The Influence of Gender and Sex Hormones in the Development of Translational and Experimental Pulmonary Arterial Hypertension

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Author Declaration

In declaration, the entire contents of this thesis have been solely written by me. All experimental data has been self-generated with the exception of immunohistochemistry images, which were performed in collaboration with Margaret Nilsen. No contents of this thesis have been previously submitted for a Higher Degree. All research was performed in Professor Margaret R. MacLean's lab as part of the Institute of Cardiovascular and Medical Sciences, College of Medical, Veterinary and Life Sciences in the University of Glasgow.

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Audrey F. Wright; Kevin White; Margaret Nilsen & Margaret R. MacLean. Estrogen Receptor Alpha Mediates The Development of Hypoxia Induced Pulmonary Arterial hypertension in Female Mice. *American Journal of Respiratory and Critical Care Medicine*. 2012; 185: A3436

Audrey F. Wright; Margaret Nilsen & Margaret R. MacLean. Castration Has No Effect On Pulmonary Haemodynamics In The Development Of Pulmonary Arterial Hypertension. *American Journal of Respiratory and Critical Care Medicine*. 2013; 187: A4634

Publications

Kirsty M. Mair; Anne Katrine Johansen; **Audrey F. Wright**; Emma Wallace & Margaret R. MacLean. (2014). Pulmonary Arterial Hypertension: Basis of Sex Differences in Incidence and Treatment Response. *Br J Pharmacol;* **17** (3); 567-579.

Yvonne Dempsie, Neil A. MacRitchie, Kevin White, Ian Morecroft, **Audrey F. Wright**, Margaret Nilsen, Lynn Loughlin, Kirsty M. Mair, Margaret R. MacLean. (2013). Dexfenfluramine and the oestrogen-metabolizing enzyme CYP1B1 in the development of pulmonary arterial hypertension. *Cardiovasc Res*; **99**(1): 24-34

Kevin White, Yvonne Dempsie, Margaret Nilsen, **Audrey F. Wright**, Lynn Loughlin, Margaret R. MacLean. (2011). The serotonin transporter, gender, and 17β oestradiol in the development of pulmonary arterial hypertension. *Cardiovasc Res*; **90**(2): 373-382

List of Abbreviations

2-OHE	2-hydroxyestrdaiol
2-ME	2-methoxyestradiol
4-OHE	4-hydroxyestradiol
4-ME	4-methoxyestradiol
5HIAA	5-hydroxyindole acetic acid
5-HT	5-Hydroxytryptamine
5-HTP	5-hydroxytrypthophan
16-OHE1	16α-hydroxyestrone
17β-HSD	17β-Hydroxysteroid Dehydrogenase
α-SMA	Alpha-smooth muscle actin
AC	Adenylate Cyclase
AF	Activation Function
AF-1	Activation Function-1
Akt	Protein Kinase B
ALK-1	Activin receptor-like kinase-1
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
APAH	Associated Pulmonary Arterial Hypertension
ApoE	Apolipoprotein E
AR	Androgen Receptor
ARE	Androgen Response Element

ATP	Adenosine 5’ Triphosphate
BMPR2	Bone Morphogenetic Protein Receptor 2
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium Ion
$[Ca^{2+}]_i$	Intracellular Calcium
Cl^-	Chlorine Ion
CaM	Calmodulin
cAMP	Cyclic 3’, 5’ adenosine monophosphate
CAS	Castration
CCBs	Calcium Channel Blockers
cGMP	Cyclic Guanosione 3’, 5’ Monophosphate
CO_2	Carbon Dioxide molecule
COMT	Cathecol-O-Methyltransferase
COPD	Chronic Obstructive Pulmonary Disorder
CTEPH	Chronic thromboembolic pulmonary hypertension
CYP450	Cytochrome P450
CYP1B1	Cytochrome P450 1B1
DAG	1,2-Diacylglycerol
DBD	DNA Binding Domain
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMEM	Dulbeccos Modified Eagle Medium

E_1	Estrone
E_2	Estradiol
E_3	Estriol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
eNOS	Endothelial Nitric Oxide Synthase
ELISA	Enzyme Linked Immunosorbent Assay
EPC	Endothelial Progenitor Cells
ER	Estrogen Receptor
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
ERA	Endothelin Receptor Antagonists
ERE	Estrogen Response Element
ERK	Extracellular signal-Regulated Protein Kinase
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
ET-1	Endothelin-1
ETA	Endothelin Receptor A
ETB	Endothelin Receptor B
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FSH	Follicle Stimulating Hormone

GDP	Guanosine Diphosphate
GnRH	Gonadotropin Releasing Hormone
GPCR	G-protein Coupled Receptor
GPER	G-Protein Coupled Estrogen Receptor
GTP	Guanosine triphosphate
H_2O_2	Hydrogen Peroxide
HDL	High Density Lipoprotein
HIF1a	Hypoxia Inducible Factor-1a
HIV	Human Immunodeficiency Virus
HPAH	Heritable Pulmonary Arterial Hypertension
HPG	Hypothalamic-Pituitary-Gonadal Axis
HPV	Hypoxic Pulmonary Vasoconstriction
HR	Heart Rate
IP3	Inositol 1,4,5 trisphosphate
IPAH	Idiopathic Pulmonary Arterial Hypertension
K^+	Potassium Ion
$[K^+]_i$	Intracellular Potassium
KCl	Potassium Chloride
Kv	Voltage Gated Potassium Channel
LBD	Ligand Binding Domain
LDL	Low Density Lipoprotein
LH	Lutenising Hormone

LV	Left Ventricle
LV+S	Left Ventricle plus Septum
LVH	Left Ventricular Hypertrophy
MAO	Monoamine Amine Oxidase
MAPK	Mitogen-Activated Protein Kinase
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
mRNA	Messenger Ribonucleic Acid
Mts1	Calcium Binding Protein S100A4
Na^+	Sodium Ion
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NANC	Non-Adrenergic-Non-Cholinergic
NH2	Amine Group
NIH	National Institutes of Health
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSCLC	Non-Small Cell Lung Cancer
NYHA	New York Heart Association
O_2	Oxygen molecule
OH	Hydroxyl Group
P_a	Arterial Pressure

P_A	Alevolar Pressure
P_v	Venous Pressure
PAEC	Pulmonary Arterial Endothelial Cell
PAF	Pulmonary Artery Fibroblast
PAH	Pulmonary Arterial Hypertension
PAP	Pulmonary Arterial Pressure
PASMCs	Pulmonary Artery Smooth Muscle Cells
PCH	Pulmonary capillary hemangiomatosis
PDE	Phosphodiesterase
PDE-5	Phosphodiesterase Type-5
PDGF	Platelet Derived Growth Factor
PGI_2	Prostacyclin
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PH	Pulmonary Hypertension
PLCβ	Phospholipase Cβ
PP2A	Protein phosphatase 2A
PPAR-γ	Peroxisome Proliferator-Activated Receptor-gamma
PPHN	Persistent Pulmonary Hypertension of the Newborn
PVOD	Pulmonary veno-occlusive hypertension
PVR	Pulmonary Vascular Resistance
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction

REVEAL	The Registry to Evaluate Early and Long-term Pulmonary Arterial Hypertension Disease Management
ROCK	Rho Kinase
RTK	Receptor tyrosine kinases
ROS	Reactive Oxygen Species
RV	Right Ventricle
RV/LV+S	Ratio of Right Ventricle over Left Ventricle plus Septum
RVH	Right Ventricular hypertrophy
RVSP	Right Ventricular Systolic Pressure
SAP	Systemic Arterial Pressure
SERM	Selective Estrogen Receptor Modulator
SERT	Serotonin Transporter
SEM	Standard Error Mean
SHAM	Sham Operated Control
SHBG	Sex Hormone Binding Globulin
SHR	Spontaneously Hypertensive
siRNA	small interfering RNA
SLC6A4	Serotonin Transporter Gene
SMC	Smooth Muscle Cell
SOCC	Store Operated Calcium Channel
SULT	Sulphotransferase
TGF-β1	Transforming Growth Factor Beta-1

TMD	Transmembrane domain
TPH	Trypthophan Hydroxylase
VEGF	Vascular Endothelial Growth Factor
VMAT	Vesicular monoamine transporter
vWF	Von Willebrand
WHO	World Health Organisation
WT	Wildtype

Abstract

Pulmonary arterial hypertension (PAH) is a progressive and debilitating disease characterised by increases in pulmonary vasoconstriction and excessive remodelling of the pulmonary arteries. Together, these processes lead to sustained elevations in pulmonary arterial pressure, right heart failure and eventual death if left untreated. Despite the number and variety of treatment options available, the survival rate in incident and prevalent cases of PAH remains poor. Therefore, a better understanding of the pathobiology of PAH is required to generate novel therapeutic approaches with improved efficiency in patients. In PAH there is a well described gender bias. Women are consistently reported to represent up to 75% of the total PAH population; however, the reasons for this female predominance remain unclear. Recently, estrogen has been implicated as a major risk factor, for example, elevated estrogen levels and alterations in estrogen metabolism are closely correlated with PAH development in females. The role of testosterone in PAH is currently under investigated.

Effects of estrogen are mediated through two classical estrogen receptors (ER)-α and –β, or the novel G-protein-coupled estrogen receptor (GPER). Expression of all of these receptors is identified in pulmonary vasculature, including in smooth muscle and endothelial cells. The role they play in PAH pathogenesis in females is largely undetermined. Given the diverse effects of estrogen described in the pulmonary vasculature during PAH, for example, proliferative effects in pulmonary artery smooth muscle cells (PASMCs), we hypothesised that estrogen receptors play an integral role in PAH in females. To examine this, we used both translational and experimental studies to characterise ERs in PAH. Chronic hypoxic male and female mice, and mice over-expressing the serotonin transporter ($SERT^+$ mice), which demonstrate female susceptibility, were used to investigate the effects of an ERα antagonist *in vivo*. GPER knockout mice were also investigated in chronic hypoxia. *In situ* and *in vitro* studies in human PASMCs with ER agonists and antagonists added clinical relevance to our findings. In addition, testosterone manipulation was investigated in male mice by castration *in vivo*.

Immunohistochemistry, immunoblotting and qRT-PCR analysis demonstrated that ERα was increased in PASMCs and pulmonary arteries from female PAH patients and chronic hypoxic mice, respectively. On the other hand, ERβ was decreased in PAH and hypoxia. It was also observed that females expressed higher levels of ERα in PAH compared to males whereas ERβ was lower in females. PAH was assessed by measuring right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH) and pulmonary vascular remodelling and muscularisation. Chronic hypoxia induced-pulmonary hypertension (PH) was attenuated in female mice dosed with the ERα antagonist MPP, shown by marked reductions in RVSP and pulmonary vascular remodelling. Hypoxic male mice remained unaffected by MPP treatment. Spontaneous PH and chronic hypoxia induced-PH observed in female $SERT^{+}$ mice were reversed by treatment with MPP. Immunoblotting and qRT-PCR analysis revealed that the possible mechanism involved in the beneficial effect of MPP in females *in vivo* involved restoring the dysfunctional bone morphogenetic protein receptor-2 (BMPR2) axis observed in PAH. This effect was only observed in female mice. In addition, chronic hypoxia induced-PH in male and female mice was unaffected by GPER deletion. Expression of GPER between female non-PAH controls and PAH patients was unchanged.

In isolated human PASMCs estrogen induced proliferation was inhibited by MPP, but not PHTPP or G15, an ERβ and GPER antagonist, respectively. The ERα agonist, PPT stimulated proliferation of human PASMCs. Both estrogen and PPT induced proliferation was dependent on downstream PI3K/Akt and ERK MAPK activity.

In males, testosterone deprivation by surgical castration had no effect on chronic-hypoxia induced PH. RVSP, RVH and pulmonary vascular remodelling were unchanged in hypoxic castrated mice relative to sham controls. Testosterone levels, assessed by enzyme linked immunosorbent assay (ELISA) demonstrated no effects of hypoxia on plasma testosterone levels. Testosterone levels were approximately halved by castration. qRT-PCR analysis showed that in mouse lung there were also no difference in expression of the androgen receptor (AR) and 5α-reducatse, the testosterone metabolising enzyme. Testosterone had no effect on proliferation of human PASMCs, although its primary metabolite, dihydrotestosterone (DHT), stimulated proliferation in a dose-dependent manner.

In summary of these findings, we have identified an ERα-dependent mechanism of PAH in females, but not in males. ERα is noticeably increased in female human PASMCs from PAH patients compared to male PAH patients. Additionally, ERα activation in female human PASMCs leads to proliferation driven by PI3K/Akt and ERK MAPK activation. Treatment with an ERα antagonist attenuated the development of chronic hypoxia induced-PH in females but not males, and reversed PH in $SERT^{+}$ female mice. We demonstrate that the mechanism attributed to the beneficial effect of MPP *in vivo* involved restoration of the dysfunctional BMPR2 signalling axis. Our results suggest that increased ERα expression may drive PAH development in females. Furthermore, we demonstrate that ERα does not play a key role in the development of hypoxia induced-PH in male mice. In addition we conclude that testosterone does not contribute to chronic hypoxic-PH observed in males. We suggest that altered local synthesis and metabolism in the lung and right ventricle may however, facilitate progression of established PAH in males and worsening survival rates. Overall, our results provide evidence for ERα in PAH development and implicate targeting ERs as a novel therapeutic target in PAH treatment.

Chapter 1.

Introduction

1.1 The Pulmonary Circulation

1.1.1 Structural Organisation

The function of the pulmonary circulation is primarily gas exchange facilitating oxygen (O_2) uptake and removal of carbon dioxide (CO_2). The structural and functional organisation of the pulmonary circulation reflects this purpose. The pulmonary circulation continuously receives the entire cardiac output from the right ventricle which receives mixed venous blood draining into the right atria from the systemic circulation via the inferior and superior vena cava. The pulmonary artery originates in the right ventricle where it bifurcates to become the *left* and *right* pulmonary artery, both of which extend into the hilum of their corresponding lungs. The anatomy of the *left* lung is subdivided in to two segments, the superior and inferior lobes separated by the oblique fissure and each lobe receives a branch from the left pulmonary artery. The right lung is divided into three lobes (superior, middle and inferior) separated by interlobular fissures and each is supplied with a branch resulting from the bifurcation of the *right* pulmonary artery. Each of these five lobes can be further subdivided anatomically into bronchopulmonary segments and contain its own segmental bronchi and corresponding pulmonary arterial branch. Each segment is functionally and anatomically distinct and as a consequence can function as a single entity meaning a single bronchopulmonary segment can be removed without affecting surrounding segments (Sealy et al. 1993). Ultimately, the branching pattern differs between the left and right lung. Distal branching in each lung continues in parallel series with the bronchial tree until the terminal alveoli are reached equivalent to fifteen orders of branching (Figure 1-1).

1.1.2 Functional Organisation

The primary lung function is closely related to structural and functional changes in the intima and media of pulmonary arteries as the branching system converges in the terminal alveoli. In humans, the smallest noncapillary blood vessels are defined as an order 1 vessel. This numbering continues with each proximal branch point until the main pulmonary artery (order 15) is reached

known as the Diameter-Defined Strahler's system (Mandegar et al. 2004). The proximal elastic arteries (orders 15-13; >1000μm diameter) are highly compliant and typically have an internal diameter greater than 1mm with increased elastic laminae in the tunica media to facilitate compliance. The more distal muscular arteries (orders 13-4) are typically between 100 and 1000μm in diameter. They progressively lose compliance with continual branching as a direct consequence of increased smooth muscle and decreased laminae in the tunica media. Phenotypically these arteries are defined by the predominance of smooth muscle in the media essential for blood pressure maintenance (HEATH & EDWARDS 1958). As the branching continues into orders 4-1 (<200μm diameter), medial smooth muscle becomes completely diminished and the resulting vessels are extremely thin-walled composed of endothelial cells and pericytes (undifferentiated smooth muscle cells) which function to facilitate blood-gas exchange in the alveoli (Figure 1-2).

The human lung has a network of over 300 million arteries, veins and capillaries throughout the 15 orders of branching defined as the capillary network. Each capillary has an internal diameter less than 10μm and supplies blood to several alveoli. The capillary network functionally facilitates blood oxygenation therefore a diffuse network of capillaries is essential across the lung. Following completion of circulation through the pulmonary capillaries, the re-oxygenated blood enters the pulmonary venous circulation via the venules and pulmonary veins until the main *left* and *right* pulmonary veins in corresponding lungs enter into the left atria.

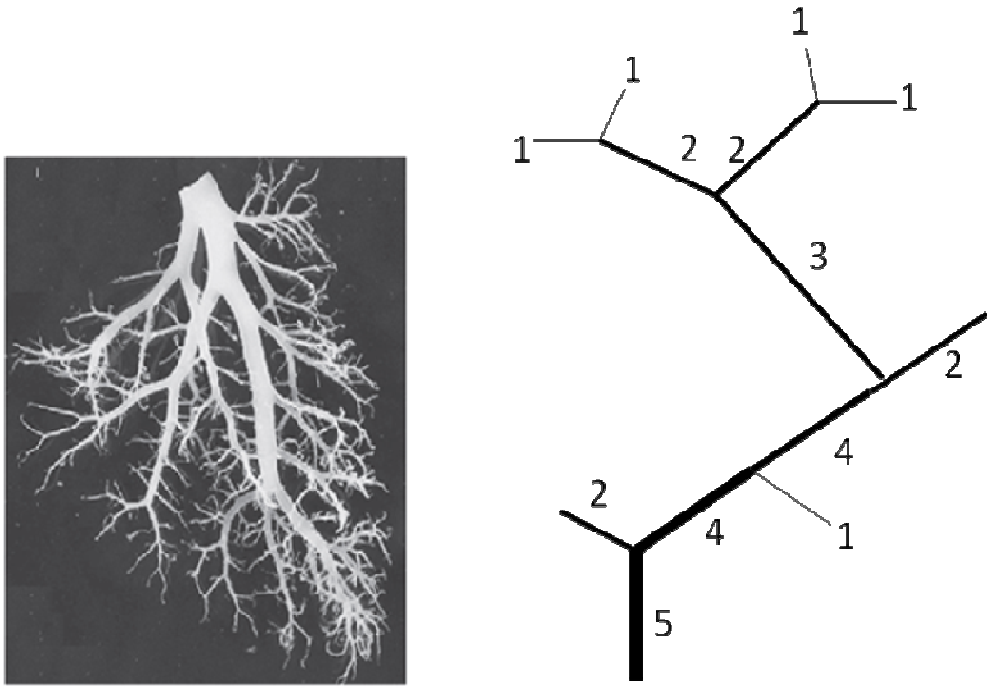


Figure 1-1: Branching of pulmonary vasculature.
Left, a typical lung cast is shown of pulmonary arterial branching. *Right,* Distal branching in each lung reaching the alveoli equates to 15 orders of branching in the Strahler model.

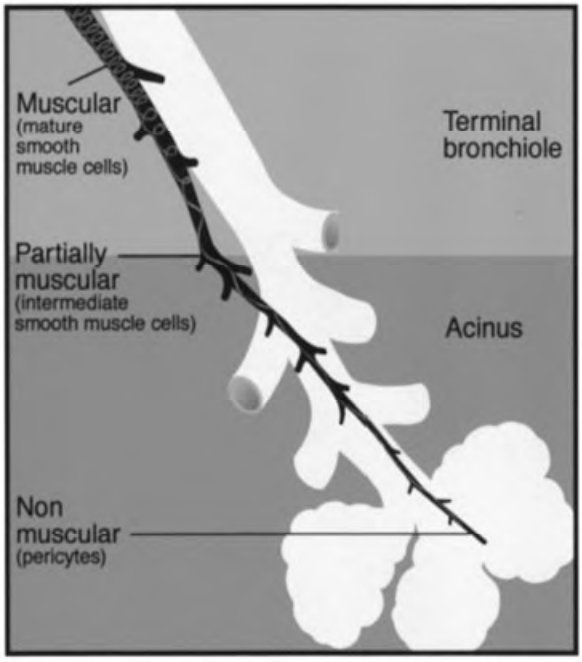


Figure 1-2: Musculature in the pulmonary arteries.
In the distal pulmonary arteries there is a complete loss of smooth muscle in the media replaced by thin walls composed of endothelial cells and pericytes. Adapted from (MacLean et al. 2000).

1.1.3 **Structure of the Pulmonary Vascular Wall**

The architecture of the normal pulmonary vasculature is engineered to ensure a high compliance, low resistance network providing an extensive surface area to facilitate gas exchange. In general the pulmonary vasculature has three concentric layers, *adventitial*, *medial* and *intimal* layers each composed of phenotypically distinct cells (Figure 1-3). The outermost *adventitial* layer exists as a collagen matrix and contributes to the structural integrity of the vessel wall. In this layer, pulmonary artery fibroblasts (PAFs) are the predominant cell type and have been identified to play an important role in response to environmental stimuli (Stenmark et al. 2006). The longitudinal *medial* layer comprises several populations of pulmonary artery smooth muscle cells (PASMCs) including immature and differentiated smooth muscle cells with an underlying elastic layer (Stenmark & Frid 1998). The medial layer is the predominant layer in the vascular wall capable of regulating vascular tone and blood pressure as PASMCs are the only cell type that produces a contractile response on stimulation. The innermost *intimal* layer exists as a monocellular endothelial layer attached to an underlying connective tissue matrix, termed the basement membrane. The pulmonary artery endothelial cells (PAECs) surround the lumen and are the only cell type in constant physical contact with blood flow therefore are proposed to be crucial in monitoring and regulating the luminal environment via release of various factors (Aaronson et al. 2002).

