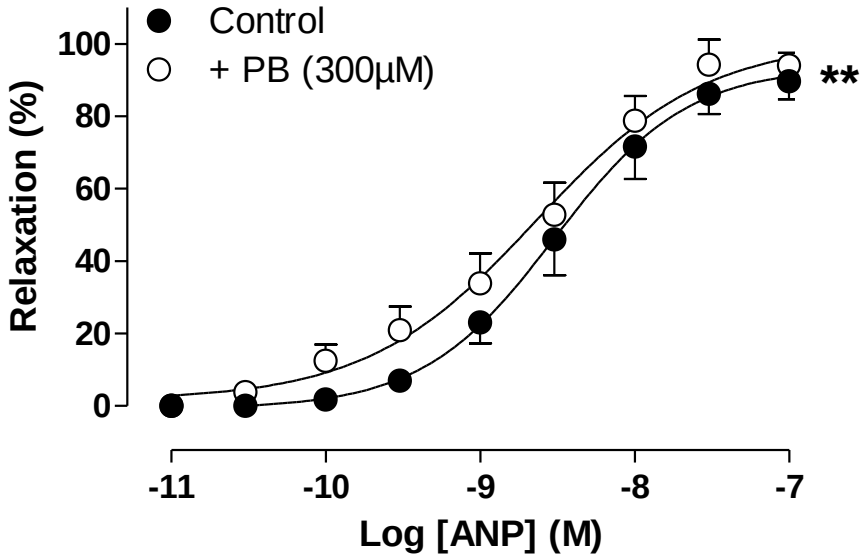


Figure 18. Concentration-response curves to ANP in mouse aortic rings in the absence and presence of PB.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. **P<0.01 v control. n=7-9.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-8.51 ± 0.091	93.71 ± 4.40
+ PB (300 µM)	-8.64 ± 0.12	100.80 ± 5.71

Table 14. Log EC_{50} and E_{max} values for ANP-induced relaxation of mouse aortic rings in the absence and presence of PB.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. n=7-9.

EFFECT OF CNP IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF MK571

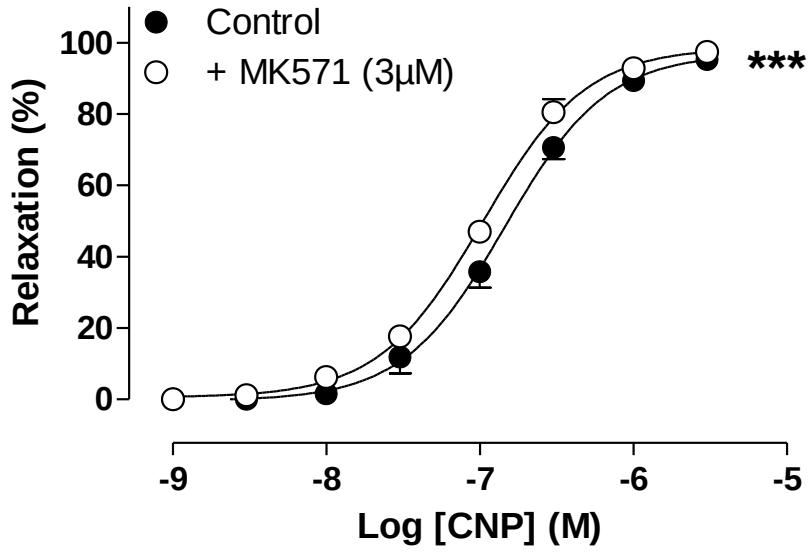


Figure 19. Concentration-response curves to CNP in mouse aortic rings in the absence and presence of MK571.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571. ***P<0.001 v control. n=6-7.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-6.84 ± 0.0.041	96.96 ± 2.96
+ MK571 (3 µM)	-6.98 ± 0.034	98.67 ± 2.28

Table 15. Log EC_{50} and E_{max} values for CNP-induced relaxation of mouse aortic rings in the absence and presence of MK571.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571. n=6-7.

EFFECT OF CNP IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF PB

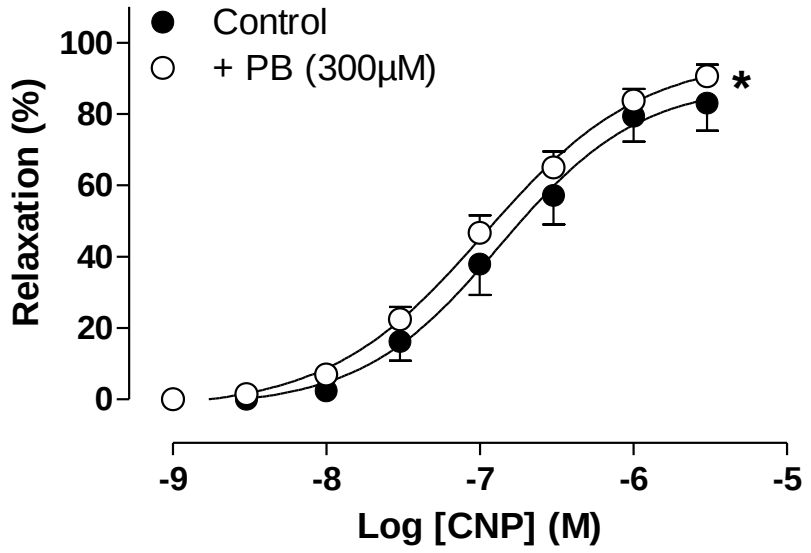


Figure 20. Concentration-response curves to CNP in mouse aortic rings in the absence and presence of PB.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. *P<0.05 v control. n=8-11.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-6.87 ± 0.14	88.50 ± 8.94
+ PB (300 µM)	-6.97 ± 0.08	95.97 ± 5.32

Table 16. Log EC_{50} and E_{max} values for CNP-induced relaxation of mouse aortic rings in the absence and presence of PB.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. n=8-11.

EFFECT OF Sp-NO IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF MK571

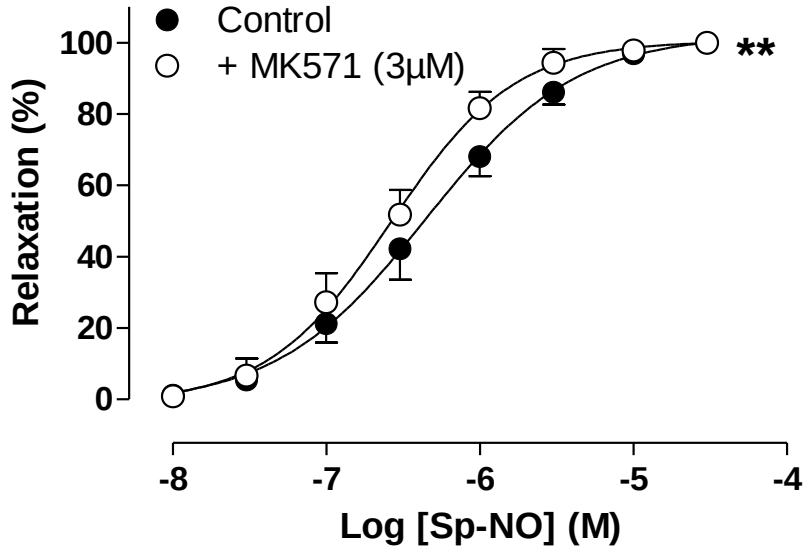


Figure 21. Concentration-response curves to Sp-NO in mouse aortic rings in the absence and presence of MK571.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571 **P<0.01 v control. n=5.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-6.36 ± 0.07	102.40 ± 3.85
+ MK571 (3 µM)	-6.58 ± 0.03	100.50 ± 1.41

Table 17. Log EC_{50} and E_{max} values for Sp-NO-induced relaxation of mouse aortic rings in the absence and presence of MK571.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571. n=5.

EFFECT OF Sp-NO IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF PB

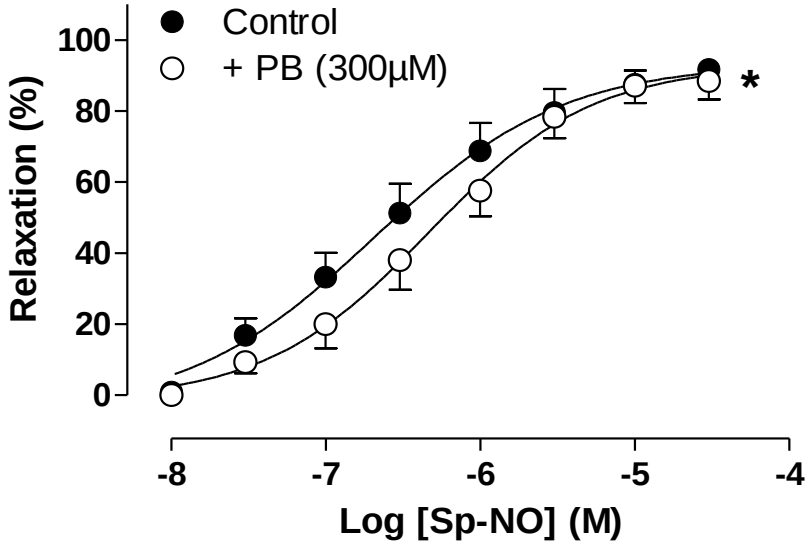


Figure 22. Concentration-response curves to Sp-NO in mouse aortic rings in the absence and presence of PB.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. *P<0.05 v control. n=7-8.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-6.69 ± 0.12	93.43 ± 5.65
+ PB (300 µM)	-6.33 ± 0.12	93.08 ± 5.98

Table 18. Log EC_{50} and E_{max} values for Sp-NO-induced relaxation of mouse aortic rings in the absence and presence of PB.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. n=7-8.

EFFECT OF Iso IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF MK571

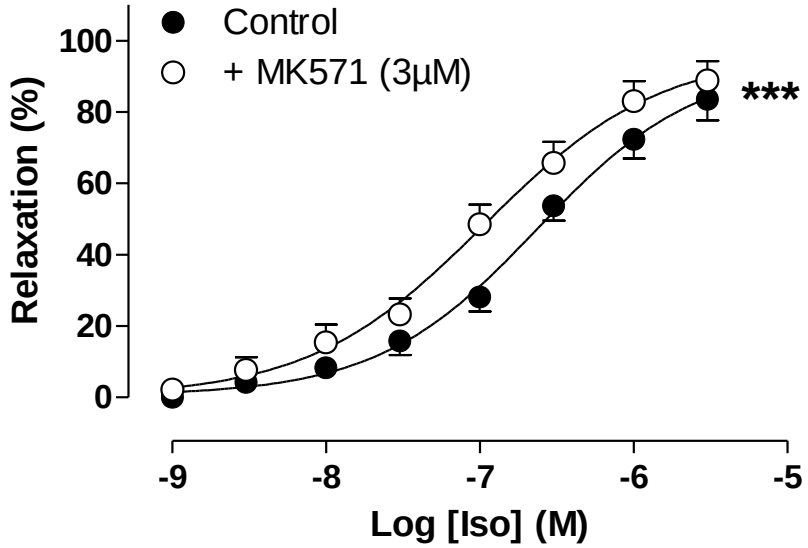


Figure 23. Concentration-response curves to Iso in mouse aortic rings in the absence and presence of MK571.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of MK571. ***P<0.001 v control. n=7-9.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-6.63 ± 0.14	94.03 ± 9.21
+ MK571 (3 µM)	-6.97 ± 0.13	96.65 ± 8.23

Table 19. Log EC_{50} and E_{max} values for Iso-induced relaxation of mouse aortic rings in the absence and presence of MK571.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone by Iso in the absence and presence of MK571. n=7-9.

EFFECT OF Iso IN MOUSE AORTIC RINGS IN THE ABSENCE AND PRESENCE OF PB

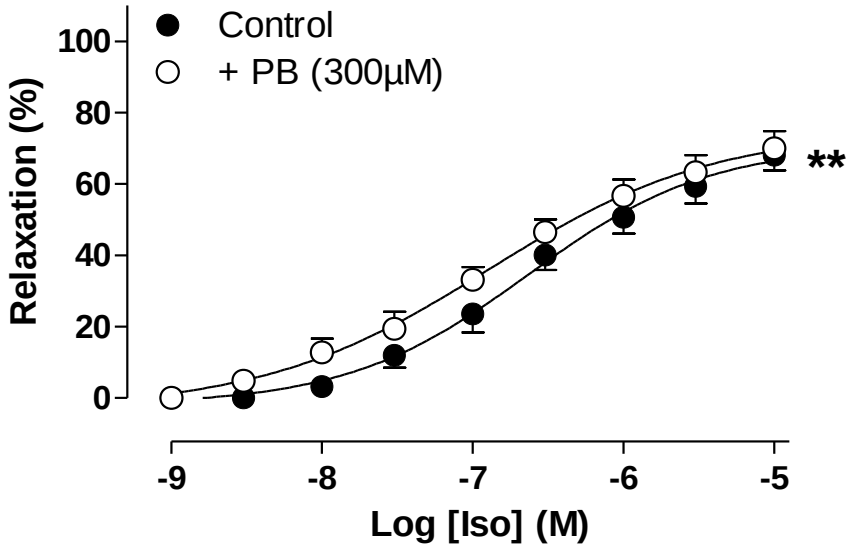


Figure 24. Concentration-response curves to Iso in mouse aortic rings in the absence and presence of PB.

Relaxation is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. **P<0.01 v control. n=7-8.

	Log EC_{50} (M)	**E_{max} (%)**
Control	-6.64 ± 0.13	70.93 ± 5.62
+ PB (300 µM)	-6.90 ± 0.16	75.12 ± 6.86

Table 20. Log EC_{50} and E_{max} values for Iso-induced relaxation of mouse aortic rings in the absence and presence of PB.

Log EC_{50} is expressed as mean ± SEM [M]. E_{max} is expressed as mean ± SEM percentage reversal of PE-induced tone in the absence and presence of PB. n=7-8.

3.2 Effect of MRP inhibition on vascular smooth muscle cell (VSMC) proliferation

Having demonstrated that MRP inhibition augmented cGMP- (and cAMP-) dependent relaxations of isolated aorta, the objective of these experiments was to determine whether MRP inhibition also reduces VSMC proliferation by enhancing cGMP signalling. Human CASMCs were used due to their relevance to human cardiovascular disease (e.g. CAD). Routine culture of hCASMCs produced a uniform monolayer when confluent which contained “hill and valley” formations characteristic of SMCs (Figure 25). Cells seeded at an initial density of 30,000 cells/well grew at an exponential rate under control conditions without becoming confluent (Figure 26). MK571 (30 nM-30 µM) concentration-dependently inhibited hCASMC proliferation compared to control (Figure 26); 30 µM MK571 completely arrested cell growth and changed cell morphology (Figure 26 & Figure 25). Based on these data, a sub-threshold concentration of MK571 (30 nM) was selected to study effects of MRP inhibition on the anti-proliferative effects of the NO-donor D-NO and ANP. D-NO was chosen due to its relatively long half-life (20 h) at 37 ºC compared to other NO donors such as Sp-NO (39 min) and SNP (2 min) since treatments were changed once every 24 h (Keefer, 2005). D-NO (10 µM) and ANP (1 µM) were chosen due to their ability to elicit sub-maximal inhibition of human VSMC proliferation (Bubb *et al*., 2014). D-NO and ANP alone did not significantly alter hCASMC proliferation, although a tendency for both to reduce mean fold-change in cell number was observed (Figure 27 & Figure 28). An essentially identical result was gleaned using the sub-threshold concentration of MK571 (30 nM; Figure 27 & Figure 28). However, when the sub-threshold concentration of MK571 was combined with either ANP (1 µM) or D-NO (10 µM) a significantly enhanced inhibitory effect on hCASMC proliferation was observed compared to control (Figure 27 & Figure 28). Moreover, MK571 combined with D-NO significantly inhibited hCASMC growth compared to D-NO alone (Figure 27), although a similar potentiation was not seen with MK571 plus ANP compared to ANP alone (Figure 28).

REPRESENTATIVE IMAGES OF HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS IN THE ABSENCE AND PRESENCE OF MK571

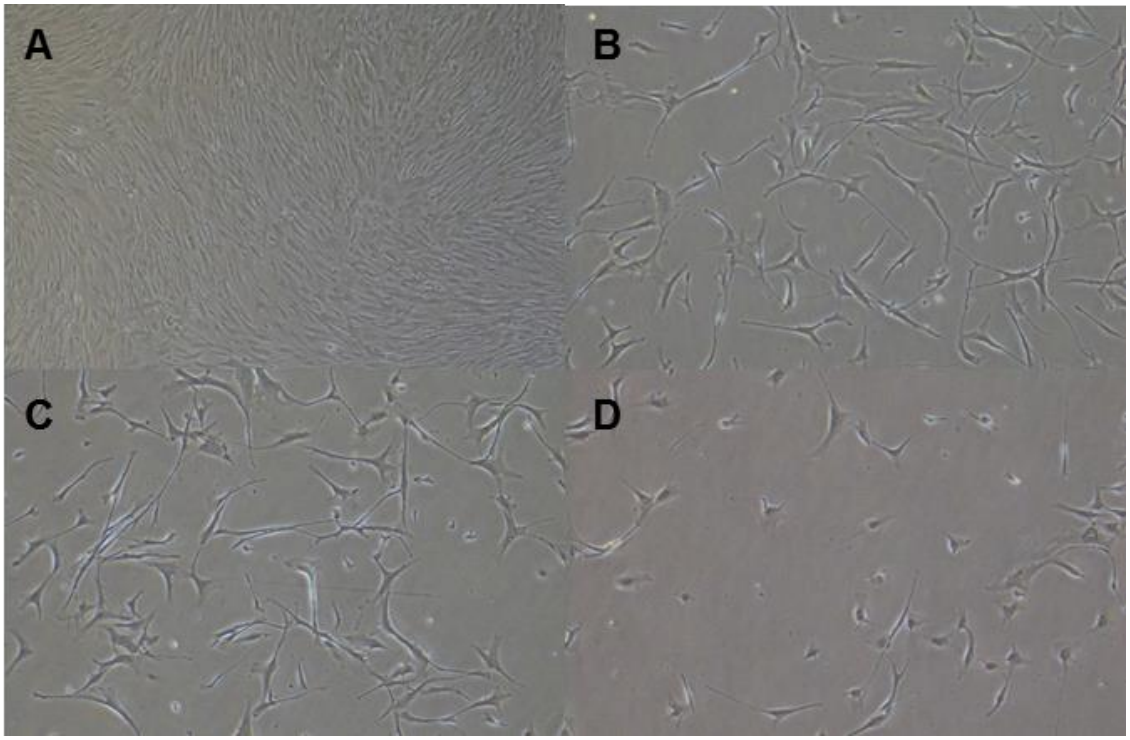


Figure 25. Representative images of hCASMC under routine culture and after seeding in the absence and presence MK571.

Representative images of confluent hCASMCs under routine culture (**A**); hCASMC 24 h after seeding (30,000 cell/well) treated with sterile ddH_2O (control; **B**), MK571 (3 µM; **C**) or MK571 (30 µM; **D**). x4 objective used for image **A**. x20 objective used for image **A**

EFFECT OF MK571 ON THE PROLIFERATION OF HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS

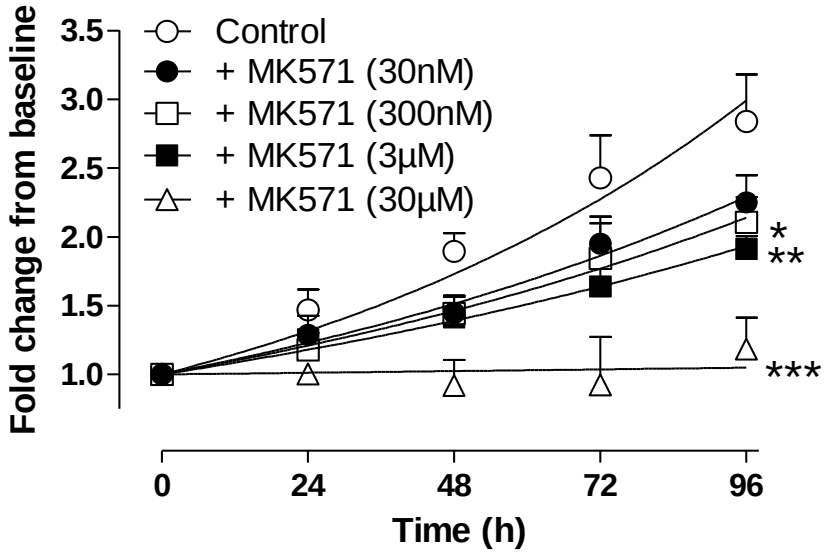


Figure 26. Effect of increasing concentrations of MK571 on hCASMC proliferation.

Proliferation of hCASMC in the absence (control) or in the presence of increasing concentrations of MK571 (30 nM, 300 nM, 3 µM, 30 µM). Data are expressed as mean ± SEM fold change in cell number compared to 0 h. *P<0.05, **P<0.01, ***P<0.001 v control. n=6-10 observations from 3-5 separate experiments**.**

EFFECT OF D-NO ON THE PROLIFERATION OF HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS IN THE ABSENCE AND PRESENCE OF MK571

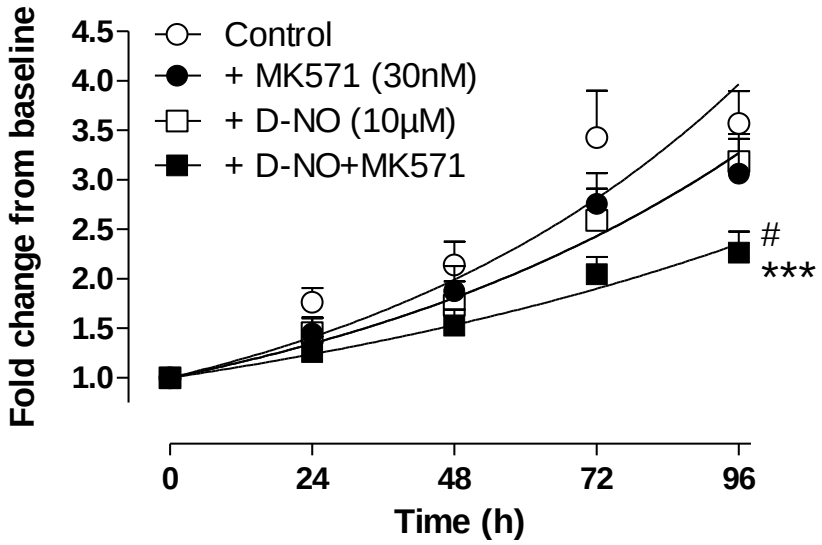


Figure 27. Effect of D-NO in the absence and presence or MK571 on hCASMC proliferation.

Proliferation of hCASMC in the absence (control) or in the presence of MK571 (30 nM), D-NO (10 µM) or MK571 plus D-NO. Data are expressed as mean ± SEM fold change in cell number compared to 0 h. ***P<0.001 v control. $^{\#}$P<0.05 v D-NO. n=10 observations from 5 separate experiments**.**

EFFECT OF ANP ON THE PROLIFERATION OF HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS IN THE ABSENCE AND PRESENCE OF MK571

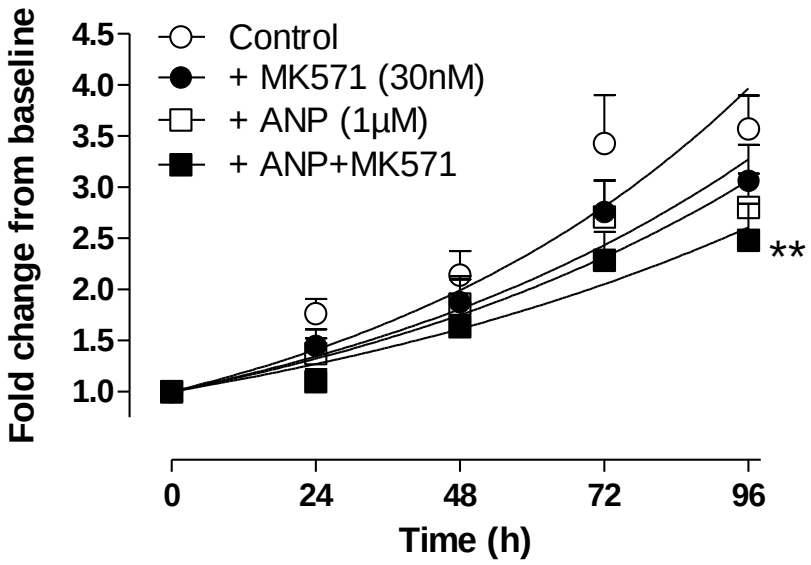


Figure 28. Effect of ANP in the absence and presence or MK571 on hCASMC proliferation.

Proliferation of hCASMC in the absence (control) or in the presence of MK571 (30 nM), ANP (10 µM) or MK571 plus ANP. Data are expressed as mean ± SEM fold change in cell number compared to 0 h. **P<0.01 v control. n=10 observations from 5 separate experiments**.**

3.3 Effect of MRP inhibition on intra and extra - cellular cGMP concentrations

In the previous set of experiments I showed that MRP blockade is able to augment the anti-proliferative effects of NO and NPs. To investigate whether MRP inhibition augments VSMC growth through effects on cGMP dynamics/transport, intracellular and extracellular cGMP concentrations were measured in hCASMC at the 24 h time point, under identical conditions employed for the proliferation assays described above. Human CASMC grown under control conditions show an essentially equal intra and extra -cellular production of cGMP (Figure 29). In hCASMCs treated with MK571 (30 nM, 300 nM and 3 µM), the intra- and extra- cellular cGMP concentrations did not differ significantly from control (Figure 26). Following 24 h treatment with D-NO (10 µM) in the absence or presence of MK571 (30 nM) intra-, or extra- cellular cGMP concentrations did not significantly change compared to control (Figure 30). However, 24 h treatment with ANP (1 µM) produced a marked, statistically-significant (14.91 ± 3.82 fmol/well; $P<0.05$) increase in extracellular cGMP compared to control (3.79 ± 0.56 fmol/well), which showed a trend towards being blunted when combined with MK571 (30 nM; 11.76 ± 3.17 fmol/well; $P>0.05$ v control; Figure 31). Importantly, the intra:extra-cellular ratio of cGMP revealed a significant decrease with ANP treatment (0.37 ± 0.05 intra:extra –cellular ratio; $P<0.01$) compared to control (1.33 ± 0.20 intra:extra –cellular ratio) which was significantly ($P<0.05$) attenuated by the presence of MK571 (30nM; 0.69 ± 0.13 intra:extra –cellular ratio; $P<0.05$ v ANP; Figure 31).

EFFECT OF MK571 ON INTRA- AND EXTRA- CELLULAR cGMP CONCENTRATIONS IN HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS

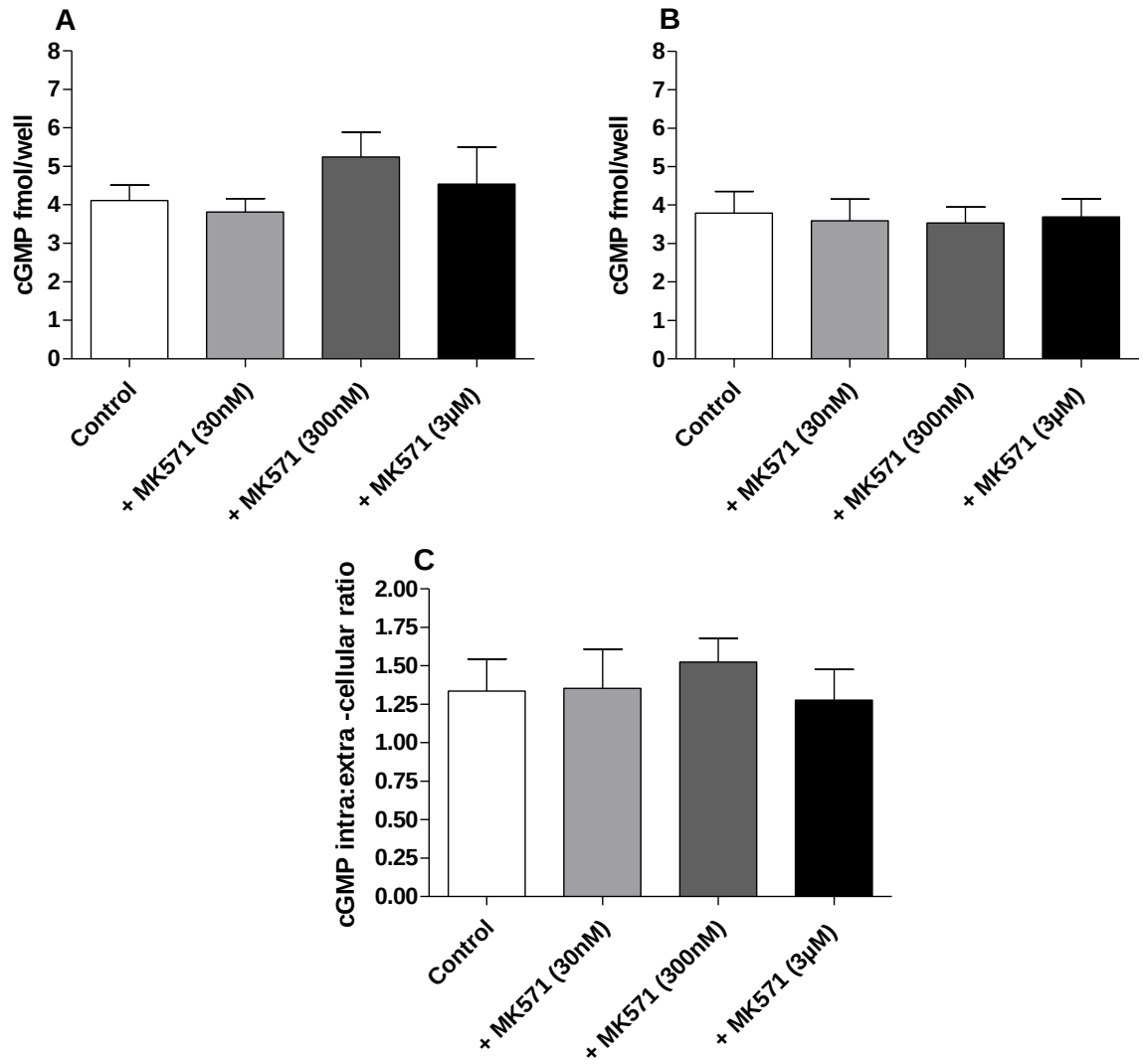


Figure 29. Effect of MK571 on hCASMC cGMP concentrations.

Intracellular (**A**) & extracellular (**B**) cGMP concentrations and intra:extra –cellular cGMP ratio (**C**) in hCASMC in the absence or presence of increasing concentrations of MK571 for 24 h. Data are expressed as mean ± SEM [cGMP] in fmol/well (**A** & **B**), or cGMP intra/extra -cellular ratio (**C**). n=8-12.

EFFECT OF D-NO ON INTRA- AND EXTRA- CELLULAR cGMP CONCENTRATIONS IN HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS IN THE ABSENCE AND PRESENCE OF MK571

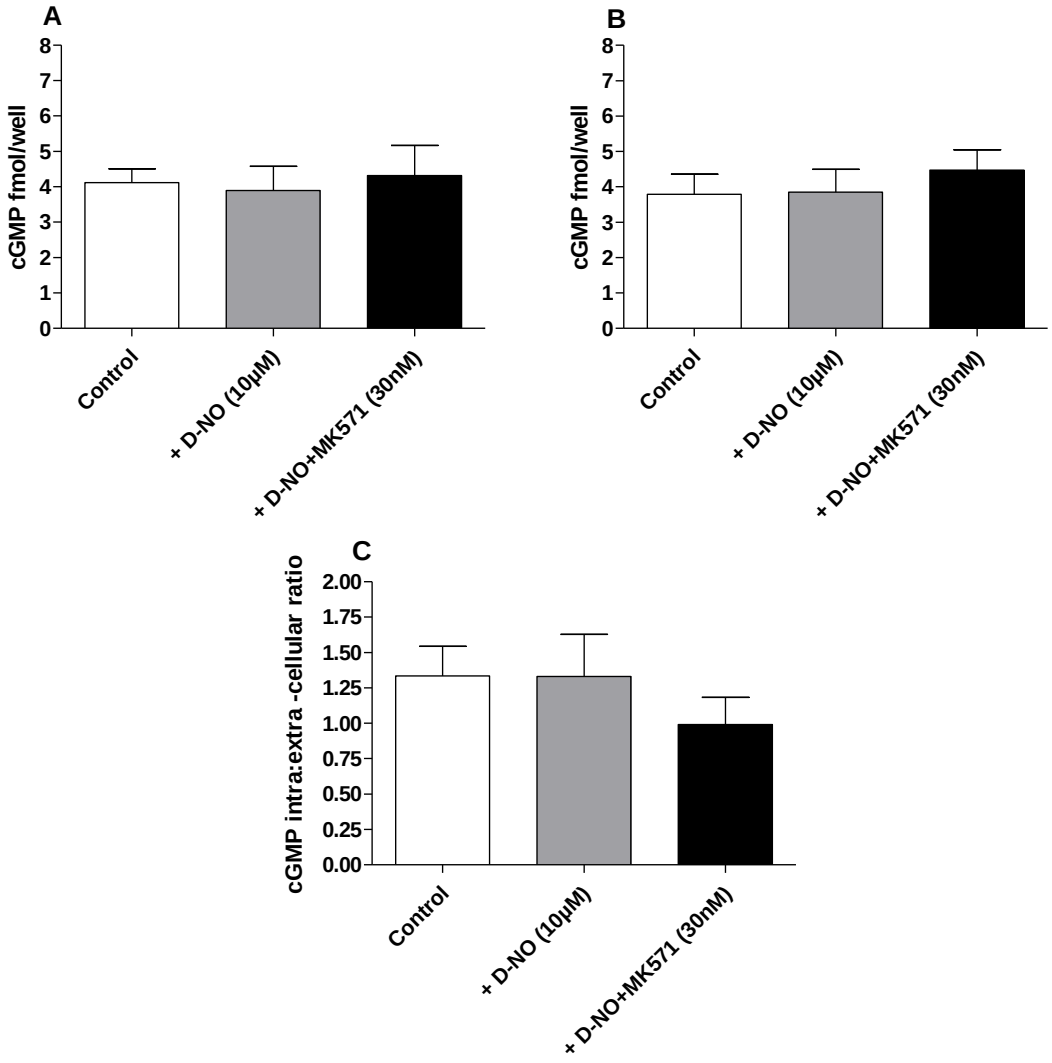


Figure 30. Effect of D-NO on hCASMC cGMP concentrations in the absence and presence of MK571.