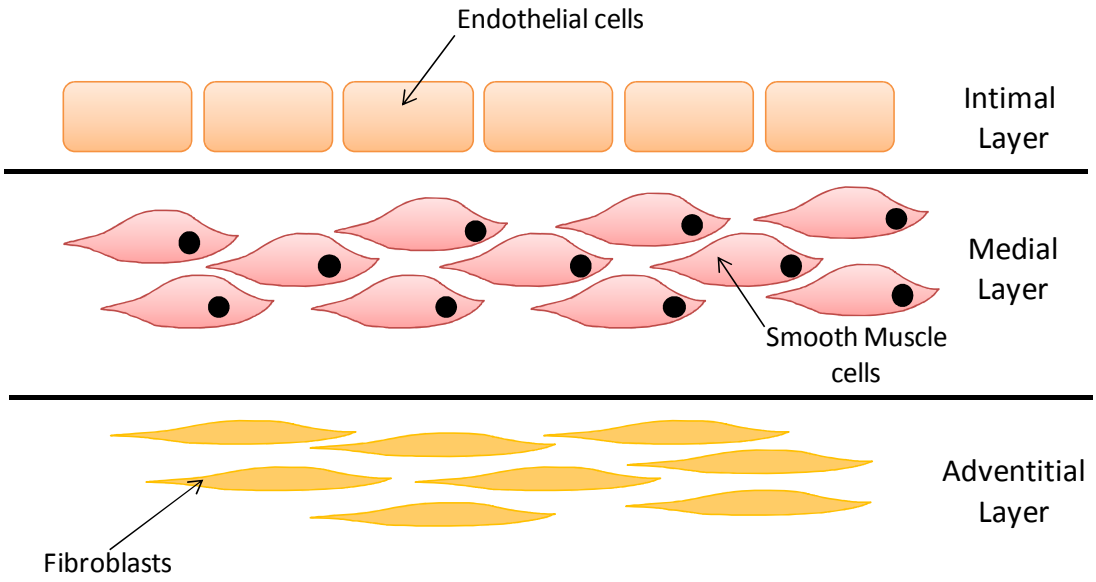


Figure 1-3: Cell types in the pulmonary vascular wall.
Endothelial, smooth muscle cells, and fibroblasts compose the intimal, medial and adventitial layers of the pulmonary vascular wall, respectively. Upon stress, for example hypoxia, immune cells such as macrophages, monocytes and dendritic cells, infiltrate the endothelial monolayer and stimulate pulmonary vascular remodelling.

1.1.4 Function of the Pulmonary Circulation

In the adult human circulation, both the left and right ventricles are arranged in parallel. Therefore, pulmonary blood flow is exactly equal to that of the left ventricle cardiac output and pulmonary arterial pressures (PAP) are directly related to cardiac output (Mandegar et.al. 2004). The pulmonary circulation is normally a high flow, low-resistance, low-pressure system that transports blood into the pulmonary micro-circulation where the most important function is gas exchange. Essentially, deoxygenated blood arriving from the systemic circulation is oxygenated by the rapid unloading of excess CO_2 and subsequent binding of O_2 molecules to haemoglobin, which resides within the red blood cells. This maintains metabolic processes throughout the body.

In addition to gas exchange, the lung also has important non-respiratory functions. This includes a primary defence system whereby the pulmonary circulation acts as a physical barrier by filtering and preventing the passage of inhaled foreign bodies and pathogens from the respiratory system to the cardiovascular system (Comroe, Jr. 1966). Within the cardiovascular system, it also acts as a physical barrier to prevent the passage of potentially lethal thrombi and embolic occlusion of essential arterial beds which may otherwise lead to infarction. The pulmonary circulation is also proposed to function as a blood reservoir (Comroe, Jr. 1966). The pulmonary vessels normally contain up to 500ml of blood and most of this can be rapidly mobilised to supply the left ventricle and maintain cardiac output.

1.1.5 Control of the Pulmonary Circulation

Major structural and functional differences exist between the systemic and pulmonary circulation. The pulmonary circulation is a high flow, low-resistance, low pressure system to avoid the consequences of Starling forces which would otherwise flow the lung with oedema fluid. Whilst in the systemic circulation, approximately 80% of vascular resistance is maintained by small muscular arterioles, resistance in the pulmonary circulation is relatively evenly distributed (MacLean et.al. 2000). The pulmonary arteries contain less medial smooth muscle and elastin and therefore a thinner wall allowing for greater

distensibility (Kilner 2004). As a consequence, PAP is typically 24/9mmHg and significantly lower than systemic arterial pressure (SAP), which is typically 120/80mmHg (Morgan et al. 2004). Similarly, the pressure gradient throughout the pulmonary circulation is 7-9mmHg, and up to ten-fold lower than those which exist in the systemic circulation.

1.1.6 Pulmonary Vascular Resistance

The pulmonary vascular resistance (PVR) is defined as the total peripheral resistance of flow which must be overcome to maintain continuous blood flow through the pulmonary arteries. The laws of physics therefore predict that in blood flow through the pulmonary vasculature, PVR is inversely proportional to the fourth power of the radius of the lumen. In other words, even small changes in the lumen size in pulmonary arteries can significantly change PVR and therefore PAP. Intimal thickening and narrowing of the lumen in diseases such as pulmonary hypertension consequently results in sustained increases in PAP.

1.1.7 Passive Regulation and Distribution of Blood Flow

The relatively low pulmonary pressures combined with the mid-entry point of the artery into the lung and gravity contributes to an uneven distribution of blood flow throughout the pulmonary circulation. For example, when upright, pulmonary blood flow to the apex of the lung is extremely low, whilst blood flow to the base of the lung is greatly increased. Passive pressure distribution can be explained by subdividing the lung into three zones determined by the relative values of the pulmonary arterial pressure (P_a), pulmonary venous pressure (P_v), an alveolar pressure (P_A) (Figure 1-4). Zone 1 describes the upper portion of the lung, the apex, where blood flow is extremely low, and this can be explained because the apex alveolar pressure is greater than both the arterial and venous pressures ($P_A > P_a > P_v$), resulting in collapse of the highly compliant vasculature. In zone 1, alveolar dead space occurs because the region is still being ventilated but not perfused. In Zone 2, the mid-portion of the lung, arterial pressure is greatest, however alveolar pressure still exceeds venous pressure ($P_a > P_A > P_v$) and therefore blood flow remains impaired. As a result, in zone 2, the perfusion pressure and vessel recruitment for the pulmonary circulation increases. Zone 3

at the base of the lung is below heart-level and therefore both the arterial pressure and venous pressures exceed alveolar pressure ($P_a > P_V > P_A$) which allows the vessels to be maximally distended at all times. During periods of increased blood flow there is substantial recruitment of the pulmonary vasculature in zone 1 and 2 allowing an even distribution of blood flow throughout the lung (Harf et al. 1978).

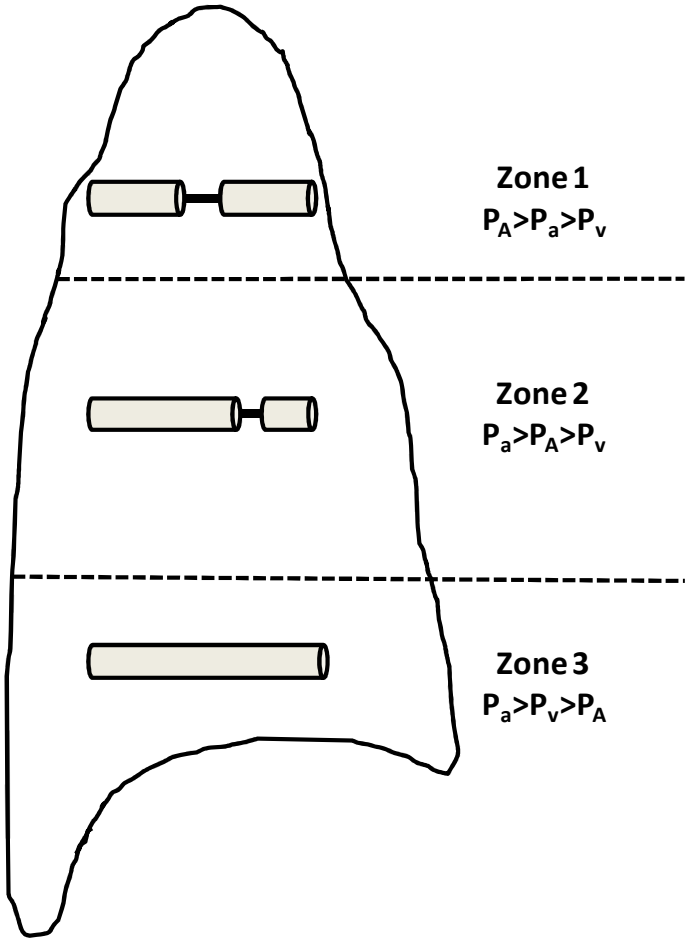


Figure 1-4: Passive distribution of blood flow through the lung.
Each lung is divided into three zones as determined by pulmonary arterial pressure (P_a), pulmonary venous pressure (P_v) and pulmonary alveolar pressure (P_A).

1.1.8 **Active Regulation and Distribution of Blood Flow**

In addition to passive distribution and regulation of blood flow in the pulmonary circulation, the active regulation is also an important determinant in pulmonary arterial pressure. Active factors include circulating hormonal influences, respiratory gases and sympathetic nerves. These factors all influence pulmonary vascular tone by affecting PVR.

The pulmonary circulation is innervated by both the sympathetic (adrenergic) and parasympathetic (cholinergic) branches of the autonomic nervous system (Dawson 1984). In addition, nonadrenergic noncholinergic (NANC) nerves regulate vascular tone (Kubota et al. 1988). Stimulation of the sympathetic nervous system results in increased neuron firing, increased PVR, and as a consequence increased PAP (Kadowitz et al. 1974). The pulmonary vasculature, particularly the PASMCs expresses α- and ß-adrenoceptors, where stimulation by noradrenaline produces vasoconstriction and vasodilation, respectively (Hyman et al. 1986; Hyman et al. 1990).

In contrast, the parasympathetic branch of the autonomic nervous system appears much less dense in the pulmonary arteries compared to the sympathetic innervations (Downing & Lee 1980). Additionally, release of the post-ganglionic neurotransmitter acetylcholine has less of an effect on pulmonary vascular tone and PVR or PAP (Murray et al. 1986). Functional NANC nerves have also been identified in pulmonary arteries, although the role of NANC nerves in the regulation of vascular tone remains to be described *in vivo*. It has been observed however, in isolated pulmonary arteries that nitric oxide mediates relaxation which is completely unaffected by adrenergic and cholinergic blockade (Scott & McCormack 1999).

1.2 Pulmonary Arterial Hypertension

1.2.1 **Classification**

Pulmonary arterial hypertension (PAH) is a debilitating disease characterised by excessive vasoconstriction and progressive remodelling in pulmonary arteries

leading to obliteration of pulmonary arteries and resulting in right heart failure and eventual death. PAH is typically diagnosed with right heart catheterisation and if mean pulmonary arterial pressure (mPAP) exceeds 25mmHg at rest (normal mPAP value at rest is 12-16mmHg). However, due to the diverse aetiology of PAH, initial diagnosis can be complicated and particularly in the context of a patient with multisystem disorder. For this reason, classification of different groups/forms of PAH has been developed. In the latest consensus meeting, the World Health Organisation (WHO) produced the Dana Point (2008) classification (Rosenkranz & Erdmann 2008) which led to well defined PAH groups. Importantly, the subgroups of PAH share a common phenotypic pathobiology (Voelkel & Cool 2004). The current Dana Point is listed in Table 1-1. Group 1 PAH is categorised into idiopathic PAH (IPAH), heritable PAH (HPAH), drug- and toxin-induced, and associated PAH (APAH), as well as persistent pulmonary hypertension of the new born (PPHN). In the developing world, PAH can also be associated with sickle cell disease and schistosomiasis, although PAH due to these diseases is rare in the Western World (Peacock et al. 2013). Furthermore, non-arterial pulmonary hypertension can be classified into the following groups: pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH) (Group 1'); pulmonary hypertension owing to left heart disease (Group 2); pulmonary hypertension owing to lung disease and/or hypoxia (Group 3); chronic thromboembolic hypertension (CTEPH) (Group 4), and pulmonary hypertension with unclear multi-factorial mechanisms (Group 5).

Despite well defined groups of PAH, symptoms develop gradually and patients typically remain undiagnosed. This can be a result of the generic signs and symptoms associated with PAH development and patients present asymptomatic with mild to moderate PAH (Simonneau et al. 2009). Only in late-stage/severe PAH are clinical signs apparent such as dyspnoea, angina-like chest pain, and fatigue. The severity of PAH in patients is also categorised using the New York Heart Association (NYHA) functional classification system (class I-IV) (Table 1-2). The functional classification of a patient is considered both at rest and during physical activity and is determined by their physical limitations. For example, in mild/early-stage PAH, patients appear asymptomatic and reside within class I whereas those most affected (late-stage PAH with right heart failure) are class

IV. The NYHA (class I-IV) system is important in both the choice of PAH therapy and an accurate predictor of patient mortality.

Table 1-1: World Health Organisation (WHO) Clinical Classifications of Pulmonary Hypertension.
Updated Dana Point, 2008. BMPR2: bone morphogenetic receptor type 2; ALK-1: activin receptor like-kinase-1; HIV: human immunodeficiency virus.

Group 1: Pulmonary Arterial Hypertension (PAH)

- 1.1 Idiopathic
- 1.2 Heritable
  - 1.2.1 BMPR2
  - 1.2.2 ALK-1, endoglin (with or without hereditary hemorrhagic telangiectasia)
  - 1.2.3 Unknown
- 1.3 Induced by drugs and toxins
- 1.4 Associated with PAH
  - 1.4.1 Connective tissue diseases
  - 1.4.2 HIV infection
  - 1.4.3 Portal hypertension
  - 1.4.4 Congenital heart disease
  - 1.4.5 Schistosomiasis
  - 1.4.6 Chronic haemolytic anaemia
- 1.5 Persistent pulmonary hypertension of the newborn

Group 1': Pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH)

Group 2: Pulmonary hypertension due to left heart disease

- 2.1 Systolic dysfunction
- 2.2 Diastolic dysfunction
- 2.3 Valvular disease

Group 3: Pulmonary hypertension due to lung diseases and hypoxemia

- 3.1 Chronic obstructive pulmonary disease (COPD)
- 3.2 Interstitial lung disease
- 3.3 Other pulmonary diseases with mixed restrictive and obstructive patterns
- 3.4 Sleep-related breathing behaviour
- 3.5 Alveolar hypoventilation disorders
- 3.6 Chronic exposure to high altitudes
- 3.7 Developmental abnormalities

Group 4: Chronic thromboembolic pulmonary hypertension (CTEPH)

Group 5: Pulmonary hypertension with unclear or multi-factorial mechanisms

- 5.1 Hematologic disorders: myeloproliferative disorders, splenectomy
- 5.2 Systemic disorders: sarcoidosis, pulmonary Langerhands cell histiocytosis, lymphangioleiomyomatosis, neurofibromatosis, vasculitis
- 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
- 5.4 Other: tumoral obstruction, fibrosing mediastinitis, chronic kidney failure on dialysis

Table 1-2: Current WHO/NYHA Functional Classification of patients with Pulmonary Hypertension

Current World Health Organisation (WHO) / New York Heart Association (NYHA) Classification of functional status of patients with pulmonary hypertension

Class I	**Patients with pulmonary hypertension but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnoea or fatigue, chest pain, or near syncope**
Class II	Patients with pulmonary hypertension resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class III	Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class IV	Patients with pulmonary hypertension with inability to perform any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnoea and/or fatigue may be present at rest and increased by almost any physical activity.

1.2.2 **Epidemiology and Prognosis**

PAH is a rare disease with an annual incidence of approximately 2-3 per million of the population (Frost et al. 2011; Humbert et al. 2006; Ling et al. 2012). The recent REVEAL Registry demographics suggest that the modern PAH patient population in the US with WHO Group I PAH (including idiopathic, familial, or associated with collagen vascular disease, congenital systemic-to-pulmonary shunts, portal hypertension, drugs or toxins, HIV infection, and miscellaneous conditions [such as splenectomy or hemoglobinopathies]) is older (mean age at diagnosis, 47 years) and has a higher female preponderance (Badesch et al. 2010; Frost et.al. 2011; Thenappan et al. 2010). The female to male ratio reported is as strong as 4.3:1 amongst the total PAH group (Walker et al. 2006) and 4.1:1 in the IPAH and APAH subcategories (Badesch et.al. 2010). This female prevalence has increased since the NIH Registry in the mid-1980s (Rich et al. 1987) and in most clinical trials, approximately three-quarters of patients are females. The reported incidence of PAH also appears to comprise a majority of patients in the IPAH and APAH category where the most common underlying associated conditions are connective tissue disease and congenital heart disease (Badesch et.al. 2010).

Observed survival rates in the PAH patient population are 83%, 67% and 58% at 1, 2 and 3 years, respectively (Humbert et al. 2010a) although younger patients (aged <50 years) are reported to have better survival rates (Ling et.al. 2012). Mortality rates in PAH are closely associated with right ventricular haemodynamic function and exercise limitation, however building evidence also shows that male sex is associated with poorer survival (Humbert et.al. 2010a; Humbert et al. 2010b; Sztrymf et al. 2008). It may therefore be important to consider the influence of gender on disease occurrence and outcomes. Moreover, the survival in incident PAH (newly diagnosed) is worse than in prevalent patients and survival outcomes also vary considerably among different aetiologies of PAH. For example, systemic sclerosis associated PAH patients have poorer survival than those with IPAH (Benza et al. 2012; Humbert et.al. 2010b). Whilst survival rates have improved in PAH in comparison with the established NIH Registry, recent studies indicate that PAH nonetheless remains a progressive, fatal disease despite advances in diagnosis and therapies (Humbert et.al. 2010a; Thenappan et.al. 2010).

1.2.3 Current Treatment Options in PAH

Patients in the modern treatment era respond better to PAH therapies; however the 5 year survival rate of 65% remains troubling (Thenappan et al. 2012). Treatment of PAH is initiated according to the clinical aetiology and functional impairment (NYHA Class I-IV) of each patient. Current therapeutic options target the increased pulmonary vascular resistance (PVR) associated with elevated vasoconstriction in the pulmonary arteries and aim to re-establish the balance between vasoconstriction and vasodilation. These include targeting chronically impaired prostacyclin and nitric oxide synthesis, and over-production of endothelin (Figure 1-5). Whilst some of them also have anti-proliferative properties and offer prevention of remodelling, none of the currently available treatments initiate regression of remodelling and the disease remains incurable. In addition, calcium channel blockade can be used to attenuate pulmonary artery vasoconstriction. In PAH, combinations of these treatments provide the most effective therapeutic approach.

1.2.3.1 Prostanoid Therapy

Prostacyclin, synthesised in pulmonary artery endothelial cells, is a potent vasodilator, inhibits platelet aggregation and has anti-proliferative properties (Geraci et al. 1999; Hoshikawa et al. 2001). Endothelial dysfunction is well described in PAH and in line with this, prostacyclin synthase is reported to be reduced in PAH patients (Christman et al. 1992; Galie et al. 2003) resulting in a deficiency of prostacyclin (PGI_2) in small- and medium-sized pulmonary arteries (Tuder et al. 1999). Prostanoid therapy has been a mainstay in the treatment of PAH for over a decade. Currently there are three FDA (Food and Drug Administration) approved prostanoids: epoprostenol, treprostinil and iloprost.

Epoprostenol is the first FDA approved prostanoid and is the first line of treatment for critically ill class III and IV symptoms because it is the most rapidly effective therapy. Given intravenously, epoprostenol improves NYHA functional class, exercise tolerance, haemodynamics and survival and quality of life in PAH (Barst et al. 1996; McLaughlin et al. 2002; Sitbon et al. 2002). Epoprostenol is a short acting vasodilator which requires chronic intravenous infusion, a major

limitation in its use. Common side effects observed include headache, jaw pain, flushing, nausea, diarrhoea, skin rash and musculoskeletal pain.

Treprostinil is a modern generation prostanoid with greater stability and a longer half life, and can be administered subcutaneously, intravenously, or as inhaled therapy. Subcutaneous administration of treprostinil is most widely used and improves exercise capacity and haemodynamics in all aetiologies of PAH in a dose-dependent manner. The intravenous and inhaled forms of treprostinil however, show greater tolerability and maintain improvements in haemodynamics (Gomberg-Maitland et al. 2005; Laliberte et al. 2004; McLaughlin et al. 2010). Side effects with intravenous treprostinil are similar to that of intravenous epoprostenol. Subcutaneous treprostinil is also associated with pain and erythema at the site of injection.

The most recently FDA approved prostanoid is inhaled iloprost. As iloprost dilates both systemic and pulmonary vasculature, inhalational administration and nebuliser therapy may bypass side effects associated with intravenous prostanoids. Iloprost shows considerable improvements in NYHA functional class and survival in patients with IPAH, CTEPH and PAH associated with connective-tissue diseases (Ewert et al. 2011; Goldsmith & Wagstaff 2004). Due to the short half-life, lasting approximately 30-60 minutes, iloprost is taken six to nine times daily (Hoeper et al. 2000; Olschewski et al. 2002).

1.2.4 **Endothelin Antagonists**

In the dysfunctional endothelium, increased production of endothelin 1 (ET-1) stimulates vasoconstriction and proliferation of smooth muscle cells in pulmonary arteries (McCulloch et al. 1998). In addition, plasma levels of ET-1 are known to be increased in PAH and correlate with severity of PAH and prognosis (Rubens et al. 2001). Effects of ET-1 are mediated through two main endothelin receptors, (ET_A and ET_B), both of which are G-protein coupled receptors. ET_A receptors are found primarily in the smooth muscle cells and are associated with vasoconstriction and proliferation in pulmonary arteries (Zamora et al. 1993). On the other hand, ET_B is mainly found in endothelial cells and to a lesser extent in smooth muscle cells (Hori et al. 1992; Zamora et.al. 1993). In the lung, ET_B receptor is involved in ET-1 clearance and stimulates the release of

nitric oxide and PGI_2 which leads to pulmonary artery vasodilation (Fukuroda et al. 1992). Following this, ET_A receptor antagonists therefore provide the best endothelin based therapy for treating PAH.

Currently, both selective and non-selective ET receptor antagonists (ERA) are approved for PAH therapy. Bosentan is an oral non-selective ERA proven to improve haemodynamics in APAH (Sitbon et al. 2005) and portopulmonary hypertension (Hoeper et al. 2005) with NYHA functional class II and III. Bosentan is associated with increased hepatic function, syncope and flushing and should be monitored carefully.

Sitaxsentan and ambrisentan are selective ET_A antagonists and, in theory, offer more selective blockade of ET-1 vasoconstrictor effects whilst maintaining vasodilator properties through ET_B. Improved exercise capacity and sustained improvements in haemodynamics were observed with both sitaxsentan and ambrisentan and approved for therapy in NYHA functional class II-IV (Barst et al. 2006; Klinger et al. 2011); however sitaxsentan treatment has subsequently been withdrawn owing to fatal liver failure and acute hepatitis.

Interestingly, it has been reported recently that women with PAH exhibit a greater clinical benefit from ERAs than men in terms of improvements in exercise capacity (Gabler et al. 2012). Gender influences in treatment-response requires further investigation.

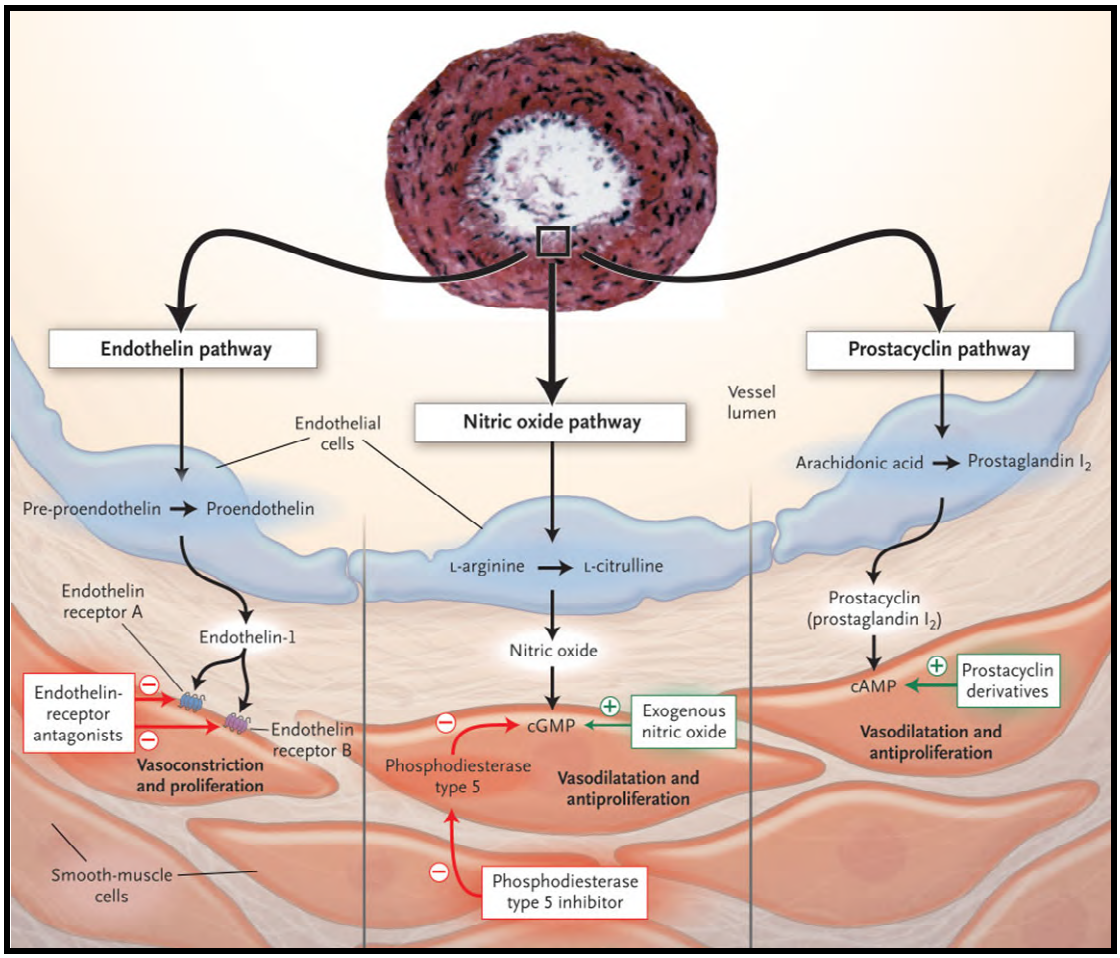


Figure 1-5: Targets of current treatment options in PAH therapy.
Current PAH treatments target impaired NO (phosphodiesterase type 5 inhibitors) and prostacyclin synthesis (prostacyclin analogues), and over-production of endothelin (Endothelin receptor antagonists) to re-establish a balance between vasoconstriction and vasodilation. Adapted from (Humbert et al. 2004).

1.2.4.1 **Phosphodiesterase Type-5 Inhibitors**

Nitric oxide (NO) is a potent endogenous, endothelium derived vasodilator that directly relaxes PASMCs through stimulation of soluble guanylate cyclise and increased production of intracellular cyclic guanosine monophosphate (cGMP). Phosphodiesterase type-5 (PDE-5) degrades cGMP and is abundantly expressed in the lung with increased activity in PAH (Black et al. 2001). Inhibition of this process using PDE-5 inhibitors such as sildenafil or tadalafil improves vasodilation. In addition, these treatments have anti-proliferative properties (Wharton et al. 2005). Indeed, both sildenafil and tadalafil have demonstrated acute and long-term beneficial effects in patients with PAH with improvements in NYHA functional class and exercise capacity reported (Galie et al. 2005; Galie et al. 2009). Currently, these are the only two FDA approved oral PDE-5 inhibitors for the treatment of functional classes II-III. Minor adverse effects of PDE-5 inhibitors generally include headache, flushing and dyspepsia.

1.2.4.2 **Calcium Channel Blockers**

Calcium channel blockers (CCBs) are utilised in PAH patients who respond positively to an acute vasoreactivity test with inhaled nitric oxide, intravenous adenosine, or intravenous epoprostenol. The pulmonary vasculature is only responsive to local mediators in approximately 5-10% of all PAH patients, therefore CCBs are of limited use. In PASMCs, CCBs reduce intracellular Ca^{2+} and cellular hyperpolarisation resulting in pulmonary vascular smooth muscle relaxation. Clinical trials using CCBs in PAH demonstrate improved survival rates in patients (Rich et al. 1992). The agents most commonly used are long-acting nifedipine, diltiazem, or amlodipine.

1.2.4.3 **Combination Therapy**

Combination therapy is generally considered most effective in treatment of PAH and maximises clinical benefit by targeting different mechanisms of action (Benza et al. 2007). For example, adjunctive therapy with sildenafil or bosentan has produced marked improvement in patients receiving prostacyclin therapy (Hoeper et al. 2003). Overall, combination therapy aims to increase efficacy and reduce toxicity to improve functional status and quality of life in patients who are unresponsive to monotherapy.

1.2.5 Future Perspectives in the Treatment of PAH

Current treatments for PAH improve symptoms and reduce severity of the haemodynamic disorder, however, gradual deterioration in a patient's condition is still an issue and often necessitates a lung transplant after years of therapy. For this reason, new therapeutic options have been investigated and emerging trends pharmacologically target the vascular remodelling process with anti-mitogenic, pro-endothelial function, pro-angiogenic, and anti-oxidative actions. In addition, regeneration using cell therapy is a novel and interesting therapeutic option.

1.2.5.1 Tyrosine Kinase Inhibitors

Recently, platelet-derived growth factor (PDGF) has been identified as a potent mitogen in pulmonary arteries contributing to pulmonary vascular remodelling and the progression of PAH (Barst 2005). PDGF exists in five isoforms (PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, and PDGF–AB) which share structural and functional properties with other growth factors such as vascular endothelial growth factor and exert their effects through two receptors, PDGFR-α and –ß (Andrae et al. 2008; Fredriksson et al. 2004). The PDGFRs belong to a family of transmembrane receptor tyrosine kinases (RTKs) and dimerisation of these receptors leads to an increase in kinase activity, for example Ras/MAPK, PI3K and phospholipase Cγ activity resulting in activation of mitogenic, pro-migratory, and anti-apoptotic genes which contribute to the vascular remodelling process. Indeed, PDGF stimulates proliferation of PASMCs (Schermuly et al. 2005) and over-expression of PDGF mRNA was demonstrated in lungs from patients with severe PAH (Humbert et al. 1998). *In vitro*, the small molecule RTK inhibitor ST1571 (Imatinib) was found to inhibit PDGF-related migration, proliferation and gene transcription in human PASMCs (Pullamsetti et al. 2012). Moreover, in two experimental models of PAH, Imatinib reverses established PAH (Schermuly et.al. 2005). RTKs are at an early stage of clinical evaluation in PAH patients. A phase II clinical study showed Imatinib reduced PVR, and improved cardiac output in a PAH treatment group (Hoeper et.al. 2000); however due to serious adverse events, for example subdural haematomas, Imatinib was withdrawn in 2013. The rationale behind use of RTKs

in PAH is very strong; however, the need to develop a safe and effective molecule is proving difficult.

1.2.5.2 **Cell Based Therapies**

It has been identified that PAEC apoptosis contributes to vascular cell proliferation in the lung and stimulates to the formation of intimal lesions (Taraseviciene-Stewart et al. 2001). Disruption of endothelial integrity in pulmonary arterioles contributes to endothelial dysfunction associated with abnormal vasomotor tone and increased smooth muscle cell growth and arterial remodelling. The recent interest in the role of apoptosis in the pathogenesis of PAH raises the possibility that novel treatments targeted toward repairing or regenerating lung microvascular endothelial cells. This may provide greater potential for reversing both structural and functional vascular abnormalities in established PAH. Indeed, cell-based gene delivery is effective in preventing monocrotaline-induced PAH using a variety of different therapeutic transgenes, including VEGF (Campbell et al. 2001), prostacyclin synthase, and endothelial nitric oxide synthase (eNOS) (Campbell et al. 1999). In addition, endothelial progenitor cells (EPCs) have been explored as a potential source for neovascularisation and intravenous infusion of EPCs in patients with IPAH has beneficial effects on exercise capacity and pulmonary haemodynamics (Wang et al. 2007). Perhaps, the combination of progenitor cells with the over-expression of therapeutic transgenes can provide a synergistic effect in the vasculature and promote regeneration of the endothelium.

1.3 Pathobiology of PAH

PAH is a complex disease with a multifactorial pathobiology. Pulmonary vascular proliferation, vasoconstriction, and in situ thrombosis are all involved in the pulmonary vascular remodelling process that underlies severe PAH. Within the pulmonary arteries, vasoconstriction initiated by hypoxia, elevations in cytoplasmic Ca^{2+} or decreased voltage-gated K^{+} channel depolarisation, is recognised to play a pivotal role in increasing pulmonary vascular resistance (PVR) and, hence, elevated pulmonary arterial pressure (PAP) (Mandegar et.al. 2004) (Figure 1.6). Sustained pulmonary vasoconstriction can influence PASMC hypertrophy and hyperplasia (Hishikawa et al. 1994) and vascular remodelling

involving all components of the vascular pulmonary vascular wall (smooth muscle cells, endothelial cells, and fibroblasts) ensues. Together, the hyperproliferative and vasoconstrictive pulmonary arteries exert an excessive burden on the pressure-intolerant right ventricle. Initially, the right ventricle undergoes compensatory hypertrophy to accommodate increased after-load and to overcome the downstream pressure, however over time this leads to right sided heart failure which is the primary cause of mortality in PAH patients (Humbert et.al. 2004).

1.3.1 Pulmonary Vasoconstriction

Pulmonary vasoconstriction is an important contributor to the elevated PVR and PAP observed in PAH. In particular, hypoxic pulmonary vasoconstriction (HPV) is a major determinant of PAH development. HPV is an important intrinsic adaptive mechanism unique to the lung which redirects the blood flow from poorly ventilated areas of the lung into a better ventilated area to improve ventilation-perfusion matching and to maximise oxygenation (Madden et al. 1992). In instances of chronic obstructive pulmonary disease (COPD) and high altitude pulmonary oedema, sustained alveolar hypoxia drives a PAH phenotype (Mandegar et.al. 2004). The mechanism by which hypoxia stimulates pulmonary vasoconstriction is unclear, although studies point to several possible pathways.

1.3.1.1 Hypoxic Pulmonary Vasoconstriction and Ca^{2+} Homeostasis

The extent of pulmonary vasoconstriction is dependent on the levels of cytosolic Ca^{2+} and activity of voltage-gated L-type Ca^{2+} channels which are regulated by membrane potential and Ca^{2+} sensitivity of the contractile apparatus (actin and myosin). Smooth muscle contraction is directly triggered by a rise in cytosolic free Ca^{2+} concentration which forms a complex with calmodulin (CaM). The Ca^{2+}/CaM complex interacts with contractile proteins, actin and myosin, and activates myosin light chain kinase (MLCK), which in turn, phosphorylates the myosin light chain (MLC). This process stimulates the activation of myosin ATPase to generate energy and the subsequent formation of crossbridges leading to smooth muscle contraction and vasoconstriction (Somlyo & Somlyo 1994) (Figure 1-7). On the other hand, relaxation of smooth muscle cells is initiated by a decrease in intracellular Ca^{2+} $[Ca^{2+}]_i$ due to Ca^{2+} uptake by the sarcoplasmic

reticulum Ca^{2+}-ATPase pump (SERCA) within smooth muscle cells and the Ca^{2+} extrusion by Na^{+}-Ca^{2+} exchanger. In addition, smooth muscle cell contraction is dependent on regulation of myosin light chain phosphatase (MLCP) activity. The decreased $[Ca^{2+}]_i$ initiates dissociation of the Ca^{2+}/CaM complex and the phosphorylated MLC is dephosphorylated by MLCP (Somlyo & Somlyo 1994) (Figure 1-7).

Intracellular Ca^{2+} is elevated in PASMCs exposed to hypoxia (Wang et al. 2005) and in line with this, during acute hypoxia a biphasic contraction is reported to coincide with elevated levels of Ca^{2+} (Robertson et al. 2000) which is sustained during hypoxia and is reversed following exposure to normoxia (Robertson et.al. 2000). Depolarisation and the opening of L-type Ca^{2+} channels are reported to account for the influx of Ca^{2+} during hypoxia (Bakhramov et al. 1998). Indeed, inhibition of these channels has been shown to attenuate hypoxia induced-vasoconstriction in pulmonary arteries (Jin et al. 1992; Leach et al. 1994). The PASMCs are proposed to be the cell type responsible for monitoring the hypoxic environment in the vasculature. This is supported by the observation that HPV still occurs in endothelium-denuded pulmonary arteries (Marshall & Marshall 1992) (Figure 1-8).

1.3.1.2 **Hypoxic Pulmonary Vasoconstriction and K^+ homeostasis**

Voltage-gated K^+ channels also play an important role in the regulation of vascular tone and work in concert with L-type Ca^{2+} channels to mediate contraction. In a resting smooth muscle cell, the membrane is permeable to K^+ and voltage-activated K^+ channels allow an efflux of K^+ out of the cell initiating membrane hyperpolarisation and the closure of voltage-gated L-type Ca^{2+} channels. Closure of K^+ channels on the other hand, leads to depolarisation and increased $[Ca^{2+}]_i$.

At rest, the PASMC membrane is more permeable to K^+, however during hypoxia K^+ currents are inhibited resulting in depolarisation of the PASMC membrane and an influx of Ca^{2+} via L-type Ca^{2+} channels (Yuan et al. 1993; Yuan et al. 1998). In PAH, altered activity of K^+ channels is reported resulting in sustained membrane depolarisation. In PASMCs, downregulation of $K_v1.5$ and $K_v2.1$ channels is

observed in rats with chronic hypoxia-induced PAH (Michelakis et al. 2002b) and $K_v1.5$ expression is reduced in patients with IPAH (Yuan et.al. 1998).

1.3.1.3 **Hypoxic Pulmonary Vasoconstriction and Reactive Oxygen Species**

The redox hypothesis of HPV suggests that redox-sensitive membrane K_v channels are decreased via adaptive mitochondrial derived reactive oxygen species (ROS). Generation of ROS occurs during hypoxia shifting the cellular balance toward a more reduced state and initiates depolarisation of the PASMC membrane and consequently pulmonary vascular contraction (Michelakis et al. 2002a). NADPH-oxidases, the enzymes that function to generate superoxide, have also been proposed as possible oxygen sensors of HPV. Evidence suggests that NADPH-oxidase is activated in response to hypoxia in PASMCs (Marshall et al. 1996) and that the generation of superoxide and formation of hydrogen peroxide (H_2O_2) mediate HPV in the lung (Weissmann et al. 1998). This is confirmed by the ability of NADPH-oxidase inhibitors to attenuate HPV in intact lungs (Weissmann et al. 2000). Whilst it is suggested the role of ROS is pivotal in HPV, their exact role still remains unclear as there is no consensus to whether ROS production is increased or decreased in hypoxia.

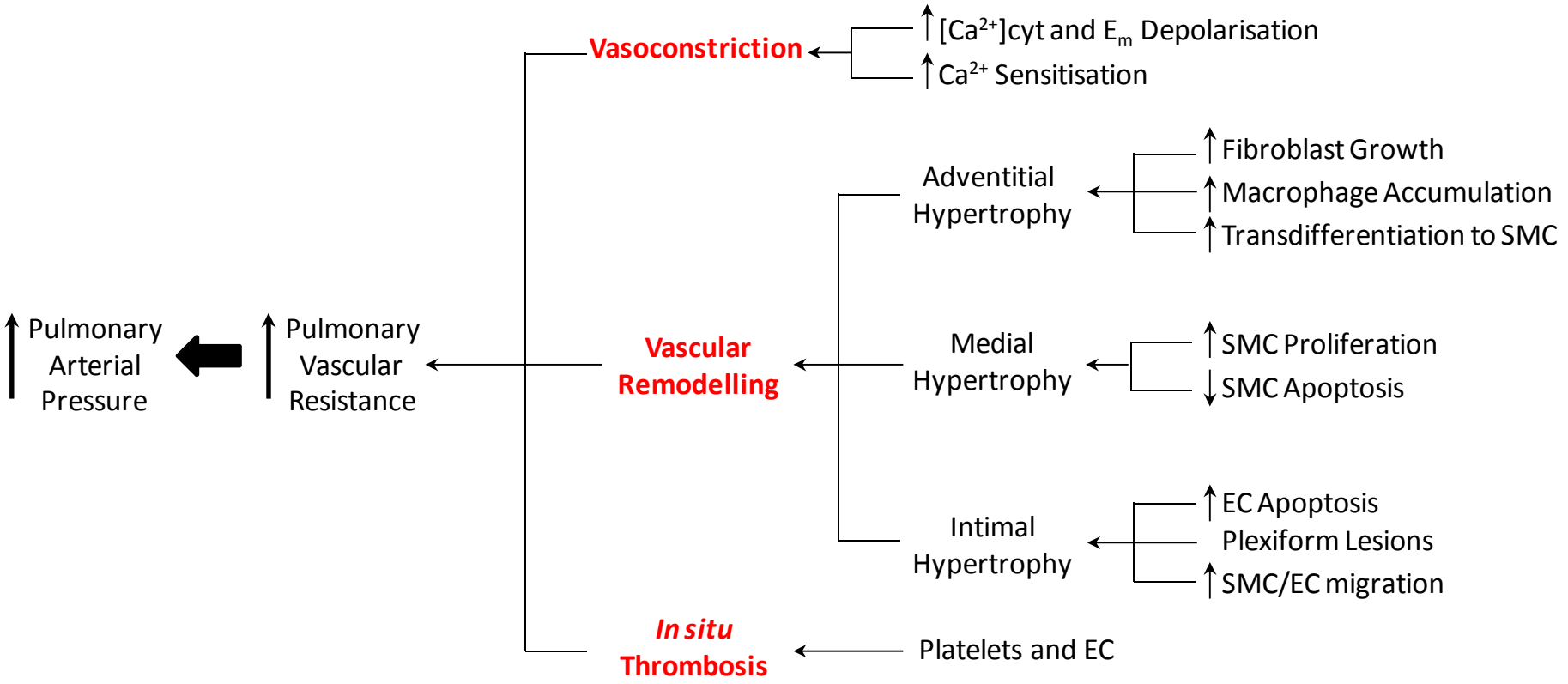


Figure 1-6: Pathophysiological processes involved in PAH development and progression.
Vasoconstriction, vascular remodelling, and in situ thrombosis contribute to the development of and sustained elevations in pulmonary vascular resistance (PVR) observed in PAH leading to increases in pulmonary arterial pressure (PAP). Cyt=cytosolic; E_m= extracellular membrane; SMC=smooth muscle cell; EC=endothelial cell. Adapted from (Mandegar et.al. 2004).