Intracellular (**A**) and extracellular (**B**) cGMP concentrations and intra:extra –cellular cGMP ratio (**C**) in hCASMC under control conditions or treated with or D-NO (10 µM) in the absence or presence of MK571 (30 nM). Data are expressed as mean ± SEM [cGMP] in fmol/well (**A** & **B**), or cGMP intra/extra -cellular ratio (**C**). n=8-12.

EFFECT OF ANP ON INTRA- AND EXTRA- CELLULAR cGMP CONCENTRATIONS IN HUMAN CORONARY ARTERY SMOOTH MUSCLE CELLS IN THE ABSENCE AND PRESENCE OF MK571

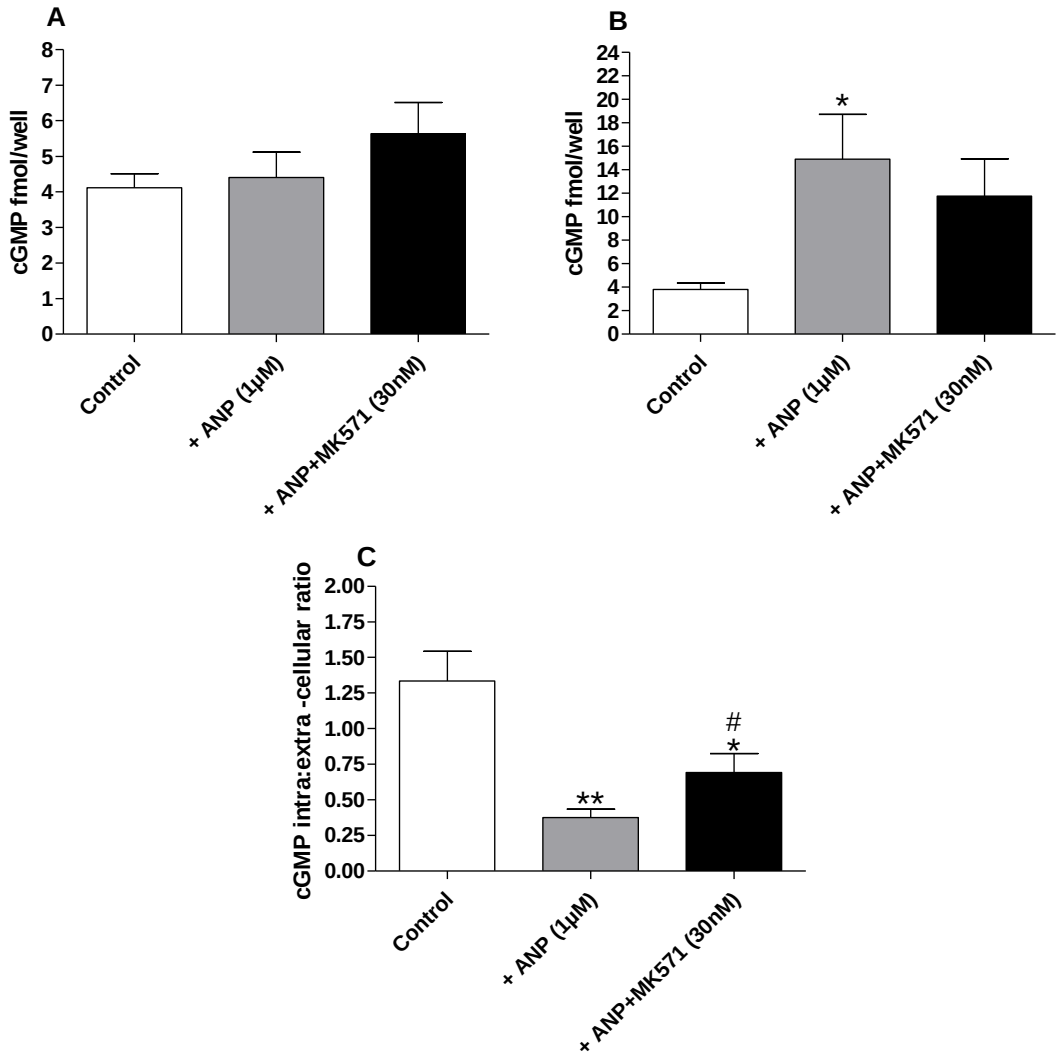


Figure 31. Effect of ANP on hCASMC cGMP concentrations in the absence and presence of MK571.

Intracellular (**A**) and extracellular (**B**) cGMP concentrations and intra:extra –cellular cGMP ratio (**C**) in hCASMC under control conditions or treated with ANP (1 μM) in the absence or presence of MK571 (30 nM). Data are expressed as mean ± SEM [cGMP] in fmol/well (**A** & **B**), or cGMP intra/extra –cellular ratio (**C**). *P<0.05, **P<0.01 v control. $^{\#}$P<0.05 v ANP. n=8-12.

3.4 Effect of MRP inhibition on acute changes in blood pressure

My *in vitro* observations in isolated blood vessels and cells suggest that inhibition of MRPs augments the vasorelaxant and anti-proliferative activity of NO and ANP, and that this is underpinned, at least in the case of ANP, by a change in the intra:extra cellular -cGMP ratio (i.e. cGMP efflux via MRPs). These data identify MRPs as playing a key role in regulating cGMP biology in the cardiovascular system, particularly in the case of natriuretic peptides. To determine if such an influence was also functionally apparent *in vivo*, the effect of MRP inhibition on NO- and ANP- induced acute changes in blood pressure was explored in anaesthetised mice.

Administration of 0.0001, 0.001, 0.01, 0.1, 1 and 3 mg/kg MK571 caused a small decrease in MABP that was indistinguishable compared to saline (Figure 32). Accordingly, the highest dose (3 mg/kg) MK571 was used in subsequent experiments to assess the effect of MRP inhibition on haemodynamic responses to NO and ANP. The NO-donor SNP (1, 3, 10 µg/kg; i.v. bolus) and ANP (1, 10, 100 µg/kg; i.v. bolus) were chosen due to their ability to elicit dose-dependent reductions in MABP (Madhani *et al*., 2006). SNP produced a dose-dependent decrease in MABP (1 µg/kg: △MABP = -4.42 ± 1.34 mm Hg; 3 µg/kg: △MABP = -11.83 ± 1.63 mm Hg; 10 µg/kg: △MABP = -23.85 ± 1.59 mm Hg; Figure 33) which was not significantly altered when combined with MK571 (3 mg/kg; Figure 33). ANP also produced dose-dependent decreases in MABP (1 µg/kg: △MABP = -2.79 ± 1.46 mm Hg; 10 µg/kg: △MABP = -7.92 ± 1.98 mm Hg; 100 µg/kg: △MABP= -11.07 ± 1.64 mm Hg; Figure 34); however, in this case the hypotensive response to ANP was significantly ($P<0.01$) enhanced when combined with MK571 3 mg/kg; (1 µg/kg: △MABP = -10.37 ± 2.00 mm Hg; 10 µg/kg: △MABP = -11.72 ± 2.66 mm Hg; 100 µg/kg: △MABP = -17.20 ± 1.91 mm Hg; Figure 34).

Administration of MK571 at the doses above caused a small decrease in HR but

this was indistinguishable compared to saline (Figure 32). SNP alone produced a significant, dose-dependent increase in HR which was not significantly altered when combined with MK571 (Figure 33). ANP alone did not produce dose-dependent changes in HR and this was not significantly altered when combined with MK571 (Figure 34).

EFFECT OF MK571 ON BLOOD PRESSURE AND HEART RATE IN ANAESTHETISED MICE

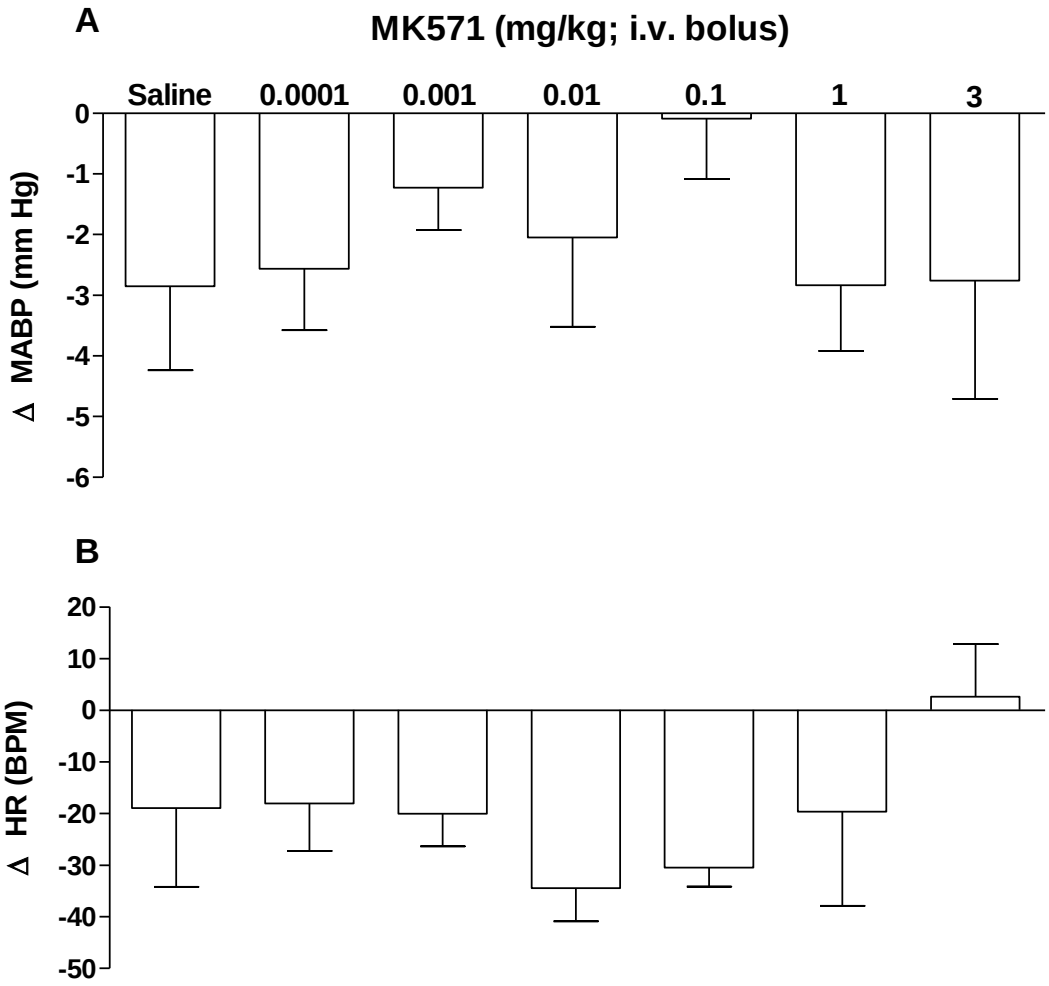


Figure 32. Effect of MK571 on blood pressure and heart rate in anaesthetised mice.

Mean arterial blood pressure (MABP; **A**) and heart rate (HR; **B**), in anaesthetised mice treated with vehicle (saline) or increasing doses of MK571 (0.001-3 mg/kg; i.v. bolus). Data are expressed as mean ± SEM. n=5.

EFFECT OF SNP ON BLOOD PRESSURE AND HEART RATE IN THE ABSENCE AND PRESENCE OF MK571 IN ANAESTHETISED MICE

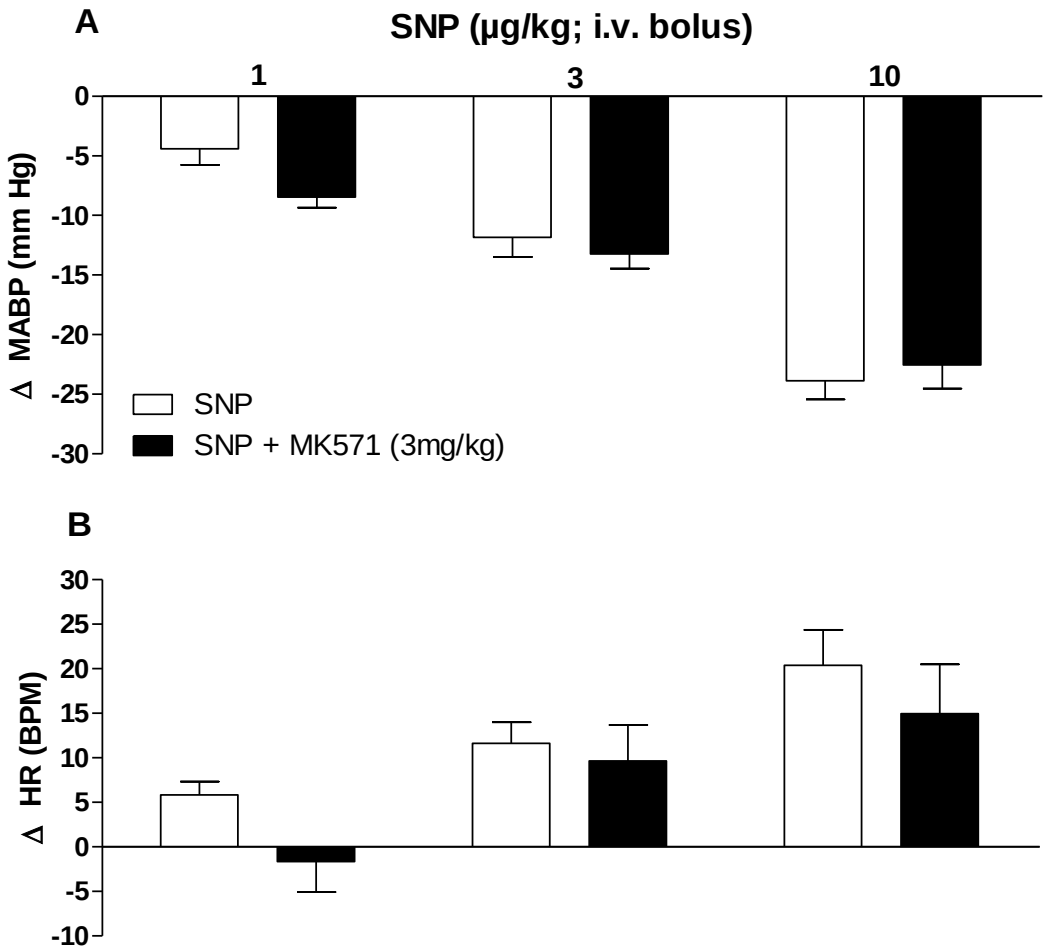


Figure 33. Effect of SNP on blood pressure and heart rate in the absence and presence of MK571 in anaesthetised mice.

Mean arterial blood pressure (MABP; **A**) and heart rate (HR; **B**), in anaesthetised mice treated with SNP (1, 3, 10 µg/kg; i.v. bolus) in the absence and presence of MK571 (3 mg/kg; i.v. bolus). Data are expressed as mean ± SEM. n=6.

EFFECT OF ANP IN THE ABSENCE AND PRESENCE OF MK571 ON BLOOD PRESSURE AND HEART RATE IN ANAESTHETISED MICE

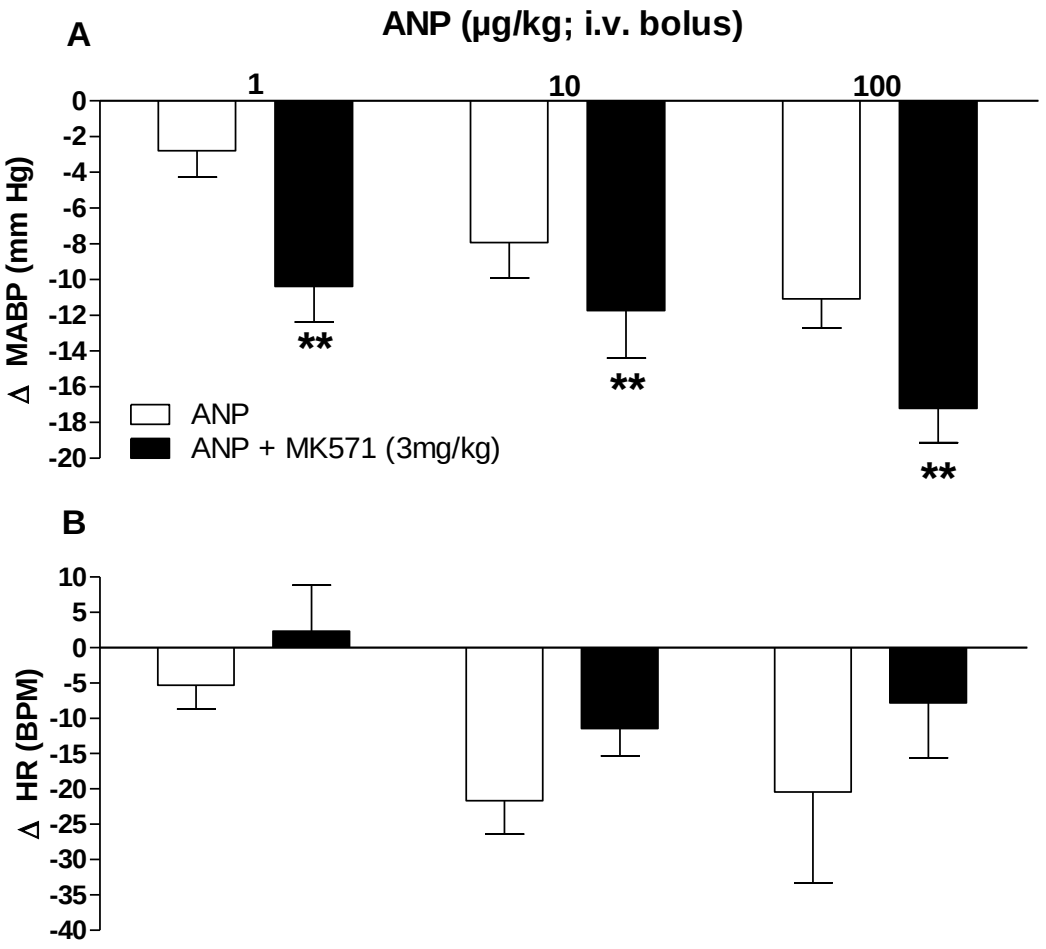


Figure 34. Effect of ANP on blood pressure and heart rate in the absence and presence of MK571 in anaesthetised mice.

Mean arterial blood pressure (MABP; **A**) and heart rate (HR; **B**), in anaesthetised mice treated with ANP (1, 10, 100 µg/kg; i.v. bolus) in the absence and presence of MK571 (3 mg/kg; i.v. bolus). Data are expressed as mean ± SEM. **P<0.05 v ANP alone. n=5.

3.5 Effect of MRP inhibition on blood pressure in conscious, telemeterised mice

Since my initial *in vivo* studies in anaesthetised mice suggested that MRP inhibition with MK571 can acutely enhance the hypotensive activity of ANP, I proceeded to conduct investigations to determine if a more chronic administration of MK571 was able to modulate blood pressure *in vivo*. Male WT C57/BL6 mice were implanted with a telemetry probes, allowing continuous measurement of MABP, HR and activity. Data shown in this section are over a 24 h period. Baseline recording were made over one weekend and then mice administered MK571 (25 mg/kg/day) in the drinking water 12 h before haemodynamic parameters were recorded a second time the following weekend.

3.5.1 Mean arterial blood pressure (MABP)

Mice had a normal circadian rhythm with respect to MABP during the 24 h time period (Figure 35). There were no significant differences in MABP over the whole 24 h or during either the 12 h light or dark phase in the absence or presence of MK571 (Figure 36).

3.5.2 Heart rate (HR)

Mice exhibited a normal HR circadian rhythm over the 24 h time period (Figure 35). HR was significantly ($P<0.05$) reduced in mice treated with MK571 (24 h period: 564.60 ± 5.16 bpm; light phase: 512.6 ± 5.72 bpm) compared to baseline (24 h period: 581.1 ± 5.15 bpm; light phase: 532.7 ± 5.21 bpm), during the light (inactive) phase and over the whole 24 h period, but not during the dark (active) phase (Figure 36).

3.5.3 Activity

Mice exhibited a normal circadian rhythm of activity over the 24 h time period with marked increases in the dark phase, as expected (Figure 35). Activity tended to be reduced when treated with MK571 over the whole 24 h period and during the light and dark phases (Figure 36). However, it was only significantly ($P<0.05$) reduced during the dark phase (10.32 ± 0.57 a.u., 8.83 ± 0.47 a.u., baseline and MK571 treated, respectively; Figure 36).

EFFECT OF MK571 ON THE CIRCADIAN RHYTHM OF BLOOD PRESSURE, HEART RATE AND ACTIVITY IN CONSCIOUS TELEMETERISED MICE

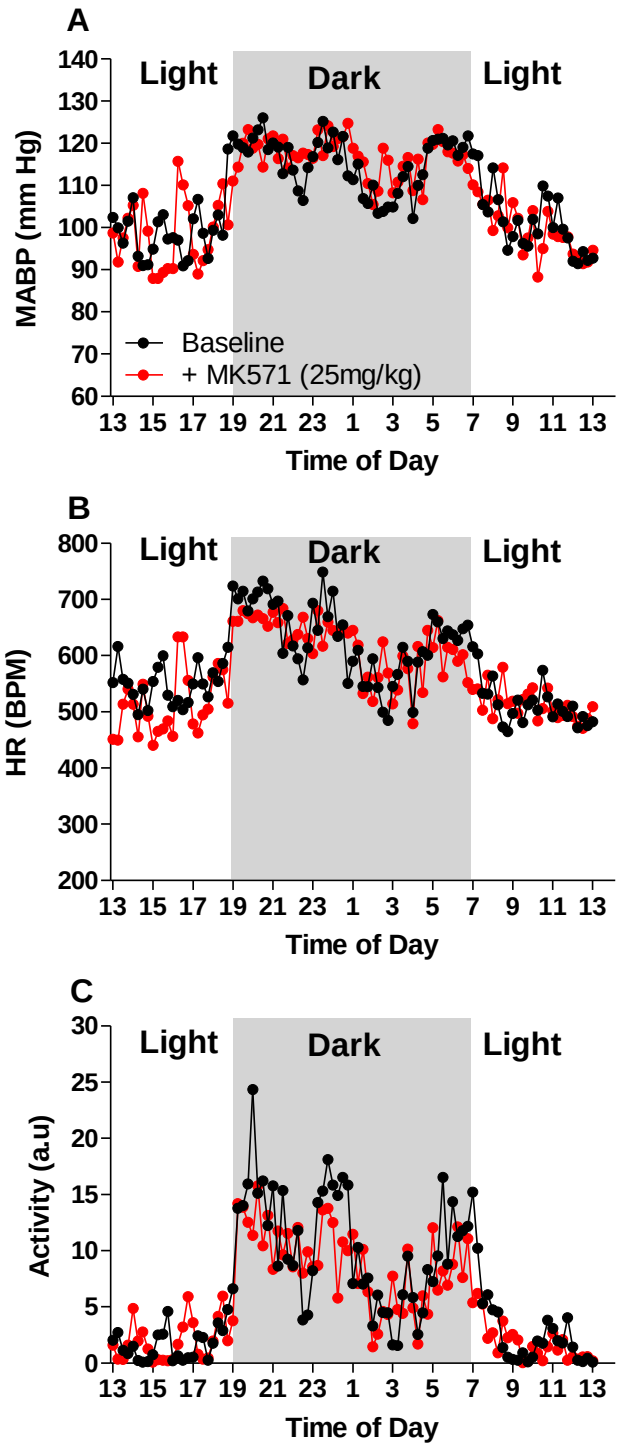


Figure 35. Effect of MK571 on the circadian rhythm of blood pressure, heart rate and activity in conscious telemeterised mice.

MABP (**A**), HR (**B**), and activity (**C**) in mice under control conditions (normal drinking water; baseline) or treated with MK571 (25 mg/kg/day; p.o). n=4.

EFFECT OF MK571 ON BLOOD PRESSURE, HEART RATE AND ACTIVITY IN CONSCIOUS TELEMETERISED MICE

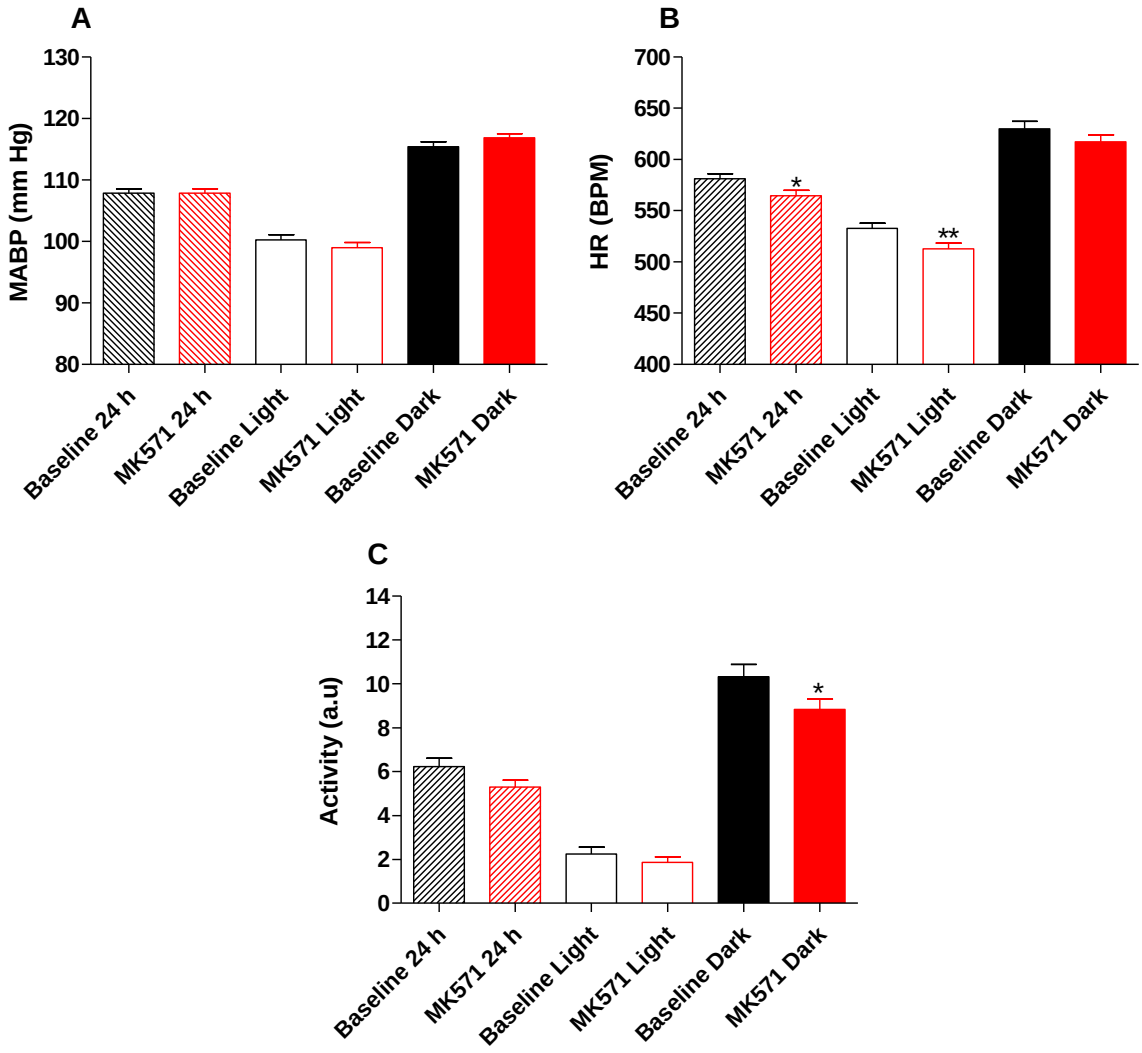


Figure 36. Effect of MK571 on blood pressure, heart rate and activity in conscious telemeterised mice.

MABP (**A**), HR (**B**), and activity (**C**) in mice under control conditions (normal drinking water; baseline) or treated with MK571 (25 mg/kg/day; p.o.; for 24 h). Data represented as mean ± SEM over 24 h. *P<0.05, **P<0.01, v baseline. n=4.

CHAPTER 4

RESULTS 2

CHAPTER 4: RESULTS 2

4 Investigation of the role of CNP and its cognate receptor NPR-C in experimental PH

4.1 Optimising the mouse hypoxia plus SU5416 model of PH

As outlined in the introduction, there are several animal models of PH that are utilised to replicate the human condition. The one I chose to use is an adaptation of a classic model of PH, that associated with chronic hypoxia (10 % O_2), except that additionally I administered the VEGFR-2 inhibitor, SU5416. This model was originally described in rats Taraseviciene-Stewart *et al*., (2001) and produces pathohistological pulmonary vascular lesions which are progressive and non-reversible (upon return to normoxia), and therefore more akin to the human disease than chronic hypoxia alone or indeed other animal models of PH; as such, the hypoxia plus SU5416 model is largely considered the gold standard for this disease (Abe *et al*., 2010). A mouse hypoxia plus SU5416 model was first described by Ciuclan *et al*., (2011), involving the combination of chronic hypoxia (3 weeks) with repeat doses of SU5416 which generated a more severe phenotype; higher RVH (Fulton's Index), RVSP and the presence of pulmonary vascular "complex" lesions. Furthermore, this report confirmed that the pulmonary vascular remodelling contained markers of EC apoptosis/SMC proliferation and when the mice were returned to normal laboratory conditions they retained PH pathology; this again more closely mimics the disease found in humans and in sharp contrast to chronic hypoxia only (reversible pathology and predominantly SMC proliferation not EC apoptosis).

Since this model had not been utilised in our laboratory previously, initial studies

were designed with the goal of optimising the protocol. SU5416 is available commercially (Tocris Bioscience, Bristol, UK), but due to its considerable cost, in-house synthesis was conducted by Prof. D. Selwood (UCL, London, UK) according to a published method (Sun *et al*., 1998). In a first set of studies, I compared the efficacy of equal doses of SU5416 sourced from either Tocris ('Tocris SU5416') or synthesised by Prof Selwood ('DS SU5416') in the hypoxia plus SU5416 experimental mouse model of PH described above (Ciuclan *et al*., 2011). This approach allowed me to assess the relative potency of both SU5416 preparations and concomitantly optimise the hypoxic exposure.

Male C57/BL6 mice were injected once a week, for 3 weeks with SU5416 (20 mg/kg; s.c.) and exposed to varying periods of hypoxia (10% O_2, normobaric, 21 or 25 days).

4.1.1 Body weight (BW)

Under control conditions mice steadily gained weight over the complete 35 day period (Figure 37). However, animals exposed to hypoxia plus SU5416 rapidly (within 3 days) lost body weight (BW; Figure 37). This drop in BW was maintained at a constant level for the whole 35 day period and did not differ significantly between the two SU5416 sources (Figure 37). After 21 days hypoxia, once mice were returned to normoxia, they gained weight at a steady rate, comparable to that observed under normoxic/control conditions (Figure 37). Thus, after a further 14 days in normoxia, mouse BW had returned to the value at the start of the experiment (Figure 37). This effect of hypoxia plus SU5416 on mouse body weight mirrored that observed originally by Ciuclan *et al*., (2011).

4.1.2 Right ventricular systolic pressure (RVSP)

Mice administered SU5416 from either source and exposed to 21 days hypoxia developed significantly ($P<0.001$) increased RVSP (46.9 ± 2.28 mm Hg & 46.7

± 3.08 mm Hg, Tocris & DS SU5416, respectively) compared to normoxia controls (33.8 ± 0.61 mm Hg; Figure 38). A further 14 days hypoxia (35 days hypoxia in total) resulted in similar RVSP values to that observed at 21 days that remained significantly greater than controls (Figure 38). Returning the mice to normoxia following 21 days hypoxia ('Reversal') resulted in a decrease in RVSP (Figure 38). In this setting, mice treated with Tocris SU5416 (41.2 ± 0.98 mm Hg) maintained an RVSP significantly ($P<0.05$) greater than control (Figure 38), whereas in animals receiving DS SU5416 the RVSP (38.1 ± 0.97 mm Hg) was no longer statistically higher than normoxic mice (Figure 38). However, overall there were no significant differences in RVSP under any conditions when comparing animals treated with either Tocris SU5416 or DS SU5416 (Figure 38). These change in RVSP are similar to those originally reported by Ciuclan *et al*., (2011).

4.1.3 Mean arterial blood pressure (MABP)

MABP was not significantly altered by hypoxia, nor the SU5416 from either source, under any experimental conditions (Figure 38).

4.1.4 Right ventricular hypertrophy (RVH)

RVH followed a similar pattern to that seen with RVSP (Figure 39). After 21 days hypoxia, RVH was significantly increased (as measured by the RV:[LV+S] ratio; Figure 39). A further increase in RVH was not observed when 35 days hypoxia was compared to 21 days, but at 35 days RVH remained significantly increased compared to control (Figure 39). Mirroring effects on RVSP, RVH was also decreased in the reversal group and no longer significantly greater than control (Figure 39). The effect I observed of hypoxia plus SU5416 on RVH relates closely to what was reported by Ciuclan *et al*., (2011). Importantly, no significant difference in RVH was observed between Tocris and DS SU5416. Thus, since these data established that SU5416 synthesised by Prof. Selwood was functionally equivalent to that commercially available, the former source

was used for all subsequent experiments. Moreover, since 21 days hypoxia produced an essentially identical phenotype to 35 days hypoxia, the former time period was employed for further study.

4.1.5 Pulmonary vascular remodelling

The lungs of mice exposed to 5 weeks hypoxia plus SU5416, which is the most severe form of this model, were inspected for the presence of complex lesions and occluded vessels, a hallmark of PAH found in humans and in the rat hypoxia plus SU5416 model. No complex lesions or occluded vessels were found in these mouse lungs (data not shown).

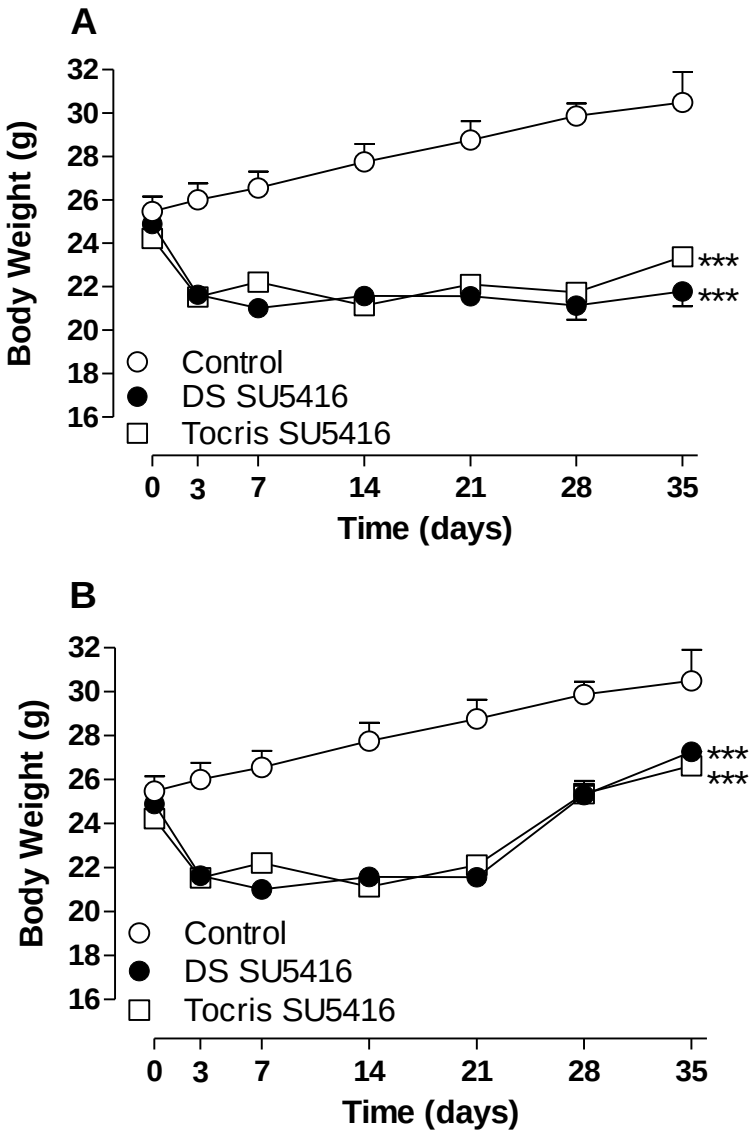


Figure 37. Effect of hypoxia plus SU5416 on body weight.

Body weight at days 0, 3, 7, 14, 21, 28 and 35 of mice administered vehicle (Control), SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) synthesised in-house (DS SU5416) or purchased from Tocris (Tocris SU5416) and exposed to either normoxia (21 % O_2; control) or hypoxia (10 % O_2) for 35 days (**A**), or 21 days hypoxia followed by 14 days normoxia (**B**). Data represented as mean ± SEM. ***P<0.001 v control. n=8-21.

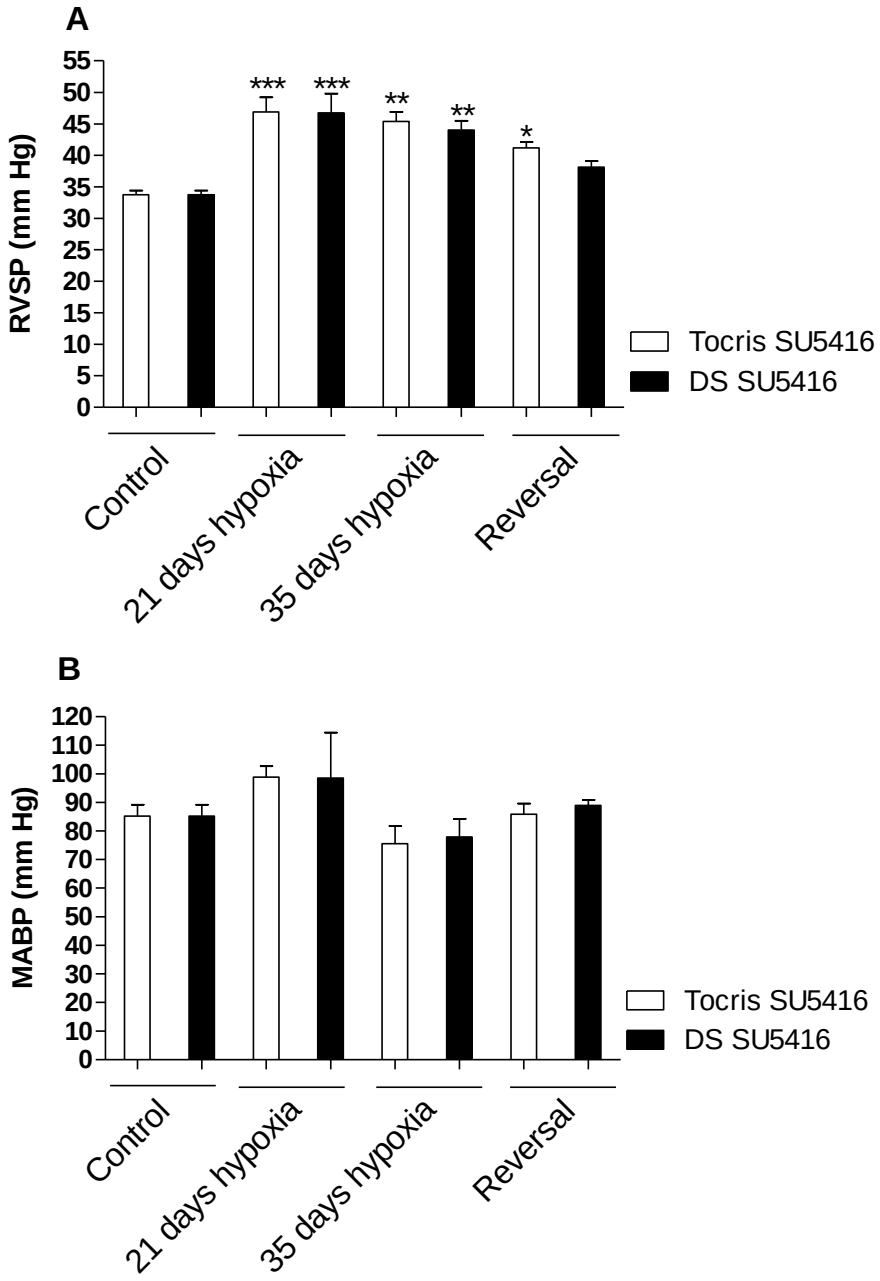


Figure 38. Effect of hypoxia plus SU5416 on haemodynamics.

Right ventricular systolic pressure (RVSP; **A**) and mean arterial blood pressure (MABP; **B**) in mice administered vehicle (control), SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) synthesised in-house (DS SU5416) or purchased from Tocris (Tocris SU5416) and exposed to either normoxia (21 % O_2; control) for 35 days, hypoxia (10 % O_2) for 21 or 35 days, or 21 days hypoxia followed by 14 days normoxia (reversal). Data represented as mean ± SEM. ***P<0.001, **P<0.01, *P<0.05 v control. n=3-7.

EFFECT OF HYPOXIA PLUS SU5416 ON RIGHT VENTRICULAR HYPERTROPHY

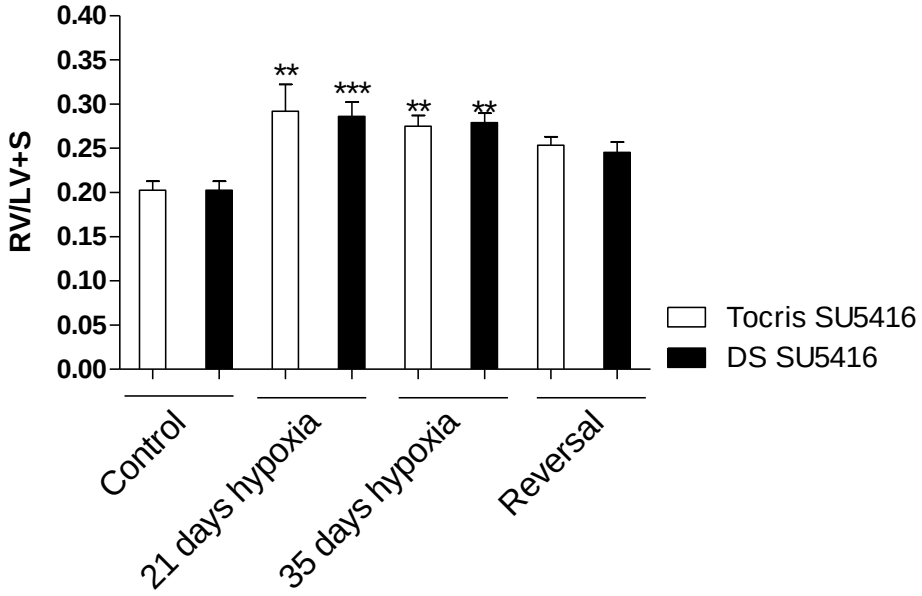


Figure 39. Effect of hypoxia plus SU5416 on right ventricular hypertrophy.

Right ventricle to left ventricle plus septum ratio (RV/[LV+S]) in mice administered vehicle (control), SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) synthesised in-house (DS SU5416) or purchased from Tocris (Tocris SU5416) and exposed to either normoxia (21 % O_2; control) for 35 days, hypoxia (10 % O_2) for 21 or 35 days, or 21 days hypoxia followed by 14 days normoxia (reversal). Data represented as mean ± SEM. **P<0.01, ***P<0.001 v control. n=3-7.

4.2 Effect of endothelial-specific deletion of CNP on the development of experimental pulmonary hypertension

Previous work from our laboratory demonstrated that endothelium-derived CNP plays a pivotal role in regulating local vascular tone, systemic blood pressure, and the reactivity of platelets and leukocytes (Moyes *et al*., 2014). In the pulmonary circulation, infusion of CNP has been reported to be ineffective in reversing the increased pressure associated with pulmonary hypertension (Casserly *et al*., 2011). However, as CNP is metabolised rapidly by peptides in the pulmonary circulation, particularly NEP (Kenny *et al*., 1993), I designed experiments to discover if endogenous CNP played a pathophysiological role in the development of PH. To achieve this goal, I exploited the endothelial-specific CNP knockout (ecCNP KO) mouse developed by our lab (Moyes *et al*., 2014). For this set of investigations, ecCNP KO and WT littermate mice (male and female; 16 week old; C57/BL6 background) were injected once a week, for 3 weeks, with SU5416 (20 mg/kg; s.c.) and exposed to 21 days hypoxia (10 % O_2, normobaric).

4.2.1 Body weight (BW)

4.2.1.1 Male

In the first 3 days both WT and ecCNP KO mice lost BW, similar to that observed during the optimisation experiments and previously reported by Ciuclan *et al*., (2011; Figure 40). Subsequently, the BW of both groups remained steady for the entire 21 day hypoxic period (Figure 40). Although WT (30.93 ± 0.68 g) mice were on average marginally heavier than KO animals (29.43 ± 0.55 g) the percentage loss in BW between day 0 and 21 was comparable (WT: -11.2 ± 3.0 %; KO: -10.2 ± 1.7 %; P>0.05; Figure 40).

4.2.1.2 Female

Female WT (normoxic 24.68 ± 0.85 g) were also heavier than ecCNP KO (normoxic 23.03 ± 0.85 g; P>0.05; Figure 41). Both WT and KO mice lost BW steadily over the first 7 days of hypoxia which then remained constant for the remainder of the experiment (Figure 41). As for the male animals, the percentage decrease in BW between day 0 and 21 was not significantly different between WT and KO (-14.5 ± 2.16 %, -12.3 ± 4.45 % WT and KO respectively; P>0.05; Figure 41).

4.2.2 Right ventricular systolic pressure (RVSP)

4.2.2.1 Male

Hypoxia significantly increased RVSP in male WT (37.7 ± 1.26 mm Hg; P<0.01) and ecCNP KO (40.8 ± 1.56 mm Hg; P<0.001) mice compared to their corresponding normoxic controls (29.8 ± 0.93 and 28.7 ± 1.56 mm Hg, WT and KO, respectively). Although, there was no significant difference in RVSP between WT and KO mice exposed to hypoxia plus SU5416, even when comparing change in RVSP for both WT (∆7.9 ± 1.26 mm Hg) and KO (∆12.1 ± 1.57 mm Hg) animals (Figure 46 and Figure 42).

4.2.2.2 Female

The RVSP in female WT (38.2 ± 1.76 mm Hg, P<0.001) and ecCNP KO (38.2 ± 1.52 mm Hg, P<0.001) mice increased significantly, compared to normoxic controls (28.3 ± 0.87 and 28.4 ± 2.11 mm Hg, WT and KO, respectively), in response to hypoxia plus SU5416 regardless of genotype (Figure 43). Again, there was no significant difference in the magnitude of this RVSP increase when comparing WT (∆9.9 ± 1.77 mm Hg) with KO (∆9.8 ± 1.52 mm Hg; Figure 47).

4.2.3 Mean arterial blood pressure (MABP)

4.2.3.1 Male

MABP was essentially identical, regardless of genotype, between normoxia and hypoxia plus SU5416 (Figure 42).

4.2.3.2 Female

An identical profile was observed in female WT and ecCNP KO mice. There was no significant difference in MABP between female WT and ecCNP KO mice in either control (normoxic) or hypoxic plus SU5416 conditions (Figure 43). This is at odds with previous data produced by our lab (Moyes *et al*., 2014) that shows female ecCNP KO mice are hypertensive compared to WT littermates, although a more sensitive BP measuring method was utilised (telemetry in conscious mice) in those studies compared to that used here (invasive catheter in anaesthetised mice).

4.2.4 Right ventricular hypertrophy (RVH)

4.2.4.1 Male

Hypertrophic responses in the RV in both male and female WT and ecCNP KO mice mirrored that observed with RVSP. Exposure to hypoxia plus SU5416 resulted in significant ($P<0.05$, $P<0.01$, WT and KO, respectively) increases in RVH in male animals (RV/[LV+S]: 0.25 ± 0.009, 0.26 ± 0.016, WT and KO, respectively) compared to control (RV/[LV+S]: 0.19 ± 0.008, 0.20 ± 0.006, WT and KO, respectively; Figure 44). Genotype did not significantly affect RV size under normoxic or hypoxic conditions (Figure 44). These findings are substantiated when change in RV/[LV+S] between normoxic and hypoxic

conditions for both WT (∆0.059 ± 0.009) and KO (∆0.061 ± 0.016) mice is calculated (Figure 46).

4.2.4.2 Female

Both WT (RV/[LV+S]: 0.20 ± 0.004) and ecCNP KO (RV/[LV+S]: 0.22 ± 0.01) female mice developed significantly ($P<0.05$ for both WT and KO) increased RVH under hypoxic conditions (RV/[LV+S]: 0.26 ± 0.01, 0.26 ± 0.02, WT and KO respectively; Figure 45). Paralleling results in the males, genotype did not alter RV under a normoxic or hypoxic environment even when change in RV/[LV+S] is calculated for both WT (∆0.053 ± 0.013) and KO (∆0.048 ± 0.016; Figure 45 & Figure 47).

4.2.5 Pulmonary vascular remodelling

4.2.5.1 Male

Pulmonary vascular remodelling in both male and female ecCNP WT and KO mice showed a predominance of partially- and fully- muscularised vessels and with no complex lesions or occluded vessels (Table 21). Male ecCNP KO mice had a slightly higher percentage of fully-muscularised vessels (54.14 ± 2.27 %) than WT (47.19 ± 2.70 %). Although, genotype did not significantly alter the proportion of non-, partially- and fully- muscularised vessels (Figure 48).

4.2.5.2 Female

Akin to the observations in the male mice, female ecCNP KO did not exhibit significant differences in pulmonary vessel muscularisation, despite KO (56.10 ± 3.90 %) mice having a slightly higher proportion of fully-muscularised vessels than WT (49.53 ± 3.63; Figure 49). Lungs were inspected for the presence of complex lesions and occluded vessels but none were found (Table 21).

EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON BODY WEIGHT IN MALE MICE WITH PULMONARY HYPERTENSION

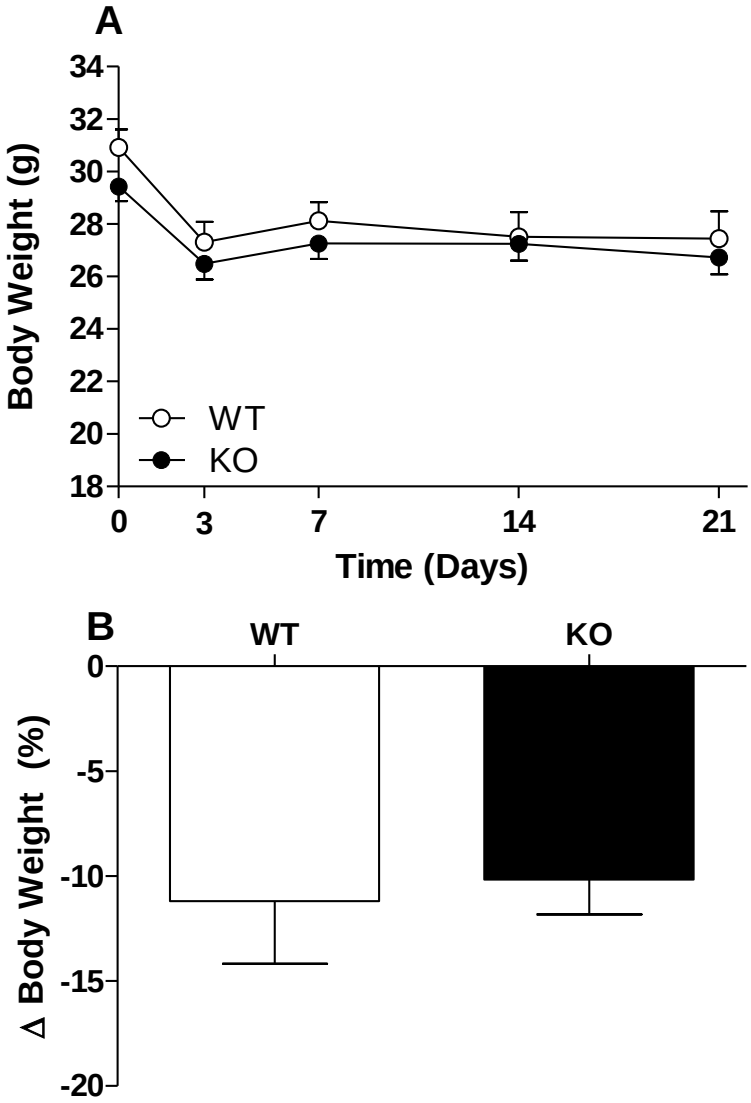


Figure 40. Effect of endothelium-specific CNP deletion on body weight in male mice with pulmonary hypertension.

Body weight (g) at day 0, 3, 7, 14 and 21 (**A**) and change in body weight between day 0 and 21 (%; **B**) in male endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. n=7-14.

EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON BODY WEIGHT IN FEMALE MICE WITH PULMONARY HYPERTENSION

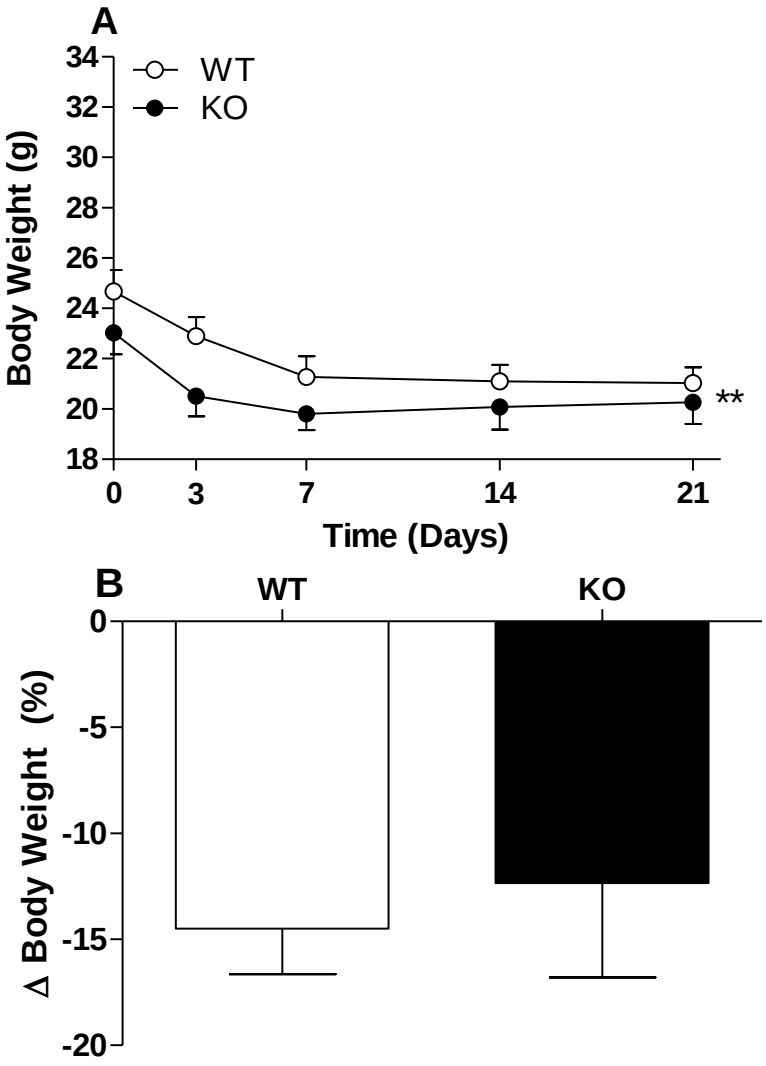


Figure 41. Effect of endothelium-specific CNP deletion on body weight in female mice with pulmonary hypertension.

Body weight (g) at day 0, 3, 7, 14 and 21 (**A**) and change in body weight between day 0 and 21 (%; **B**) in female endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. **P<0.01 v WT. n=9-12.

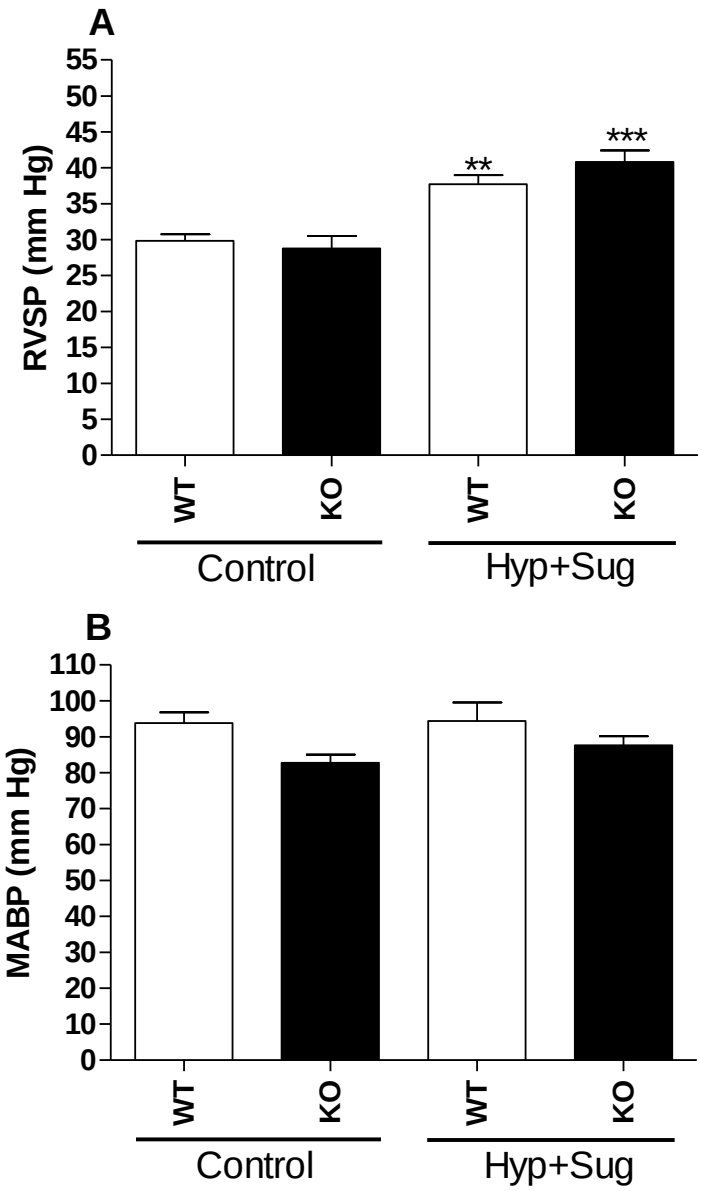


Figure 42. Effect of endothelium-specific CNP deletion on haemodynamics in male mice with pulmonary hypertension.

Right ventricular systolic pressure (RVSP; **A**) and mean arterial blood pressure (MABP; **B**) in male endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. ***P<0.001 v KO, **P<0.01 v WT. n=6-13.

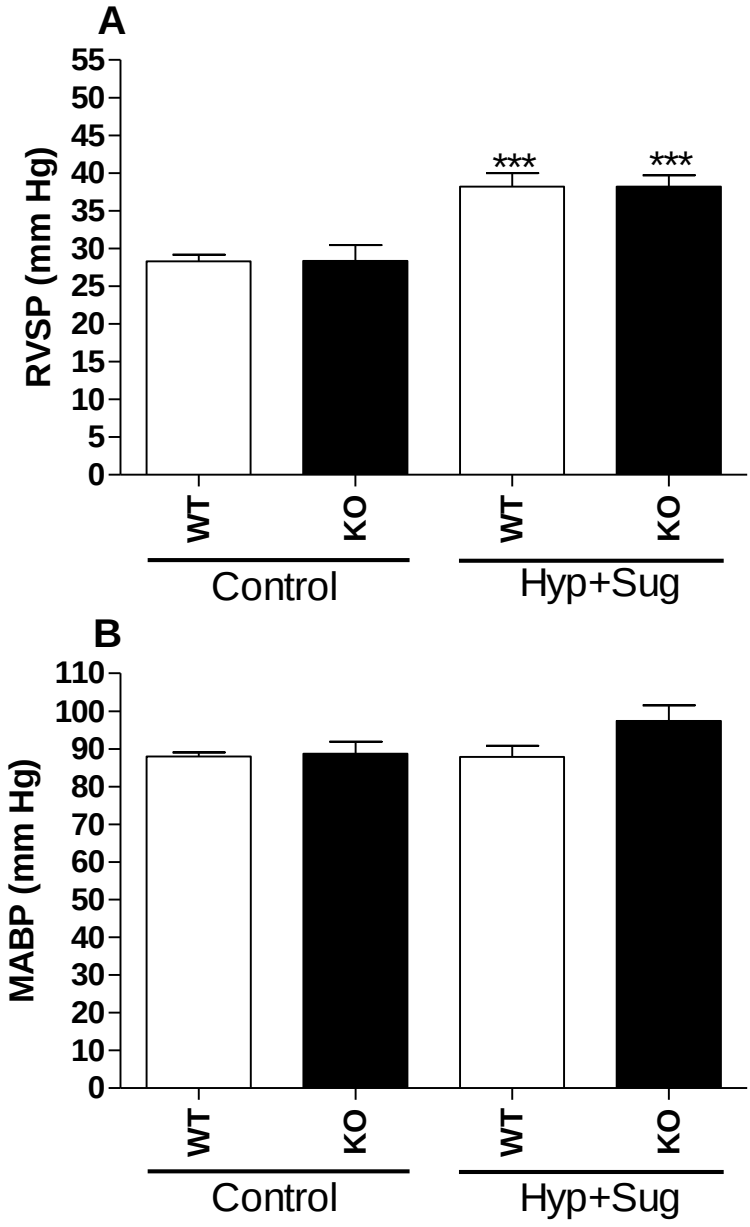


Figure 43. Effect of endothelium-specific CNP deletion on haemodynamics in female mice with pulmonary hypertension.

Right ventricular systolic pressure (RVSP; **A**) and mean arterial blood pressure (MABP; **B**) in female endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14 or vehicle). Data are represented as mean ± SEM. ***P<0.001 v WT, KO. n=7-13.

EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON RIGHT VENTRICULAR HYPERTROPHY IN MALE MICE WITH PULMONARY HYPERTENSION

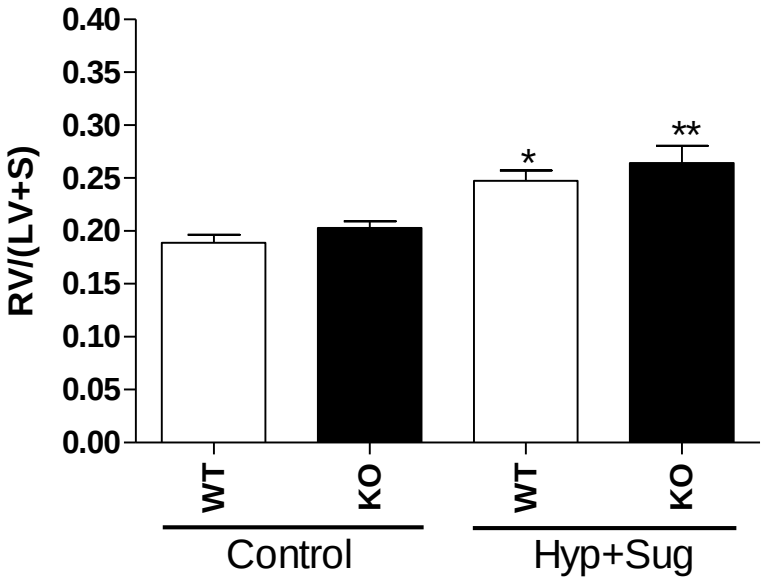


Figure 44. Effect of endothelium-specific CNP deletion on right ventricular hypertrophy in male mice with pulmonary hypertension.

Right ventricle to left ventricle plus septum ratio (RV/[LV+S]) in male endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. *P<0.05 v WT, **P<0.01 v KO. n=7-13.

EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON RIGHT VENTRICULAR HYPERTROPHY IN FEMALE MICE WITH PULMONARY HYPERTENSION

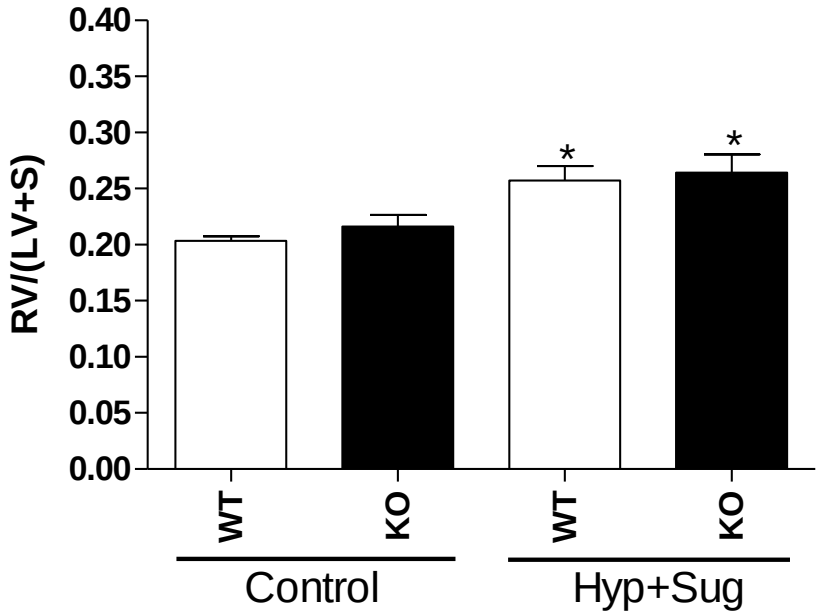


Figure 45 Effect of endothelium-specific CNP deletion on right ventricular hypertrophy in female mice with pulmonary hypertension.

Right ventricle to left ventricle plus septum ratio (RV/[LV+S]) in female endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. *P<0.05 v WT, KO. n=8-14.

EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON CHANGE IN RIGHT VENTRICULAR SYSTOLIC PRESSURE AND HYPERTROPHY IN MALE MICE WITH PULMONARY HYPERTENSION

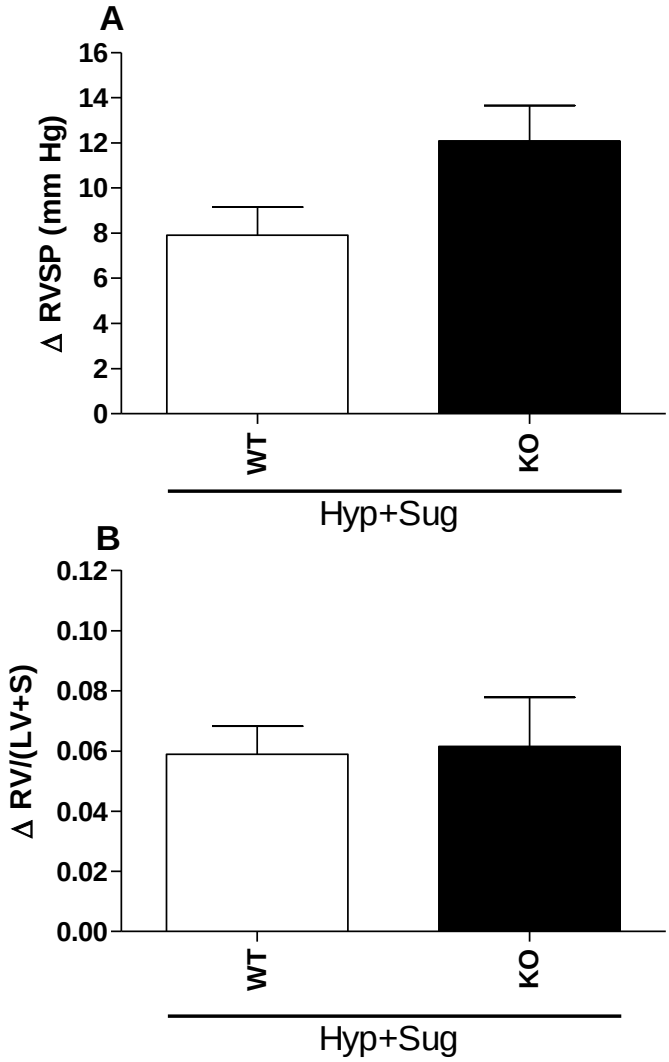


Figure 46. Effect of endothelium-specific CNP deletion on change in right ventricular systolic pressure and hypertrophy in male mice with pulmonary hypertension.

Change in right ventricular systolic pressure (RVSP; **A**) and right ventricle to left ventricle plus septum ratio (RV/[LV+S]; **B**) in male endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. n=7-13.

EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON CHANGE IN RIGHT VENTRICULAR SYSTOLIC PRESSURE AND HYPERTROPHY IN FEMALE MICE WITH PULMONARY HYPERTENSION

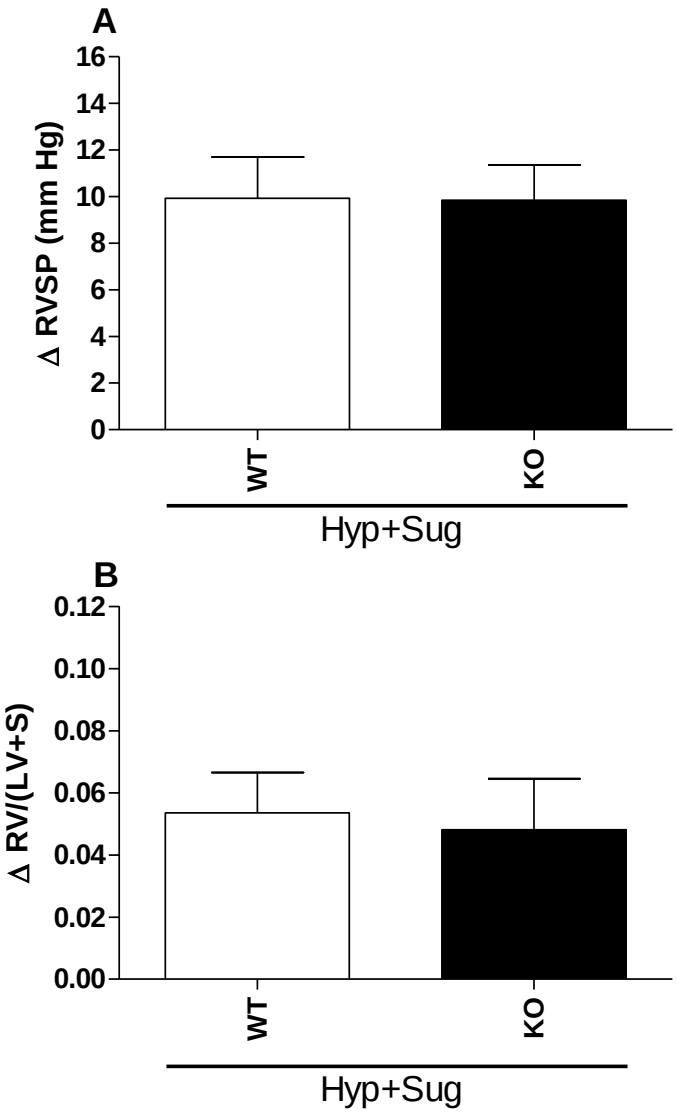


Figure 47. Effect of endothelium-specific CNP deletion on change in right ventricular systolic pressure and hypertrophy in female mice with pulmonary hypertension.

Change in right ventricular systolic pressure (RVSP; **A**) and right ventricle to left ventricle plus septum ratio (RV/[LV+S]; **B**) in female endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. n=10-13.

EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON VASCULAR REMODELLING IN MALE MICE WITH PULMONARY HYPERTENSION

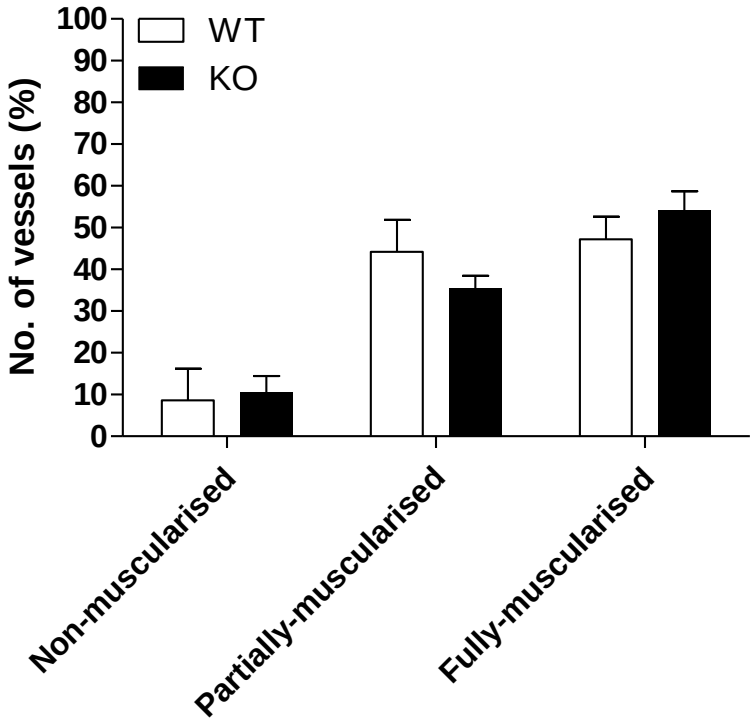


Figure 48. Effect of endothelium-specific CNP deletion on vascular remodelling in male mice with pulmonary hypertension.

Number of vessels (%; <100 μM in diameter) within the pulmonary vasculature that are non-muscularised (<25 % α-SMA stain), partially-muscularised (25-75 % α-SMA stain) and fully-muscularised (>75 % α-SMA stain) in male endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. n=3.

EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON VASCULAR REMODELLING IN FEMALE MICE WITH PULMONARY HYPERTENSION

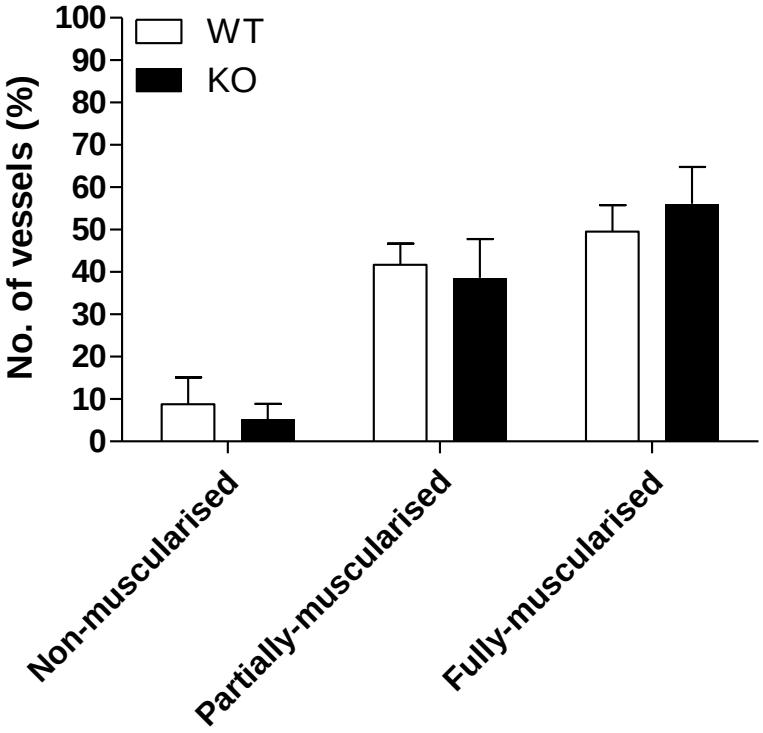


Figure 49. Effect of endothelium-specific CNP deletion on vascular remodelling in female mice with pulmonary hypertension.

Number of vessels (%; <100 μM in diameter) within the pulmonary vasculature that are non-muscularised (<25 % α-SMA stain), partially-muscularised (25-75 % α-SMA stain) and fully-muscularised (>75 % α-SMA stain) in female endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. n=4.

REPRESENTATIVE IMAGES OF THE EFFECT OF ENDOTHELIUM-SPECIFIC CNP DELETION ON VASCULAR REMODELLING IN MALE AND FEMALE MICE WITH PULMONARY HYPERTENSION

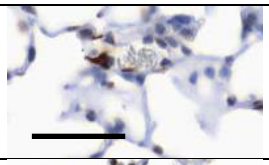
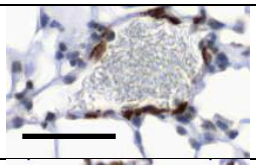
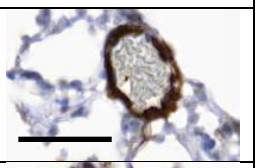
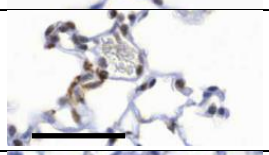
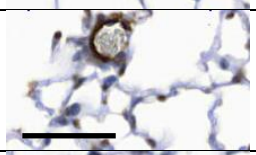
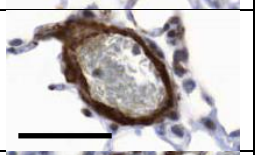
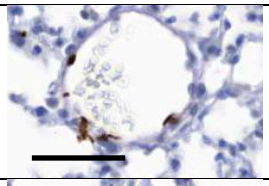
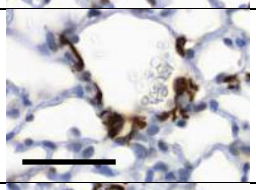
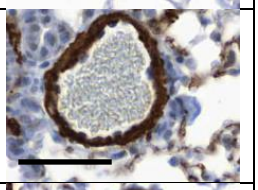
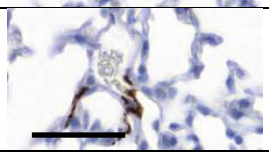
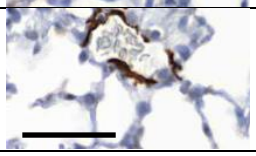
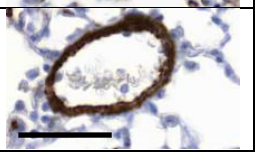
	Non-muscularised	Partially-muscularised	Fully-muscularised
Male ecCNP WT			
Male ecCNP KO			
Female ecCNP WT			
Female ecCNP KO			

Table 21. Representative images of endothelium-specific CNP deletion on vascular remodelling in male and female mice with pulmonary hypertension.

Representative images of vessels within the pulmonary vasculature that are non-muscularised (<25 % α-SMA stain), partially-muscularised (25-75 % α-SMA stain) and fully-muscularised (>75 % α-SMA stain) in male and female endothelium-specific CNP knockout (ecCNP KO) mice and wild-type (WT) littermates exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14). H & E stain (blue) and α-SMA stain (brown). Images taken at x20 magnification. Scale bar = 50 μm.

4.3 Effect of global deletion of NPR-C on the development of experimental pulmonary hypertension

Recent work by our laboratory has assigned a critical signalling role to NPR-C in the systemic circulation (Moyes *et al*., 2014) in addition to its well-established function to clear natriuretic peptides (Maack *et al*., 1987). In particular, the endothelial dysfunction and hypertension observed in ecCNP KO animals appears to be recapitulated, at least in part, in global NPR-C deleted mutants. Data from the previous set of experiments indicated that endothelium-derived CNP does not play a prominent role in regulating pulmonary vascular reactivity in the context of PH. However, since other cellular sources of CNP may be important in this pro-proliferative disorder (e.g. cardiomyocyte, macrophage, fibroblast, VSMC), and the possibility that alternate NPs (i.e. ANP and/or BNP; released in patients with PH) might also activate NPR-C, I proceeded to examine the phenotype of global NPR-C KO mice in the hypoxia plus SU5416 model of PH. Of note, although I originally hypothesised that cGMP-dependent processes might underlie any beneficial activity of CNP, the demonstrated importance of NPR-C in the systemic circulation and the shortage of usable NPR-B KO offspring (due to significantly reduced survival) targeted this line of investigation towards mice with NPR-C gene deletion.

The same methodology was used in these experiments; male and female, 16 week old, NPR-C KO and WT littermates were injected once a week, for 3 weeks, with SU5416 (20 mg/kg ; s.c.) or vehicle, and exposed to 21 days hypoxia (10% O_2, normobaric) normoxia (21% O_2).

4.3.1 Body weight (BW)

4.3.1.1 Male

Male NPR-C KO (34.4 ± 0.95 g) mice were slightly heavier than their WT (31.91 ± 0.69 g) counterparts at the start of the experiment. Both WT and KO animals lost BW in the first 3 days which then remained relatively stable for the remainder of the experiment, which was also observed by Ciuclan *et al*., (2011) and in optimisation experiments (Figure 50). Comparison of the BW between male NPR-C KO and WT revealed a significant ($P<0.01$) difference over the entire time course of the experiment, with WT always having a lower BW (Figure 50). This is despite KO mice having an elongated, narrowed bone structure, and lean physique observed in this strain (Bordicchia *et al*., 2012; Matsukawa *et al*., 1999). However, the percentage change in BW from the start to the end of the experiment was not significantly different between genotypes (-15.5 ± 1.8 %, -16.7 ± 2.4 % WT and KO, respectively; Figure 50).

4.3.1.2 Female

Female WT (26.21 ± 0.66 g) mice were heavier than KOs (23.03 ± 0.85 g) at day 0 (Figure 51). KO animals steadily lost weight over the first 3 days of hypoxia but subsequently BW stabilised for the remainder of the experiment (Figure 51). A similar pattern was observed in WT mice; BW fell within the first 3 days, which then remained constant for the balance of the experiment (Figure 51). Over the whole time course, WT mice were significantly and consistently heavier than KO littermates ($P<0.001$; Figure 51). However, when comparing percentage loss in BW from the start to the end of the experiment there is no significant difference between genotypes (-12.6 ± 2.3, -10.8 ± 2.8 %; WT and KO, respectively; Figure 51).

4.3.2 Right ventricular systolic pressure (RVSP)

4.3.2.1 Male

Male WT and NPR-C KO mice demonstrated a similar response to hypoxia plus SU5416 with respect to RVSP. NPR-C KO mice had a slightly lower RVSP (26.9 ± 1.88 mm Hg) under control (normoxic) conditions when compared to WT (28.6 ± 1.22 mm Hg). Hypoxia plus SU5416 resulted in a significant increase in RVSP in both WT (36.6 ± 1.60 mm Hg; $P<0.01$) and KO (38.0 ± 1.73 mm Hg; $P<0.001$) mice (Figure 52). The magnitude of the change was not significantly altered by genotype (∆8.0 ± 1.60, ∆11.0 ± 1.73 mm Hg, WT and KO, respectively; Figure 66).

4.3.2.2 Female

Female NPR-C KO mice developed a significant ($P<0.001$) increase in RVSP when exposed to hypoxia plus SU5416 compared to control (26.5 ± 1.22 mm Hg to 39.1 ± 2.67 mm Hg), although this response was not significantly different to WT (27.8 ± 1.61 mm Hg to 36.6 ± 1.60 mm Hg; Figure 53). There is also no significant difference comparing the increase in RVSP between normoxic and hypoxic plus SU5416 conditions for both WT (∆8.8 ± 1.60 mm Hg) and KO (∆12.6 ± 2.67 mm Hg) mice (Figure 67).

4.3.3 Mean arterial blood pressure (MABP)

4.3.3.1 Male

Male NPR-C KO mice had significantly ($P<0.05$) lower MABP (77.0 ± 1.53 mm Hg) under control conditions than WT littermates (90.3 ± 3.03 mm Hg; Figure 52), consistent with previous reports (Matsukawa *et al*., 1999; Moyes *et al*.,

2014). This relative profile was mirrored when both WT (88.7 ± 2.28 mm Hg) and KO (75.2 ± 2.82 mm Hg) animals were exposed to hypoxia plus SU5416 (Figure 52).

4.3.3.2 Female

Female WT (90.5 ± 1.58 mmHg) and NPR-C KO (85.2 ± 0.95 mmHg) mice had similar MABP under control (normoxic) conditions or when exposed to hypoxia plus SU5416 (98.9 ± 2.43 mm Hg, 90.8 ± 4.63 mm Hg; WT and KO, respectively; Figure 53).

4.3.4 Right ventricular hypertrophy (RVH)

4.3.4.1 Male

Hypoxia plus SU5416 produced a significant ($P<0.001$) increase in RV mass, when normalised to LV+S, in NPR-C male KO mice (RV/[LV+S]: 0.18 ± 0.060 to 0.26 ± 0.018 in normoxia and hypoxia, respectively; Figure 54). However, under the same conditions, WT littermates did not exhibit a significant increase in RVH (RV/[LV+S]: 0.20 ± 0.011 to 0.23 ± 0.008 in normoxia and hypoxia, respectively; Figure 54). When comparing change in RVH between normoxia and hypoxia plus SU5416, KO (Δ0.081 ± 0.018) RV/[LV+S] ratio was significantly ($P<0.05$) increased compared to WT (Δ0.025 ± 0.011; Figure 56).

4.3.4.2 Female

Right ventricular hypertrophy in female NPR-C KO mice and WT counterparts followed the same pattern observed with RVSP (Figure 55). Female NPR-C KO mice under control (normoxic) conditions have similar RV/[LV+S] ratio (0.19 ± 0.005) compared to WT (0.20 ± 0.008). When exposed to hypoxia plus SU5416, female NPR-C KO develop a significant RVH (0.27 ± 0.043; $P<0.001$)

that is mirrored by WT (0.24 ± 0.027; P<0.01; Figure 55). Similar to that observed in males, female NPR-C KO mice (∆0.087 ± 0.018) developed a significantly (P<0.05) greater increase in RVH compared to WT mice (∆0.045 ± 0.009; Figure 57).

4.3.5 Pulmonary vascular remodelling

4.3.5.1 Male

Male NPR-C WT and KO mice showed similar pulmonary vascular remodelling after hypoxic plus SU5416 (Table 22), with no observed presence of complex lesions or occluded lesions. Both genotypes developed mainly partially- and fully- muscularised vessels with minimal non-muscularised vessels (Figure 58). Male NPR-C KO mice had slightly more fully-muscularised vessels (54.1 ± 2.70 %) than WT (47.2 ± 2.27 %), although this did not reach statistical significance (Figure 58).

4.3.5.2 Female

A similar pattern was observed in female WT and NPR-C KO mice, with no complex lesions or occluded lesions (Table 22). These animals developed similar levels of non-, partially- and fully- muscularised vessels after exposure to hypoxia plus SU5416 (Figure 59). However, there were no significant differences between WT and KO mice (Figure 59).

EFFECT OF GLOBAL NPR-C DELETION ON BODY WEIGHT IN MALE MICE WITH PULMONARY HYPERTENSION

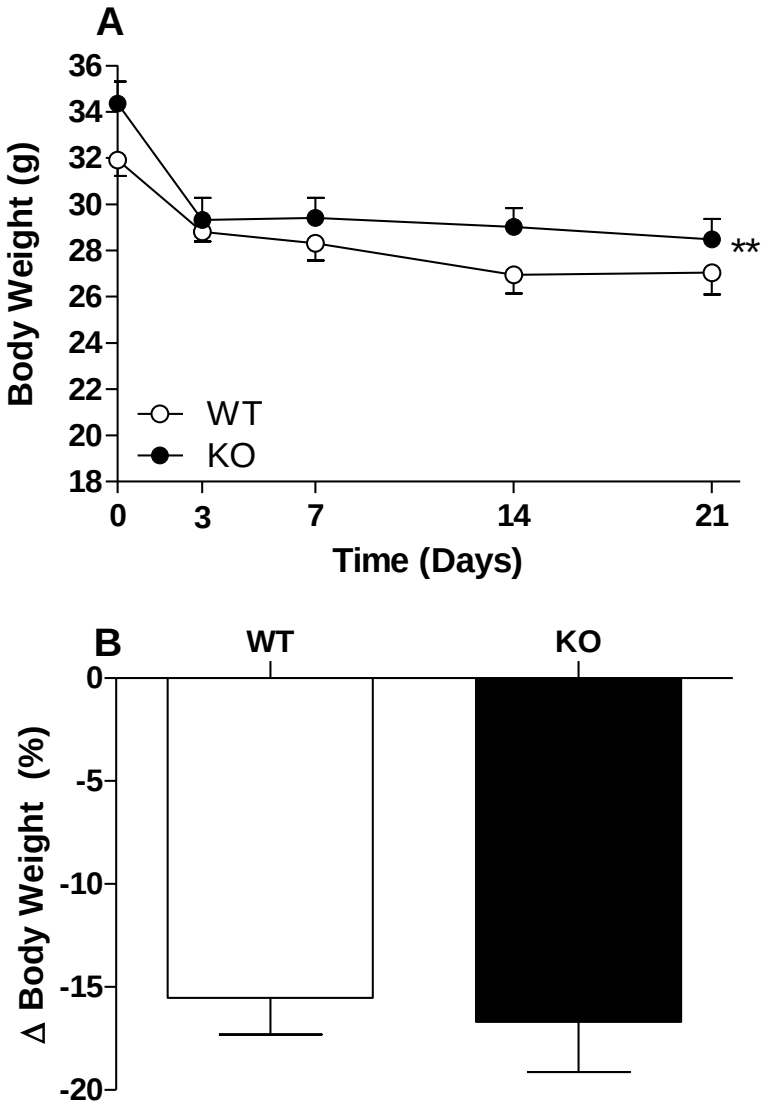


Figure 50. Effect of global NPR-C deletion on body weight in male mice with pulmonary hypertension.

Body weight at day 0, 3, 7, 14 and 21 (g; **A**) and change in body weight between day 0 and 21 (%; **B**) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. **P<0.01 v WT. n=7-11.

EFFECT OF GLOBAL NPR-C DELETION ON BODY WEIGHT IN FEMALE MICE WITH PULMONARY HYPERTENSION

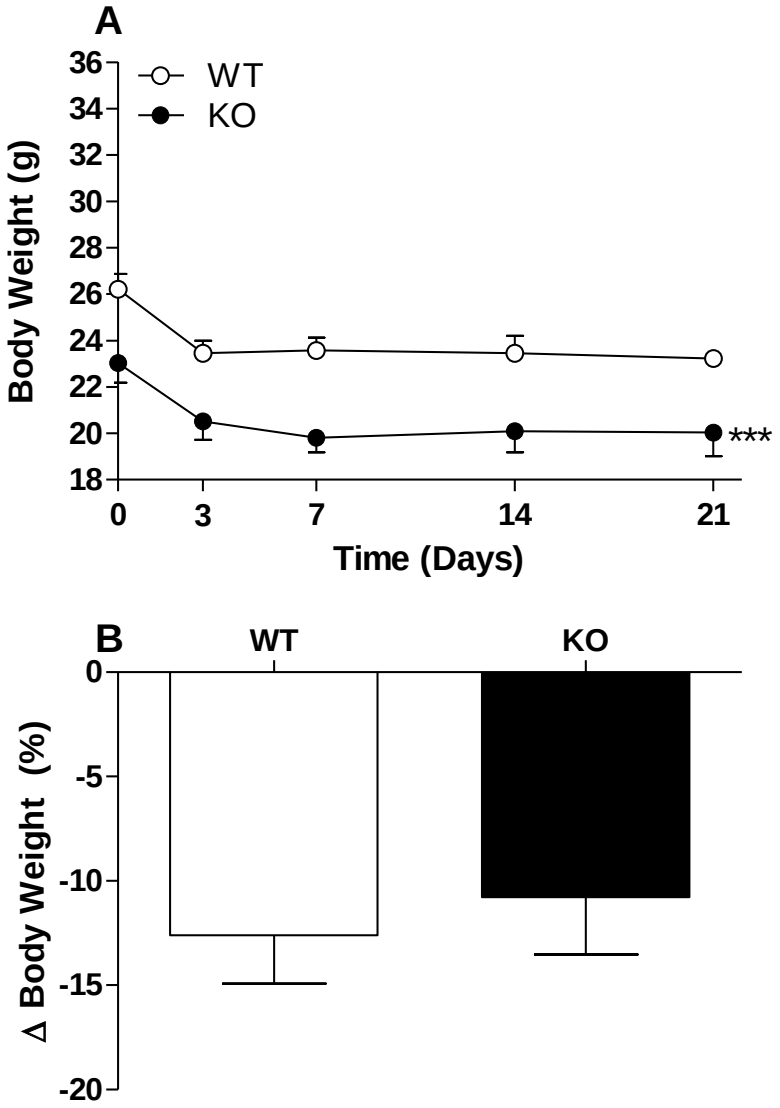


Figure 51. Effect of global NPR-C deletion on body weight in female mice with pulmonary hypertension.

Body weight at day 0, 3, 7, 14 and 21 (g; **A**) and change in body weight between day 0 and 21 (%; **B**) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. ***P<0.001 v WT. n=6-11.

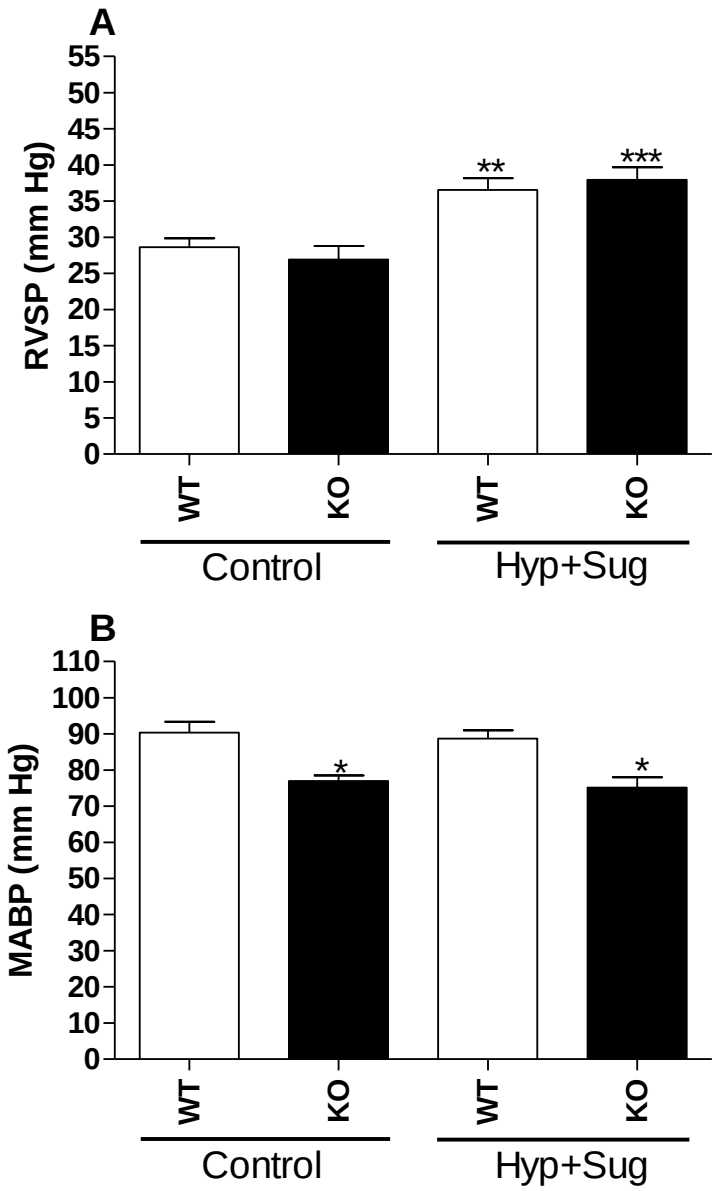


Figure 52. Effect of global NPR-C deletion on haemodynamics in male mice with pulmonary hypertension.

Right ventricular systolic pressure (RVSP; **A**) and mean arterial blood pressure; **B**) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. *P<0.05 v WT, **P<0.01 v WT, ***P<0.001 v KO. n=5-11.

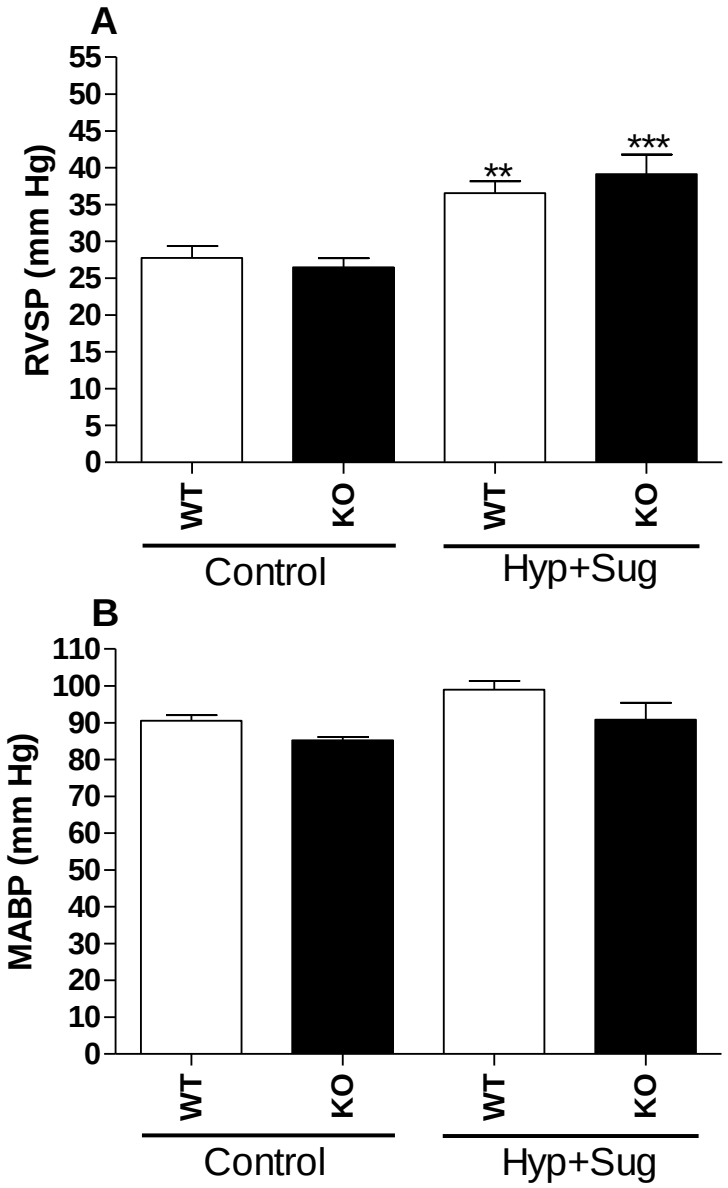


Figure 53. Effect of global NPR-C deletion on haemodynamics in female mice with pulmonary hypertension.

Right ventricular systolic pressure (RVSP; **A**) and mean arterial blood pressure; **B**) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. **P<0.01 v WT, ***P<0.001 v KO. n=5-11.

EFFECT OF GLOBAL NPR-C DELETION ON RIGHT VENTRICULAR HYPERTROPHY IN MALE MICE WITH PULMONARY HYPERTENSION

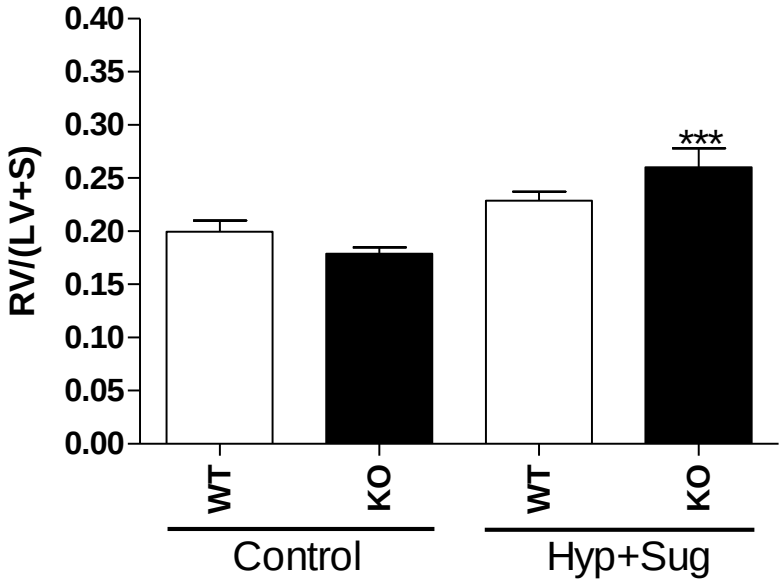


Figure 54. Effect of global NPR-C deletion on right ventricular hypertrophy in male mice with pulmonary hypertension.