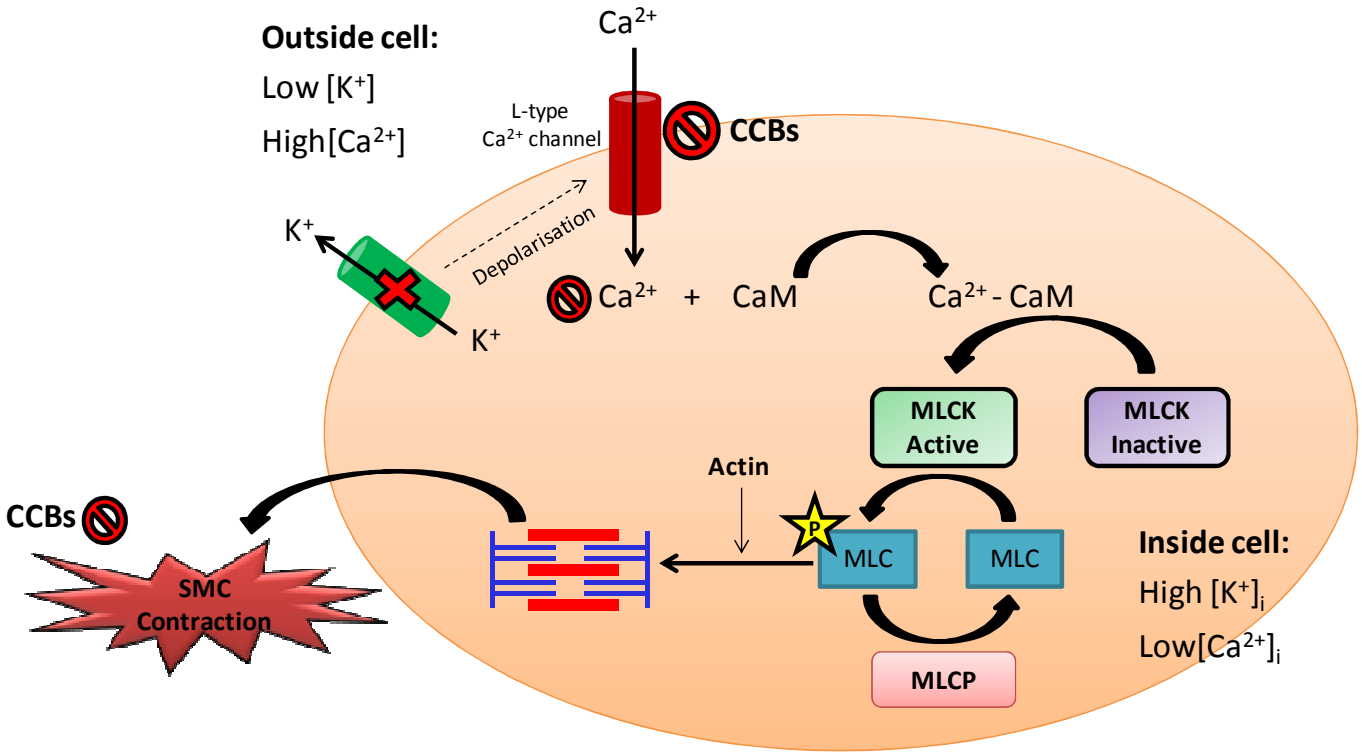


Figure 1-7: Signal transduction mechanisms underlying smooth muscle cell contraction.
Intracellular Ca^{2+} is a major determinant of smooth muscle cell (SMC) contraction. As free Ca^{2+} enters the SMC it forms a complex with calmodulin (CaM). The CaM complex then activates myosin light chain kinase (MLCK) which subsequently phosphorylates myosin light chain (MLC). Myosin light chain phosphatase (MLCP) inactivates this complex. This stimulates ATPase activity and initiates cross-bridging between actin and myosin and contraction. SMC contraction is also initiated by inhibition of K^+ efflux by voltage-activated K^+ channels leading to sustained depolarisation. Calcium channel blockers (CCBs) used in PAH treatment inhibit L-type Ca^{2+} channels and Ca^{2+} entry thereby reducing intracellular Ca^{2+} signalling and SMC contraction.

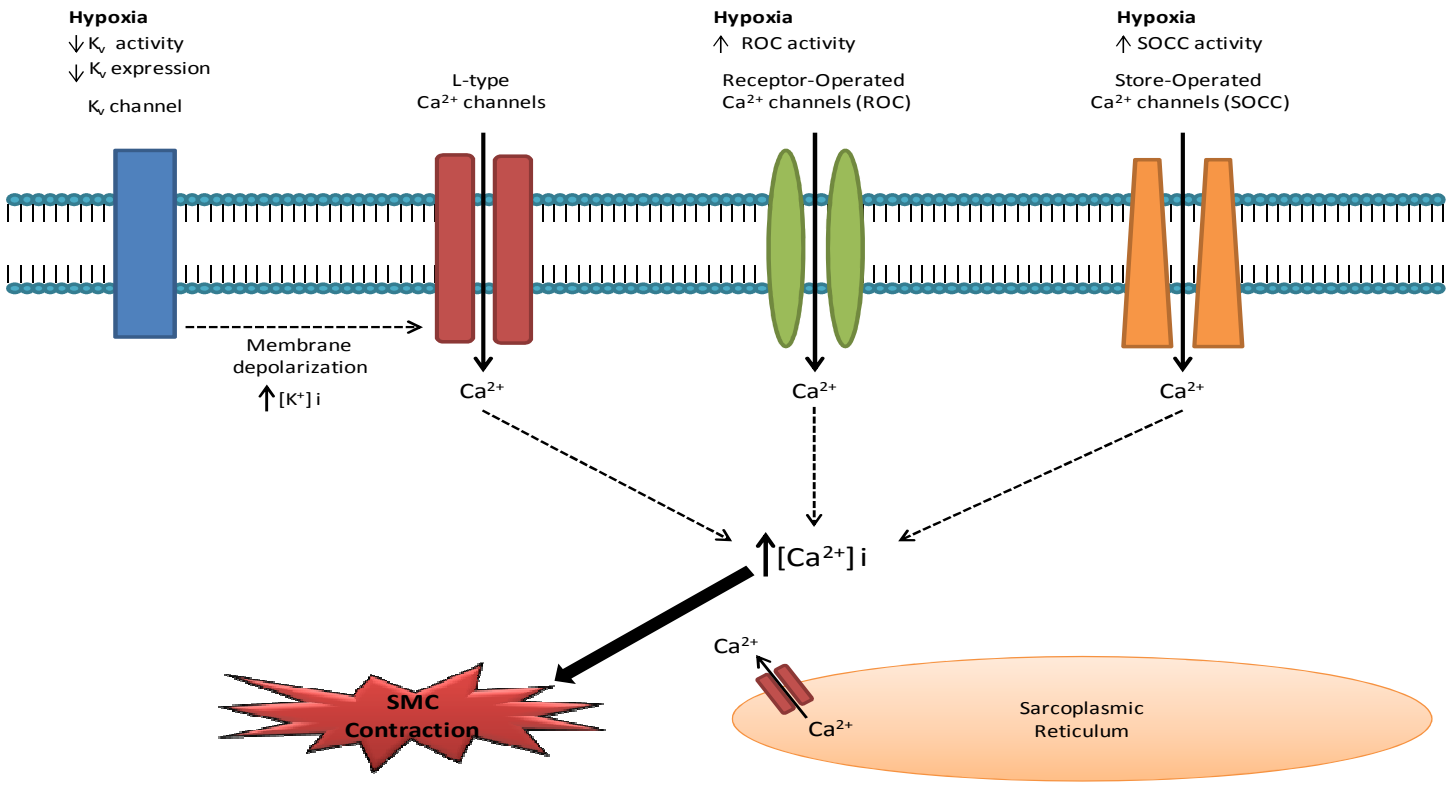


Figure 1-8: Proposed mechanisms involved in hypoxic pulmonary vasoconstriction
Hypoxia results in decreased activity and expression of Kv channel function leading to elevated intracellular K^+ and membrane depolarization. Increased Ca^{2+} occurs via influx via L-type Ca^{2+} channels. Increased activity of receptor-operated Ca^{2+} channels (ROC) and store-operated Ca^{2+} channels further contribute to increased intracellular Ca^{2+}. Together, these mechanisms lead to smooth muscle cell contraction and if sustained will promote proliferation.

1.3.2 Pulmonary Vascular Remodelling

Under normal conditions, the structure of the pulmonary arterial wall is maintained by a fine balance between proliferation and apoptosis of PASMCs, PAECs and fibroblasts. In PAH this balance is disrupted, and PAH is characterised by intimal thickening and muscularisation of the distal, previously non-muscular pulmonary arteries, resulting in a narrowed lumen and subsequent impairment of blood flow. This process is termed pulmonary vascular remodelling and is considered the hallmark in PAH (Tuder et al. 2009). Pulmonary vascular remodelling involves all three cell types in the vascular wall and arises from an increase in proliferation combined with suppression of apoptosis (Mandegar et.al. 2004).

1.3.2.1 The Role of Smooth Muscle Cells in Pulmonary Vascular Remodelling

Smooth muscle cells underlie the pulmonary vascular remodelling process. Smooth muscle hypertrophy and hyperplasia play an integral role in intimal and medial thickening which is widely observed in mild/early-stage PAH (Figure 1-9). In addition, smooth muscle cells and fibroblasts begin to produce increased matrix proteins, including collagen and elastin, within the media and adventitia contributing to medial thickness (Jeffery & Morrell 2002). During PAH, changes in the phenotype of PASMCs are also described, for example, PASMCs taken from PAH patients exhibit a more proliferative phenotype (Eddahibi et al. 2001). An increased migratory smooth muscle cell phenotype is also described. One common feature to PAH is the distal progression of smooth muscle cells into the normally non-muscular small peripheral pulmonary arteries resulting in muscularisation of the terminal portion of the pulmonary artery (MacLean et.al. 2000). Migration from the media to the subendothelial layer results in a layer of cells situated between the endothelium and the internal elastic lamina, termed the neointima (Yi et al. 2000). In the neointima, the smooth muscle cell phenotype alters from contractile to synthetic and secretes excessive extracellular matrix proteins contributing significantly to increased vascular resistance (Olschewski et al. 2001).

1.3.2.2 **The Role of Endothelial Cells in Pulmonary Vascular Remodelling**

In severe and end-stage PAH, another important form of vascular remodelling is observed involving disorganised proliferation and apoptosis of endothelial cells leading to formation of 'plexiform lesions' (Cool et al. 1997; Tuder et al. 1994). Plexiform lesions (Figure 1-9) are typically seen arising from smaller resistance arteries (200-400μm) distal to the bifurcation site and are described as complex, glomeruloid-like vascular structures (Cool et al. 2005; Sakao et al. 2009). They appear to have a complex inflammatory microenvironment distinct from remodeled arteries in PAH lungs. The continuous proliferation and sprouting of vascular channels is associated with up-regulation of hypoxia and shear stress-induced angiogenic mediators, such as HIF1a, VEGF-α and TGF-β1 (Jonigk et al. 2011). In addition, loss of tumour suppressor gene peroxisome proliferator-activated receptor-γ (PPAR-γ), is reported in plexiform lesions of PAH patients (Ameshima et al. 2003). PPAR-γ is antiproliferative and anti-inflammatory (Jiang et al. 1998) and has previously been shown to mediate an important protective role in experimental PAH (Hansmann et al. 2008). Decreased production of vasodilators (Tuder et.al. 1999) and increased production of vasoconstrictors (Giaid et al. 1993) is also described in these plexiform lesions. Regardless of the histopathologic subtype involved, pulmonary vascular remodelling always results in obstruction of the lumen and the impairment of blood flow leading to increased vascular resistance.

Importantly experimental animal models of PAH fail to recapitulate the severe vascular phenotype in PAH with neointimal formation and plexiform lesions. The role plexiform lesions play in the pathogenesis of human PAH is therefore controversial. However, the discovery of novel animal models of PAH appears to show endothelial cell proliferation, luminal obliteration and severe PAH as a result of plexiform lesions and neointimal changes. For example, S100A4/Mts1 protein over-expressing mice (Dempsie et al. 2011; Greenway et al. 2004), schistosomiasis murine model (Crosby et al. 2010) and administration of VEGF receptor antagonist Sugen 5416 + hypoxia rat model (Abe et al. 2010). Since these lesions appear to have morphology similar to human plexiform lesions, a better understanding of human pathology is achievable.

1.3.2.3 **Downstream Signalling Involved in Vascular Remodelling**

Akt, or protein kinase B, is a serine/threonine-specific protein kinase that plays a critical role in cell apoptosis, proliferation and migration. In addition, Akt signalling is a critical mediator of cardiac hypertrophy, survival and stress. It is recognised that the survival signal mediated by various growth factors and cytokines involves phosphatidylinositol 3'-kinase (PI3K)/Akt signal transduction pathway. Vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) can induce PI3K activity in aortic endothelial cells (Guo et al. 1995; Xia et al. 1996) and PASMCs (Goncharova et al. 2002). Recent evidence has also highlighted an important role of increased thrombin-induced Akt phosphorylation in PASMCs in IPAH and CTEPH resulting in increased proliferation and leading to medial hypertrophy and vascular remodelling (Ogawa et al. 2013). Interestingly, Akt phosphorylation is also regulated by estrogen. Reduced Akt signal transduction by estrogen reverses remodeling secondary to PH (Nadadur et al. 2012) and significantly reduces Akt signalling during right heart failure in PH (Nadadur et al. 2012).

The mitogen-activated protein kinase (MAPK)/ extracellular signal-related kinase (ERK) pathway is also crucially involved in vascular remodeling and cardiac hypertrophy. MAPK cascades are highly conserved signal transduction pathways and are believed to regulate cell and myocyte growth in response to developmental signals or physiologic and pathologic stimuli (Meng et al. 2011). Previous studies have demonstrated that ERK and p38 MAPK expression are increased in cardiac hypertrophy (Altamirano et al. 2009; Streicher et al. 2010). In the pulmonary vasculature, MAPK/ERK also plays a central role in smooth muscle cells in response to mechanical, hypoxic and growth-factor induced stimuli, in particular promoting PASMC growth (Mandegar et al. 2004). Estrogen is also involved in regulation of ERK and leads to a decline in ERK1 phosphorylation in right heart failure PH (Nadadur et al. 2012). Collectively, this shows that PI3K/Akt and MAPK/ERK pathways play a pivotal role in regulating PASMC proliferation and migration and cardiac hypertrophy.

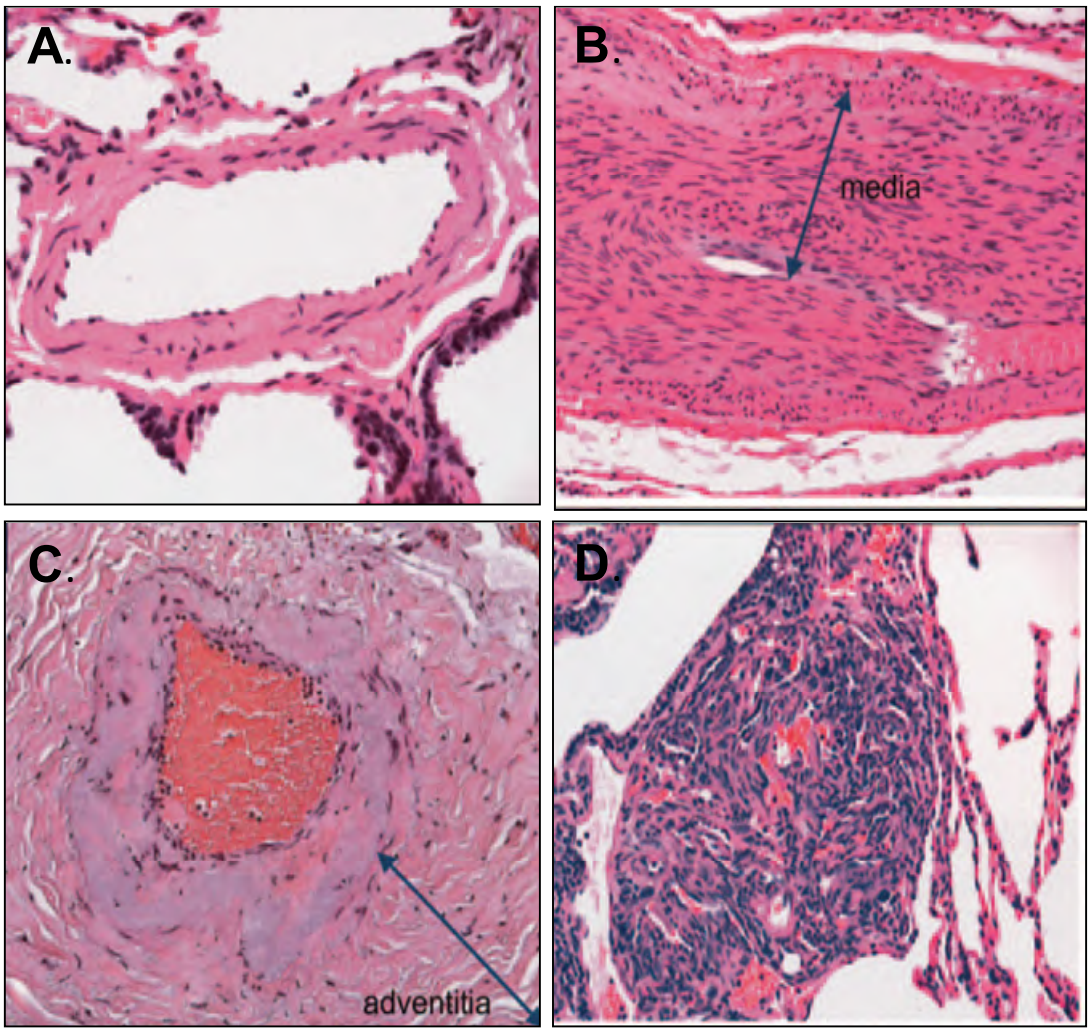


Figure 1-9: Histopathological changes observed in pulmonary vascular remodelling in human PAH.
Top Left, A: a normal pulmonary artery. *Top Right*, B: smooth muscle cell hypertrophy and proliferation leading to medial hyperplasia typically observed in mild/moderate human PAH. *Bottom Left*, C: adventitial fibrosis observed in moderate PAH. *Bottom Right*, D: a plexiform lesion characterised by lumen obliteration observed in severe/end-stage PAH. Adapted from (Cool et.al. 2005).

1.4 Genetic Basis of Pulmonary Arterial Hypertension

1.4.1 Bone-Morphogenetic Protein Receptor-Type 2

PAH is a disease with an underlying genetic susceptibility. The term heritable PAH (HPAH) has recently been adopted to account for PAH in families in which a specific genetic mutation infers susceptibility to disease development. The inheritance pattern in HPAH is described as autosomal dominant implying each child of an affected individual is at a 50% risk of inheriting the mutant allele (Thompson & McRae 1970). Like IPAH, the development of HPAH has a marked female predominance and in addition exhibits a genetic anticipation. That is, patients with a HPAH typically present at earlier ages (Loyd et al. 1995) and are more likely to be female. A candidate gene approach lead to the discovery of the first gene associated with HPAH. A marker for HPAH localised on chromosome 2q31-32 (Nichols et al. 1997) was identified as a receptor in the TGF-β superfamily, bone-morphogenetic protein receptor-type 2 (BMPR2) (Lane et al. 2000). Mutations in BMPR2 are responsible for approximately 80% of HPAH cases (Morrell 2010). However, an incomplete penetrance is described as only 10-20% of mutation carriers develop PAH (Newman et al. 2004). This strongly suggests the presence of modifying genetic or environmental factors that infer an increased or decreased risk. Indeed, alterations in estrogen metabolism and estrogen metabolites are associated with disease penetrance in female patients with a BMPR2 mutation (Austin et al. 2009).

Although BMPR2 mutations and subsequent dysfunctional TGF-β signalling represent the majority of HPAH cases, alternative pathogenic mutations in the TGF-β family underlie PAH susceptibility. Particularly mutations in activin receptor-like kinase 1 (ALK-1) are observed in hemorrhagic telangiectasia (HHT) and individuals present with PAH that is clinically and histopathologically indistinguishable from other forms of HPAH (Machado et al. 2009). Patients with ALK-1 mutations, and therefore defective Smad signalling, typically have shorter survival times compared to BMPR2 carriers and non-carriers (Girerd et al. 2010).

1.4.2 Normal BMP/BMPR2 and TGF-β Signalling

BMPs are the largest group of cytokines within the TGF-β superfamily. They are primarily involved in regulating growth, differentiation and apoptosis in smooth

muscle cells. When co-expressed with type I BMP receptor, BMPR2 has high affinity for ligands BMP-2, -4, and -7. Receptor-ligand binding stimulates phosphorylation of Smad1, -5 and -8, which in canonical signalling, then associate with Smad-4 in the cytoplasm to translocate to the nucleus (Morrell et al. 2009) (Figure 1-10). The co-Smad complex is involved in regulation of transcription of Smad-responsive genes, for example the inhibitor of DNA binding family of proteins (Ids) (Miyazono & Miyazawa 2002). Id proteins are basic helix-loop-helix transcription factors that lack a DNA binding domain. Four members of the Id family (Id1-4) have been identified in mammalian cells. They are encoded by separate genes and demonstrate different expression patterns, hence they have various functions in several different organs. For example, Id2 expression is high in immune cells and developing epithelial cells, whereas Id1 and Id3 are expressed in developing lung mesenchyme (Rudarakanchana et al. 2002). Id1 and Id3 are now known to be the most abundantly expressed Id transcripts in PASMCs and are regulated by BMP-4 and -6 (Yang et al. 2013). Id signalling and other downstream signalling complexes from Smad are switched off by Smad ubiquitination and regulatory factors (Smurfs) (Shi et al. 2004).