Right ventricle to left ventricle plus septum ratio (RV/[LV+S]) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. ***P<0.001 v KO. n=6-15.

EFFECT OF GLOBAL NPR-C DELETION ON RIGHT VENTRICULAR HYPERTROPHY IN FEMALE MICE WITH PULMONARY HYPERTENSION

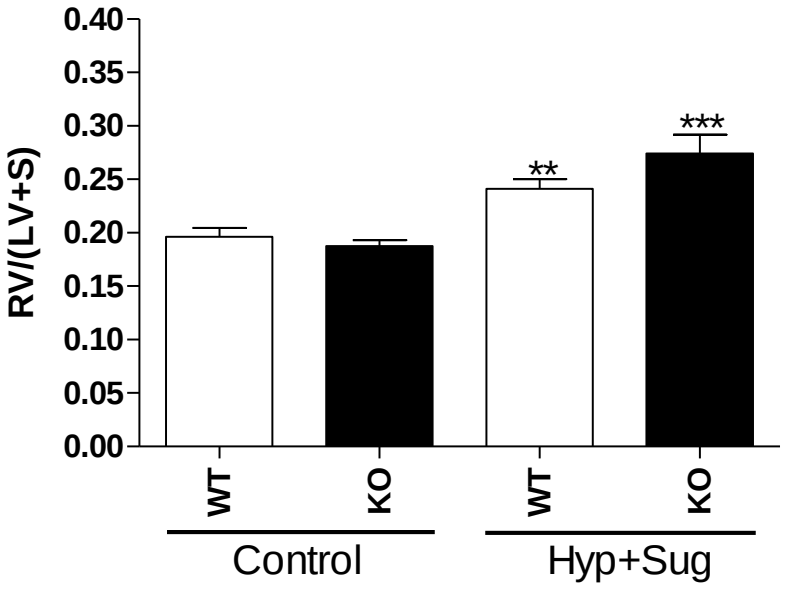


Figure 55. Effect of global NPR-C deletion on right ventricular hypertrophy in female mice with pulmonary hypertension.

Right ventricle to left ventricle plus septum ratio (RV/[LV+S]) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. **P<0.01 v WT, ***P<0.001 v KO. n=6-12.

EFFECT OF GLOBAL NPR-C DELETION ON CHANGE IN RIGHT VENTRICULAR SYSTOLIC PRESSURE AND HYPERTROPHY IN MALE MICE WITH PULMONARY HYPERTENSION

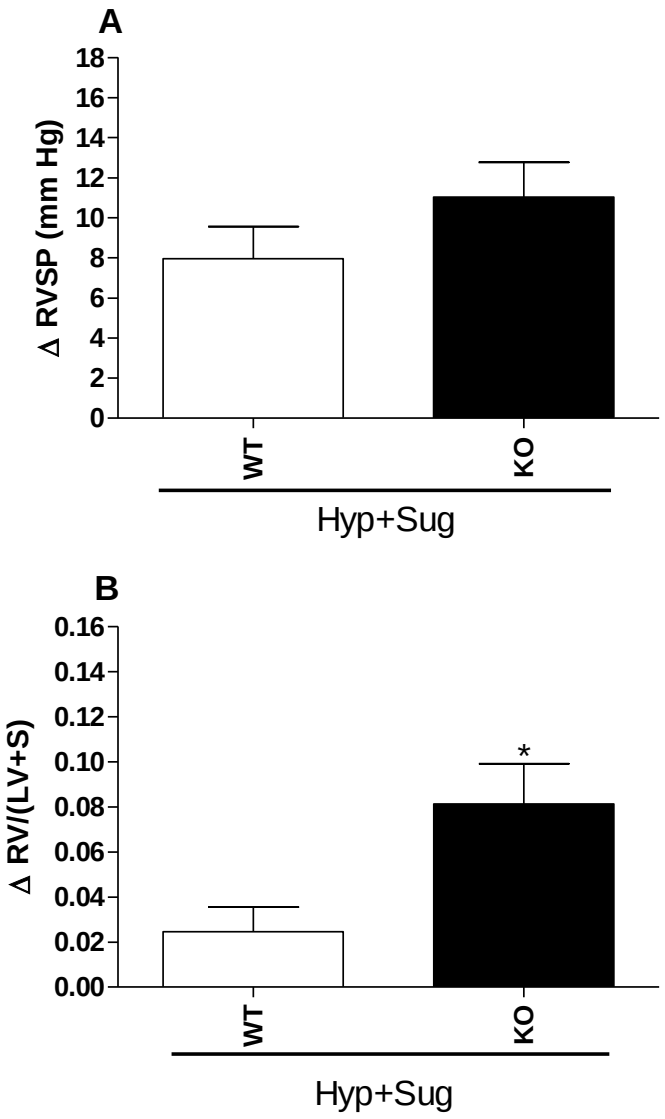


Figure 56. Effect of global NPR-C deletion on change in right ventricular systolic pressure and hypertrophy in male mice with pulmonary hypertension.

Change in right ventricular systolic pressure (RVSP; **A**) and right ventricle to left ventricle plus septum ratio (RV/[LV+S]; **B**) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. *P<0.05 v WT. n=8-9.

EFFECT OF GLOBAL NPR-C DELETION ON CHANGE IN RIGHT VENTRICULAR SYSTOLIC PRESSURE AND HYPERTROPHY IN FEMALE MICE WITH PULMONARY HYPERTENSION

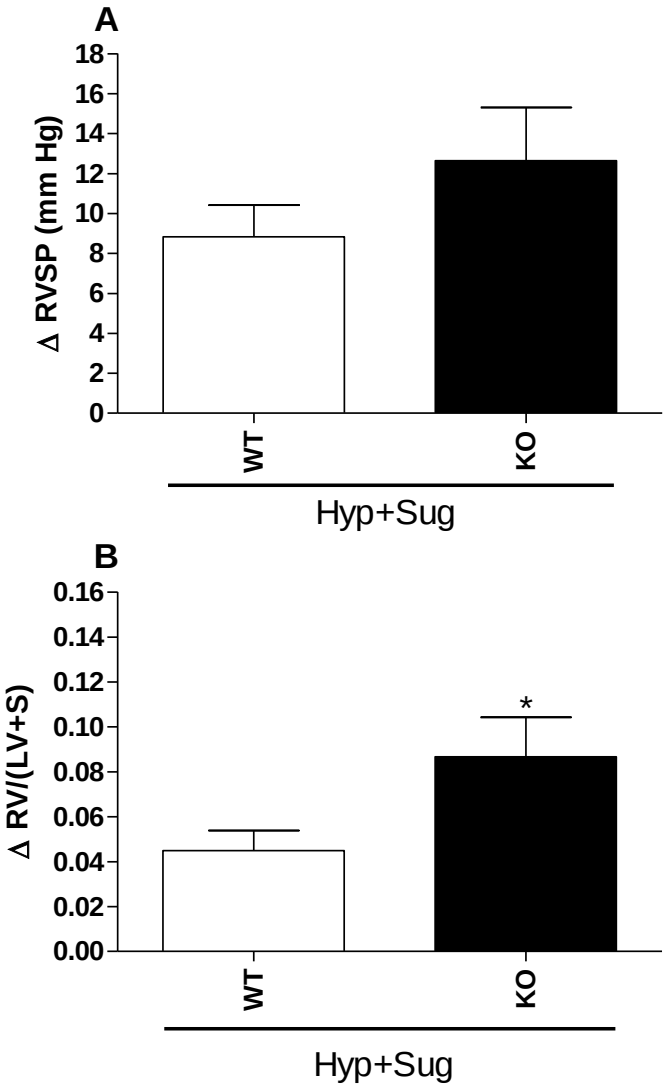


Figure 57. Effect of global NPR-C deletion on change in right ventricular systolic pressure and hypertrophy in female mice with pulmonary hypertension.

Change in right ventricular systolic pressure (RVSP; **A**) and right ventricle to left ventricle plus septum ratio (RV/[LV+S]; **B**) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. *P<0.05 v WT. n=5-9.

EFFECT OF GLOBAL NPR-C DELETION ON VASCULAR REMODELLING IN MALE MICE WITH PULMONARY HYPERTENSION

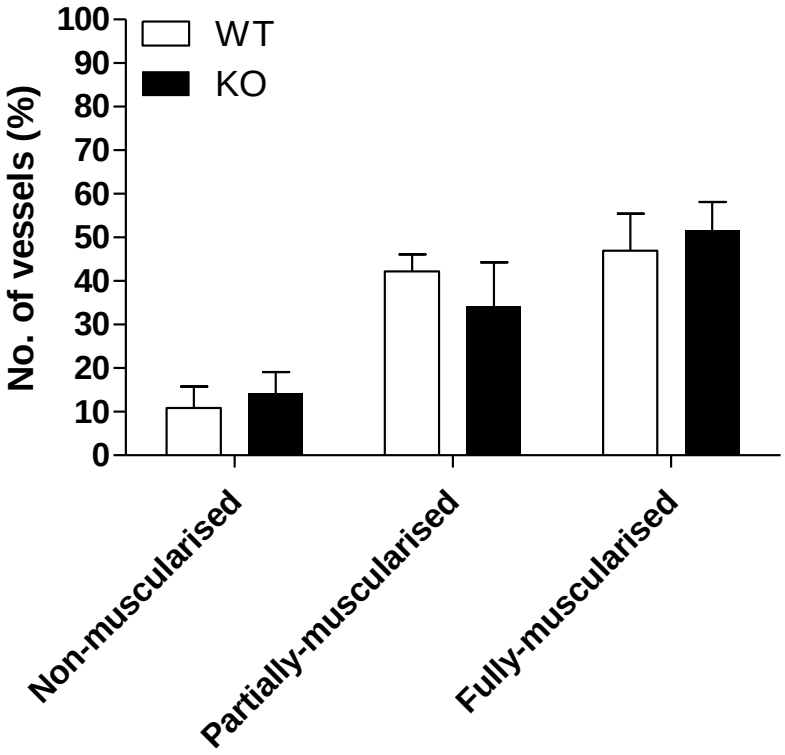


Figure 58. Effect of global NPR-C deletion on vascular remodelling in male mice with pulmonary hypertension.

Number of vessels (%; <100 μM in diameter) within the pulmonary vasculature that are non-muscularised (<25 % α-SMA stain), partially-muscularised (25-75 % α-SMA stain) and fully-muscularised (>75 % α-SMA stain) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. n=5-6.

EFFECT OF GLOBAL NPR-C DELETION ON VASCULAR REMODELLING IN FEMALE MICE WITH PULMONARY HYPERTENSION

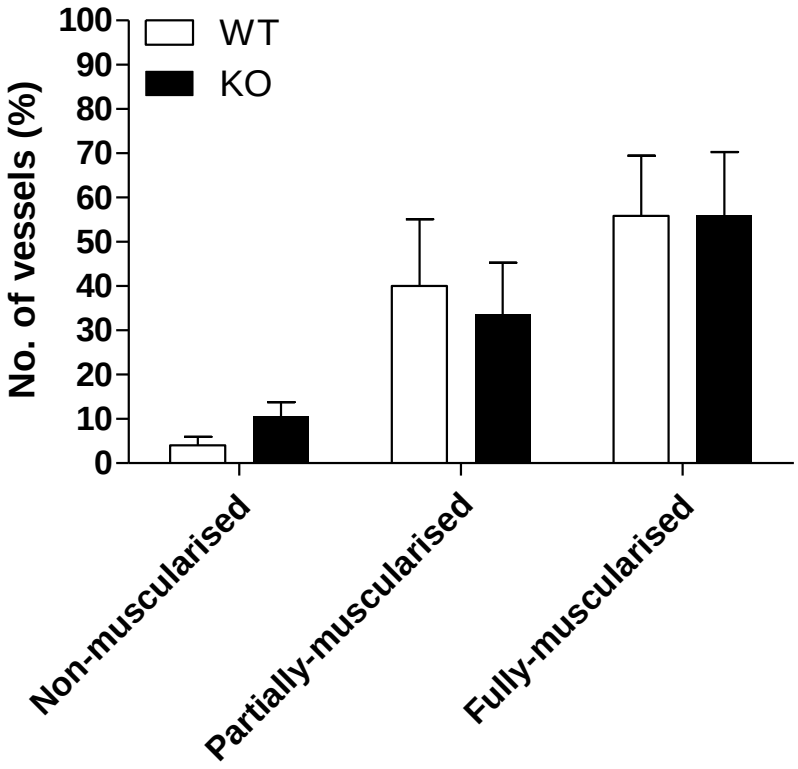


Figure 59. Effect of global NPR-C deletion on vascular remodelling in female mice with pulmonary hypertension.

Number of vessels (%; <100 μM in diameter) within the pulmonary vasculature that are non-muscularised (<25 % α-SMA stain per vessel), partially-muscularised (25-75 % α-SMA stain per vessel) and fully-muscularised (>75 % α-SMA stain per vessel) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates under control conditions (normoxia; 21 % O_2) or exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14) or vehicle. Data are represented as mean ± SEM. n=4-5.

REPRESENTATIVE IMAGES OF GLOBAL NPR-C DELETION ON VASCULAR REMODELLING IN MALE AND FEMALE MICE WITH PULMONARY HYPERTENSION

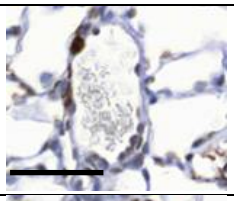
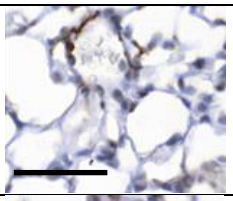
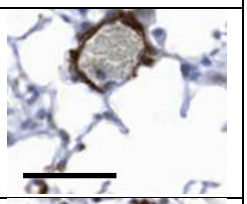
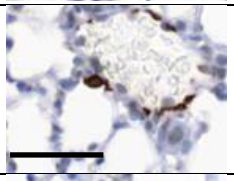
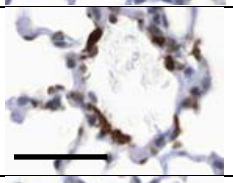
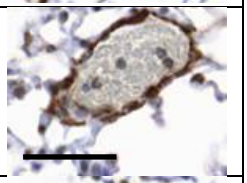
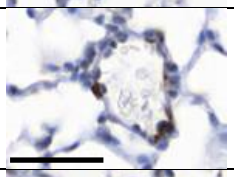
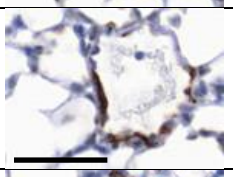
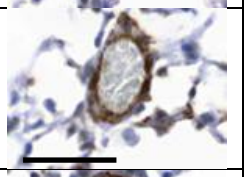
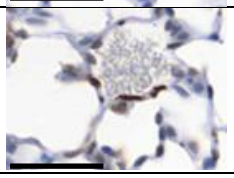
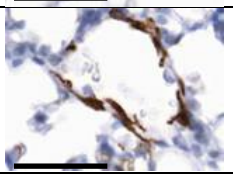
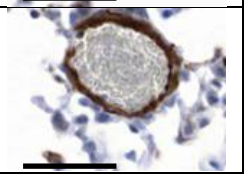
	Non-muscularised	Partially-muscularised	Fully-muscularised
Male NPR-C WT			
Male NPR-C KO			
Female NPR-C WT			
Female NPR-C KO			

Table 22. Representative images of global NPR-C deletion on vascular remodelling in male and female mice with pulmonary hypertension.

Representative images of vessels within the pulmonary vasculature that are non-muscularised (<25 % α-SMA stain), partially-muscularised (25-75 % α-SMA stain) and fully-muscularised (>75 % α-SMA stain) in male and female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates exposed to hypoxia (10 % O_2; 21 days) plus SU5416 (20 mg/kg; s.c. at days 0, 7 & 14). H & E stain (blue) and α-SMA stain (brown). Images taken at x20 magnification. Scale bar = 50 μm.

4.4 Effect of global deletion of NPR-C on the development of bleomycin-induced lung fibrosis and secondary pulmonary hypertension

Data gleaned from the hypoxia plus SU5416 experimental model of PH indicated that NPR-C might play a significant role in the development of RVH. In addition, CNP signalling has been shown to exert beneficial (i.e. anti-fibrotic) effect in several models of fibrosis (e.g. Kimura *et al*., 2016; Murakami *et al*., 2004; Soeki *et al*., 2005). Therefore, I designed experiments to assess whether genetic deletion of NPR-C affected disease severity in a second model of PH, which is associated with bleomycin-induced lung fibrosis.

In this complementary model of PH secondary to pulmonary fibrosis, male and female NPR-C KO, and age matched WT littermates, received a single oropharyngeal (o.p.) instillation, under light isoflurane anaesthesia, of Bleo-Kyowa® (Bleomycin Sulphate; Bleo) weight adjusted (1 µl to 1 g of body weight), resulting in individual mice receiving 4 mg/kg (4,000 IU or 4 USP) dissolved in saline. Control mice received saline (o.p.) only. The mice were then left in normal housing conditions for 14 days before haemodynamic measures were taken.

4.4.1 Body weight (BW)

4.4.1.1 Male

Male WT (34.9 ± 0.92 g) mice were slightly heavier than NPR-C KO (32.3 ± 0.96 g) littermates consistent with previously published work (Matsukawa *et al*., 1999; Figure 60). Following administration of Bleo, both WT and KO mice lost weight at a steady rate throughout the 14 day period (Figure 60). Immediate reductions in BW after lung instillations of Bleo have previously been reported,

with the extent of BW loss proportional to dose (Failla *et al*., 2006; Murakami *et al*., 2004). Although, BW of WT and NPR-C KO animals were significantly ($P<0.01$) different across the 14 day time course (with KO mice always having lower BW), the percentage change in BW from day 0 to day 14 was almost identical for WT (-16.3 ± 4.1 %) and KO (-16.8 ± 4.8 %) mice (Figure 60).

4.4.1.2 Female

In the females, WT mice (28.7 ± 0.83 g) were marginally heavier than NPR-C KO (27.9 ± 0.94 g) littermates at the start of the study (Figure 61). Both WT and NPR-C KO mice steadily lost BW for the first 7 days, after which the loss in BW tapered and remained constant until the end of the experiment (Figure 61). BW throughout the experiment was not significantly different between WT and KO animals, although WTs were consistently heavier than KOs. Percentage change in BW between day 0 and 14 was similar between WT and NPR-C KO mice (-8.6 ± 3.0, -6.8 ± 1.7 %, respectively; Figure 61).

4.4.2 Right ventricular systolic pressure (RVSP)

4.4.2.1 Male

Control (normoxic) male WT mice had an essentially identical RVSP (28.6 ± 1.22 mm Hg) compared to NPR-C KO animals (26.9 ± 1.88 mm Hg; Figure 62). After being treated with bleo, RVSP increased significantly in both WT ($P<0.001$) and KO ($P<0.001$) mice. Although, the magnitude of this response was marginally greater in NPR-C KO mice (43.2 ± 2.79 mm Hg) compared to WT (38.7 ± 0.70 mm Hg) there was no significant difference between the two genotypes (Figure 62). However, when comparing the change in RVSP between normoxia and hypoxia plus SU5416, NPR-C KO (Δ16.3 ± 2.79 mm Hg) mice developed a significant increase ($P<0.05$) compared to WT mice (Δ10.1 ± 0.67 mm Hg; Figure 66).

4.4.2.2 Female

Female WT and NPR-C KO mice had very similar RVSP under control conditions (27.8 ± 1.6 mm Hg and 26.5 ± 1.2 mm Hg, respectively). These values increased significantly after treatment with bleomycin (WT: 33.0 ± 1.11 mm Hg; KO 37.3 ± 0.94 mm Hg; $P<0.05$ and $P<0.001$, respectively; Figure 63). Of note, bleomycin-treated NPR-C KO mice had significantly ($P<0.05$) higher RVSP than WT animals (Figure 63). This significant difference was reflected when change in RVSP was calculated (Δ5.3 ± 1.11, Δ10.8 ± 0.94 mm Hg, WT and KO respectively; $P<0.001$; Figure 67).

4.4.3 Mean arterial blood pressure (MABP)

4.4.3.1 Male

Male NPR-C KO mice tended to have a lower MABP (77.0 ± 1.53 mm Hg) compared to their WT littermates (90.3 ± 3.03 mm Hg) under basal (control) conditions, as I showed in the hypoxia model (Figure 62). After administration of bleomycin, the MABP in WT (77.4 ± 4.12 mm Hg) mice dropped significantly ($P<0.01$) whereas blood pressure in the NPR-C KO animals underwent little change (77.5 ± 2.68 mm Hg) possibly because it is inherently low (Figure 62). Nonetheless, MABP is not significantly different between WT and NPR-C KO mice either under control conditions or after exposure to bleomycin (Figure 62).

4.4.3.2 Female

Female NPR-C KO mice had marginally lower MABP (85.2 ± 0.96 mmHg) than WT (90.5 ± 1.58 mmHg) under control (normoxic) conditions (Figure 63). This remained unchanged in both WT (90.6 ± 1.96 mmHg) and KO (86.4 ± 1.87 mm Hg) animals following bleomycin administration (Figure 63).

4.4.4 Right ventricular hypertrophy (RVH)

4.4.4.1 Male

Subsequent to administration of bleomycin, male WT and NPR-C KO mice exhibited a trend towards an increase in RVH but this didn't reach statistical significance (Figure 64). RV/(LV+S) ratio was increased in WT mice with bleomycin treatment (0.20 ± 0.011 to 0.24 ± 0.017; Figure 64) with an analogous change observed in NPR-C KO animals (0.18 ± 0.060 to 0.23 ± 0.087; Figure 64). This was also the case when calculating change in RV/(LV+S) ratio for WT (∆0.036 ± 0.017) and KO (∆0.049 ± 0.009) mice (Figure 66).

4.4.4.2 Female

A similar lack of RVH was also observed in female WT animals undergoing bleomycin treatment. RV/(LV+S) ratio increased marginally in WT mice (0.20 ± 0.008 to 0.21 ± 0.004) but not to a statistically significant degree (Figure 65). This is in sharp contrast to NPR-C KO mice in which a greater, significant ($P<0.001$) increase was observed (0.19 ± 0.006 to 0.24 ± 0.007), which was also significantly ($P<0.05$) larger than WT (Figure 65). This finding was accentuated when change in RV/(LV+S) ratio from normoxia to hypoxia plus SU5416 was calculated (∆0.015 ± 0.005, ∆0.049 ± 0.007, WT and KO, respectively; $P<0.001$; Figure 67).

4.4.5 Lung weight

4.4.5.1 Male

The lung weight in male WT mice (0.176 ± 0.011 g) was similar to NPR-C KO

(0.163 ± 0.011 g) under control conditions (Figure 68). Treatment with bleomycin caused lung weight to increase significantly in WT (0.328 ± 0.033 g; P<0.001) and NPR-C KO (0.176 ± 0.011 g; P<0.01) mice compared to control (Figure 68). However, there was no significant differences between genotype in this regard (Figure 68). The same pattern was observed when lung weight was normalised to BW; both WT and NPR-C KO mice developed significantly (WT: 0.0044 ± 0.006 to 0.0121 ± 0.002; KO: 0.0048 ± 0. to 0.014 ± 0.002; P<0.01, P<0.05 WT and KO respectively) increased lung weight/BW ratios (Figure 68).

4.4.5.2 Female

Female NPR-C KO animals had slightly heavier lungs (0.157 ± 0.006 g) compared to WT littermates (0.148 ± 0.005 g) under control conditions (Figure 69). When administered bleomycin, both WT and NPR-C KO mice develop a significant (P<0.001 for both) increase in lung weight (Figure 69). Importantly, NPR-C KO mice developed a significantly (P<0.05) greater increase (0.377 ± 0.030 g) than WT (0.268 ± 0.020 g) animals (Figure 69). This was also observed when lung weight was normalised to account for changes in BW. Control WT lung weight/BW ratio was 0.005 ± 0.003 and increased significantly (P<0.01) to 0.010 ± 0.001, whilst KO mice increased from 0.005 ± 0.001 to 0.016 ± 0.002 (P<0.001); the change in KO is also significantly (P<0.01) larger than WT (Figure 69).

4.4.6 Pulmonary vascular remodelling

4.4.6.1 Male and female

Male and female NPR-C WT and KO mice dosed with bleomycin developed obvious pulmonary vascular remodelling characterised by areas of fibrosis rich in collagen deposits (Table 23). However, time constraints entailed that the degree of fibrosis was not quantified.

EFFECT OF GLOBAL NPR-C DELETION ON BODY WEIGHT IN MALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

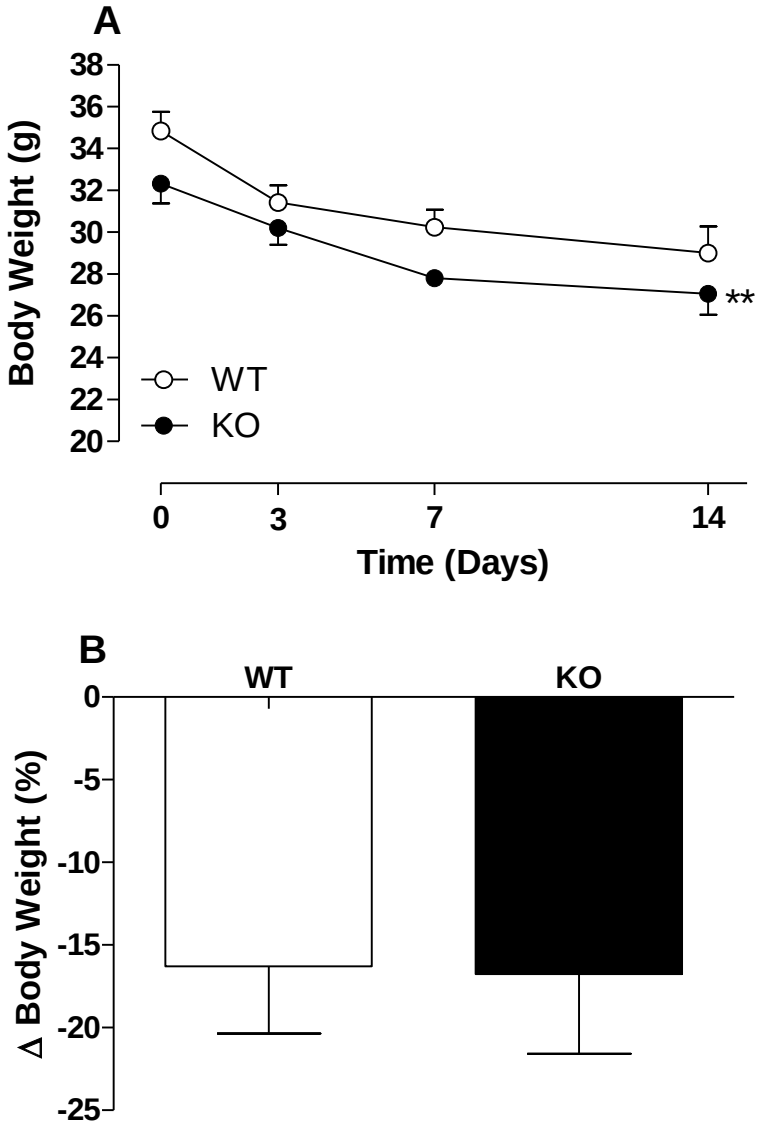


Figure 60. Effect of global NPR-C deletion on body weight in male mice with bleomycin-induced pulmonary hypertension.

Body weight at day 0, 3, 7 and 14 (g; **A**) and change in body weight between day 0 and 14 (%; **B**) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. **P<0.01 v WT. n=10-14.

EFFECT OF GLOBAL NPR-C DELETION ON BODY WEIGHT IN FEMALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

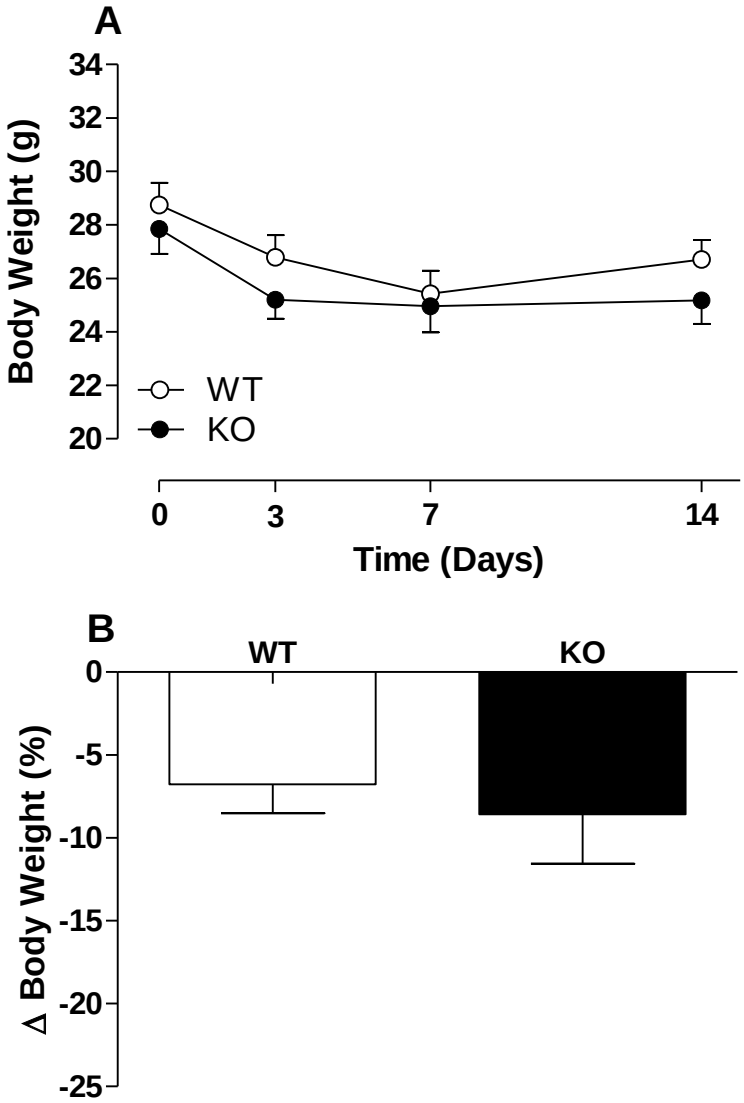


Figure 61. Effect of global NPR-C deletion on body weight in female mice with bleomycin-induced pulmonary hypertension.

Body weight at day 0, 3, 7 and 14 (g; **A**) and change in body weight between day 0 and 14 (%; **B**) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. n=14-16.

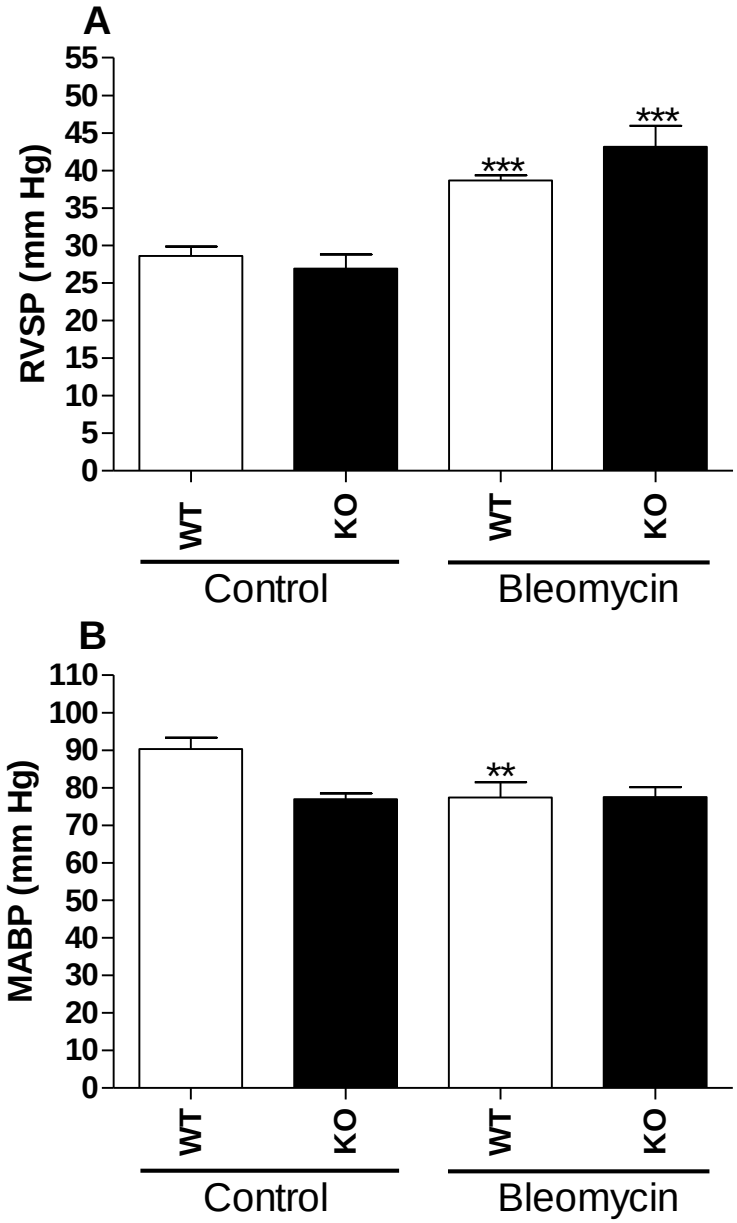


Figure 62. Effect of global NPR-C deletion on haemodynamics in male mice with bleomycin-induced pulmonary hypertension.

Right ventricular systolic pressure (RVSP; **A**) and mean arterial blood pressure (MABP; **B**) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. **P<0.01 v WT, ***P<0.001 v control WT, KO. n=4-11.

EFFECT OF GLOBAL NPR-C DELETION ON HAEMODYNAMICS IN FEMALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

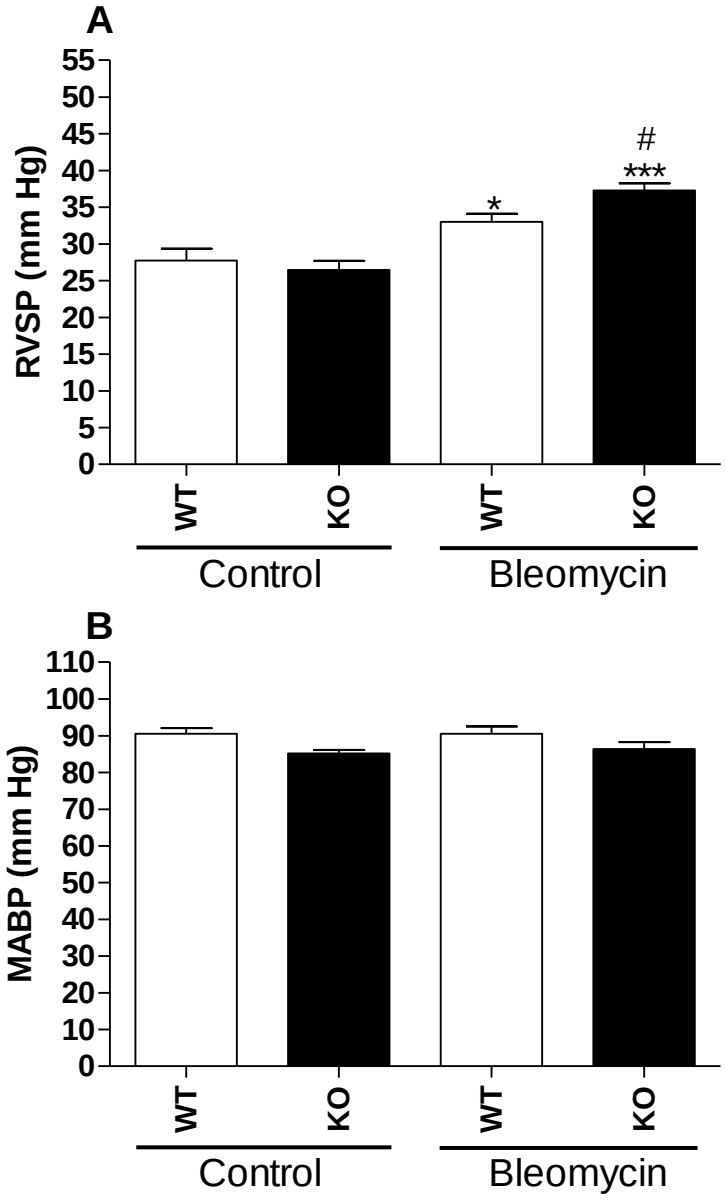


Figure 63. Effect of global NPR-C deletion on haemodynamics in female mice with bleomycin-induced pulmonary hypertension.

Right ventricular systolic pressure (RVSP; **A**) and mean arterial blood pressure (MABP; **B**) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. *P<0.05 v WT, ***P<0.001 v KO control; #P<0.05 v Bleo WT. n=8-15.

EFFECT OF GLOBAL NPR-C DELETION ON RIGHT VENTRICULAR HYPERTROPHY IN MALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

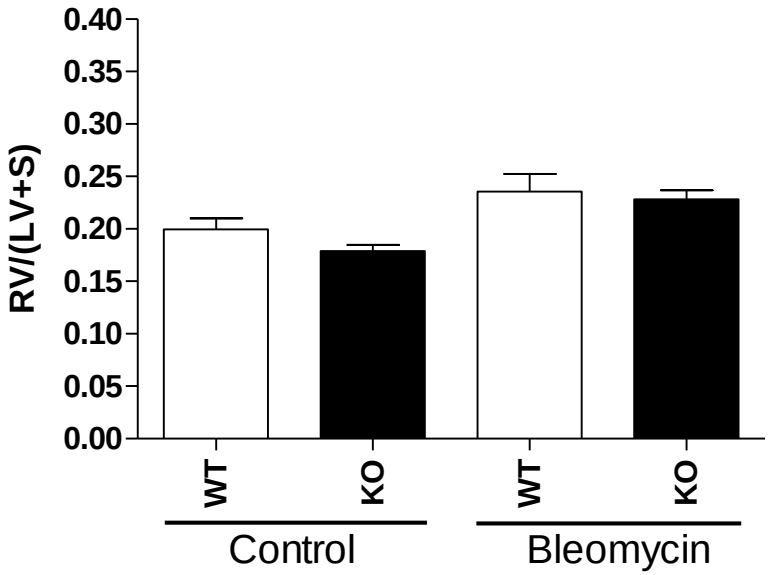


Figure 64. Effect of global NPR-C deletion on right ventricular hypertrophy in male mice with bleomycin-induced pulmonary hypertension.

Right ventricle to left ventricle plus septum ratio (RV/[LV+S]) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. n=6-15.

EFFECT OF GLOBAL NPR-C DELETION ON RIGHT VENTRICULAR HYPERTROPHY IN FEMALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

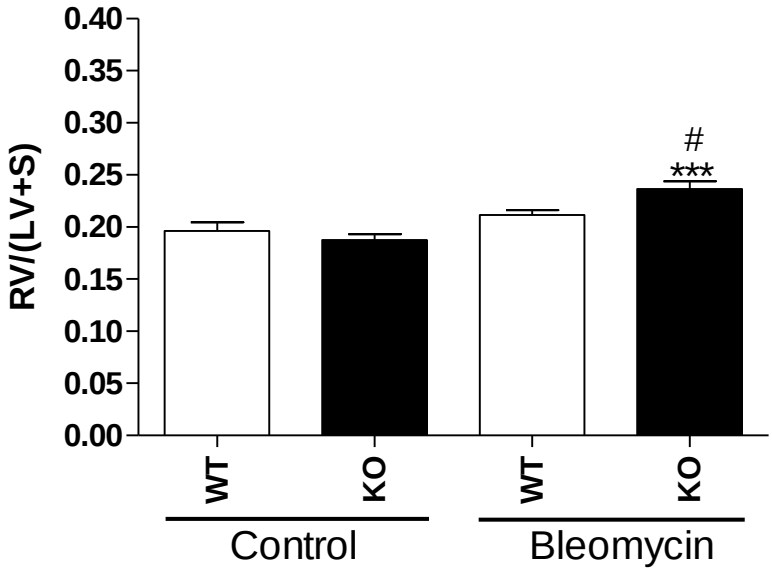


Figure 65. Effect of global NPR-C deletion on right ventricular hypertrophy in female mice with bleomycin-induced pulmonary hypertension.

Right ventricle to left ventricle plus septum ratio (RV/[LV+S]) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. ***P<0.001 v KO, *P<0.05 v Bleo WT. n=11-15.

EFFECT OF GLOBAL NPR-C DELETION ON CHANGE IN RIGHT VENTRICULAR SYSTOLIC PRESSURE AND HYPERTROPHY IN MALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

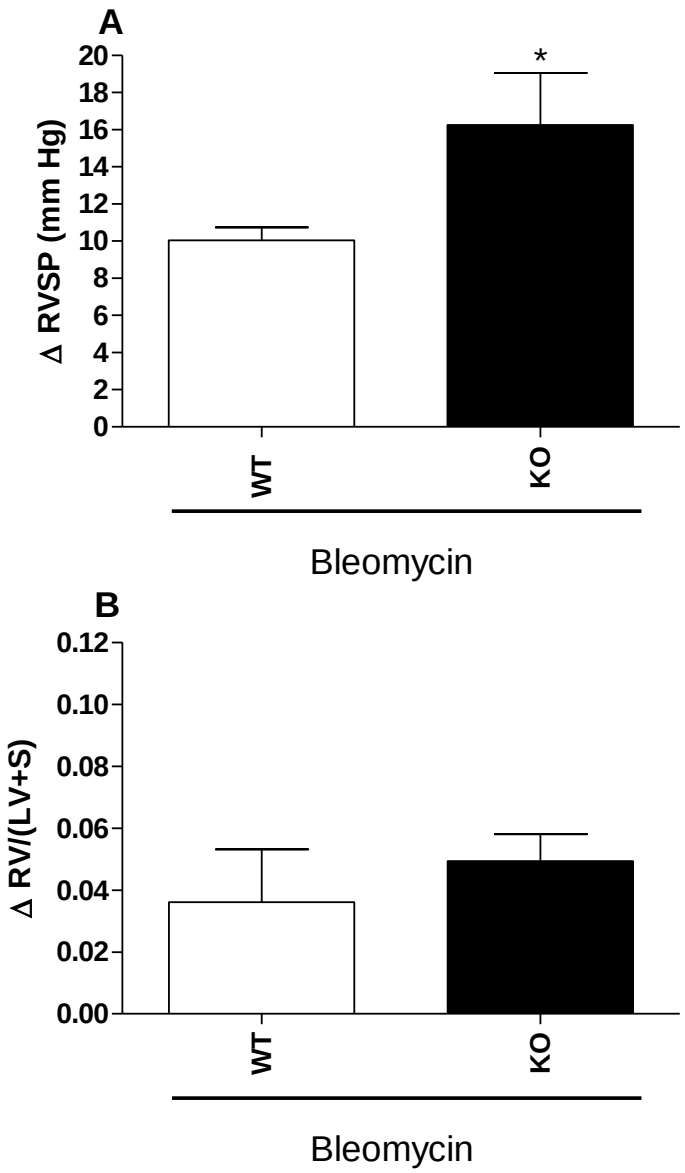


Figure 66. Effect of global NPR-C deletion on change in right ventricular systolic pressure and hypertrophy in male mice with bleomycin-induced pulmonary hypertension.

Change in right ventricular systolic pressure (RVSP; **A**) and right ventricle to left ventricle plus septum ratio (RV/[LV+S]; **B**) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. *P<0.05 v WT. n=10-11.

EFFECT OF GLOBAL NPR-C DELETION ON CHANGE IN RIGHT VENTRICULAR SYSTOLIC PRESSURE AND HYPERTROPHY IN FEMALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

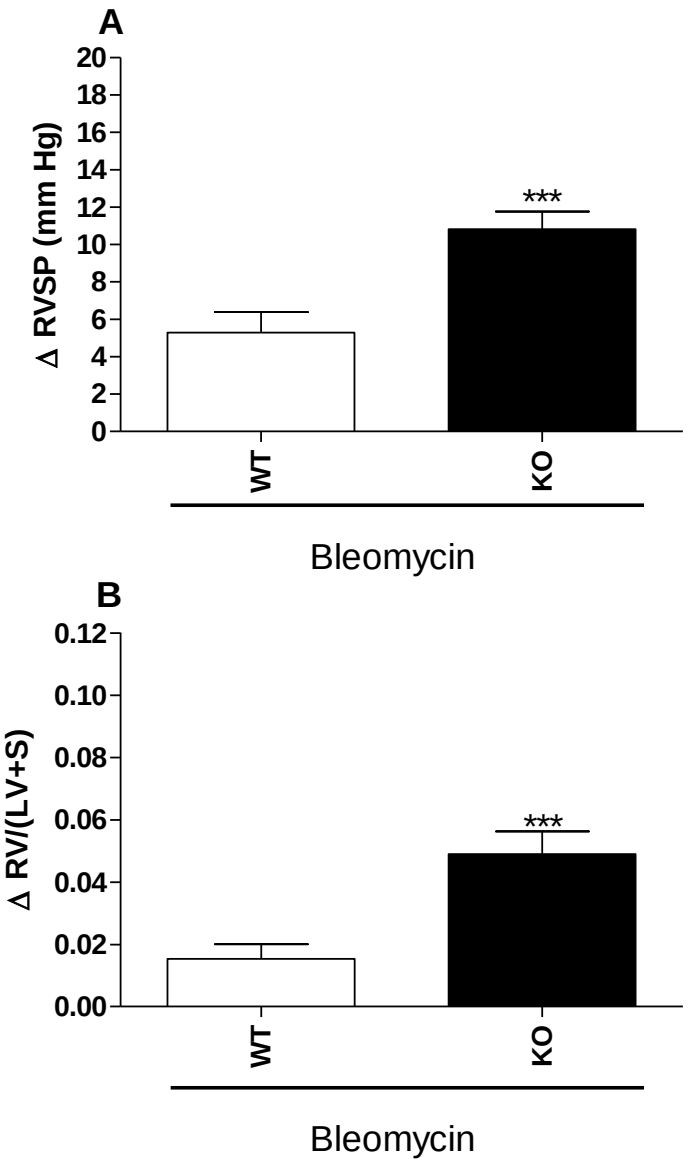


Figure 67. Effect of global NPR-C deletion on change in right ventricular systolic pressure and hypertrophy in female mice with bleomycin-induced pulmonary hypertension.

Change in right ventricular systolic pressure (RVSP; **A**) and right ventricle to left ventricle plus septum ratio (RV/[LV+S]; **B**) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0) . Data are represented as mean ± SEM. ***P<0.001 v WT. n=14-15.

EFFECT OF GLOBAL NPR-C DELETION ON LUNG WEIGHT IN MALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

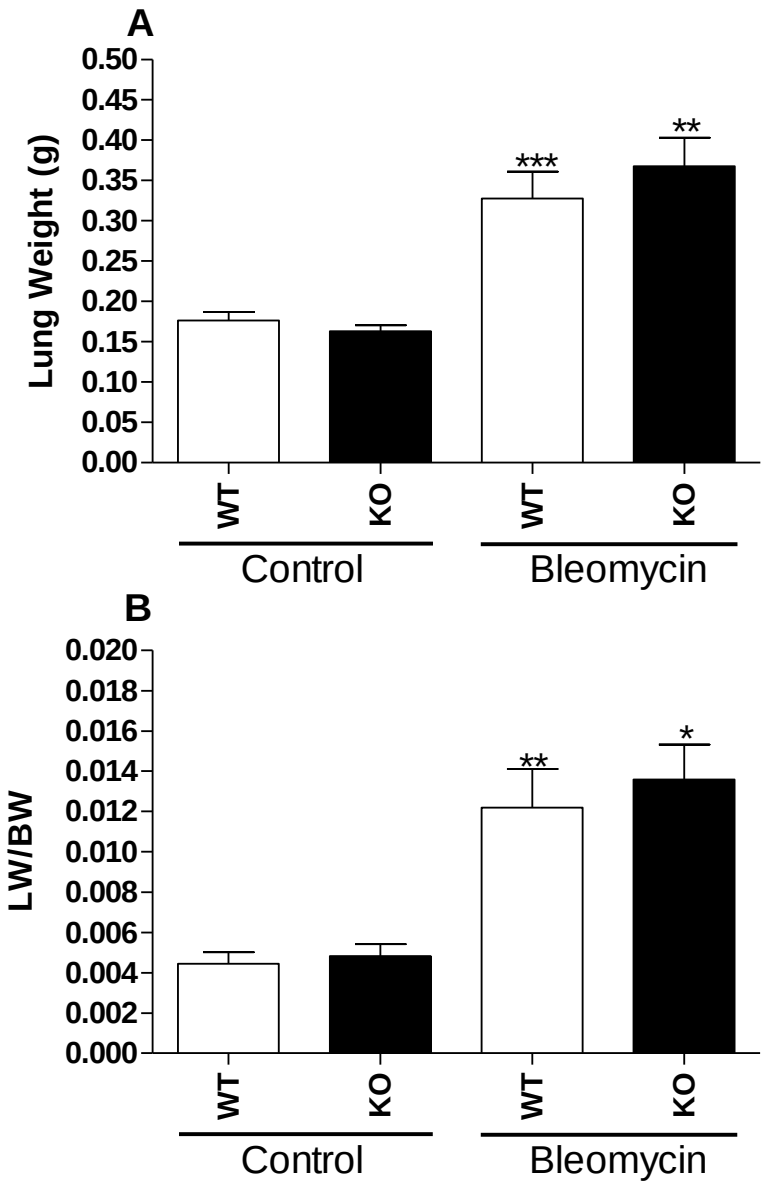


Figure 68. Effect of global NPR-C deletion on lung weight in male mice with bleomycin-induced pulmonary hypertension.

Lung weight (g; **A**) and lung weight to body weight ratio (LW/BW; **B**) in male natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. ***P<0.001 v WT, **P<0.01 v KO (**A**). **P<0.01 v WT, *P<0.05 v KO (**B**). n=3-10.

EFFECT OF GLOBAL NPR-C DELETION ON LUNG WEIGHT IN FEMALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

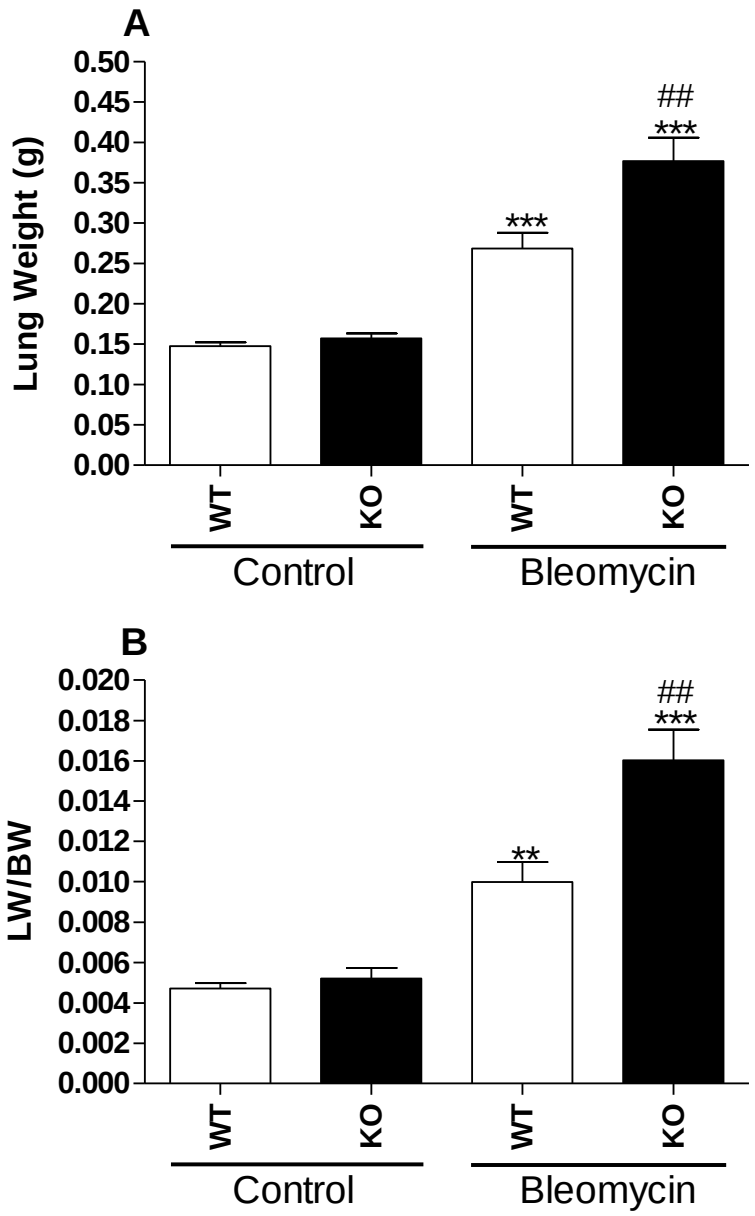


Figure 69. Effect of global NPR-C deletion on lung weight in female mice with bleomycin-induced pulmonary hypertension.

Lung weight (g; **A**) and lung weight to body weight ratio (LW/BW; **B**) in female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates administered bleomycin (4 mg/kg; o.p.; day 0) or saline (at day 0). Data are represented as mean ± SEM. ***P<0.001 v control WT, KO (**A**). ***P<0.001 v KO; **P<0.01 v WT (**B**). ###P<0.001 v Bleo WT (**A** & **B**). n=5-16.

REPRESENTATIVE IMAGES SHOWING THE EFFECTS OF GLOBAL NPR-C DELETION ON LUNG FIBROSIS IN MALE AND FEMALE MICE WITH BLEOMYCIN-INDUCED PULMONARY HYPERTENSION

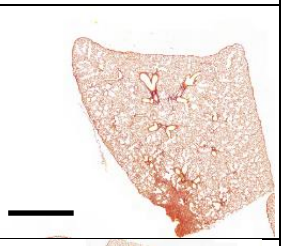
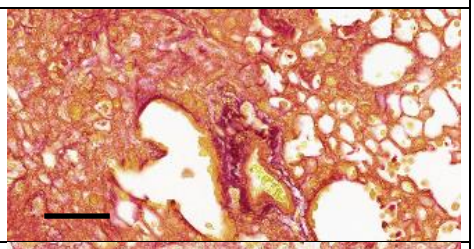
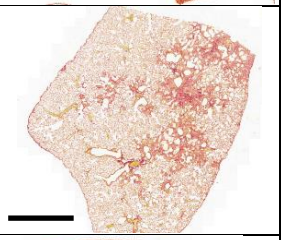
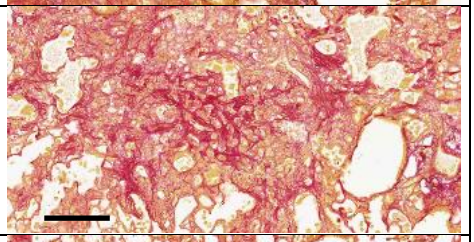
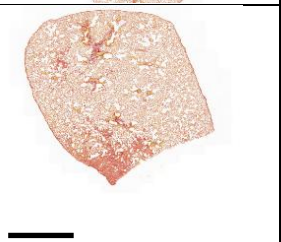
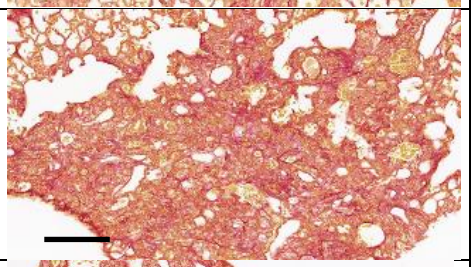
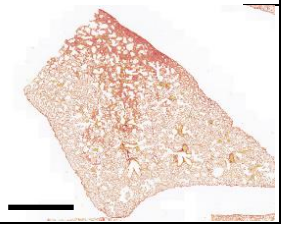
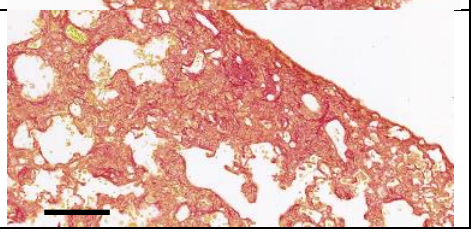
	x1 magnification	x10 magnification
Male NPR-C WT		
Male NPR-C KO		
Female NPR-C WT		
Female NPR-C KO		

Table 23. Representative images showing the effects of global NPR-C deletion on lung fibrosis in male and female mice with bleomycin-induced pulmonary hypertension.

Representative images of lung fibrosis in male and female natriuretic peptide receptor-C knockout (NPR-C KO) mice and wild-type (WT) littermates 14 days following administration of bleomycin (4 mg/kg; o.p.; day 0). Picrosirius red (PSR) stain (red). Scale bar at x1 magnification = 2 mm. Scale bar at x10 magnification = 200 µm.

CHAPTER 5

DISCUSSION

CHAPTER 5: DISCUSSION

5.1 Summary of key findings

I have shown that MRPs contribute to cyclic GMP signal termination in the vasculature contributing to the regulation of homeostasis. Specifically, I have found MRP inhibition alone relaxes mouse aortic rings, and potentiates relaxations induced by both sGC and pGC activation, with a preference toward pGC. In addition, MRP inhibition significantly attenuates cGMP efflux by hCASMCs resulting from pGC activation, which is not seen with sGC stimulation or MRP inhibition alone. Despite this, MRP inhibition *per se* concentration-dependently attenuates hCASMC proliferation and enhances the anti-proliferative effects of both pGC and sGC -generated cGMP. I have also provided evidence for a functional role for MRPs in regulating vascular responses *in vivo*. Whilst MRP inhibition itself does not reduce BP, blockade of cGMP efflux does significantly enhance reductions in BP triggered by pGC activation (but not sGC). These data demonstrate MRPs contribute to the regulation of cGMP-dependent vascular homeostasis, with a preference toward membrane produced cGMP rather than cytosolic, in accord with cGMP signalling compartmentalisation.

Using male and female endothelial cell-specific CNP KO mice, in a well-established hypoxia plus SU5416 model of PH, I have found that EC-derived CNP does not contribute to the development of PH in either sex. However, I found that the CNP receptor, NPR-C, is essential for innate protection against the development of significant RVH in both male and female mice using the same experimental model of PH (using global NPR-C KO animals), although a similar effect against increased RVSP is not seen. My results also demonstrate that NPR-C is essential in the protection against the development of bleomycin-induced PH. Specifically, the absence of NPR-C causes significant increases in RVSP in both male and female mice, and greater RVH and lung weights in females. In sum, CNP derived from endothelial cells, specifically, is not

essential in the protection against PH pathogenesis; however, its receptor, NPR-C, appears to be crucial in maintaining normal pulmonary and cardiac function in this disease.

5.2 The role of MRPs in vascular homeostasis

The intracellular second messenger cGMP is a well characterised, central player in the regulation of cardiovascular homeostasis (Francis *et al*., 2010; Tsai and Kass, 2009). Increasing intracellular concentrations of cGMP relaxes vessels, inhibits platelet aggregation and prevents VSMC proliferation. Maintenance of intracellular cGMP concentrations is thought to consist of a finely-tuned balance between cGMP generation by GC enzymes and degradation by PDEs. Cellular extrusion of cyclic GMP has been appreciated for many years, but with the general opinion that it's of lesser importance than PDEs in terminating cGMP signalling; this has led to this mechanism being largely ignored in terms of cGMP dynamics and cardiovascular function (Davoren and Sutherland, 1963; Mercapide *et al*., 1999). Recently, MRPs have been identified as the transport mechanism responsible for the cellular efflux of cAMP and cGMP. As a consequence, this has led to the hypothesis that MRPs contribute to the regulation of cyclic nucleotide dependent vascular homeostasis. Indeed, there is mounting evidence that MRP-dependent cAMP efflux has a broad array of cardiovascular effects; inhibition of VSMC growth and platelet aggregation improved cardiac contractility, reduced cardiac fibrosis, and as a therapeutic target in PH (Borgognone and Pulcinelli, 2012; Claude *et al*., 2015; Hara *et al*., 2011; Sassi *et al*., 2014, 2008). However, there is a paucity of evidence defining cGMP-dependent cardiovascular effects of MRPs. Therefore, in this thesis I have investigated functional cGMP-dependent biological effects in the vasculature modulated by MRPs.

One key limitation in this field is the lack of potent and selective pharmacological MRP inhibitors. Some have circumvented this crucial caveat

by utilising siRNA to reduce expression of specific MRPs within different cell-types, in addition to studies using MRP4 and MRP5 KO mice. However, in the absence of these techniques and/or transgenic models, this illustrates a need to use structurally-distinct MRP inhibitors to reassure as to target specificity. Specifically-designed, selective MRP inhibitors have not yet been developed, but compound screening aimed at finding novel competitive inhibitors of this transporter protein family have been successful. During such screenings the MRP inhibitors MK571 and probenecid were identified. MK571 is an inhibitor of MRP4 (Chen *et al*., 2002; Jedlitschky *et al*., 2000; Reid *et al*., 2003) and considered by most to be a specific inhibitor of this particular isoform, despite other studies showing effective MRP5 inhibition by this compound (Reid *et al*., 2003). In contrast, probenecid is a relatively selective inhibitor of MRP5 over MRP4 (Jedlitschky *et al*., 2000; Reid *et al*., 2003). Although, some evidence shows that at the same concentrations probenecid can inhibit MRP4 equally well as MRP5 (Chen *et al*., 2002; van Aubel *et al*., 2002). In sum, MK571 and probenecid are likely to have both MRP4 and MRP5 inhibitory activity and therefore the effect of MRP inhibition I observed may have come about from either MRP4 or MRP5 or simultaneous MRP4/MRP5 inhibition.

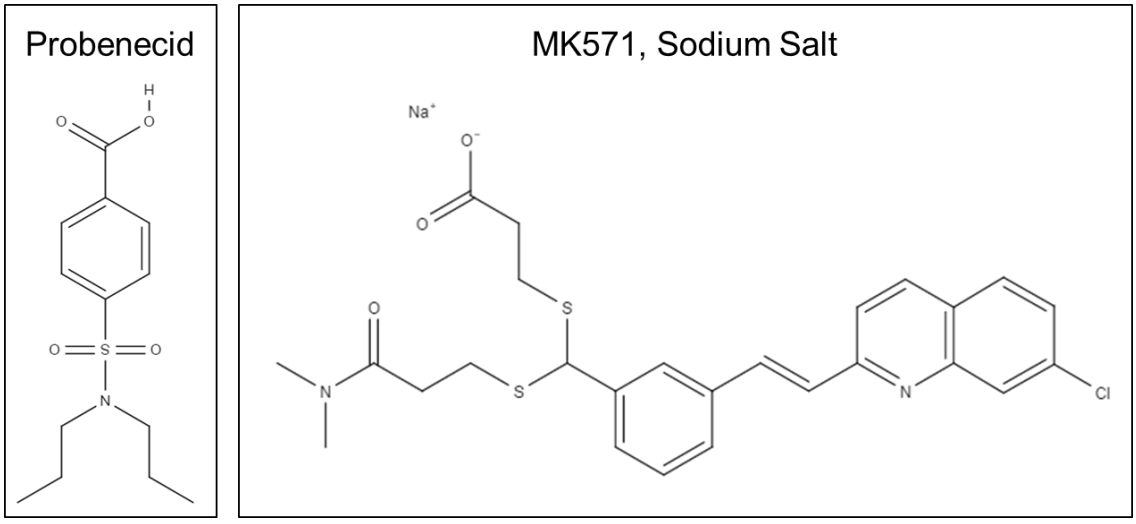


Figure 70. Molecular formulae of MK571 and probenecid.

Molecular formulae demonstrating the structural distinction between multidrug resistance protein inhibitors probenecid and MK571.

Although, structurally-distinct inhibitors of MRPs (Figure 70), these compounds also affect alternate mechanisms such that possible off-target effects should therefore be considered.

MK571 was originally developed as a leukotriene D4 (LTD4) receptor (also known as a cysteinyl leukotriene receptor-1, $CysLT_1$) antagonist, for the treatment of asthma (Jones *et al*., 1989; Kips *et al*., 1991; Young, 1991). This drug was later dropped for a more potent candidate compound, MK-0476, currently prescribed by the name Montelukast. MK571 was originally identified as an inhibitor of MRPs because of its structural similarity to the leukotrienes, which are well characterised substrates of MRP1 (Gekeler *et al*., 1995; Leier *et al*., 1996, 1994). MK571 prevents the contraction and proliferation of airway smooth muscle and VSMCs caused by leukotriene D4 (Panettieri *et al*., 1998). Moreover, MRP1, another transporter of the ABCC family, can extrude leukotrienes contributing to inflammation and oxidative stress -mediated VSMC proliferation. However, these actions should not have complicated interpretation of my data because leukotrienes were not added to any of my experimental setups and the experimental conditions utilised did not involve oxidative stress and inflammation. Thus, it is reasonable to assume that antagonism or transport of leukotrienes would not have complicated interpretation of my findings (Cole, 2014; Mueller *et al*., 2008; Widder *et al*., 2007).

Probenecid was first synthesised in 1949 and designed to reduce the renal clearance of penicillin prescribed to soldiers during World War II with the aim of reducing the dose prescribed and thus the cost. Since then, probenecid has been found to reduce the clearance, therefore increase the plasma concentration and half-life of a multitude of antibiotics making it highly effective as an antibiotic adjuvant. The mode of action of probenecid is as a non-specific inhibitor of OAT proteins, which also reduce the reabsorption of uric acid, explaining the use of probenecid as a treatment for gout (Roch-Ramel and Guisan, 1999). During studies investigating the mechanism behind cGMP efflux from cells, probenecid was found to inhibit the transport process, leading to the hypothesis that cGMP efflux was due to a kind of OAT (Hamet *et al*., 1989). Therefore, probenecid may have affected my results by inhibiting OATs and not

MRPs as I assumed. OATs are mainly expressed in excretory organs such as the kidney, gastrointestinal tract and liver, which reflect their overall essential role in the distribution and excretion of numerous endogenous metabolic products and exogenous organic anions. However, they are not found in vascular tissue to any extent and therefore the effects I observed in mouse aortic rings were most likely due to MRP inhibition (Riedmaier *et al*., 2012).

Subthreshold concentrations of both MK571 and probenecid were established and utilised in vascular reactivity studies such that they inhibited MRPs without inducing a vascular effect themselves. Therefore, it should be noted that the magnitude of the shifts described within this thesis are concentration-dependent, with a higher concentration of inhibitor potentially eliciting an enhanced response. Practically, however, the use of a higher concentration of MRP inhibitor results in an inability to contract the tissue to PE, preventing effective study. A larger vascular effect of MRP inhibition has already been shown by (Krawutschke *et al*., 2015), in mouse aortic ring assays where relaxations to the NO donor, GS-NO, when pre-incubated with 10 μM MK571; this resulted in a larger leftward shift when compared to the relaxations to Sp-NO in my studies using 3 μM MK571. Thus, whilst the increases in potency observed in my functional pharmacological studies are modest, the beneficial effects of MRP inhibitors I describe are almost certainly underestimates of the maximum attainable outcome.