1.4.3 Consequences of Dysfunctional BMP/BMPR2 and TGF-β Signalling

A critical reduction in BMPR2 expression and function due to mutations as observed in HPAH, leads to deficient Smad signalling. The growth inhibitory effects of BMPs have been shown to be Smad1 dependent (Yang et al. 2005). In particular, a loss of signalling by the Smad1/5 pathway in response to BMP2 and BMP4 is described following BMPR2 mutation and therefore a reduction in smooth muscle cell apoptosis (Yu et al. 2005). Knockdown of BMPR2 with siRNA has also been demonstrated to increase the susceptibility of pulmonary artery endothelial cells to apoptosis (Teichert-Kuliszewska et al. 2006). Additionally, in experimental PH, heterozygous $BMPR2^{+/-}$ mice when chronically infused with serotonin, develop more severe PH compared with wildtype littermates (Long et al. 2006). Thus BMPR2 dysfunction also increases the susceptibility to PH when exposed to other environmental stimuli. In the presence of BMPR2 mutations, the disruption of the normal Smad signalling pathway can also lead to activation of alternate p38 mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathways which lead to smooth muscle cell proliferation and

inhibition of apoptosis (Yu et al. 2005). It has been identified that Id1 and Id3 induction is dependent on BMPR2 as mutations in BMPR2 reduce the BMP-4 and -6 stimulated inductions of Id1 and Id3 in PASMCs (Yang et al, 2013). Recently it has been shown that agents enhancing BMP/Smad/Id signalling are effective at restoring the growth suppressive effects of BMP in BMPR2 mutant cells (Yang et al. 2010; Yang et al. 2013).

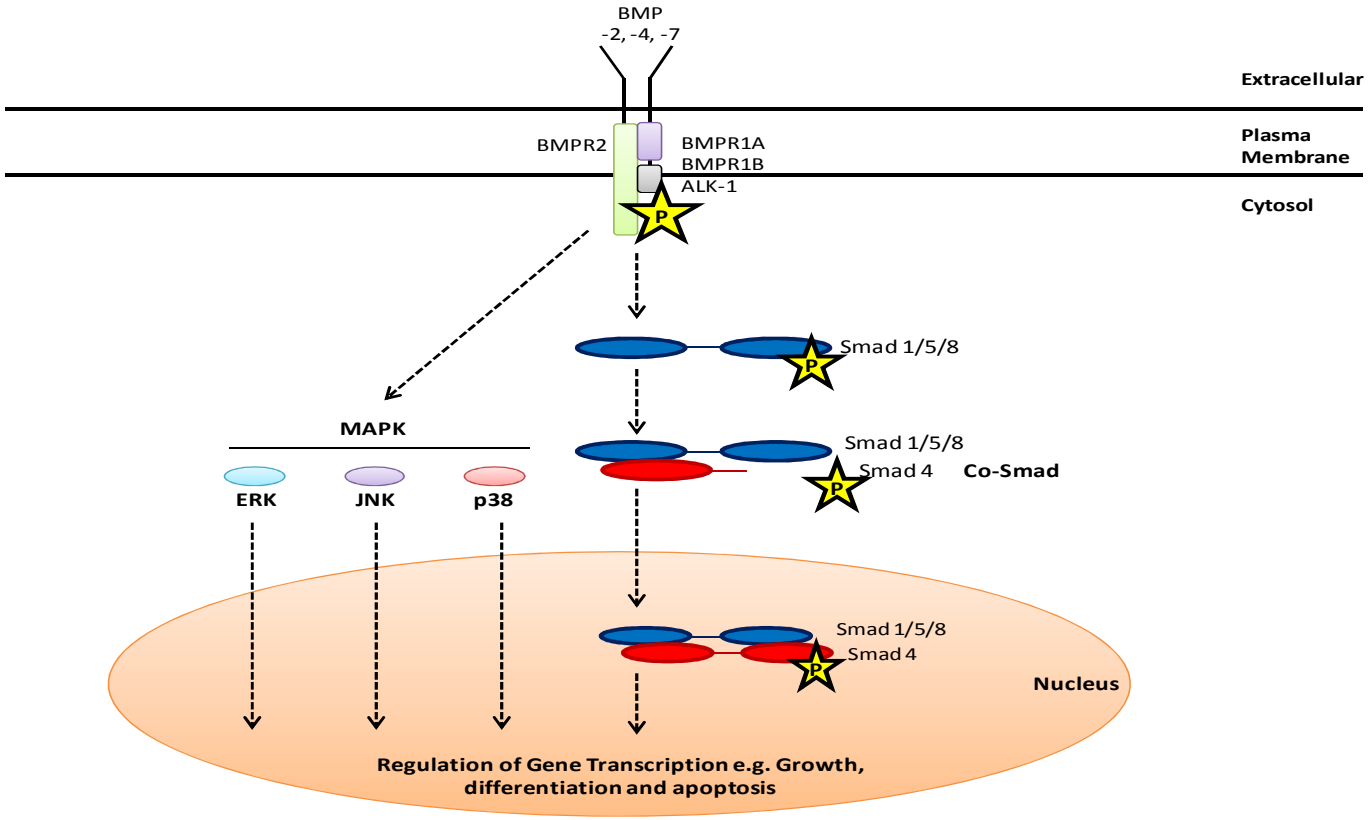


Figure 1-10: BMPR2 and downstream signalling cascade. When co-expressed with type 1 BMP receptor (BMPR1), BMPR2 has high affinity for ligands BMP-2, -4 and -7. In canonical signalling, ligand-receptor activation stimulates phosphorylation of Smad-1, -5 and -8 and this complex further dimerises with Smad-4 to form a co-Smad. This co-Smad complex then translocates to the nucleus and regulates transcription of genes involved in growth, differentiation and apoptosis. Dysfunctional BMPR2 mutations in HPAH can lead to activation of an alternate downstream signalling pathway and activation of mitogen activated protein kinases (MAPK) and as a consequence smooth muscle cell proliferation and inhibition of apoptosis.

1.4.4 Serotonin Transporter

Additional genes also account for HPAH cases. In particular polymorphisms in the serotonin transporter (SERT) may influence development of PAH in the presence or absence of BMPR2 mutations. SERT is encoded by the solute carrier family 6 member 4 (SLC6A4) gene located on chromosome 17 position 17q11.2. Long (L) and short (S) functional polymorphisms are found within the promoter region of SERT. The S allele results in reduced SLC6A4 transcription and therefore reduced SERT protein expression whereas the L allele is associated with a two- to threefold higher rate of gene transcription (Lesch et al. 1996). Indeed, the LL allele SERT polymorphism has been identified in a small cohort of IPAH patients (Eddahibi et.al. 2001). SERT plays an integral role in development of both experimental and human PAH where it is responsible for mitogenic and vasoconstrictive actions of serotonin (Eddahibi et al. 1999; MacLean et al. 1996). Mice over-expressing the human SERT gene construct ($SERT^+$ mice) develop severe PAH (MacLean et al. 2004). In line with this, mice with targeted over-expression of SERT in the PASMCs develop PAH (Guignabert et al. 2006), whilst mice devoid of the SERT gene are protected against the development of hypoxia-induced PAH (Eddahibi et al. 2000).

1.5 Serotonin Biosynthesis and Metabolism

Serotonin (5-hydroxytryptamine, 5-HT) was identified chemically in 1948 following its isolation from serum, originating in platelets (RAPPORT et al. 1948). Localisation of serotonin was subsequently found as a major neurotransmitter in the central nervous system and in the periphery in the gastrointestinal tract. The intestinal enetrochromaffin cells are the main site of serotonin synthesis with over 80% of peripheral synthesis originating in the gastrointestinal tract. The remaining 20% is synthesised in other cell types including serotonergic neurons and PAECs (Hoyer et al. 2002). The highest concentration of serotonin is found in the platelets which act as a store for up to 99% of total peripheral serotonin via the serotonin transporter (SERT). Although circulating serotonin is very low (i.e. <1nM) it controls many important physiological actions, in the cardiovascular system (Berger et al. 2009), the brain (e.g. control of respiration, memory, nociception and behaviour) and the intestine (Hoyer et.al. 2002).

Endogenous serotonin arises from biosynthesis from the precursor amino acid tryptophan (Figure 1-11). Decarboxylation of tryptophan is catalysed by the enzyme tryptophan hydroxylase (TPH) to produce 5-hydroxytryptophan which undergoes further decarboxylation by the aromatic L-amino acid decarboxylase finally producing serotonin. TPH is the rate-limiting enzyme in this two step biosynthetic pathway. Currently, two genes which encode for TPH have been identified: *Tph1* and *Tph2* (Walther & Bader 2003). These *Tph* isoforms share a 71% sequence homology. Despite being functionally identical, TPH2 is expressed exclusively in the brain whereas TPH1 is responsible for serotonin synthesis in the periphery (Nakamura & Hasegawa 2007).

Serotonin degradation and inactivation occurs rapidly through oxidative deamination catalysed by the enzyme monoamine oxidase (MAO) in the liver and lung. MAO exists in two isoforms, MAO-A and MAO-B, encoded by different genes. MAO-A preferentially degrades serotonin to produce 5-hydroxyindol-acetaldehyde which is subsequently oxidised to 5-hydroxyindole acetic acid (5-HIAA) by aldehyde dehydrogenase. 5HIAA is the primary metabolite of serotonin and is excreted in the urine.

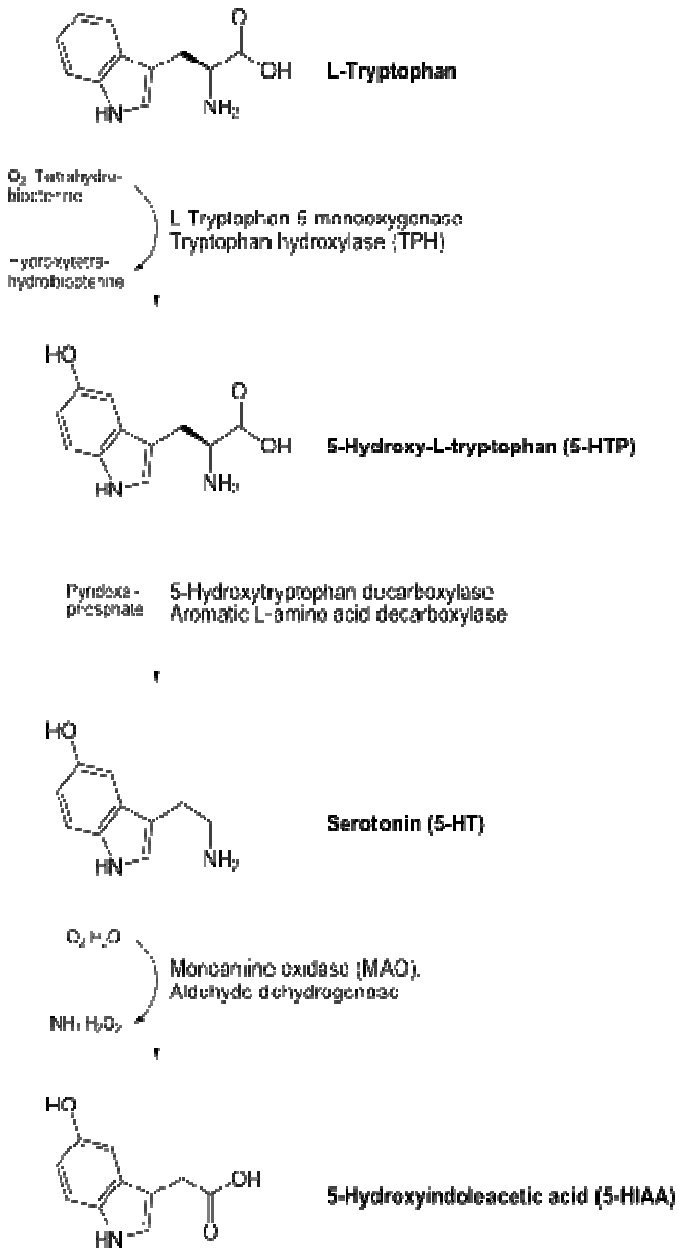


Figure 1-11: Serotonin Biosynthesis and Metabolism.
Serotonin (5-HT) is synthesised from amino acid precursor tryptophan. Conversion to 5-hydroxy-L-tryptophan is catalysed by the rate-limiting action of tryptophan hydroxylase (TPH). Subsequent conversion by non-specific decarboxylase enzymes produce serotonin. Serotonin metabolism by both monoamine oxidase and aldehyde dehydrogenase produces the primary metabolite 5-hydroxyindoleacetic acid (5-HIAA). Adapted from (Druce et al. 2009).

1.6 Physiological Functions of Serotonin

Under normal circumstances, the lung vascular bed in not exposed to excessive serotonin levels. However, there is evidence during PAH that there is decreased serotonin storage within platelets leading to enhanced plasma concentration of 'free' serotonin (Herve et al. 1995). Accumulating evidence exists describing a pivotal role for serotonin in development of experimental and human PAH (MacLean & Dempsie 2010). Within the pulmonary circulation, serotonin signalling is mediated via three distinct pathway components through which serotonin may facilitate PAH development. These are TPH-1, SERT and the 5-HT receptors, and the downstream signalling pathways activated by SERT and 5-HT receptors.

1.6.1 Tryptophan Hydroxylase-1

Peripheral synthesis of serotonin is initiated by the rate-limiting enzyme tryptophan hydroxylase-1 (TPH-1). Although TPH1 is predominantly expressed in the intestinal enterochromaffin cells, local serotonin synthesis within the PAECs mediating paracrine effects are also considered important in the periphery. Local serotonin production in the PAECs is believed to facilitate a mitogenic and vasoconstrictive microenvironment in which PASMCs promote hyperplasia. Indeed, it has recently been shown that expression of the *Tph1* gene is increased in lungs and PAECs from patients with IPAH (Eddahibi et al. 2006).

Experimentally, development of chronic hypoxia-induced PH and dexfenfluramine-induced PAH is ablated in *Tph1*$^{-/-}$ mice devoid of peripheral serotonin synthesis supporting the importance of serotonin in disease development (Dempsie et al. 2008a; Morecroft et al. 2007). Previously, in unpublished work, we have demonstrated that there is low TPH1 expression in PAECS from normoxic mice, however after exposure to hypoxia TPH1 is abundantly expressed suggesting that hypoxia induces TPH1 expression and *de novo* synthesis of serotonin. Hypoxia and mechanical stretch have previously been shown to increase TPH1 expression in rabbit lung (Pan et al. 2006). This is consistent with the observation that serotonin is overproduced in lungs and PAECs from patients with IPAH (Eddahibi et.al. 2006). Together, this evidence

indicates that peripheral serotonin plays an essential role in the development of PAH.

1.6.2 **5-HT Receptors**

The actions of serotonin in the periphery are numerous and complex. This arises from the vast number of 5-HT receptor subtypes. To date, there are 14 known structurally distinct receptors categorized into seven major subtypes (5-HT_{1-7}) (Figure 1-12). These are defined by their structure and coupling to downstream signal transduction pathways (Alexander et al. 2006). Multiple splice variants also exist for certain receptors producing several isoforms for one receptor subtype, for example 5-HT_1 comprises of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}. With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, 5-HT receptors are members of the G-protein-coupled receptor (GPCRs) superfamily (Hoyer et.al. 2002).

The 5-HT GPCRs all share a similar structural homology. They contain an extracellular N-terminus domain responsible for ligand binding, seven transmembrane α-helices, and an intracellular C-terminal domain. Ligand binding initiates activation of the associated G-protein. G-proteins exist as heterotrimeric structures composed of α, ß, and γ subunits. These are typically classified by the Gα subunit into four classes of subunits distinguished from each other by their sequence homology. These include: G_s, G_i, G_q and $G_{12/13}$ (Table 1-3). Each respective G-protein activates its own unique signal transduction pathway. The effector for both the G_s and G_i pathways is the cyclic-adenosine monophosphate (cAMP) generating enzyme adenylate cyclase (AC). In the Gs class, AC is activated and ATP catalyses conversion of cAMP, which in turn, activates cAMP-dependent protein kinase (PKA). In contrast, interaction with the G_i subunit inhibits AC activation. The effectors of the G_q and $G_{12/13}$ subunits are phospholipase Cß (PLCß) and small G-proteins, respectively. Typically, when the receptor is inactive, the receptor remains bound to an also inactive Gα-subunit bound to guanosine diphosphate (GDP). Upon ligand activation, a conformational change promotes exchange of a molecule of GDP for guanosine triphosphate (GTP). In turn, G-protein activation results in dissociation of the Gα subunit from the Gßγ complex, and both entities activate downstream signalling pathways.

1.6.2.1 **5-HT$_1$ Receptor Subtypes**

The 5-HT$_1$ receptor class comprises of five receptor subtypes: 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$ and 5-HT$_{1F}$. In humans, these receptors share 40-63% sequence homology. The latter two subtypes are represented in lower case as their physiological role remains to be determined. The 5-HT$_1$ receptors are all coupled to the G_i protein and therefore stimulation of this receptor results in inhibition of AC and cAMP production. Expression of all 5-HT$_1$ receptors has been identified in both the CNS and the periphery and the functional roles of 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_{1D}$ are well characterised in various tissues. Specifically in the cardiovascular system, 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors predominate (Hoyer et.al. 2002).

The 5-HT$_{1B}$ receptor is well characterised in the pulmonary circulation in both experimental and human PAH. Experimentally, the 5-HT$_{1B}$ receptor has been shown to be involved in the development of chronic hypoxia induced-PAH, as well as in the contractile responses mediated by serotonin in the pulmonary arteries (Keegan et al. 2001). Increased expression of 5-HT$_{1B}$ in pulmonary arteries from monocrotaline-treated rats also supports a role for 5-HT$_{1B}$ in PAH development (Wang et al. 2001). Indeed, 5-HT$_{1B}$ receptor knockout mice exhibit a reduction in right ventricular systolic pressure (RVSP) and pulmonary vascular remodelling following exposure to hypoxia (Keegan et.al. 2001). In addition, in small resistance pulmonary arteries from fawn-hooded rats which develop exaggerated hypoxia-induced PAH, serotonin-induced vasoconstriction is inhibited by the 5-HT$_{1B}$ selective antagonist SB224289 (Morecroft et al. 2005).

In humans, 5-HT$_{1B}$ is implicated in both serotonin-induced pulmonary arterial vasoconstriction in small and large pulmonary arteries (MacLean 1999; Morecroft et al. 1999) and also in PASMC proliferation. Moreover, pulmonary arteries from PAH patients show elevated expression of the 5-HT$_{1B}$ receptor (Launay et al. 2002). Evidence for a functional role of 5-HT$_1$ in human pulmonary arteries originated from studies which described a vasoconstrictive property of sumatriptan (a 5-HT$_{1B/D}$ receptor agonist) (Macintyre et al. 1992). Furthermore, the serotonin-induced vasoconstriction in these arteries was indeed inhibited by the 5-HT$_{1B/D}$ antagonist, GR55562, which supports 5-HT$_{1B/D}$ mediated vasoconstriction (MacLean et.al. 1996). In addition, in PAH lungs, hypoxia

reduced cGMP and eNOS levels and raised vascular tone markedly potentiate 5-HT_{1B} mediated responses in pulmonary arteries (MacLean 1999). It has also recently been demonstrated that the 5-HT_{1B} receptor can mediate proliferation in PASMCs (Lawrie et al. 2005). In the systemic circulation, serotonin mediates vasoconstriction via 5-HT_{2A} receptor (Hoyer et al. 1994), therefore the 5-HT_{1B} receptor could be a pulmonary selective target for PAH therapy.

Functional interactions exist between the 5-HT_{1B} receptors and SERT. In the pulmonary circulation, this results in heightened vasoconstriction and proliferation. Indeed, dual inhibition of both the 5-HT_{1B} receptors and SERT is more effective in preventing hypoxia-induced PH in mice than inhibition of SERT alone implicating a synergistic effect between the receptor and transporter of 5-HT (Morecroft et al. 2010). In addition, dual blockade of both 5-HT_{1B} and SERT is more effective at inhibiting serotonin-induced proliferation in both non-PAH and IPAH PASMCs (Morecroft et.al. 2010). The interaction arises from stimulation of the 5-HT_{1B} receptor which results in activation of rho small G protein and its downstream mediator ROCK. This in turn facilitates the nuclear translocation of SERT-induced phosphorylated ERK1/2 and transcriptional regulation of proliferative genes including GATA-4 and cyclin D1 (Liu et al. 2004).

1.6.2.2 **5-HT_2 Receptor Subtypes**

The 5-HT_2 class of receptor share 46-50% sequence homology and couple preferentially to G_q, activating PLC to increase the formation of inositol 1,4,5 trisphosphate (IP3) and 1,2-diacylglycerol (DAG). In turn, these second messengers increase cytosolic Ca^{2+} (Hoyer et.al. 2002). The 5-HT_{2A} receptor is widely expressed in the CNS and periphery, and has been implicated in vasoconstriction and proliferation in PASMCs in PAH (Dempsie et al. 2008b). 5-HT_{2A} receptors are also demonstrated to be involved in platelet aggregation and thrombosis (Nagatomo et al. 2004).