My data show that both MK571 and probenecid concentration-dependently relax mouse aortic rings, suggesting an inherent functional role of MRPs in regulating vascular tone. Collectively, MK571 and probenecid significantly increased the potency of Sp-NO, ANP and CNP -induced relaxations in pre-contracted mouse aortic rings, with a preference toward pGC activation. The innate ability of MRP inhibitors *per se* to relax vessels probably resulted from intrinsic turn-over of cGMP production by either sGCs or pGCs. The endothelium-dependent vasodilator ACh produced significant relaxations confirming the presence of an intact, functional endothelium. The presence of an intact endothelium in aortic ring segments is known to enhance extracellular concentrations of cGMP (33-fold higher) compared to denuded vessels, which

results from eNOS activity, release of NO from ECs, which in-turn activates sGC present in VSMCs (Schini *et al*., 1989). Surprisingly, I was unable to see potentiation of the dilator response to ACh in my studies. This could be due to cGMP signal compartmentalisation within VSMCs; specifically, that ACh-dependent NO release stimulates VSMC sGC but the cGMP signal generated isn't large enough to be affected by MRPs. Schini *et al*., (1989), showed that treatment of rat aortic rings with ACh causes a peak in tissue cGMP concentration (1343 ± 380 fmol pg^{-1} DNA) after 1 min, but that this concentration decreases by approximately 86 % when measured at 5 min. This observation suggests that the initial cGMP signal following addition of ACh is degraded rapidly by PDEs, perhaps in subcellular locations not under the influence of MRPs. Interestingly, the same study reported that significant egress of cGMP is observed, but only after 60 min. This gives rise to the possibility that the rate of MRP-driven efflux of cGMP is too slow to affect the functional response to ACh. However, the vasorelaxant response to pharmacological addition of NO (via an NO-donor), rather than through endothelium-dependent eNOS activation, is increased in the presence of MK571. One explanation for this discrepancy is that ACh relaxes mouse aorta through an alternative, NO-independent mechanism. However, whilst this is likely to be true in the resistance vasculature where EDHF predominates (Villar *et al*., 2007), endothelium-dependent NO release by ACh is the principal vasodilatory mechanism in the aorta (Chataigneau *et al*., 1999; Scotland *et al*., 2005). An alternative argument is that, in contrast to the capacity of ACh to generate NO in specific areas of the cytosol, an exogenous NO donor produces considerably higher concentrations of NO which leads to maximal activation of the entire sGC pool. This idea is alluded to by Schini *et al*., (1989), since increasing concentrations of ACh do not elevate extracellular cGMP whereas the NO donor, SNP, results in almost a ten-fold increase in extracellular cGMP concentrations. These data imply that more cytosolic cGMP is generated by pharmacological concentrations of NO, spilling over into the membrane region and resulting in increased extrusion by MRPs (Figure 71). In comparison, some studies have reported that cGMP efflux following sGC stimulation (by SNP) is more effective than via pGC stimulation (using ANP; Mercapide *et al*., 1999). However, the data from these studies may not best represent what occurs

biologically as the concentration of both SNP and ANP used was pharmacological rather than physiological (500 µM), entailing that upon stimulation with SNP the cGMP generated may have “spilled over” into the membrane area increasing its rate of extrusion, in comparison to a normal endogenous signal which would result in a contained cytosolic cGMP “cloud” (Figure 71).

In functional reactivity studies MRP inhibition demonstrated a preference toward potentiating pGC-stimulated vessel relaxations compared with sGC activation. Recent innovation has permitted the study of real-time production of cGMP in VSMC, visualised and quantified using an intracellular cGMP FRET sensor. In these cells, MRP inhibition (using MK571) significantly enhances the magnitude of intracellular cGMP generated from both sGC and pGC activation; however, cGMP levels stay maximal for longer periods with pGC activation, compared to sGC where the signal declines relatively rapidly (Krawutschke *et al*., 2015). These data indicate that MRPs are more important in regulating membrane-localised cGMP “pools”, rather than cytosolic cGMP, which instead is predominantly PDE regulated (Fischmeister *et al*., 2006; Krawutschke *et al*., 2015). This contributes to the understanding of cGMP signalling compartmentalisation and the preferential effect of MRP inhibition to membrane, compared to cytosolic, -generated cGMP. My data support this notion but also go further to show this dichotomy from a functional standpoint, in which MRP inhibition had a greater effect on vessel relaxation in response to natriuretic peptides than NO donors.

If this difference between sGC and pGC signalling with respect to MRPs can be explained by subcellular co-localisation, currently there is little or no data defining MRP protein-protein interactions with pGCs. However, MRP4 is localised in caveolin-1-enriched membrane fractions in VSMCs, implying that proximity is likely (Sassi *et al*., 2008). Moreover, both NPR-A and NPR-B co-localise with caveolin-3 in cardiac homogenates (Doyle *et al*., 1997; Horikawa *et al*., 2011), and NPR-A co-localises with caveolin-1 in lung homogenates (Chen *et al*., 2012). Therefore, one might hypothesise that pGCs and MRPs could be co-localised in micro-domains, meaning the effect of cGMP egression would

have a greater effect on pGC than sGC -signalling. Furthermore, the cGMP downstream effecter molecules PKG and PDEs can also be present either in cytosolic or particular subcellular membrane fractions meaning that the co-localisation of MRPs, pGCs, PKG and PDEs within caveolae/microdomains forms a highly regulated cGMP signalling environment through precisely controlling concentrations of intracellular cGMP to elicit particular cellular functions (Francis *et al*., 2010). In some situations this phenomenon may also be of relevance to sGC because of its ability to translocate to the plasma membrane via the chaperone Hsp^{90} (Agullo *et al*., 2005; Venema *et al*., 2003). In addition to membrane trafficking by Hsp^{90}, a fraction of endogenous sGC within the normal heart is found in caveolin-3 (Tsai *et al*., 2012) and caveolin-1 (Linder *et al*., 2005) -rich micro-domains. When present in the caveolae the enzyme is protected against oxidation that can occur from vascular injury, which inactivates the catalytic haem domain on the β subunit; therefore, the enzyme remains active compared to its cytosolic counterparts (Tsai *et al*., 2012). Overall, this demonstrates that not only can MRP inhibition potentiate pGC produced cGMP, but in disease environments characterised by oxidative stress, MRP inhibition may also potentiate cGMP generated from membrane located sGC, amplifying the signal, thus expanding the therapeutic potential of MRP inhibitors (Schnabel and Blankenberg, 2007; Figure 71). Interestingly, in the case of probenecid the potency of Sp-NO was significantly inhibited in my functional pharmacological studies; this almost certainly results from direct inhibition of sGC. This characteristic of probenecid has already been described in cultured porcine kidney epithelial and rat fibroblast cells; the mechanism is thought to involve blocking of the interaction between two unidentified, heat-stable, high molecular weight factors that each facilitate the production of cGMP by sGC (Patel *et al*., 1995). This appears to be a sGC specific phenomenon as ANP stimulated cGMP is not affected by probenecid (Hamet *et al*., 1989).

Determining which specific MRP isoform (MRP4 or MRP5) is responsible for the effects I observed on vascular reactivity is difficult. The accepted cyclic nucleotide transporting specificity of MRP4 and MRP5 is controversial but is likely to play a key role in determining the pathways regulated by both cAMP and cGMP efflux. MRP4 is universally accepted as a predominantly cAMP

transporting protein despite the fact that MRP4 inhibition results in elevated intracellular cGMP levels (Hara *et al*., 2011; Sassi *et al*., 2008). MRP4 has a lower K_m value (9.69 ± 2.3 µM) for cGMP compared to cAMP (44.5 ± 5.8 µM) suggesting it may actually have a preference for cGMP over cAMP (Chen *et al*., 2001). However, the V_{max} for cAMP extrusion (4.14 ± 0.40 pmol/mg/min) is approximately twice as high as cGMP (2.01 ± 0.34 pmol/mg/min; Chen *et al*., 2001). This suggests at low cAMP concentrations MRP4 transports cGMP but at high cAMP concentrations, cAMP itself is egressed. This theory appears to be supported in practice by studies that show increasing cAMP levels decrease cGMP eggression, presumably via competition (Hamet *et al*., 1989; Patel *et al*., 1995). In comparison, MRP5 is commonly accepted as a predominantly cGMP transporting protein because of its significantly lower K_m and higher V_{max} values for cGMP (K_m = 2.1 ± 0.2 µM; V_{max}/K_m = 2100 µl x mg^{-1} protein x min^{-1}) compared to cAMP (K_m = 379 ± 24 µM; V_{max}/K_m = 90 µl x mg^{-1} protein x min^{-1}; Jedlitschky *et al*. 2000). Therefore, in the context of my reactivity studies I hypothesise that both MRP4 and MRP5 efflux cGMP simultaneously upon sGC and pGC activation, with MRP5 being the more favoured protein because of its superior efficiency in exporting cGMP. Whereas, vasodilatation elicited by increased cAMP, such as those I observed with Iso, is likely to be more dependent on MRP4, with MRP5 playing a minor role.

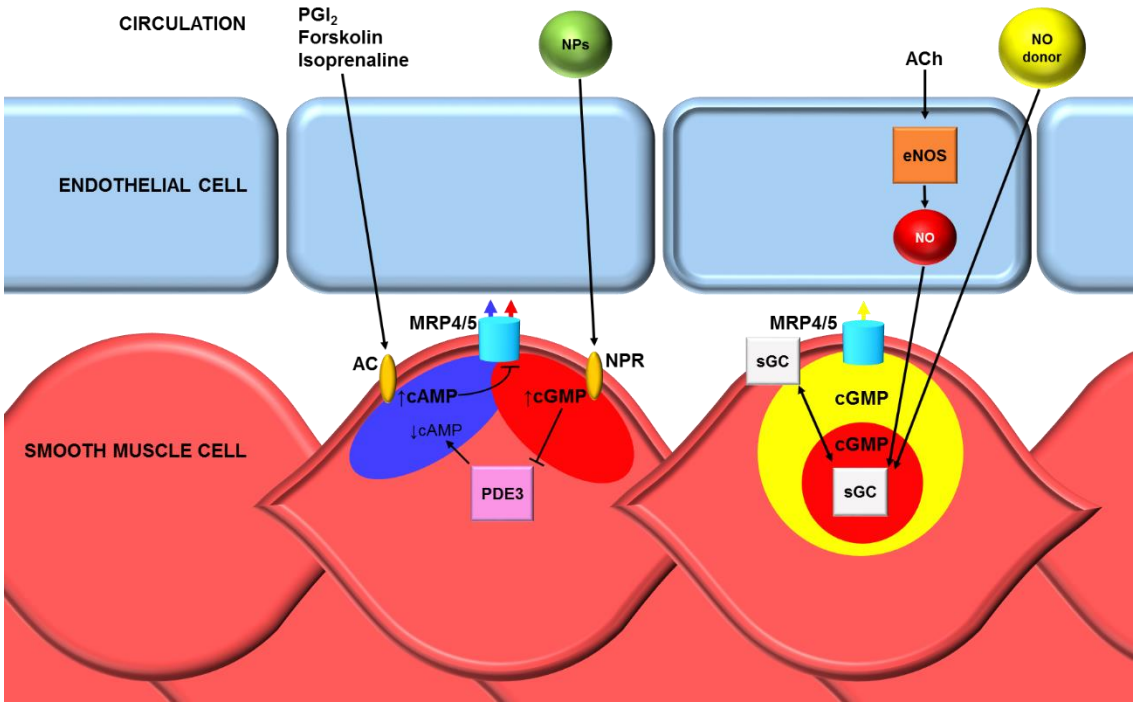


Figure 71. An illustration of the multiple interactions multidrug resistance proteins have with cGMP and cAMP elevating and degrading enzymes within the vasculature.

Endothelial nitric oxide synthase (eNOS), soluble guanylyl cyclase (sGC), nitric oxide (NO), prostacyclin (PGI_2), natriuretic peptides (NPs), multidrug resistance protein (MRP), phosphodiesterase (PDE), adenylyl cyclase (AC), natriuretic peptide receptor (NPR), acetylcholine (ACh).

This notion of simultaneous cAMP and cGMP transport by MRPs is complemented by the finding that both MRP4 and MRP5 contain two substrate-binding sites; MRP4 having a high affinity cAMP substrate-binding site, MRP5 a high affinity cGMP binding site, and both proteins a low affinity dual cAMP and cGMP binding site (Sager and Ravna, 2009). This entails that, depending on local concentration, both cyclic nucleotides can be transported by either one of these proteins. This theory is likely to be true because the MRP substrate-binding pocket is known to bind more than one substrate at one time, based on export kinetics of native and conjugated substrates (Deeley and Cole, 2006; Schumacher *et al*., 2004). This facet gives MRPs a broad substrate-transporting capability (Sharom, 2008). However, such kinetic variations have not yet been reported for cyclic nucleotide transport (Deeley and Cole, 2006).

Specific cell and tissue expression of MRP4 and MRP5 will also determine their role in VSMC relaxation. In the vasculature, MRP4 and MRP5 are both expressed on ECs; however, MRP4 is considered by some to be the only MRP expressed on VSMCs, and MRP5 mainly an EC located protein (Sassi *et al*., 2008). This has led to the assumption that all VSMC effects are elicited by MRP4 specifically, although there is plenty of evidence to show MRP5 expression on VSMC in addition to ECs (Dazert *et al*., 2003; Meissner *et al*., 2007; Mitani *et al*., 2003; Xu *et al*., 2004). Evidence supports the thesis that both MRP4 and MRP5 are expressed on VSMC and contribute to vessel reactivity since reduction of MRP5 expression using siRNA in rat pial arteriolar smooth muscle enhances sGC stimulated relaxations and decreases extracellular cGMP levels, demonstrating a specific functional effect of MRP5, albeit in the cerebral vasculature (Xu *et al*., 2004). Overall, these findings indicate that MRP4 and MRP5 are capable of regulating vascular homeostasis, with the inhibition of both proteins likely to elicit an additive, more advantageous effect. However, to elucidate the precise role of each in vascular reactivity will necessitate the use of MRP4 and MRP5 KO mice (Borst *et al*., 2007).

A similar pattern of activity with MRP inhibition was observed in parallel experiments in cultured hCASMC. I found concentration-dependent reductions in proliferation with MK571 alone and, in addition, a subthreshold concentration of MK571 potentiated the anti-proliferative effects of ANP and D-NO, with a preference toward sGC activation (in contrast to the vascular reactivity data). Similar to the vascular reactivity experiments, the ability of MRP inhibition *per se* to attenuate VSMC proliferation is likely due to intrinsic cGMP production, supported by MK571 alone generating small increases (~10 % in 15 min) in intracellular cGMP in VSMCs as measured by FRET (Krawutschke *et al*., 2015); when sustained over several days this could potentially have inhibited VSMC proliferation. In addition, MRP4 expression has been found to be significantly increased in proliferating hCASMCs compared to quiescent cells, increasing the quantity of cGMP (and cAMP) egressed; thus, an anti-proliferative effect of MRP inhibition can be achieved (Sassi *et al*., 2008). To emphasise the effect of MRP inhibition on intracellular cGMP concentrations and VSMC proliferation,

previous studies have shown that in primary hCASMCs rendered deficient in MRP4 or MRP5 using siRNA, there is a significant increase in the intra:extra -cellular cGMP and cAMP ratios under basal conditions, that results in a concomitant attenuation of growth (Sassi *et al*., 2008). However, in these studies the authors concluded that this was mainly a cAMP-driven phenomenon because the use of the PKG inhibitor, KT5823, did not affect proliferation.

My data demonstrate that MRPs possess the capacity to extrude cGMP in terms of functional vasorelaxation and VSMC proliferation, but I didn't observe changes in VSMC intra:extra -cellular cGMP ratios in the presence of MK571 alone. The anti-proliferative effect of the NO donor, D-NO, was significantly enhanced when combined with MK571; however, I observed no difference in the VSMC intra:extra -cellular cGMP ratio. In contrast, the extracellular cGMP concentrations after pGC activation with ANP showed a substantial, significant increase, which was attenuated by MRP inhibition. This reflects my findings in vascular reactivity studies where MRPs appear to be preferentially coupled to pGC. The apparent lack of difference in VSMC intra:extra -cellular cGMP ratio with MRP inhibition alone may have resulted from not allowing sufficient time for cGMP to accumulate under basal conditions, since my measurements were performed after 24 h compared with those of Sassi *et al*., (2008) after 72 h. In addition, smaller levels of intra:extra -cellular cGMP may have been detectable using a more sensitive technique such as FRET. This may also explain the lack of difference in the VSMC intra:extra -cellular cGMP ratio with D-NO, where significant differences in proliferation were noted over the whole 96 h growth period but changes in intra:extra -cellular cGMP after 24 h may have been too small to measure by ELISA. In addition, signal compartmentalisation may have come into play, where less of the cGMP signal generated from sGC activation was under the influence of MRPs compared with that of pGC activation (Figure 71).

The vasodilatory effect of MRP inhibition alone may only be an isolated vessel phenomenon, because MK571 did not significantly affect MABP in anaesthetised and conscious mice, despite the compound having a relatively long plasma half-life (two to three hours in humans; Margolskee, 1991) and the

dose used (25 mg/kg/day) having previously been shown to effectively reverse experimental PH (Hara *et al*., 2011). However, MK571 did exhibit a preference to potentiate pGC activation evident by significantly enhanced reductions in systemic BP elicited by acute bolus doses of ANP (but not NO). This suggests in situations where endogenous circulating levels of NPs are increased (e.g. heart failure, MI and PH) that MRP inhibition will potentiate reductions in BP, and the lack of an observable effect of MRP inhibition on MABP, in conscious mice, may be due to low basal circulating concentrations of circulating NPs. This should not be a surprise as the MRP4, MRP5 and MRP4/MRP5 double KO mice all lack an obvious phenotype, suggesting under basal conditions MRPs aren't essential to vascular homeostasis (Borst *et al*., 2007). However, longer-term MRP4 deficiency results in cardiac hypertrophy, as observed in aging (9 month old) MRP4 KO mice, which is not apparent in the same strain at 3 months (Sassi *et al*., 2012). Thus, in health this mechanism may not be crucial to the maintenance of cardiovascular homeostasis *in vivo*, whereas its role may be triggered in cardiovascular disease characterised by enhanced circulating levels of NPs (e.g. heart failure, PH and MI; Potter *et al*., 2006).

Overall, my data describe in detail the role of MRPs in regulating several facets of vascular homeostasis, vessel reactivity, VSMC proliferation and BP *in vivo*, by augmenting cGMP signalling. Furthermore, this thesis provides further evidence to support the importance of MRPs in contributing to cGMP compartmentalisation through the ability of MK571 to favour pGC over sGC activation.

5.2.1 Effect of MRPs in cardiovascular disease

A key observation of the potential impact of MRPs in cardiovascular disease was made by Hara *et al*., (2011), who showed that MRP4 expression is significantly increased in the pulmonary arteries of PH patients and in the lungs of mice exposed to hypoxia. Furthermore, MRP4 expression is increased in proliferating VSMCs compared to quiescent cells, suggesting in vascular diseases characterised by VSMC hyperplasia, MRPs could be reducing the

anti-proliferative effect of cyclic nucleotides (Sassi *et al*., 2008). Also, MRP5 expression is increased in blood vessels and cardiomyocytes in the hearts of patients with ischaemic cardiomyopathy (Dazert *et al*., 2003). Overall, this demonstrates that in cardiovascular diseases (e.g. PH), MRP expression increases substantially, similar to that observed with PDEs, blunting the anti-proliferative and blood pressure lowering potential of endogenous NO and NP signalling (Wharton *et al*., 2005). Therefore, inhibiting MRPs *per se* to treat cardiovascular disorders could pose an attractive therapeutic target, in addition to also being an effective adjuvant to other cGMP elevating agents.

Indeed, the potential power of MRP inhibition alone can be predicted when considering the increased levels of circulating ANP, BNP and CNP in patients with cardiovascular diseases such as PH, MI and congestive heart failure (Kaiser *et al*., 2015; Potter *et al*., 2006). Synergy between cGMP elevating agents (e.g. PDE and NEP inhibitors) has been well characterised in experimental models of PH and fibrosis (Baliga *et al*., 2014, 2008). Indeed, cGMP/cAMP -elevating combination therapies have been shown to be effective in patients with PH (e.g. epoprostenol and sildenafil; Simonneau *et al*., 2008). This gives credence to the idea that combination of NP elevating agents (e.g. NEP inhibitors and/or PDE inhibitors) with MRP inhibitors is also likely to be of therapeutic benefit. Indeed, the link between two such mechanisms, cGMP degradation due to PDEs and efflux by MRPs, has been demonstrated through greater elevations in VSMC intracellular cGMP concentrations in the presence of MRP inhibitors when combined with PDE inhibitors such as sildenafil and IBMX (Hara *et al*., 2011; Krawutschke *et al*., 2015; Sassi *et al*., 2008). However, the relationship between PDEs and MRPs is not straight-forward. Firstly, treatment with the PDE5 inhibitor, sildenafil, in normoxic WT mice and PASMCs from patients with PH results in a significant increase in MRP4 expression, suggesting a compensatory mechanism to cope with the increased intracellular concentrations of cGMP, which may contribute to tachyphylaxis (Hara *et al*., 2011). This observation is particularly pertinent as PDE5 inhibitors are already prescribed to patients with PH and therefore could result in increased MRP expression and reduced therapeutic value. Second, the PDE5 inhibitor sildenafil and the PDE3 inhibitor trequinsin have each been identified as inhibitors of

MRPs (Chen *et al*., 2002; Jedlitschky *et al*., 2000; Reid *et al*., 2003). This observation has muddied the waters somewhat when measuring increases in intracellular cGMP; are changes due to a PDE inhibition, MRP inhibition or both? This observation leads to the assumption that possible concomitant inhibition of PDE5 and MRPs may contribute to the current therapeutic benefit of sildenafil, and combining cGMP efflux inhibitors with elevators should be explored. Indeed, to support this concept of combination therapy, in the heart PDE9 is the predominant isoform that degrades cGMP generated by NPs, whereas PDE5 favours NO (Lee *et al*., 2015). My data show that MRPs also favour NP generated cGMP over NO/sGC. Therefore, in diseases characterised by elevating responses by NPs appear more advantageous, inhibiting MRPs may pose a more attractive therapeutic target (e.g. heart failure, PH and MI; Potter *et al*., 2006)

Other synergistic effects of cyclic nucleotide transport by MRPs have been observed in VSMCs (and ECs). Here, increasing intracellular cAMP concentrations through adenylyl cyclase activation using forskolin causes an increase in intracellular cGMP due to attenuation of cGMP eggression (Hamet *et al*., 1989). Further evidence of this phenomenon is provided by studies from (Krawutschke *et al*., 2015) in which human VSMC intracellular cGMP levels were increased by the β-adrenoceptor agonist, isoproterenol (isoprenaline), but not by inhibition of PDE3 with cilostamide. This reflects, somewhat, the same cyclic nucleotide cross-talk observed with PDEs and would naturally lead to the prediction that cAMP based treatments such as the IP receptor agonists treprostinil, epoprostenol and iloprost, used in the treatment of PH could be exercising further therapeutic effect by additionally elevating intracellular VSMC cGMP levels because of cAMP directly inhibiting MRPs (Figure 71).

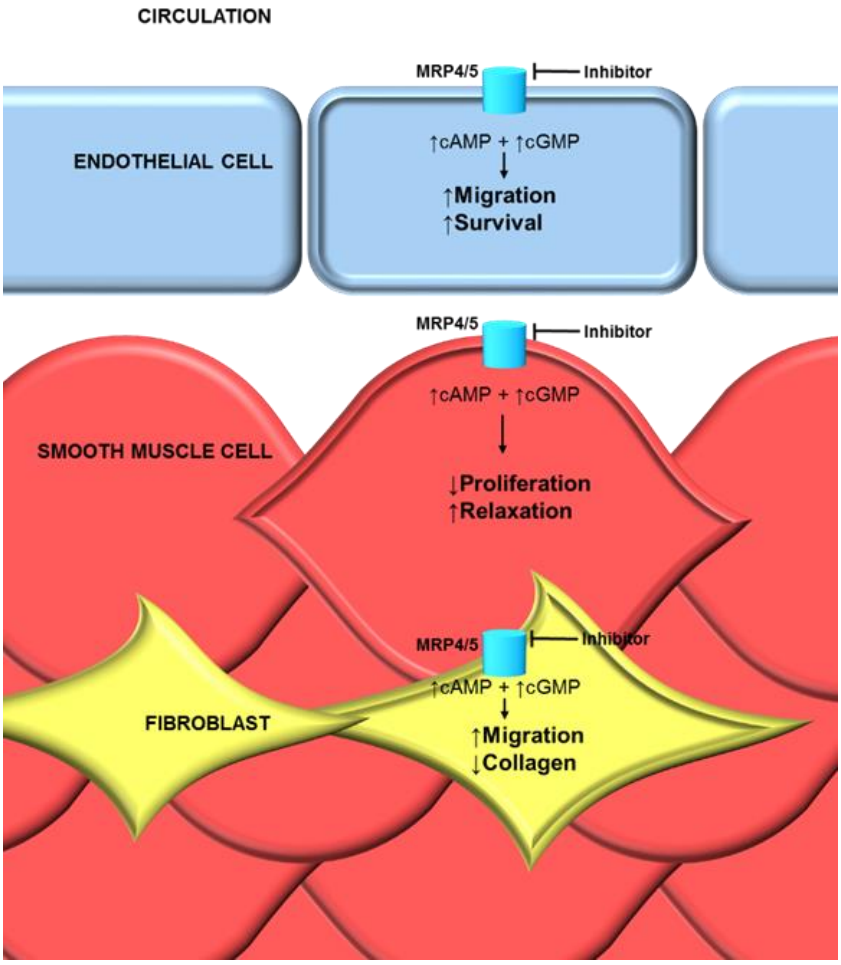


Figure 72. An illustration of the potential cardiovascular benefits of MRP inhibition.

Cyclic adenosine-3',5'-monophosphate (cAMP), cyclic guanosine-3',5'-monophosphate (cGMP), multidrug resistance protein (MRP).

Multidrug resistance proteins may also exert an effect on the process of angiogenesis and repair from vascular injury. Fibroblasts from MRP4 KO mice migrate faster and exhibit improved wound healing capability compared to WT littermates, which is due to higher intracellular cGMP (and cAMP) concentrations (Sinha *et al*., 2013). Indeed, MRP4 knockdown in EC results in enhanced migration and survival because of increased intracellular cyclic nucleotide levels (Tagami *et al*., 2010; Figure 72). The pro-angiogenic factor VEGF, present at high levels in PH, is implicated in this process as it leads to decreased EC MRP4 expression (Tagami *et al*., 2010). Overall, this intimates that MRPs may play a key role in vascular cell migration, which could be particular pertinent in disease states. For example, in fibrotic disorders including

PH, pulmonary fibrosis and cardiac fibrosis, MRP inhibition in fibroblasts could not only enhance migration and therefore wound repair, but also prevent excessive fibrosis by attenuating myofibroblast differentiation which is responsible for synthesis and deposition of extracellular matrix, specifically collagen (Camelliti *et al*., 2005; Figure 72). Increasing intracellular cAMP (Swaney *et al*., 2005), and cGMP (Baliga *et al*., 2014) through PDE5 and NEP inhibition, has already been demonstrated to reduce fibrosis. Dual prescription of IP receptor agonists with either PDE5 inhibitors or sGC stimulators is already in use for the treatment of PH; therefore, targeting both types of cyclic nucleotide simultaneously through MRP inhibition poses an obvious advantage (Simonneau *et al*., 2013).

In sum, my thesis contributes to the understanding of the cGMP-dependent effects of MRPs in vascular homeostasis by demonstrating their ability to modulate vascular reactivity, VSMC proliferation and BP *in vivo*. This suggests that the use of MRP-targeted therapy poses an attractive option for the treatment of cardiovascular disease, and quite possibly could already be in play in the treatment of PH due to off-target effects of PDE inhibitors.

5.3 The role of C-type natriuretic peptide and its cognate receptor natriuretic peptide receptor-C in pulmonary hypertension

Pulmonary hypertension is a devastating and severe pan-vasculopathy with a seven year survival rate upon diagnosis. The pathogenesis of PH appears to initiate with endothelial dysfunction and develops in to a complex multifaceted disease, which particularly benefits from a combination therapy paradigm (Gali *et al*., 2013). CNP is a powerful vasoprotective peptide acting through both NPR-C and NPR-B. CNP has been found to be particularly beneficial in the protection against vascular diseases (Moyes *et al*. 2014); however the pharmacodynamic effect of CNP in PH is controversial with some studies demonstrating beneficial effects (Itoh *et al*., 2004; Kimura *et al*., 2016; Murakami *et al*., 2004) and others no effect at all (Casserly *et al*., 2011). The vascular actions of CNP are considered to be through NPR-B stimulation and cGMP-dependent mechanisms, the latter of which is exploited in the treatment of PH. Additionally, CNP is secreted by endothelial cells in response to shear stress and inflammogens, both of which are increased in PH (Wilkins, 2012). Thus, experiments were designed to tease out the exact role of endogenously generated CNP in PH by using ecCNP KO mice in the hypoxia plus SU5416 experimental model. Furthermore, to investigate through which receptor CNP initiates any potential beneficial effect I utilised global NPR-C KO mice in the same preclinical model of PH. Furthermore, since CNP has been demonstrated to exert a pronounced anti-fibrotic capability (Kimura *et al*., 2016; Murakami *et al*., 2004; Soeki *et al*., 2005), I utilised the bleomycin-induced model of PH and investigated the effect of global NPR-C KO on pathogenesis.

5.3.1 The role of endothelial cell-derived C-type natriuretic peptide in pulmonary hypertension

5.3.1.1 Hypoxia plus SU5416 model of PH

To investigate the role of CNP in PH I utilised an animal model that has recently been reported to best recapitulate the characteristic of this disease in mice. The hypoxia plus SU5416 model of PH, originally reported by Ciuclan *et al*., (2011), is described as a severe model of PH best mirroring the histopathological features and progressive nature of the disease found in humans. In addition, this murine model was described to be similar to that published by Taraseviciene-Stewart *et al*., (2001) in rats, which has been thoroughly characterised and accurately represents the disease found in patients (Abe *et al*., 2010). Therefore, as I had mice available to me that were either deficient in CNP from ECs specifically or NPR-C globally, it logically led me to use this murine model to investigate the role of each of these proteins in the development of PH. Firstly, as this model hasn't previously been used in our lab I performed experiments to determine whether the PH phenotype reported could be reproduced in our lab.

In WT mice exposed to hypoxia plus SU5416, as described by Ciuclan *et al*., (2011) I observed large increases in RVSP and RVH indicative of PH. When mice were removed from the chronic hypoxic insult there was a reduction in RVSP and RVH indicating reversal of this pathology. These results suggest that without the continuous insult of hypoxia the mice recover spontaneously, such that the PH phenotype is not irreversible and progressive like the hypoxia plus SU5416 model in rats, and more importantly what's observed in patients. In addition, I also investigated whether a longer period of chronic hypoxia (5 weeks instead of three) resulted in a more severe phenotype. Although, 5 weeks hypoxia did not cause further increases in RVSP and RVH, the values remained essentially the same as at the 3 week time-point, suggesting that the hypoxic insult sustains pathology. In addition, to determine whether this model

was more akin to the disease characterised by the development of severe complex lesions and occluded vessels I qualitatively analysed lungs from mice exposed to 5 weeks hypoxia plus SU5416. I assumed this extended model would exhibit a more severe pathology than 3 weeks hypoxia, thus increasing the chance of detecting signs of advanced vascular lesion. However, I did not detect any complex lesions or occluded vessels in the lungs of these mice. In conclusion, using this model I was able to recapitulate the findings originally published by Ciuclan *et al*., (2011); this model generates a severe PH phenotype whilst the animals are continuously exposed to hypoxia but, in contrast to the original findings of Ciuclan *et al*., (2011), this phenotype reverses when animals are removed from hypoxia. In addition, I did not detect the presence of complex lesions or occluded vessels derived from hyper-proliferative ECs suggesting that this model may not completely recapitulate the human disease as originally concluded. In fact, my observations are mirrored by those of Vitali *et al*., (2014) who whilst performing an in-depth investigation of this model also found that PH like pathologies, RVSP and RVH, regress once mice are returned to normoxia. Interestingly, this group found that hypoxia plus SU5416 treated mice exhibited significant vascular pruning, which is thought to arise from the development of angio-obliterative lesions, also observed in PH patients and the rat hypoxia plus SU5416 model (McLaughlin and McGoon, 2006; Taraseviciene-Stewart *et al*., 2001). However, vascular pruning or angio-obliterative lesions are not characteristics of the mouse chronic hypoxia model of PH, demonstrating advantages of the current model. In addition, the original report demonstrated that when SU5416 is combined with hypoxia in mice, it causes a shift from an apoptotic to a hyper-proliferative EC phenotype, a hallmark of PH, but not observed in hypoxia alone models. In sum, no one animal model recapitulates all the characteristics of PH (Stenmark *et al*., 2009), but I believe the combination of SU5416 with hypoxia better reflects the endothelial dysfunction found in the human disease and therefore allowed me to more appropriately investigate the vascular protective effects of CNP and NPR-C.

To ensure the continual use of this model was economically viable, I investigated possible differences in potency of SU5416 sourced commercially

compared to that synthesised in-house, the latter being significantly cheaper. I did not find any differences in the degree of PH developed when using either source of SU5416. Therefore the less expensive SU5416 (synthesised in-house) was selected and used for all subsequent experiments involving transgenic animals.

The pathophysiological effect of endothelial cell-derived CNP (ecCNP KO mouse) deletion in mice has been well characterised (Moyes *et al*., 2014). Both male and female ecCNP KO mice exhibit increased leukocyte adhesion and platelet aggregation, which contribute to an enhanced development of aortic atherosclerotic plaques and aneurysms (males only, 50 %) when compared to WT littermates. However, only female ecCNP KO mice are inherently hypertensive. These data along with those demonstrating the ability of CNP to modulate EC and VSMC growth (Khambata *et al*., 2011), suggest that endothelial cell-derived CNP is an essential regulator of vascular homeostasis. These striking findings led to the hypothesis that ecCNP plays an essential role in a disease characterised by inflammation, thrombosis, endothelial dysfunction and VSMC proliferation; an excellent example being PH.