In experimental PAH, antagonism of 5-HT_{2A} inhibits monocrotaline-induced PAH in mice (Hironaka et al. 2003), and also inhibits serotonin-induced pulmonary vasoconstriction in vessels from both normoxic and hypoxic rats (Morecroft et.al. 2005). In isolated rat PASMCs, activation of 5-HT_{2A} receptors has also been shown to directly inhibit $K_v1.5$ channels, which results in decreased K^+ cellular

efflux and depolarisation mediating vasoconstriction (Cogolludo et al. 2006). Moreover, serotonin-induced proliferation in rat pulmonary arterial fibroblasts is 5-HT_{2A} receptor mediated (Welsh et al. 2004).

In humans, the 5-HT_{2A} receptor is present in human pulmonary arteries, although it only contributes to vasoconstriction when serotonin levels are much higher than the physiological range (Morecroft et.al. 1999). Ketanserin, a selective 5-HT_{2A} receptor antagonist is a proven therapy for PAH, however it's clinical effectiveness is limited by the expression of 5-HT_{2A} in systemic arteries where it also mediates serotonin-induced vasoconstriction (Dempsie et.al. 2008a; Frishman et al. 1995). In one clinical trial, ketanserin failed to improve pulmonary haemodynamics in either primary or secondary PH (Frishman et.al. 1995).

The 5-HT_{2B} receptor has also been identified to mediate multiple effects in the pulmonary vasculature. In vivo, the development of hypoxia-induced PH is inhibited in mice deficient for the 5-HT_{2B} receptor, and similarly in mice treated with the 5-HT_{2B} selective antagonist RS-127445 (Launay et.al. 2002). In addition, this receptor is regarded to play a key role in regulating plasma serotonin levels in mice (Callebert et al. 2006). The 5-HT_{2B} receptor is also associated with promoting cell cycle progression in fibroblasts (Nebigil et al. 2000) and cell survival in cardiomyocytes (Nebigil et al. 2003) suggesting it may contribute to remodelling and cardiac hypertrophy. Albeit the 5-HT_{2B} receptor is up-regulated in pulmonary arteries removed from pulmonary hypertensive patients, loss of 5-HT_{2B} receptor function appears to predispose to dexfenfluramine-induced PAH in humans (Blanpain et al. 2003).

1.6.2.3 **Additional 5-HT Receptor Subtypes**

In addition to the well defined effects of 5-HT_1 and 5-HT_2 receptors, the expression of additional 5-HT receptors has also been observed in the pulmonary circulation. mRNA transcripts for the 5-HT_{1A}, 5-HT_{1D}, 5-HT_{3A}, 5-HT_{3B}, 5-HT_4, 5-HT_6, and 5-HT_7 receptors have been identified in rabbit pulmonary arteries (Molderings et al. 2006). Multiple physiological functions for these receptor subtypes exist in the periphery; however they appear to play a minor role in the receptor-dependent effects of serotonin in the pulmonary circulation. The

serotonin-receptor mediated effect in PAH are assumed to be driven via 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2B}.

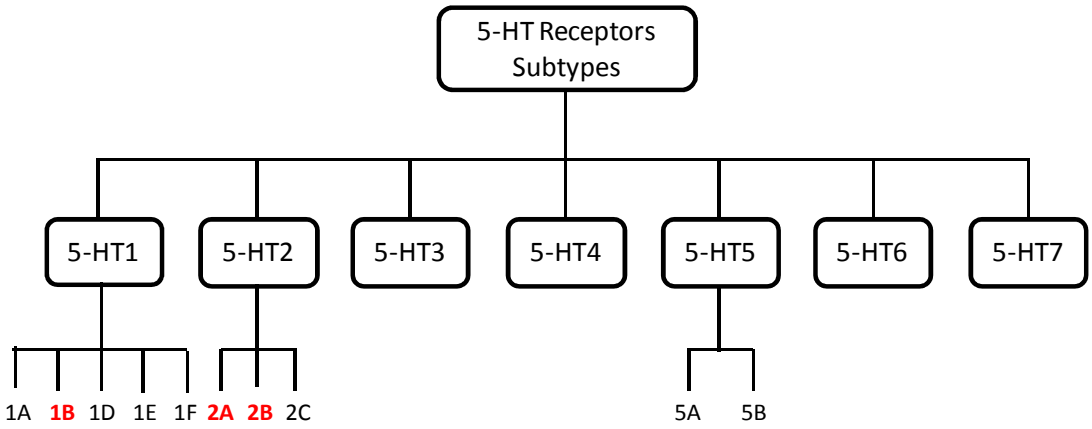


Figure 1-12: Classification of 5-HT receptor subtypes.
Those highlighted in red have been identified to mediate serotonin-induced effects in experimental and human PAH. The physiological role of 5-HT receptors -5 and -6 in pulmonary hypertension is undetermined.

Table 1-3: 5-HT receptor subtype signalling pathways.
All 5-HT receptors are G-protein-coupled receptors (GPCRs) with the exception of 5-HT3 which is a ligand gated ion channel. Activation of GPCRs activates downstream intracellular second messenger cascades.

Receptor	Type	Mechanism
5-HT1	$G_{i/o}$	↓cAMP
5-HT2	$G_{q/11}$	↑IP3 and DAG
5-HT3	Ligand-gated Na^+ and K^+ cation channel	Depolarisation
5-HT4	G_s	↑cAMP
5-HT5	$G_{i/o}$	↓cAMP
5-HT6	G_s	↑cAMP
5-HT7	G_s	↑cAMP

1.6.3 Serotonin Transporter

1.6.3.1 Structure

The serotonin transporter (SERT) is encoded by a single gene located on chromosome 17q11.2. Transcriptional regulation and function of SERT is controlled by a repetitive element of varying length in the promoter region of the gene (Ramamoorthy et al. 1998). The alleles are commonly composed of either 14 short (S) or 16 long (L) repeated elements. The L allele is associated with a two-to threefold higher rate of SERT gene transcription and increased mRNA expression, protein expression and functional activity (Lesch et.al. 1996). Structurally, SERT consists of an intracellular N terminus domain, 12 transmembrane domains (6 extracellular; 5 cytoplasmic loops) and an intracellular C-terminus (Torres et al. 2003) (Figure 1-13).

1.6.3.2 Function

SERT operates as a Na^+-dependent transporter, utilising the Na^+ concentration gradient to facilitate serotonin transport across the membrane (Torres et.al. 2003). Binding of Na^+, Cl^- and serotonin induces a conformational change in the transporter allowing exposure of the serotonin binding site to the opposite side of the membrane and thus transport of substrate and ions. Specifically the aspartic acid residue (D98) situated at transmembrane domain 1 (TMD1) is essential for serotonin recognition (Nelson 1998). Inactivation of SERT is mediated by binding to an intracellular K^+ via an active cysteine residue located on TMD3, which is consequently transported outside the cell. Typically, serotonin is transported into the cell via SERT and this direction of transport is energetically unfavourable (net loss of K^+). To compensate, the Na^+/K^+-ATPase-mediated continuous influx of K+ coupled with the continuous efflux of Na+ acts as an equilibrator to maintain the transmembrane concentration gradient.

SERT activity can also be regulated via pre- and post-translational modifications. Allelic variation is responsible for pre-translational modification leading to altered expression and activity of SERT as discussed earlier. On the other hand, post-translational modifications can be mediated by protein kinase C (PKC), protein phosphatase 2A (PP2A) and p38 (Ramamoorthy et.al. 1998). Multiple serine and threonine phosphorylation sites have been located on the cytoplasmic

domains responsible for SERT expression and trafficking from the cell membrane. Activation of PKC has been shown to phosphorylate SERT in a Ca^{2+}-dependent manner, resulting in internalisation and decreased activity. Conversely, PPA2 maintains higher levels of SERT as its inhibition results in increased SERT membrane sequestration. On the other hand, the mitogen activated protein kinase (MAPK) p38 regulates the activity in a manner distinct from PKC by regulating the delivery of SERT to the membrane and reducing SERT expression (Samuvel et al. 2005). Moreover, it is observed that serotonin can also directly decrease SERT phosphorylation (Ramamoorthy et.al. 1998). This negative feedback mechanism may act to prevent the internalisation of SERT in circumstances where extracellular serotonin levels are enhanced. Together, these studies highlight the importance of SERT regulation on expression and function.

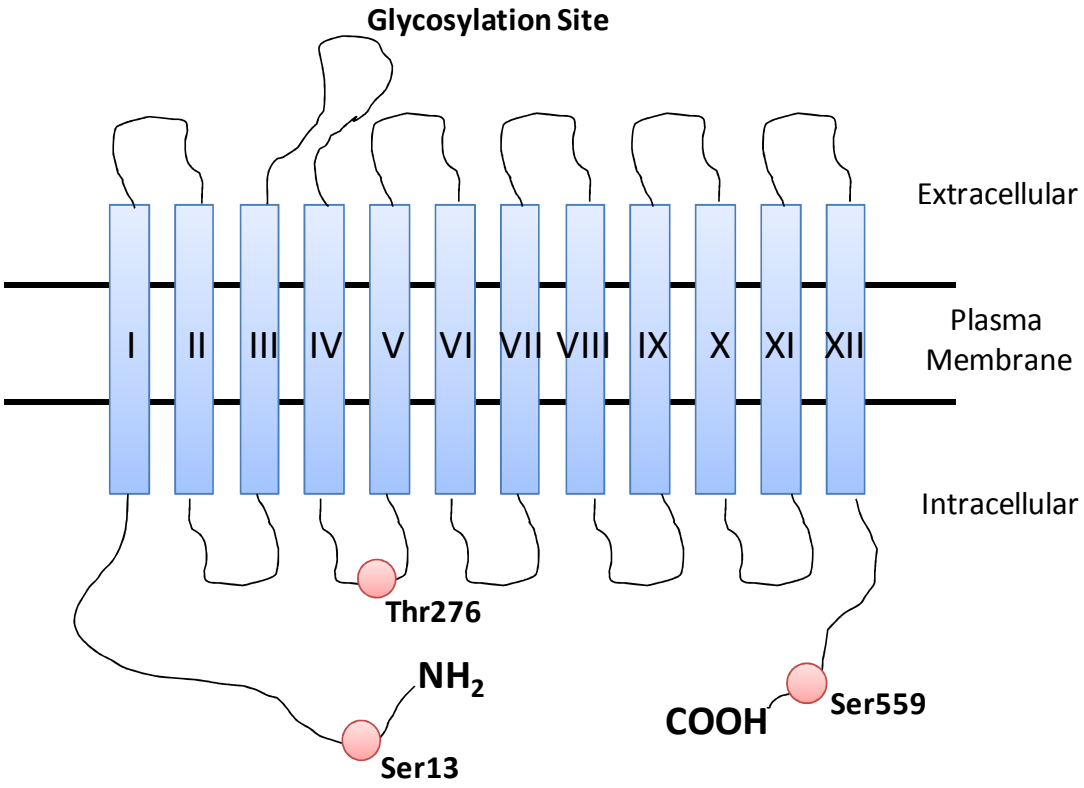


Figure 1-13: Structure of the Serotonin Transporter.
The serotonin transporter exists as an intracellular N-terminus, 12 transmembrane domains and an intracellular C-terminus. The serotonin transporter structure also contains several sites that undergo post-translational modification, shown here in red.

1.7 Serotonin Hypothesis of PAH

The 'serotonin hypothesis' of PAH arose in the 1960s when an 'epidemic' of PAH was reported in women taking the appetite suppressant drug, aminorex. In fact, aminorex was associated with a >30 fold increased incidence of PAH (Abenhaim et al. 1996; Kramer & Lane 1998). In the 1980s, the new generation of anorexigens, the fenfluramines, were also found to be associated with PAH. The anorexigens and fenfluramines were later identified to be indirect serotonergic agonists evoking release of serotonin by acting as SERT substrates (Rothman et al. 1999). Following the observation from a multicentre study that the incidence of PAH had a strong correlation with these appetite suppressant pills, there was a complete withdrawal of all aminorex/fenfluramine-based anorexigens for clinical use in obesity.

Both aminorex and fenfluramine are amphetamine-like drugs. They influence serotonin signalling by interacting with SERT. Inside the cell, these substrates compete with monoamines for vesicular sequestration via the vesicular monoamine transporter (VMAT). Once inside the cell they subsequently disrupt vesicular monoamine storage and promote serotonin release (Rothman et.al. 1999). Often the fenfluramines were co-administered with phentermine ('fen-phen') which is also a SERT substrate and MAO inhibitor. Combined, these treatments potentiated accumulation of plasma serotonin via increased serotonin release and decreased metabolism. These observations formed the basis of the 'serotonin hypothesis'.

The 'serotonin hypothesis' was consistent with reports based on elevated circulating levels of serotonin in PAH patients and individuals with platelet storage disorders who also appear susceptible to PAH (Herve et al. 1990; Herve et.al. 1995). However, controversy in the 'serotonin hypothesis' was highlighted following the observation that serotonin levels were not necessarily elevated with fenfluramine treatment. The 'fen-phen' combination was associated with decreases in plasma serotonin levels in humans (Rothman & Baumann 2002) and patients receiving fenfluramines had plasma serotonin levels within the normal physiological range (Kawut et al. 2006). This converging evidence suggests that elevated plasma serotonin levels are not essential in the development of anorexigen-induced PAH. Perhaps serotonin concentrations in local

microenvironments surrounding PASMCs and PAECs may be more indicative of susceptibility to anorexigen-induced PAH.

Experimentally the 'serotonin hypothesis' has been supported. It has been shown previously that dexfenfluramine-induced PAH is evident in wildtype mice although this effect is ablated in *Tph1*$^{-/-}$ mice. This definitively shows that dexfenfluramine mediates PAH via a peripheral serotonergic mechanism (Dempsie & MacLean 2008). Dexfenfluramine also promotes development of PAH in rats by potentiating acute hypoxic pulmonary vasoconstriction (Eddahibi et al. 1998). However, the exact role of the fenfluramines in PAH remains obscure as dexfenfluramine has also been shown to protect against the development of both hypoxia- and monocrotaline-induced PAH (Rochefort et al. 2006).

Non-serotonergic mechanisms have also been reported for fenfluramines in PAH. For example, the fenfluramine, dexfenfluramine, has been reported to directly inhibit K^+ channels (Weir et al. 1996) and increase intracellular Ca^{2+} (Reeve et al. 1999) which act to promote vasoconstriction and proliferation. In addition, metabolism of dexfenfluramine produces the metabolite nordexfenfluramine which exhibits agonistic activity at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors (Rothman & Baumann 2002). The roles of the 5-HT_{2A} and 5-HT_{2B} receptors in PAH were described previously.

1.8 Serotonin Transporter in PAH

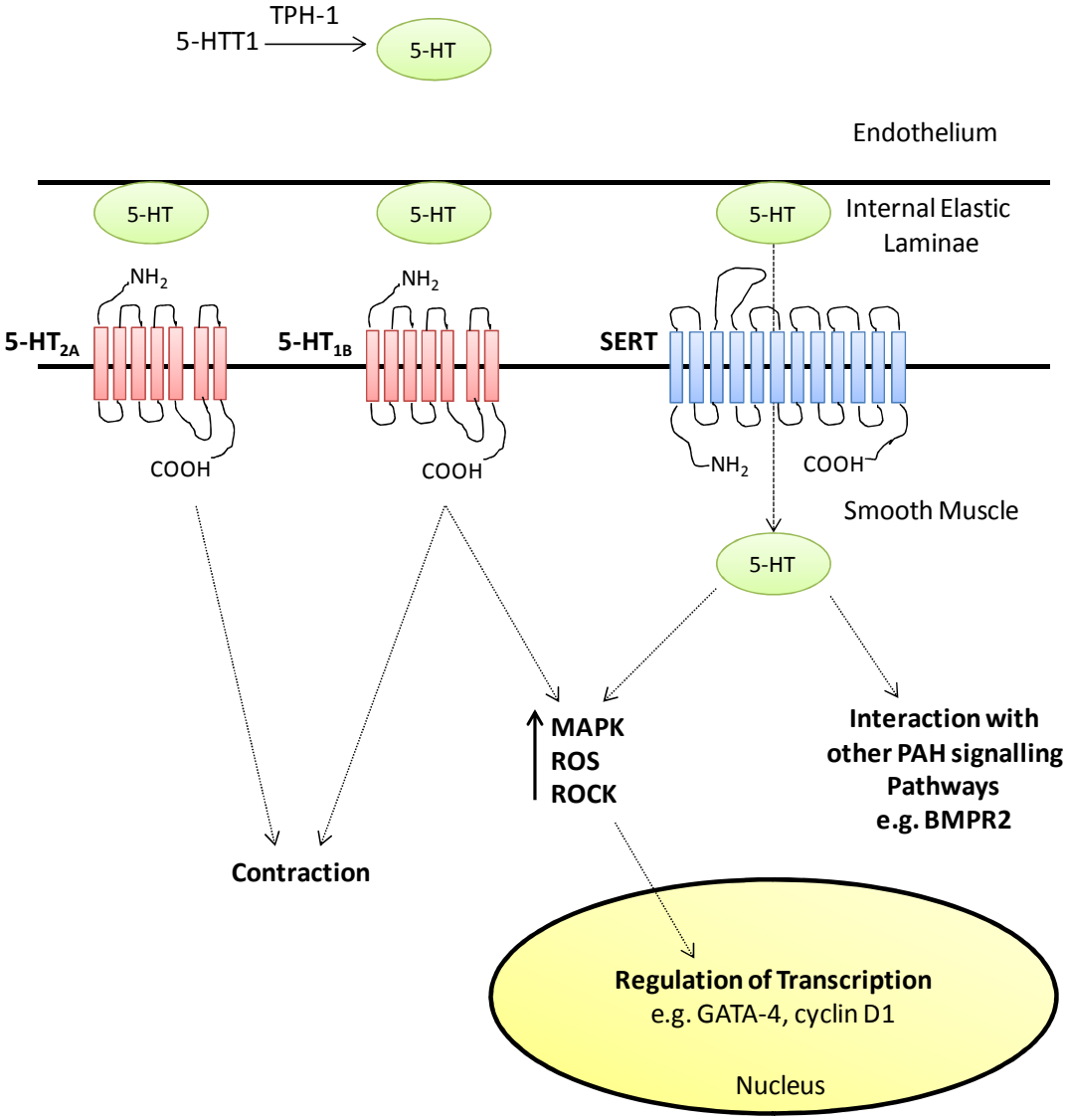
The SERT is particularly highly expressed in both platelets and lungs. Serotonin mediates its effects by binding to cell surface serotonin receptors, which initiate intracellular signalling, or it can be transported into the cell via SERT (Figure 1-14). In both experimental and human PAH, SERT is proposed to play a key role in disease pathogenesis. There is evidence suggesting a link between SERT polymorphisms with the development of PAH. In one report, the L allele variant was found to be more prevalent in the homozygous form in 65% of IPAH patients associated with increased expression and activity of SERT, compared to 27% in controls (Eddahibi et.al. 2001). Additionally, the LL genotype is observed in 56% of patients with chronic obstructive pulmonary disease associated with more severe PAH (Eddahibi et al. 2003). Patients with this polymorphism appear to have an earlier age of diagnosis and/or shorter survival rate in IPAH and HPAH

compared to those with an S-allelic polymorphism. Interestingly in HPAH, an interaction between SERT polymorphisms and BMPR2 mutations is also proposed determining susceptibility to PAH (Willers et al. 2006). Ubiquitous SERT over-expression and/or activity are observed in the pulmonary arteries and lungs from patients with both IPAH and HPAH, and secondary PAH (Eddahibi et.al. 2001). Furthermore, PASMCs derived from IPAH patients exhibit an exaggerated proliferative phenotype in response to serotonin or serum dependent on SERT activity (Eddahibi et.al. 2006). Serotonin-induced proliferation in PASMCs involves SERT-dependent generation of reactive oxygen species (ROS), activation of the extra-cellular regulated kinase (ERK) and the RhoA/ROCK pathway (Liu et.al. 2004; MacLean & Dempsie 2010). Once inside the nucleus, pERK1/2 can further phosphorylate mitogenic transcription factors such as GATA-4 and cyclin D1. The serotonin-induced proliferation in PAFs is also mediated via SERT, as citalopram can successfully block these effects (Welsh et.al. 2004). Hence, evidence suggests that internalisation of serotonin through SERT is essential for the mitogenic properties of serotonin in PASMCs and PAFs. On the other hand, SERT expression is low in PAECs and is believed not to be involved in the formation of plexiform lesions observed in severe/end-stage PAH (Eddahibi et.al. 2001).

In experimental PAH an increase in SERT mRNA levels has also been observed in lungs from rats exposed to chronic hypoxia localised to newly remodelled distal pulmonary arteries. Basal SERT expression was absent suggesting that *in vivo*, large pulmonary arteries do not phenotypically express SERT under normoxic conditions and that induction occurs in response to hypoxia (Eddahibi et.al. 1999). Indeed, mice over-expressing SERT ($SERT^+$ mice) exhibit increased RVSP and pulmonary vascular remodelling, and also develop exaggerated hypoxia-induced PAH (MacLean et.al. 2004). Interestingly, it was later identified that only $SERT^+$ female mice develop an exaggerated PAH phenotype implicating a gender susceptibility dependent on circulating serotonin. Indeed, 17β-estradiol, the main circulating female hormone, increased expression of SERT in PASMCs (White et al. 2011). Similarly, mice with targeted SERT over expression in PASMCs develop PAH (Guignabert et.al. 2006). Pulmonary arterial remodelling in $SERT^+$ mice is associated with elevated RhoA/ROCK signalling and ROCK inhibition ablates PAH in SERT+ mice (Mair et al. 2008). Conversely, mice devoid

of the SERT gene are less susceptible to the development of hypoxia-induced PAH (Eddahibi et.al. 2000). In addition, the SERT inhibitor citalopram protect against hypoxia-induced PAH in mice (Morecroft et.al. 2010) and fluoxetine attenuates monocrotaline-induced PAH in rats (Guignabert et al. 2005). Monocrotaline-induced PAH in rats is associated with an up-regulation of SERT, and the protective effects of statins described in monocrotaline-induced PAH is dependent on down-regulation of SERT (Laudi et al. 2007).

Continually building evidence supports the 'serotonin hypothesis' of PAH. Substantial evidence exists implicating serotonin signalling, particularly SERT and 5-HT receptors, in the vascular remodelling process and pulmonary arterial vasoconstriction. Targeting heightened serotonin signalling offers new pulmonary-specific therapeutic targets for PAH. Certainly, a combined approach targeting dual inhibition of the 5-HT_{1B} receptor and SERT would provide a novel and effective treatment for PAH.



1-14: Serotonin signalling in the pulmonary vasculature.
Serotonin is synthesised in the endothelium by tryptophan hydroxylase (TPH-1). Serotonin is then released and mediates effects on underlying smooth muscle via 5-HT receptors and the serotonin transporter (SERT). Specifically stimulation of 5-HT_{1B} and 5-HT_{2A} initiates smooth muscle contraction. 5-HT_{1B} can also stimulate proliferation.

1.9 Estrogen

1.9.1 Synthesis and Metabolism

The female sex hormones are steroid hormones which comprise both estrogens and progesterones. The primary source and synthesis of these hormones occurs in the ovarian follicles and the corpus luteum in the female reproductive tract and play a key role in development of secondary sex characteristics. To a lesser extent, estrogen synthesis can also take place in liver and adipose tissue and expression of estrogen-synthesising enzymes in vascular smooth muscle and endothelial cells (Tofovic 2010) implicates the importance of 'local' estrogen synthesis and autocrine/paracrine effects of estrogen in the periphery and cardiovascular tissues (Simpson & Davis 2001). The synthesis of estrogens is controlled by the hypothalamic-pituitary-gonadal axis (HPG axis) which involves various endocrine hormones secreted from the hypothalamus and pituitary gland of the brain. In particular, luteinising hormone (LH) released from the pituitary stimulates synthesis of estrogen, and follicle stimulating hormone (FSH) increases transcription of aromatase, the enzyme specific to estrogen synthesis. The continuous modulation of this group of hormones in females by positive-and negative-feedback forms the basis of the reproductive cycle. Estrogens exist as three major naturally occurring isoforms: estrone (E1), estradiol (E2) and estriol (E3). Estradiol is the predominant circulating hormone in pre-menopausal women, whilst estrone is important during the menopause and estriol during pregnancy.

The biosynthesis of estrogen is initiated by the synthesis of androstenedione from the precursor cholesterol (Figure 1-15). Androstenedione provides an intermediate stage in metabolism from which estradiol can be synthesised by two distinct pathways, either immediately or through testosterone. The cytochrome P450 enzyme CYP19A1, or aromatase, converts androstenedione to estrone, which in turn is converted to estradiol by 17β hydroxysteroid dehydrogenase –type 1 (17βHSD-1). Alternatively, reduction of androstenedione to testosterone, which requires a second 17βHSD isoform (17βHSD-2) occurs followed by aromatisation of testosterone to estradiol. The molecular biology and steroidogenesis of estrogen is well described (Payne & Hales 2004).

Estradiol is then rapidly metabolised by oxidation (Figure 1-14). Oxidative metabolism occurs primarily in the liver producing hormonally non-estrogenic metabolites ready for elimination from the body. Estradiol and estrone are in equilibrium with 17ßHSD-1, and oxidation/reduction occurs at the carbon-17 (C17) position and favours formation of estrone. Further metabolism of estradiol occurs, for example, at the C16, C4 and C2 positions producing biologically active metabolites. Several members of the cytochrome P450 (CYP450) family are essential in mediating this nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidative metabolism of estradiol. Specifically CYP1A1, CYP1A2 CYP1B1, and CYP3A4 appear critical in the formation of 2- and 4-hydroxy-derivatives. The 2-hydroxylation pathway is the major metabolic pathway in the liver whereas 4-hydroxylation constitutes a relatively minor pathway in estrogen metabolism. The 2-and 4-hydroxy-derivatives are then further converted to 2- and 4-methoxy metabolites by the catechol-O-methyltransferase (COMT) enzyme. Elimination of these metabolites can occur at either the hydroxyl- or methoxy-metabolite stage if they undergo sulphation by sulphotransferases (SULTs) to hormonally inactive water-soluble metabolites and subsequent excretion by the kidneys via urine. There are multiple pathways via which estradiol can be metabolised (Zhu & Conney 1998).

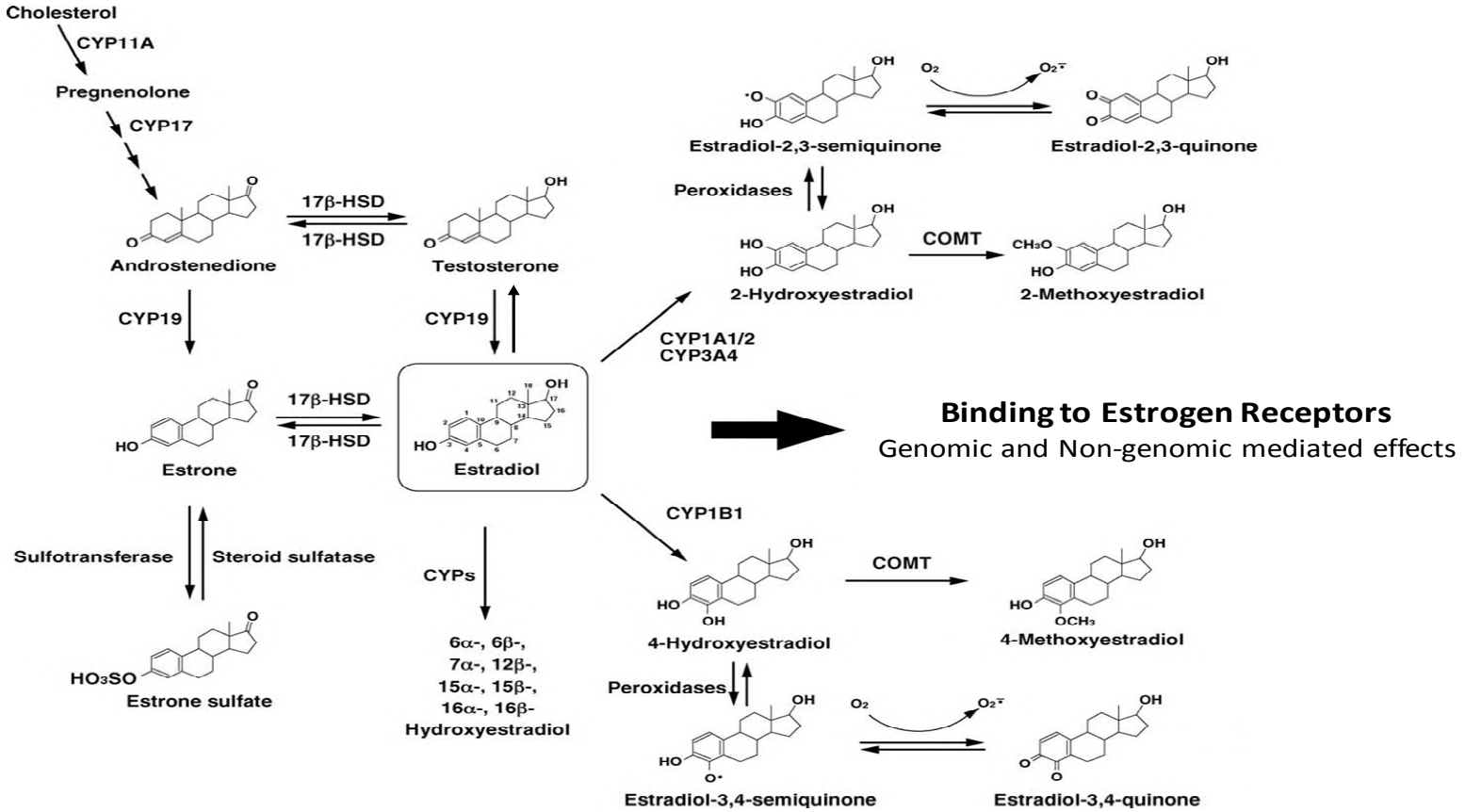


Figure 1-15: Estrogen biosynthesis and metabolism.
Androstenedione is derived from the precursor cholesterol. Estradiol is synthesised immediately by aromatase (CYP19A1) to estrone (E1) or through testosterone by 17β hydroxysteroid dehydrogenase (17βHSD-1). Further metabolism of estradiol by cytochrome P450 enzymes (CYP450) CYP1A1, CYP1A2, CYP3A4 and CYP1B1 produces 2-and 4-hydroxy-metabolites. Subsequently these are metabolised to 2- and 4-methoxy-metabolites by cathecol-O-methyltransferase (COMT). Adapted from (Tsuchiya et al. 2005).

1.9.2 **Estrogen Effects in the Systemic Circulation**

Estrogen exerts diverse cardiovascular effects in both males and females. There is a wealth of evidence implicating cardio-protective and vaso-protective effects of estrogen in cardiovascular disease. Indeed, the incidence of cardiovascular disease is higher in men than that of age-matched pre-menopausal women, although these gender differences narrow after the menopause when the protection against cardiovascular disease is lost (Meyer et al. 2006). A strong link between estrogen levels and hormone replacement therapy and cardioprotection is proposed (Rosano et al. 2003).

Estrogen intimately regulates fundamental cardiovascular functions, including blood pressure, blood flow, vasodilation and vasoconstriction, vascular inflammation and remodelling atherosclerosis through diverse effects on various components of the vascular wall, such as endothelial and smooth muscle cells. Both acute and long term vasodilator effects of estrogen are mediated in part via generation of endothelium-derived NO and are attenuated by NO synthase (NOS) inhibitors (Caulin-Glaser et al. 1997). Additionally, estrogen treatment can attenuate the vasoconstrictive properties of many mediators including phenylephrine and 5-HT in aorta and coronary arteries and decreases sympathetic nervous system –induced vasoconstriction (Dubey et al. 2004).

Experimental studies also indicate that estrogen can protect blood vessels from atherosclerosis lesion formation in apolipoprotein E (ApoE) deficient mice ($ApoE^{-/-}$) (Bourassa et al. 1996), lower plasma levels of low density lipoprotein cholesterol (LDL) and raise levels of high density lipoprotein cholesterol (HDL) (Soma et al. 1993; White 2002). Estrogen also appears to have a blood pressure lowering effect evidenced by changes in blood pressure throughout the menstrual cycle (Dunne et al. 1991) and during pregnancy (Siamopoulos et al. 1996).

Endothelial progenitor cells (EPCs) derived from bone marrow cells, are actively involved in cardiovascular homeostasis. EPCs repair endothelial damage and function and promote angiogenesis (Mayr et al. 2011). In fertile women, EPC populations are higher than in men; however, their numbers are subject to fluctuations with the hormonal cycle and fall after the menopause (Fadini et al.

2008). The number and function of EPCs may therefore reflect the degree of cardiovascular protection in females and explain the lower prevalence of cardiovascular disease in premenopausal women.

1.9.3 Estrogen Metabolite Effects in the Systemic Circulation

Catcholestradiols and methoxyestradiols also exert cardio-protective effects in the cardiovascular system. The effects of these metabolites are unlikely to involve estrogen receptors however, as catecholestradiols have approximately one-fourth the binding affinity of estradiol, and methoxyestradiols do not significantly bind to ERs (Dubey et al. 2000). Estrogen metabolites exert effects on endothelial and vascular smooth muscle cells. 2-methoxyestradiol, 2-hydroxyestradiol and 4-methoxyestradiol all inhibit migration, proliferation and collagen synthesis in human and rodent vascular smooth muscle cells. The mechanism of this inhibition involves blocking free-radical production as both 2-hydroxyestradiol and 2-methoxyestradiol are potent anti-oxidants (Seeger et al. 1997). In endothelial cells, 2-hydroxyestradiol and 2-methoxyestradiol stimulate the generation of the potent vasodilator prostacyclin and NO (Seeger et al. 1999). *In vivo*, 2-methoxyestradiol has been shown to attenuate both renal and cardiovascular injury by reducing blood pressure and cardiac hypertrophy associated with chronic NOS inhibition (Tofovic et al. 2005a).

1.9.4 Estrogen Effects in the Pulmonary Circulation

There is a striking female predominance reported in idiopathic and familial forms of PAH with females up to four times as likely to present with disease as males (Badesch et.al. 2010; Humbert et.al. 2006). *Endogenous* and *exogenous* sex hormones represent a biologically relevant potential risk factor, and in particular estrogens are implicated in PAH pathogenesis. For example, polymorphisms in aromatase, the estrogen synthesising enzyme, and ESR1/ERα, are associated with elevated estrogen levels in the lungs of female patients and predispose to portopulmonary hypertension (Roberts et al. 2009a; White et al. 2012a). Additionally, altered estrogen metabolism by CYP1B1 is implicated in idiopathic and heritable PAH (White et.al. 2012a) and CYP1B1 represents a modifier gene that drives susceptibility in females heritable PAH patients harbouring a BMPR2 mutation (Austin et.al. 2009; West et al. 2008).

Despite the well observed female susceptibility in clinical PAH, this is not translated to the currently utilised animal models of PH. In fact, males exhibit more severe monocrotaline- and hypoxia-induced PH compared to females (Rabinovitch et al. 1981) and estrogen exerts protective effects on pulmonary vasculature and improves right ventricular contractility (Giuberti et al. 2007; Lahm et al. 2012a; Resta et al. 2001). This is defined as the 'estrogen paradox' whereby the higher incidence and prevalence of PAH in females in the clinic is not represented by the animal models most commonly used to study the disease. For this reason, the observed gender bias in human PAH remains unclear.

Estrogen mediated protection in PH appears to involve several mechanisms. Estrogen is reported to inhibit induction of endothelin-1, a potent mitogen and vasoconstrictor in pulmonary arteries, following chronic hypoxia (Earley & Resta 2002), an effect which is absent in ovariectomised rats. Estrogen may also increase vascular NO production (Gonzales et al. 2001) and prostacyclin (Sherman et al. 2002). Together these effects of estrogen promote pulmonary vasodilation. This effect was confirmed in pulmonary arteries from proestrous females, characterised by physiologically high estrogen levels, whereby the vasoconstrictor response to vasoactive agents such as KCl and phenylephrine, and hypoxia was attenuated (Lahm et al. 2007). Additionally, estrogen is suggested to have inhibitory effects of Ca^{2+}-dependent mechanisms of smooth muscle cells contraction (Murphy & Khalil 2000), protein kinase C (Kanashiro & Khalil 2001) and Rho-kinase (Shimokawa & Takeshita 2005). The latter may also have important implications for inhibiting smooth muscle cell proliferation and migration.

Increasing evidence from experimental animal studies implicates a protective role of estrogen in PH. *In vivo*, estrogen ameliorates monocrotaline induced-PH in mechanisms dependent on NO and prostacyclin stimulation and a decrease in signalling through the endothelin-1 pathway (Yuan et al. 2013). Estrogen also appears important in attenuation of hypoxia induced-PH in male rats by reducing pulmonary vascular remodelling (Lahm et.al. 2012a). In the heart, estrogen also mediates RV cardioprotective effects. Estrogen induces RV angiogenesis and reverses right ventricular hypertrophy (Nadadur et al. 2012; Umar et al. 2011a), and importantly restores RV contractility and function by enhancing right ventricular ejection fraction and cardiac output (Lahm et.al. 2007; Matori et al.

2012; Umar et.al. 2011a). Since RV function is the most important prognostic factor in determining survival in PAH and women often have improved survival rates, it may not be surprising that estrogen exerts beneficial effects in the RV. Certainly, there is a correlation between estrogen levels and RV function described in humans (Ventetuolo et al. 2011).

The absence of a suitable model in which to study the female predisposition to PAH however, has limited research into the role of estrogen in PAH pathogenesis. In recent models of pulmonary hypertension dependent on serotonin, estrogen has been identified as a risk factor in pulmonary arteries. In SERT over-expressing mice ($SERT^+$), S100A4/Mts1 over-expressing mice and in dexfenfluramine-induced PH a female susceptibility is described dependent on circulating endogenous estrogen (Dempsie et.al. 2011; Dempsie et al. 2013; White et.al. 2011). In particular, estrogen was shown to increase expression of tryptophan hydroxylase -1 (TPH1), the enzyme responsible for 5-HT synthesis, and $5\text{-}HT_{1B}$ in pulmonary artery smooth muscle cells (White et.al. 2011) suggesting estrogen may stimulate a mitogenic environment in smooth muscle cells in females. The complexity of the 'estrogen paradox' suggests that multiple pathways must be involved in generating the female predisposition. For example, although in IPAH and HPAH women are over represented, estrogen appears protective in development of PAH in high-altitude natives distinct from IPAH and HPAH (Scherrer et al. 2006) and postmenopausal women with systemic sclerosis are at increased risk for development of PH (Scorza et al. 2002). The interest in addressing the gender bias in PAH is continually expanding.

1.9.5 Estrogen Metabolite Effects in the Pulmonary Circulation

Evidence also suggests that metabolites of estrogen also play an important role in pulmonary vasculature and PAH. In general it is regarded that metabolites of the 16-hydroxylation and 4-hydroxylation pathways have proinflammatory, mitogenic and angiogenic properties, whilst the 2-hyroxylation pathway produces estrogen metabolites with anti-inflammatory, antiproliferative and antiangiogenic properties (Dubey et.al. 2004) (Figure 1-16). Like estrogen, the majority of studies implicate a protective role of non-estrogenic hydroxyestradiols and methoxyestradiols metabolites in the pulmonary circulation.

In monocrotaline and hypoxia-induced PH, 2-hydroxyestradiol and 2-methoxyestradiol have been shown to attenuate pulmonary vascular and cardiac remodelling, as well as reducing mortality (Tofovic et al. 2005b; Tofovic et al. 2005c; Zhang et al. 2007). Pharmacological concentrations of 2-methoxyestradiol also inhibits serum induced proliferation in pulmonary artery endothelial cells, smooth muscles cells and fibroblasts (Tofovic et al. 2008). A recent study suggests reduced activity of the 2-hydroxylation pathway and reduced 2-hydroxyestradiol to 16α-hydroxyestrone ratios in urine in women with familial PAH (Austin et.al. 2009). In line with this, administration of exogenous 16α-hydroxyestrone to female mice induces PH (White et al. 2012). A shift toward the 16-hydroxylation pathway may therefore be pathogenic in PAH in females. Moreover, increased expression of CYP1B1, the major enzyme in the 4-hydroxylation pathway is increased in IPAH and HPAH (White et al. 2012). This may also have important implications in PAH development in females by increasing potentially mitogenic and inflammatory metabolites although the 4-hydroxylation pathway has not been investigated in experimental PH.