Male and female ecCNP WT and KO mice when exposed to hypoxia plus SU5416 developed PH evidenced by significantly increased RVSP, RVH and muscularisation of the pulmonary small arteries. However, no significant differences in RVSP, RVH and extent of muscularisation were observed between genotypes. Overall, these data show that CNP derived from ECs specifically is not essential in the development of PH using this model. This finding is surprising as endothelium-derived CNP has previously been shown to protect against the pathogenesis of vascular disease (atherosclerosis; Moyes *et al*., 2014). In addition, the importance of endothelium-derived CNP in vascular function is more evident in female ecCNP KO mice than males; for this reason I studied both sexes in the experimental model of PH but no overt changes were apparent in either sex. If anything, one would predict that female ecCNP KO mice would have exhibited a pulmonary hypertensive phenotype, assuming the higher blood pressure in the systemic vasculature would be mirrored in the pulmonary circulation. However, I did not observe a high RVSP in female

ecCNP KO mice, under normoxic conditions suggesting ecCNP in females has a disparate function between the pulmonary and systemic circulation. Furthermore, a hallmark of PH is the development of thrombi in the pulmonary circulation exacerbated by vascular lesions, this is due to enhanced platelet aggregation and inflammation that precedes vascular remodelling and predicts survival (Rabinovitch *et al*., 2014). Therefore, the significant anti-thrombotic and leukocyte adhesive properties of ecCNP should be vital to disease pathogenesis. Again, this should lead to a worsening of the PH phenotype in ecCNP KO mice. However, whilst this may potentially be the case in PH patients, this characteristic of the disease may not be best recapitulated in the mouse hypoxia plus SU5416 model. Chronic hypoxia in both rodents and humans is characterised by inflammation consisting of increased infiltration of macrophages and neutrophils, with enhanced secretion of pro-inflammatory cytokines such as TNF-α, IL-1β , IL-6, and IL-8 that increase the risk of aberrant platelet aggregation (Stenmark *et al*., 2006b). Rats and mice exposed to hypoxia plus SU5416 also have increased secretion of pro-inflammatory cytokines and leukocyte recruitment (Ciuclan *et al*., 2011; Taraseviciene-Stewart *et al*., 2001). Although differences in the inflammatory cell profile have been noted; the rat hypoxia plus SU5416 model has only a small number of macrophages in the alveolar space whereas the mouse hypoxia plus SU5416 model demonstrates a significant expression of the macrophage recruitment marker CD68, which is also found in plexiform lesions of PH patients (Rabinovitch *et al*., 2014). Regardless, using the mouse hypoxia plus SU5416 model with its pro-inflammatory phenotype should have illustrated the vital anti-inflammatory properties of EC-derived CNP present in WT mice but absent in KOs, which should have resulted in a more severe phenotype in KOs; it remains unclear why this was not observed.

Anti -thrombotic and -inflammatory characteristics of CNP have been demonstrated with CNP infusion (0.75 μg/h) in the rat monocrotaline model of PH resulting in both reversal of established disease and prevention of its progression (Itoh *et al*., 2004). Specifically, CNP decreases monocyte/macrophage infiltration and reduces expression of pro-thrombotic markers. In addition, CNP infused (2.5 mg/kg/min) prophylactically significantly

attenuates the number of macrophage, neutrophil and lymphocyte lung infiltrates caused by LPS (Kimura *et al*., 2015). In contrast, but mirroring my own findings, Casserly *et al*., (2011) reported that CNP infusion (0.75 and 2.25 µg/h) had no beneficial effect in rats exposed to either chronic hypoxia alone or hypoxia plus SU5416. When CNP was used in the monocrotaline model of PH it was shown to suppress pulmonary EC apoptosis and increase proliferation, in addition to reducing VSMC proliferation. This suggests CNP aided the maintenance of the endothelial barrier, the initial site of monocrotaline insult, inhibiting VSMC proliferation (Itoh *et al*., 2004; Rosenberg and Rabinovitch, 1988). Moreover, when VEGF is added to ECs this same pro-proliferative EC phenotype also occurs (Alon *et al*., 1995; Gerber *et al*., 1998). Furthermore, this forms the foundation for the rat and mouse hypoxia plus SU5416 model where VEGFR inhibition prevents VEGF mediated EC survival and instead encourages EC apoptosis, leading to the selection of a hyper-proliferative apoptotic-resistant EC phenotype (Taraseviciene-Stewart *et al*., 2001). Akin to VEGF, CNP has the ability to promote endothelial cell proliferation, which has been demonstrated extensively to aid neovascularisation, enhancing wound repair after vascular injury (Furuya *et al*., 1995, 1991; Khambata *et al*., 2011; Kühnl *et al*., 2005; Morishige *et al*., 2000; Ohno *et al*., 2002; Pelisek *et al*., 2006); thus, it is perplexing that I didn't observe a more severe PH-like phenotype in mice absent of EC derived CNP.

A speculative explanation for the disparity in effectiveness of exogenously administered CNP (monocrotaline v hypoxia or hypoxia plus SU5416) may depend on the type and degree of pathogenic insult that initiates these different *in vivo* models. Different vascular insults may result in different mechanisms that modulate EC and VSMC growth, which also determine the effectiveness of CNP in attenuating a phenotype in one model over another. Chronic exposure to hypoxia in general, and hypoxia plus SU5416 models of PH, results in an increased secretion of VEGF (Ciuclan *et al*., 2011; Stenmark *et al*., 2006b; Taraseviciene-Stewart *et al*., 2001). VEGF administered to ECs under routine culture significantly reduces CNP secretion and expression (Doi *et al*., 1996). Moreover, exposing VSMC to hypoxia increases expression of VEGF but not bFGF, which contrastingly increases CNP secretion by ECs (Brogi *et al*., 2008;

Suga *et al*., 1992b). Although there are many different mitogens and pro-inflammatory cytokines secreted during hypoxia that can simultaneously increase and decrease CNP secretion, these specifics illustrate that the precise mix of mitogens and pro-inflammatory cytokines generated by hypoxia determine the balance between enhanced or suppressed CNP secretion. Furthermore, it may not just be whether CNP itself is secreted but also the simultaneous secretion of vasoconstrictive, pro-vascular remodelling agents. Suga *et al*., (1992b) demonstrated a number of different mediators that significantly increase CNP secretion from EC cells such as TGF-β, bFGF, thrombin and arginine-vasopressin (AVP); however, the same factors also simultaneously increase ET-1 production suggesting that the secretion of vasodilator/anti-proliferative factors are balanced with vasoconstrictive/pro-proliferative ones. Furthermore, 8-bromo-cAMP and 8-bromo-cGMP, membrane permeable PKA and PKG activators, respectively, increase CNP secretion from ECs in the absence of increased ET-1 secretions. This suggests a potential beneficial positive feedback mechanism where by increased cAMP and cGMP production by either GC or adenylyl cyclase in response to NO, NPs or PGI_2 elevates CNP secretion. Therefore, in PH characterised by reduced cGMP and cAMP signalling, and enhanced ET-1 signalling, it is conceivable that the disease is by nature one of deficient CNP secretion and that its absence in the ecCNP KO I studied does not affect pathogenesis because CNP secretion is also reduced in WT mice.

Another point to consider is whether endothelial cell-derived CNP is the predominant source of CNP. If endothelial cell-derived CNP isn't a major player in the protection against PH pathogenesis, as suggested by my results, could CNP produced from another cell-type be more important? Elegant studies performed by Chauhan *et al*., (2003), clearly demonstrate that ACh-induced CNP secretion from resistance arteries (mesenteric) is dependent completely on the presence of an intact endothelium. However, there are alternative lines of evidence demonstrating CNP is secreted by other cell-types, which could potentially play a role in PH; these include VSMC (Casco *et al*., 2002; Kelsall *et al*., 2006; Mendonca *et al*., 2006; Naruko *et al*., 2005, 1996; Suga *et al*., 1998; Woodard *et al*., 2002), macrophages (Casco *et al*., 2002), fibroblasts (Chrisman

and Garbers, 1999; Horio *et al*., 2003) and cardiomyocytes (Del Ry *et al*., 2011; Kelsall *et al*., 2006).

Vascular SMCs could be considered ideally located to elicit beneficial autocrine and paracrine effects on CNP secretion. ECs co-cultured with VSMCs significantly increase EC secreted CNP but only when the cells are in direct contact demonstrating a reciprocal relationship between VSMCs and ECs. This effect is dependent on the release of TGF-β from VSMCs when in direct contact with ECs, and lends support to the overall importance of an intact endothelium. However, ecCNP KO mice are absent of endothelial cell-derived CNP suggesting if this mechanism was significant, a worse phenotype would have been observed in KO mice using this model (Komatsu *et al*., 1996). In addition, mitogens such as PDGF, the expression of which is increased in hypoxic mammals and PH patients, enhances CNP expression in VSMCs and ECs from humans, but does the opposite in rodents (Mendonca *et al*., 2006). This suggests that the release of mitogens such as PDGF with chronic hypoxia in rats may decrease the secretion of CNP from VSMC possibly contributing to pathogenesis, whereas in humans it appears hypoxia may increase the secretion of VSMC-derived CNP reducing disease progression. Therefore, endogenous CNP secretion from both ECs and VSMCs in mice exposed to hypoxia plus SU5416 may play an inherently menial role in preventing disease progression because secretion of this peptide is inhibited in both WT and ecCNP KO mice due to mitogen-triggered downregulation. However, similar to that observed in ECs, the combination of specific mitogens and cytokines may determine the extent to which CNP is secreted by VSMCs. Thus, it is difficult to conclude whether in the mouse hypoxia plus SU5416 model there is a compensatory effect of elevated CNP secretion from VSMCs in the absence of ecCNP, or that inherently CNP secretion is attenuated in both cell-types; regardless, this would account for no observable difference between ecCNP KO and WT mice.

Macrophage lung infiltration is a common component in the mouse hypoxia plus SU5416 model of PH, but absent in the rat model (Ciuclan *et al*., 2011; Taraseviciene-Stewart *et al*., 2001). In addition, macrophage pulmonary

infiltrates are also a common feature in PH patients (Rabinovitch *et al*., 2014). Macrophages can secrete CNP, specifically when monocytes are exposed to pro-inflammatory stimuli (e.g. TGF-β) causing them to differentiate into macrophages (Kubo *et al*., 2001). Further, Suga *et al*., (1992b) showed that co-culturing macrophages with ECs increases EC-derived CNP secretion by ~10-fold. Therefore, macrophages secrete CNP themselves and also increase EC-derived CNP secretion. The importance of one mechanism over the other as a vasoprotective effect has already been shown. Macrophages are a predominant feature in atherosclerosis (Casco *et al*., 2002) but the absence of ecCNP in mice results in enhanced atherosclerotic lesions with increased macrophage infiltration meaning that the CNP secreted by macrophages is not significant enough to compensate for the lack of EC-derived CNP (Moyes *et al*., 2014). Therefore, in my studies it would be reasonable to assume that CNP derived from macrophages did not compensate for the lack of ecCNP.

Fibroblasts are known to accumulate in the pulmonary vascular adventitia of PH patients and also during chronic hypoxia, this is followed by fibroblast migration into the intimal and medial layers, forming part of the vascular lesion through proliferation and production of extracellular matrix (Stenmark *et al*., 2006a). In fact, rat cardiac fibroblasts secrete CNP which is significantly enhanced through stimulation with TGF-β, bFGF, and ET-1 (Horio *et al*., 2003). C-type natriuretic peptide released by fibroblasts can act as an autocrine signal by inhibiting fibroblast differentiation, migration and collagen deposition (Li *et al*., 2015). Therefore, fibroblast-derived CNP is a potential source of CNP that could have blunted any detrimental effects of ecCNP deletion.

CNP is expressed and secreted by the heart, particularly in cardiac disorders such as heart failure (Kalra *et al*., 2003), suggesting that CNP acts in an autocrine fashion to attenuate cardiac remodelling. Although this could account for a lack of significant difference in RVH between WT and ecCNP KO mice in the PH model it is unlikely to account for a similar phenotype with respect to vascular remodelling. In particular, CNP does not have endocrine capabilities akin to ANP and BNP as it has a very short half-life in the circulation. Nonetheless, it would be of interest to evaluate the phenotype of

cardiomyocyte-specific CNP null mice which have recently been developed by our lab (Moyes *et al*., 2015).

The study by Casserly *et al*., (2011) demonstrating little or no effect of CNP infusion in rats exposed to hypoxia or hypoxia plus SU5416 noted significantly elevated plasma levels of CNP, implying adequate activation of NPR-B (or possibly NPR-C). This led to the assumption that the observed lack of effect was likely due to decreased receptor expression. The authors showed that NPR-B protein expression was undetectable in the lungs of rats exposed to hypoxia plus SU5416 and treated with CNP. However, Li *et al*., (1995) showed a 2-fold increase in NPR-B expression in lungs from rats exposed to 4 weeks hypoxia, suggesting either the hypoxia plus SU5416 combination or continuous CNP infusion decreases NPR-B expression. Continuous CNP infusion has been commonly used and demonstrated to be of therapeutic value in a wide range of experimental models, for example reducing intimal thickening from vascular injury (1 µg/kg/h; 5 or 14 day infusion; Furuya *et al*., 1995), decreasing Ang-II induced cardiac hypertrophy and fibrosis (0.05 µg/kg/min; 2 week infusion; Izumiya *et al*., 2012), attenuating the magnitude of cardiac infarct area and fibrosis due to experimental MI (0.1µg/kg/min; 2 week infusion; Soeki *et al*., 2005) and preventing monocrotaline induced PH (0.75 µg/hour; 4 and 8 week infusion; Itoh *et al*., 2004). Consequently, CNP infusion does not appear to attenuate either NPR-B or NPR-C signalling otherwise CNP would not have exhibited a therapeutic effect. In addition, desensitisation does not appear to occur in disease models since infusion of both ANP and BNP significantly reduce severity of PH developed from chronic hypoxia in rats (Jin *et al*., 1990; J R Klinger *et al*., 1998). In fact, exposure of rats to chronic hypoxia (4 weeks) causes a ~2-fold increase in NPR-B lung expression (Li *et al*., 1995). In sum, these data suggest that the hypoxia and SU5416 combination itself, and not CNP infusion *per se*, decreases lung NPR-B protein expression preventing any beneficial effect of CNP. Thus, in my studies, the reason underlying a lack of effect of ecCNP KO may well reside with a decrease in NPR-B expression which abrogated any beneficial effect of an increased CNP secretion in WT mice. In addition to activating NPR-B, CNP also activates NPR-C (Chauhan *et al*., 2003; Moyes *et al*., 2014; Villar *et al*., 2007). NPR-C expression has been

shown previously to be highly sensitive to hypoxia, being significantly reduced in the lungs of mice (~20 %) after just 48 h hypoxia, with a further decrease after 4 weeks (Li *et al*., 1995). Indeed, a reduction in NPR-C expression was originally alluded to by Klinger *et al*., (1994), reporting that ANP clearance was reduced in the lungs of rats exposed to 3 weeks hypoxia. This has also been shown in mice exposed to chronic hypoxia where lung NPR-C mRNA expression is significantly reduced in both WT and ANP KO mice, suggesting a reduction in NPR-C expression is independent of circulating NP concentrations and most probably due to the hypoxic insult itself (Sun *et al*., 2000). In fact it is not hypoxia *per se* that decreases NPR-C expression but rather the combination of hypoxia-responsive growth factors, FGF and PDGF, that activate tyrosine kinase receptors, decreasing NPR-C expression in rat pulmonary VSMCs (Sun *et al*., 2001). Thus, NPR-C is most highly expressed in the lung under basal conditions suggesting a prominent role in the pulmonary circulation. However, if NPR-C played a critical role in the pulmonary circulation I would have expected NPR-C KO mice to develop spontaneous PH, similar to that observed with eNOS KO mice (Steudel *et al*., 1997), but this was not the case (see 5.3.2).

In sum, the lack of an overt effect between WT and ecCNP KO mice using the murine hypoxia plus SU5416 model of PH may have been due to a compensatory increase in cardiac-derived CNP attenuating an effect on RVH and/or fibroblast-derived CNP ameliorating an effect on vascular remodelling. Moreover, decreased expression of CNP, NPR-B and NPR-C in the setting of PH entails that, in both WT and ecCNP KO mice the capacity of the peptide to elicit any beneficial effects would have been absent.

5.3.2 The role of natriuretic peptide receptor-C in pulmonary hypertension

5.4.2.1 Hypoxia plus SU5416 model of PH

Initially, I hypothesised that CNP can elicit a cGMP-dependent beneficial effect in PH via activation of NPR-B and production of cGMP. To study this possibility I planned to exploit the NPR-B KO mouse colony maintained by our lab, but the availability of such animals was markedly reduced as a result of poor fecundity and innately poor survival. However, since recent evidence points to an important signalling function for NPR-C in the heart and vasculature (Anand-Srivastava, 2005; Moyes *et al*., 2014; Rose and Giles, 2008), I decided to explore the phenotype of global NPR-C null animals. Although the signalling pathways triggered by NPR-C activation are not mediated by cGMP, rather $G_{i/o}$ G-proteins, NPR-C does possess a NP clearance function, suggesting that depletion of this receptor could increase cGMP signalling indirectly (Matsukawa *et al*., 1999).

Male NPR-C KO mice exhibit a hypotensive phenotype, which has been attributed to a reduction in NP clearance (Matsukawa *et al*., 1999; Moyes *et al*., 2014). In contrast, female NPR-C KO mice exhibit a hypertensive phenotype resulting from the role of this receptor in orchestrating the EDHF-like activity of CNP. Furthermore, male and female NPR-C KO mice demonstrate enhanced vascular wall leukocyte adhesion properties, similar to male and female ecCNP KOs (Moyes *et al*., 2014). In addition to NPR-B, NPR-C mediates the anti-proliferative effects of CNP on VSMC and pro-proliferative effects on ECs (Khambata *et al*., 2011). In sum, deletion of NPR-C increases inflammation and disables the ability of CNP to prevent aberrant VSMC mitogenesis and sustain the endothelial barrier; collectively these facets of CNP biology led to the hypothesis that the absence of NPR-C signalling would be severely detrimental to the development of PH, a disease characterised by inflammation, VSMC proliferation and endothelial cell dysfunction. Therefore, using the same mouse

hypoxia plus SU5416 model of PH described above, I investigated the effect of global deletion of NPR-C on disease severity.

Hypoxia plus SU5416 caused PH in both male and female WT and NPR-C KO mice evident by significant increases in RVSP when compared to mice under control (normoxic) conditions. These increases in RVSP were accompanied by significantly enhanced RVH in all mice except male WT. Both genotypes of either sex had a predominance of partially and fully muscularised pulmonary arterioles (<100 μm diameter) suggesting that this model was able to induce severe vascular remodelling characterised by VSMC proliferation. However, this was not affected by genotype in either sex. Despite global deletion of NPR-C not causing significant differences in RVSP and pulmonary vascular remodelling between genotypes, NPR-C KO mice did develop significantly increased RVH compared to WT. Overall, these data show that NPR-C is not essential to the development of higher RVSP and pulmonary vascular remodelling but instead this receptor contributes to the protection against RVH associated with the disease. This is surprising as both male and female NPR-C KO mice exhibit increased leukocyte adhesion to the vascular wall under basal conditions and when stimulated by the addition of IL-1β (Moyes *et al*., 2014). Therefore, as this PH model exhibits a significant inflammatory component one would expect pathogenesis to be exacerbated in NPR-C KO mice, and in both sexes.

Arguably the most important feature of NPR-C signalling in the context of PH, is its ability to inhibit VSMC proliferation but enhance EC growth (Khambata *et al*., 2011; Figure 73). Aberrant VSMC proliferation and EC apoptosis are key components of PH in humans and both the rat and mouse hypoxia plus SU5416 models (Ciuclan *et al*., 2011; Taraseviciene-Stewart *et al*., 2001; R. M. Tuder *et al*., 2007). Therefore, the ability of CNP/NPR-C signalling to oppose these pathological vasculature features would be expected to offer protection against PH pathogenesis. However, no differences in RVSP or pulmonary vascular remodelling were observed between NPR-C KO and WT mice. This could be explained by the dual functionality of NPR-C, signalling through G-proteins and NP clearance (Maack *et al*., 1987; Matsukawa *et al*., 1999); in this way, the absence of this receptor could have also led to an enhanced presence of NPs

within the vasculature, increasing the beneficial effects of pGC activation on ECs and VSMCs, balancing out the negative effects of disabled CNP/NPR-C signalling. Indeed, the plasma half-life of ANP is extended in the NPR-C KO mice (Matsukawa *et al*., 1999), both BNP and CNP also bind to this receptor (Bennett *et al*., 1991), and therefore one could envisage a substantial net beneficial effect of reduced NP clearance. However, my results offer evidence to the contrary in that both male and female NPR-C KO developed significantly higher RVH compared to their WT counterparts. There is evidence demonstrating the benefit of NPR-A and/or NPR-B activation in attenuating cardiac hypertrophy (Knowles *et al*., 2001; Langenickel *et al*., 2006) but if clearance was the predominant functional role of this receptor then RVH would have been attenuated, when in fact the opposite was true. A direct link between NPR-C signalling and cardiac hypertrophy has yet to be shown. However, there are several possible mechanisms that might explain a protective role. For example, $G_{i/o}$-driven activation of PI3K/Akt might result in eNOS phosphorylation to produce cardioprotective NO (Watts *et al*., 2013), akin to the beneficial effect of β_3-adrenoceptors (Xiao *et al*., 1999) and shown to occur in response to NPR-C activation in the vasculature (El Andalousi *et al*., 2013; Murthy *et al*., 2000, 1998). Also, inhibition of the cardiac sarcolemmal Na^+/H^+ exchanger (NHE) prevents the development of hypertrophy via downregulation of calcineurin/NFAT activation (Avkiran and Haworth, 2003); $G_{i/o}$-linked receptors downregulate NHE. Third, $G_{i/o}$-coupled receptors would inhibit adenylyl cyclase to reduce cAMP-driven hypertrophic responses (Kudej *et al*., 1997).

In addition to cardiomyocytes, NPR-C expressed on fibroblasts might also have contributed to the RVH associated with PH. NPR-C KO mice, under basal conditions, have substantial fibrosis in the right and left atria but not in the ventricles (Egom *et al*., 2015). Therefore, with an innate cardiac fibrotic phenotype it would be reasonable to assume that under high pressure, such as in those induced by the hypoxia plus SU5416 model, that NPR-C KO mice would have developed significantly greater cardiac hypertrophy and fibrosis in the RV.

In sum, global deletion of NPR-C is detrimental to the pathogenesis of PH with respect to RVH, but not pulmonary vascular pressure or remodelling. Thus, I have established a novel cardioprotective action of NPR-C that offsets the development of pressure-induced cardiac hypertrophy, although exactly how this occurs will require further investigation. The lack of effect of NPR-C deletion on vascular morphology and reactivity may be due to the dual functionality of NPR-C (clearance and signalling).

5.4.2.2 Bleomycin-induced model of PH

Since CNP has been reported to exert substantial anti-fibrotic effects in many organs, including the kidney (Hu *et al*., 2015) and liver (Tao *et al*., 1999), coupled to the anti-hypertrophic activity of NPR-C I identified in the previous set of experiments, I investigated the hypothesis that NPR-C might also prevent the development of PH secondary to lung fibrosis.

The administration of bleomycin to male and female WT and NPR-C KO mice resulted in PH evident by significant increases in RVSP in all groups. However, only female NPR-C KO mice developed significantly elevated RVH upon bleomycin administration compared to controls, which was not seen with any other sex or genotype, hinting that the pathology developed in these mice was more severe. Indeed, female NPR-C KO mice administered bleomycin had significantly higher RVSP and RVH when compared to WT. A similar exacerbation in RVSP was not observed in male NPR-C KO, compared to WT, suggesting a sex-difference with females being more prone to pulmonary fibrosis than males. This was reflected in differences in lung weight, which showed that female NPR-C KO mice had significantly elevated lung weight compared to WT in response to bleomycin, whereas no difference was observed in male mice. This was reflected in differences in lung weight; after initial bleomycin instillation lung weight correlates with collagen content (Scotton *et al*., 2013), and female NPR-C KO mice had significantly elevated lung weight compared to WT, whereas no difference was observed in male mice. However, lung weight is also determined by vascular permeability leading to pulmonary

oedema (Scotton *et al*., 2009). NPR-C contributes to the regulation of pulmonary vascular permeability (Klinger *et al*., 2013), suggesting the changes in female NPR-C KO lung weight might be due to a combination of elevated pulmonary fibrosis and/or oedema.

Although Moyes *et al*., (2014) found that both male and female NPR-C KO mice develop equally augmented inflammatory responses (e.g. increased leukocyte recruitment and atherosclerosis) I observed a profound sex-difference with respect to disease severity in experimental pulmonary fibrosis. In humans with pulmonary fibrosis males have a higher incidence and poorer survival than females, which would fit with my findings hinting at a more effective utilisation of NPR-C in females (Han *et al*., 2008). Pathological severity in the bleomycin model is known to be influenced by the female sex hormone, oestrogen, which exacerbates the detrimental phenotype (Gharaee-kermani *et al*., 2005); this is thought to be due to fibroblasts exhibiting differences in collagen and TGF-β production. Therefore, the absence of NPR-C on fibroblasts specifically might contribute to the sex-difference observed in my studies. The production of CNP is significantly enhanced in fibroblasts stimulated with TGF-β as well as other growth factors (e.g. bFGF and ET-1; Horio *et al*., 2003), and as TGF-β plays a key role in the deposition of extracellular matrix in both experimental and human pulmonary fibrosis, this likely results in a negative feedback mechanism, whereby CNP acts as a autocrine and paracrine messenger, activating NPR-C present on fibroblasts to regulate proliferation and collagen deposition, alongside BNP and possibly ANP (Huntley *et al*., 2006; Figure 73).

Although the precise mechanism through which NPR-C activation inhibits fibrosis is not known, it is unlikely to occur through modulating intracellular cAMP, unlike the beneficial effects observed in RVH. In fact, powerful fibroblast mitogens such as lysophosphatidate are known to function through $G_{i/o}$ -protein activation (van Corven *et al*., 1989) and fibroblast proliferation and extracellular matrix production is attenuated by increasing intracellular cAMP concentrations (D'armiento *et al*., 1973; Dubey *et al*., 2001; He & Grinnell, 1994; Liu *et al*., 2004). Interestingly however, whilst the α-subunit of $G_{i/o}$-proteins inhibits cAMP production, the βγ-subunit simulates phospholipase-C (PLC). C-type natriuretic

peptide, acting through NPR-C with subsequent PLC stimulation, triggers an increase in outwardly rectifying non-selective cation currents due to transient receptor potential (TRP) channels in rat fibroblasts (Rose *et al*., 2007). Furthermore, a number of different TRP channels are expressed on fibroblasts which regulate TGF-β mediated fibroblast proliferation and collagen deposition (Du *et al*., 2010; Nilius & Szallasi, 2014; Yue *et al*., 2013). Therefore, the anti-fibrotic effects of NPR-C signalling could have been mediated through modulation of the same TRP channels used to elicit pro-fibrotic effects of TGF-β (Figure 73).

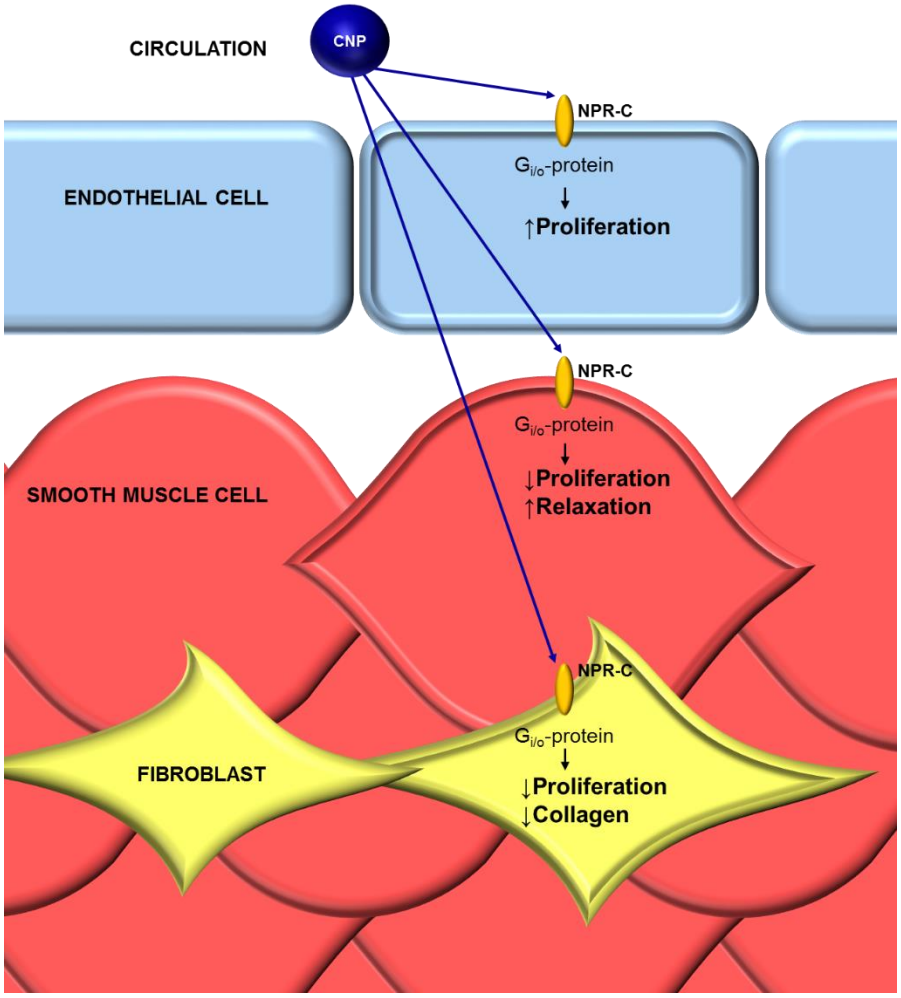


Figure 73. An illustration of the potential cardiovascular benefits of CNP. C-type natriuretic peptide (CNP), natriuretic peptide receptor-C (NPR-C), $G_{i/o}$ -protein-coupled receptor ($G_{i/o}$ -protein).

Overall, this demonstrates an initial proof of concept study that NPR-C is protective against the development of pulmonary fibrosis. This is an important observation because of the dual functionality of NPR-C (clearance and signalling) and fits well with the protective role of NPR-C in the RV I demonstrated in the hypoxia plus SU5416 model of PH. Again, in this setting of lung fibrosis, deletion of NPR-C worsens disease progression, implying that extending the half-life of NPs in the circulation is not the predominate effect; rather the signalling capacity of the receptor is more important. Pulmonary vascular remodelling was not analysed specifically, nor quantification of lung collagen content to determine differences in fibrosis between genotypes; however, NPR-C is known to maintain the EC barrier, prevent VSMC proliferation, attenuate leukocyte recruitment and reduce fibroblast proliferation, which would indicate that the absence of this capacity in the NPR-C KO would lead to an increase susceptibility to the development of pulmonary fibrosis.

5.4 Conclusions & future studies

I have demonstrated that MRPs can be targeted to regulate cGMP-dependent vascular homeostasis. Multidrug resistance proteins contribute to the tight control of intracellular cGMP concentrations mediated through active efflux of membrane located cGMP “clouds” in VSMCs; facilitating cGMP compartmentalisation by acting as an adjuvant to PDEs. These transporter proteins facilitate the termination of the cardioprotective cGMP signal generated by sGC and pGC; therefore, MRP inhibition represents a novel therapeutic paradigm for the treatment of cardiovascular diseases by enhancing NO and NP signalling.

I have also shown that endothelial cell-derived CNP is not essential to host protection against PH, although alternate cellular sources of this peptide may offset pathogenesis using the hypoxia plus SU5416 model. In contrast, NPR-C has a cardioprotective capacity in PH identifying a new avenue of enquiry toward sustaining RV function in this disease (which is the best predictor of prognosis). This receptor also exhibits both a cardio- and vaso- protective

capacity in the bleomycin-induced model of pulmonary fibrosis with secondary PH, especially in females. Therefore, the development of NPR-C agonists may provide an effective, novel treatment for PH patients.

To build on my research into the role(s) of MRPs in regulating cGMP signalling one would ideally acquire the MRP4 and MRP5 KO mice, and repeat vascular reactivity, VSMC proliferation and *in vivo* BP studies to accurately delineate the roles of these transporter proteins in vascular homeostasis. Further still, it would be worthwhile to, investigate the role(s) of MRP4 and MRP5, using pharmacological interventions and transgenic lines, in disease models associated with altered NO and/or NP signalling (e.g. stroke, MI, and heart failure). Since probenecid is already a licensed medicine, repurposing of this MRP inhibitor might prove a quick and inexpensive approach to improve the treatment of cardiovascular disease.

Further elucidation of the role(s) of NPR-C in lung fibrosis and RV remodeling is also warranted by my thesis research. In particular, one might explore the cause of the increased lung weight observed in the female NPR-C KO mice by comparing wet to dry lung weight, the extent of pulmonary oedema, and lung collagen content using high performance liquid chromatography (HPLC). To more fully investigate a role for CNP in PH it will be necessary to generate VSMC, fibroblast or cardiomyocyte cell-specific CNP KO mice to better understand how this peptide contributes to PH pathogenesis in each cell-type, thereby contributing to the overall understanding of this devastating condition. Additionally, studies using specific agonists/antagonists of NPR-B and NPR-C, and/or cell-specific NPR-B and NPR-C KO mice, would allow delineation of these two parallel pathways in pathogenesis.

CHAPTER 6

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