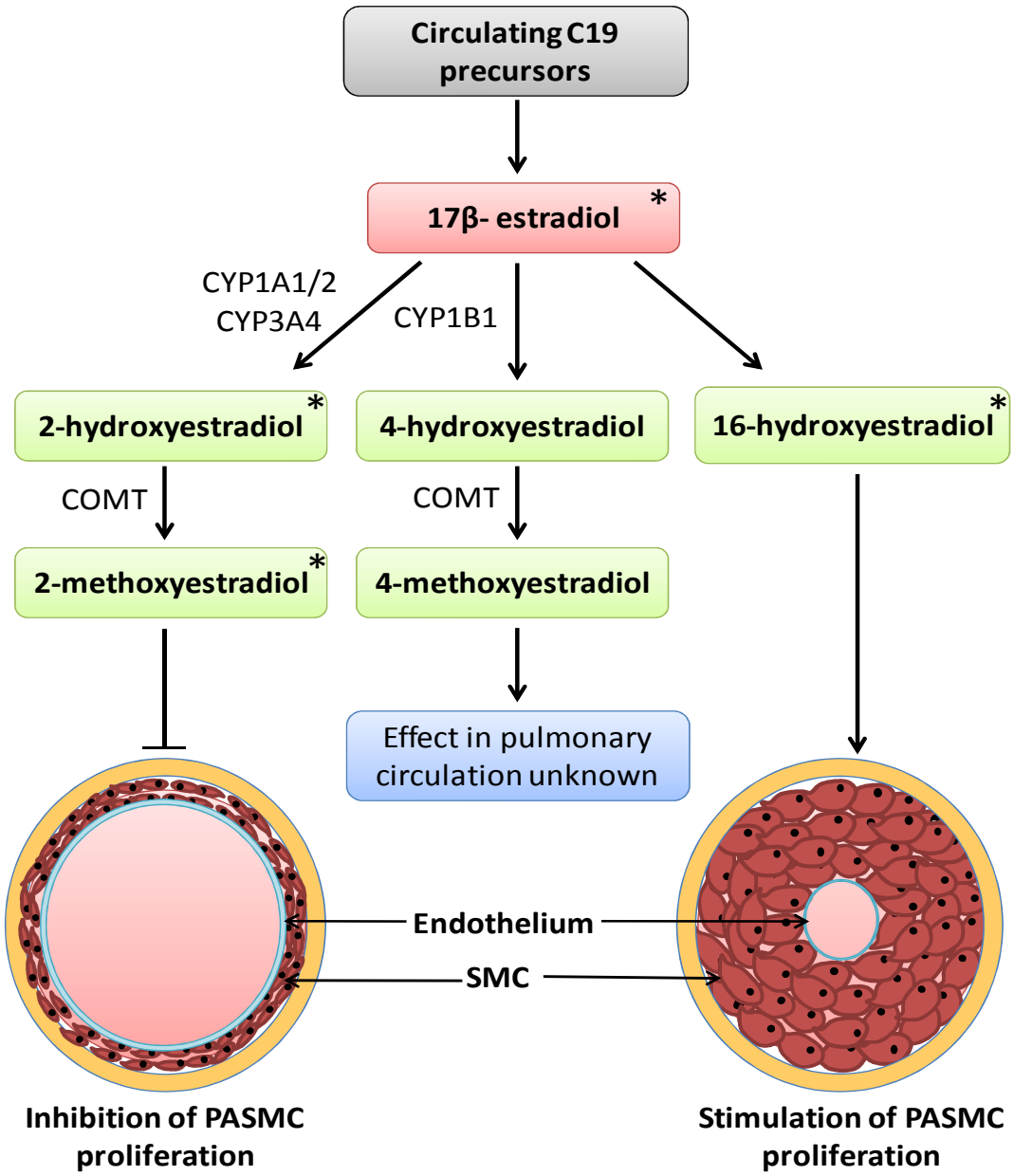


Figure 1-16: Effects of estrogen metabolites in pulmonary arteries.
17β-estradiol is metabolised by cytochrome P450 (CYP) enzymes to produce 2- and 4-hydroxyestradiol metabolites. Further metabolism by catechol-O-methyltransferase (COMT) produces 2- and 4-methoxyestradiols, respectively. Both 2-hydroxy and 2-methoxyestradiol have been shown to inhibit pulmonary artery smooth muscle cell (PASMC) proliferation, whilst 16α-hydroxyestradiol stimulates proliferation. The effect of 4-hydroxylation is on PASMC is unknown. (* studied in experimental PH). Adapted from (Mair et al, 2013).

1.10 Estrogen Receptors

The impact of estrogen physiology in reproductive functions and in wider roles including modulation of inflammation, brain and behaviour, and cardiovascular functions, is achieved through activation of cellular hormone-specific estrogen receptors (ERs). Two distinct types of signalling are described for estrogen: non-rapid 'genomic' signalling and rapid 'non-genomic' signalling (Mendelsohn 2002). In the genomic pathway, estrogens bind to intracellular ERs inducing a conformational change and results in direct regulation of gene transcription. In contrast, non-genomic signalling occurs rapidly through the activation of second messengers and signal transduction. Recent identification of ERs located on the plasma membrane may also be accountable for rapid non-genomic estrogen signalling (Aronica et al. 1994; Pietras & Szego 1977). Therefore activation of both intracellular and/or membrane receptors mediate physiological estrogen responses.

1.10.1 Classical Estrogen Receptors: Subtypes and Structure

The classical ERs belong to the steroid/thyroid superfamily of nuclear receptor transcription factors. Until 1995 it was assumed there was only one ER responsible for mediating all of the physiological and pharmacological effects of natural and synthetic estrogens. This receptor is now referred to as ERα following the cloning of the second ER, ERβ from various species (Enmark et al. 1997; Mosselman et al. 1996; Tchoudakova et al. 1999; Todo et al. 1996; Tremblay et al. 1997). ERα and ERβ are the products of separate genes, ESR1 and ESR2 on chromosome 6q25.1 and chromosome 14q23.2, respectively (Enmark et.al. 1997; Menasce et al. 1993). For ERα, at least two splice variants are recognised (ERα-36 and ERα46), whereas four splice variants exist for ERβ (ERβ2, -4, -5, and Δexon5). They are composed of three independent but interacting functional domains: the NH_2-terminal or A/B domain, the C domain or DNA-binding domain (DBD), and the D/E/F or ligand-binding domain (LBD) (Figure 1-17). The DBD is involved in DNA recognition and binding and is the most evolutionary conserved domain in ERs. It contains two zinc fingers which play a crucial role in receptor dimerisation and in binding of receptors to specific estrogen receptor response elements (EREs) in target genes.

On the other hand, the LBD of the NH_2-terminal is not highly conserved, and represents the most variable domain in both sequence and length. LBD mediates ligand binding, receptor dimerisation, and transactivation of target gene expression by binding estrogen as well as other estrogenic ligands. Transcriptional activation is facilitated by two distinct activation functions (AF), AF-1 and AF-2 in the LBD. AF-1 is constitutively active independent of the presence of a ligand but provides a more robust up-regulation of gene transcription in synergy with AF-2 (Kushner et al. 2000). Both ER isoforms show a degree of selectivity with respect to AF regions: ERα predominantly mediates transcriptional activation via AF-1 whilst ERß appears to regulate transcription via AF-2 function. Interestingly, comparison of the AF-1 domains in the two ERs has identified that ERα and ERß often have opposing actions under the same conditions in the same cells lines (Nilsson et al. 2001).

1.10.2 Classical Estrogen Receptors: Activation and Mechanism of Action

Binding of estrogens and/or pharmacological agents to the ERs induces conformational changes in the receptor and this promotes changes in the rate of transcription of estrogen regulated genes. AF-1 and AF-2 must interact with co-regulator complexes (either co-activators or co-repressors) to activate or suppress gene expression, although there are far fewer nuclear receptor co-repressors. Briefly, four major signalling mechanisms of classical ERs have been described (Figure 1-18). In classical genomic signalling, ERs must cooperate as dimers in order to translocate from the cytosol to the nucleus. Following receptor dimerisation in the cytosol upon ligand binding, activation involves dissociation from chaperone proteins followed by the direct activation of EREs in the promoters of target genes in the nucleus. As a consequence of gene regulation, protein translation is affected resulting in alterations in cell function. Alternatively, interaction with other transcription factor complexes lacking ERE-sequences, for example Fos/Jun complexes (Kushner et al. 2000) occurs in the tethered pathway via the ER dimer complex and by utilising activator-protein-1 (AP-1). Another alternative mechanism of ER action involves ligand-independent activation through other signalling pathways, for example growth factor signalling. In this instance, growth factor signalling leads to

activation of kinases that may phosphorlyate and thereby initiate dimerisation of ERs and regulation of target genes.

In addition to the classical genomic pathway of estrogen involving slow transcriptional effects, there are well depicted rapid effects occurring within seconds or minutes of estrogen addition. Although these rapid effects have been extensively studied it is still unclear whether or not classical membrane-bound ERs are involved or if there is a distinct membrane associated receptor. For example, these effects may be attributed to the third estrogen receptor, GPR30/GPER, which is a G-protein coupled receptor (Carmeci et al. 1997; Filardo et al. 2000; Moriarty et al. 2006).

1.10.3 The Novel G Protein-Coupled Receptor, GPR30/GPER: Structure and Mechanism of Action

Both genomic and rapid signalling events initiated by estrogen have solely been attributed to membrane bound classical ERs, ERα and ERβ. However, in the last few years, the discovery of a 7-transmembrane G protein-coupled receptor, GPR30/GPER, has been attributed to both rapid and transcriptional events in response to estrogen (Filardo & Thomas 2005; Nilsson et.al. 2001; Prossnitz et al. 2008). GPER was identified in multiple organs, and in ER-negative breast cancer cell lines (Carmeci et al. 1997) and as such has suggested a possible link to physiologic responses in estrogen-responsive tissues and cancers. The functional role of GPER involves the rapid activation of mitogen activated protein kinases (MAPK) (Filardo et al. 2000), PI3K activation and calcium mobilisation (Bologa et al. 2006) and hence rapid responses in target organs. For GPER-mediated transcriptional activation, GPER signalling requires epidermal growth factor receptor (EGFR) and occurs through rapid ERK1/2 phosphorylation in triggering the genomic response to estrogen leading to stimulation of *c-fos* and cyclin D1 expression (Maggiolini et al. 2004). Rapid signalling events mediated by GPER leading to transcriptional activation and ligand-dependent activation in the genomic model of ER activity may work in concert to generate estrogen mediated alterations in gene expression.

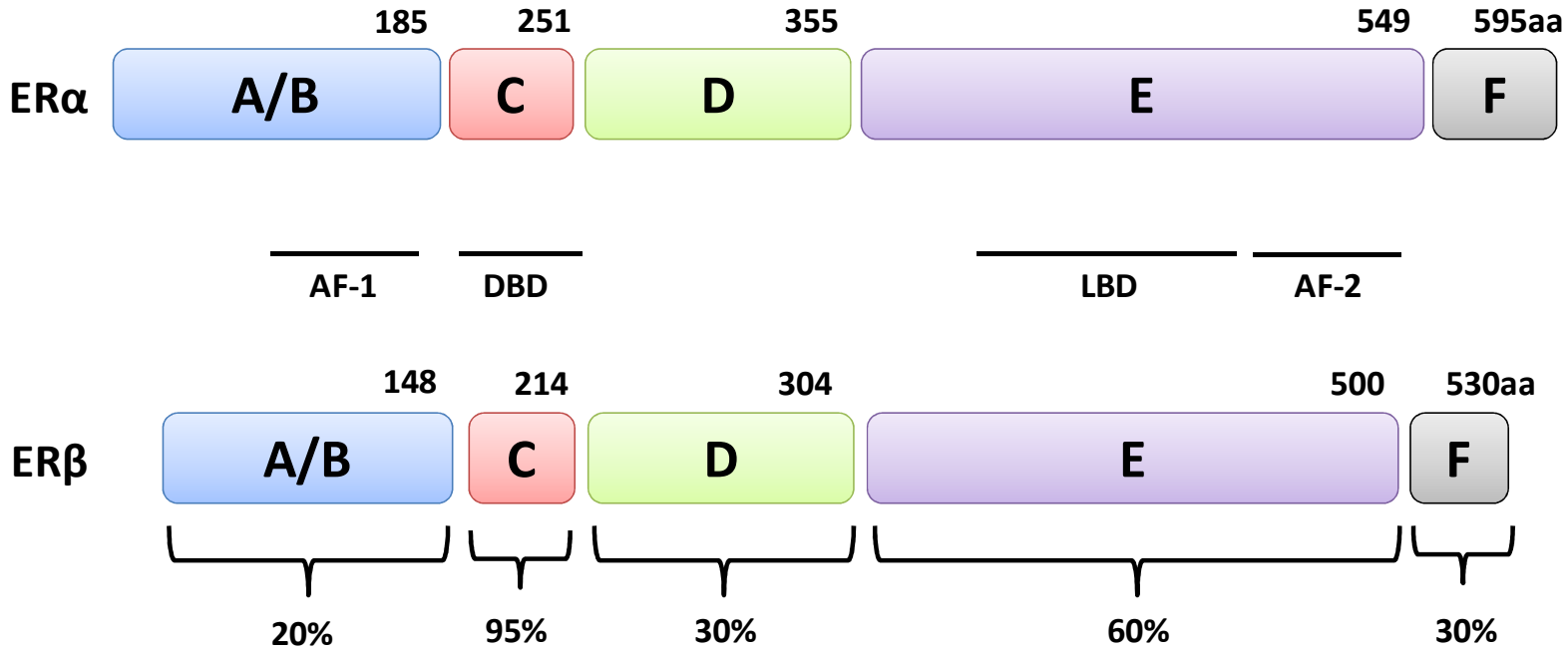


Figure 1-17: Schematic representation of estrogen receptor subtypes ERα and ERβ.
The domains of the receptor include the DNA-binding domain (DBD), the ligand-binding domain (LBD) and two transcriptional activator functions (AF-1 and AF-2). Percentage sequence homology between ERs is indicated in ERβ. The central and most conserved domain is the DBD.

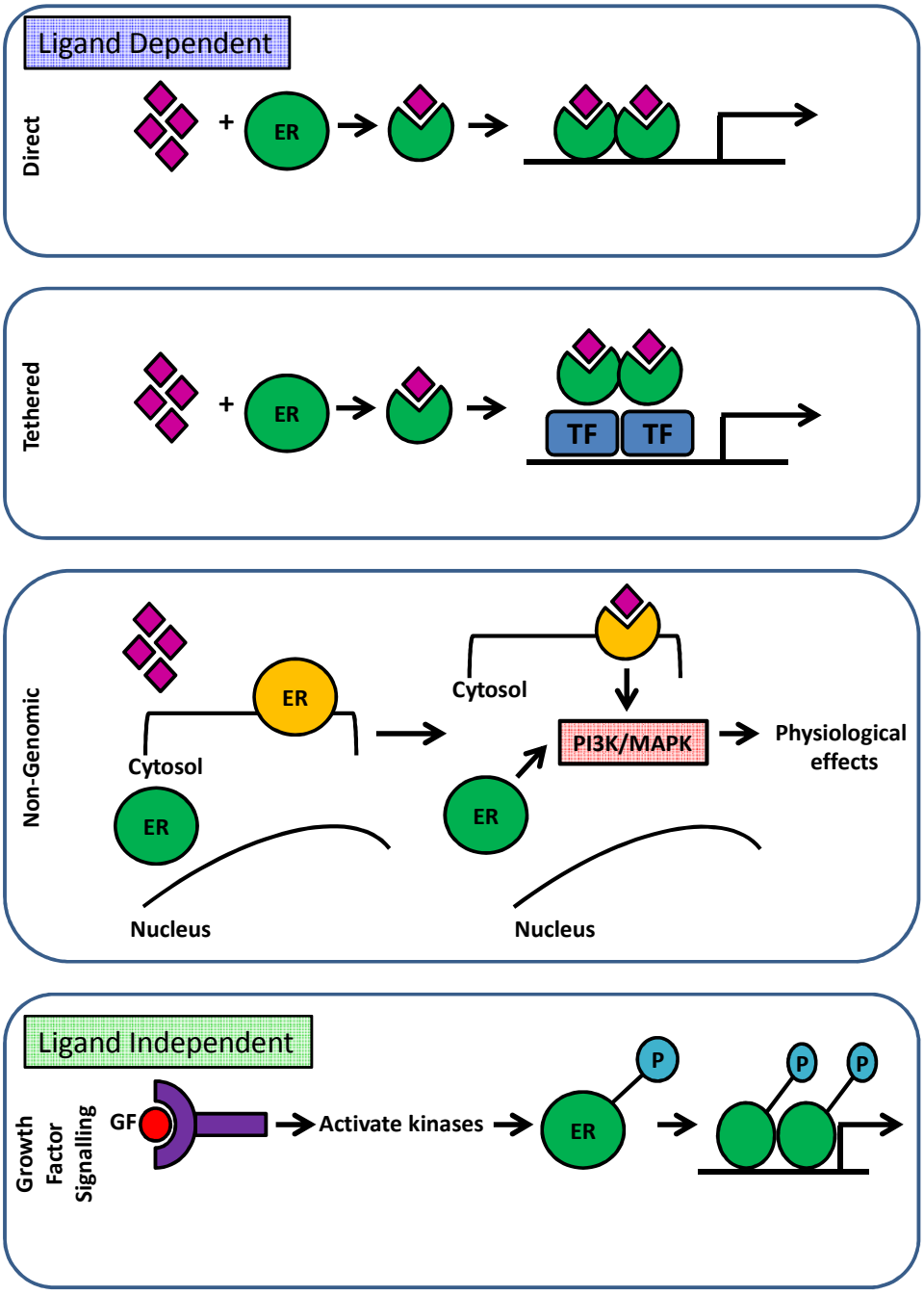


Figure 1-18: Schematic representing the possible mechanisms of action utilised by ERs. ER- estrogen receptor; TF, transcription factor; PI3K, phosphoinositide-3-kinase; MAPK, mitogen-activated protein kinase; GF, growth factor; P, phosphorylation. Adapted from (Heldring et al. 2007a)..

1.11 Estrogen Receptors in Disease

Estrogens influence many physiological processes in mammals. As well as a fundamental role in reproduction, estrogens are known to be involved in cardiovascular and respiratory health, bone integrity, immune responses and cognition and behaviour. For example, women show a significant increase in blood pressure following the onset of menopause (Dubey et al. 2002). It is therefore not surprising that ERs have a wide tissue distribution and also differ in their distribution patterns (Nilsson et.al. 2001). Given this widespread role of estrogen, pathophysiological implications are evident and a divergent role between genders in many diseases is plausible.

1.11.1 Estrogen Receptors in Cancer

Estrogen plays a central role in the development of breast cancer and cancers in other estrogen sensitive tissues such as the endometrium and ovaries (Zhu et al. 2012). ER positive breast cancer is in fact accountable for almost 75% of all breast cancers. ERα is considered pro-proliferative in certain tissues and cancers whereas ERß is more widely regarded as possessing anti-proliferative properties (Paruthiyil et al. 2004). In particular, ERα status is the most important predictor of breast cancer prognosis (Burns & Korach 2012) and several sequence variations or single nucleotide polymorphisms (SNPs) in ESR1 are associated with either an increased or a decreased risk of breast cancer (Yaich et al. 1992; Zuppan et al. 1991). In breast cancer, binding of estrogen to ERs stimulates tumour development and progression by regulating estrogen-dependent transcription of proliferative factors and mediators of cellular growth (Deroo & Korach 2006). An increase in cell division and DNA synthesis is regarded to increase risk for replication errors and detrimental mutations resulting in disruption of normal cellular processes such as apoptosis, proliferation and DNA repair. Currently, both selective estrogen receptor modulators (SERMs) and aromatase inhibitors are used in the treatment of ER-positive breast cancer.

Non-small-cell lung cancer (NSCLC) is also influenced by hormonal status in which aromatase is considered a key predictive biomarker for treatment. Both

ERα and ERβ are expressed in normal and cancerous lung epithelium and estrogen elicits gene transcription that stimulates cell proliferation and inhibits cell death (Kazmi et al. 2012). Additionally, GPER expression is enhanced in lung cancer cells and tumours compared to normal lung (Jala et al. 2012).

1.11.2 Estrogen Receptors in Cardiovascular Disease

Given that the incidence of cardiovascular disease is low in premenopausal women but increases substantially to levels comparable to men following the menopause, infers that estrogens may protect the female cardiovascular system. Functional ERs have been detected in human endothelial cells, vascular smooth muscle cells and in cardiomyocytes and it is well established that estrogen can cause vasodilation by both ER-dependent and independent mechanisms (Dubey et.al. 2002).

Polymorphisms in both ERs have been associated with cardiovascular disease. Identified polymorphisms in ERα have been linked to severity and risk of coronary artery disease (Kunnas et al. 2000; Lu et al. 2002; Pollak et al. 2004) and increased risk of myocardial infarction and blood pressure in men (Peter et al. 2005; Shearman et al. 2003). In females, reduced levels of ERα by epigenetic regulation and gene methylation have also been associated with development of coronary artery disease (Losordo et al. 1994) whereas ERα appears highly expressed in normal arteries. In carotid arteries of healthy female mice, the protective effects of estrogen in response to vascular injury are mediated by ERα (Pare et al. 2002). ERα activation in carotid artery also stimulates eNOS and NO production promoting vasodilation and inhibition of inflammation (Chambliss et al. 2000) and prevents endothelial injury. On the other hand, ERβ polymorphisms have attributed to left ventricular mass and left ventricular wall thickness in women with hypertension (Peter et.al. 2005) and with blood pressure in men (Ellis et al. 2004; Pedram et al. 2008). In line with this, anti-fibrotic effects in the heart appear ERβ dependent (Pedram et.al. 2008) and estrogen therapy can reduce cardiac hypertrophy in ERα deficient mice but is ineffective in ERβ deficient mice (Babiker et al. 2004) suggesting a crucial role for ERβ in cardiac hypertrophy. In addition, ERβ knockout mice show abnormal vascular function and hypertension as well as increased mortality (Pelzer et al.

2005; Zhu et al. 2002). Together these studies implicate a wide variety of roles for ERs in the heart and vasculature.

GPER is also widely distributed in cardiovascular tissue including the human heart (Kvingedal & Smeland 1997; Owman et al. 1996) and aortic endothelial and smooth muscle cells (Takada et al. 1997). Indeed, GPER is an important regulator of estrogen in the cardiovascular system (Olde & Leeb-Lundberg 2009). Rapid events mediated by estrogen, such as vasodilation have been attributed to GPER activation (Lindsey et al. 2011) and is regarded to be an endothelium dependent NO-derived mechanism (Broughton et al. 2010). In addition, GPER mediated regulation of blood pressure and vascular tone has been described (Haas et al. 2009; Martensson et al. 2009). GPER characterisation in cardiovascular disease remains the least well defined, however, the understanding of ERs in cardiovascular disease is consistently improving and ER targeted therapies become an increasingly viable treatment of cardiovascular disease.

1.11.3 Estrogen Receptors in Pulmonary Hypertension

Gender appears to exert regulatory effects on human lung development and in healthy and diseased lung. Expression of ERs has been identified in human lung (Mollerup et al. 2002) suggesting estrogens play an important role in the lung. In fact, ERα and ERβ are required for the formation and maintenance of full competent alveoli in female mice, but not in male mice (Massaro & Massaro 2004; Massaro & Massaro 2006). In addition, female ERβ knockout mice have abnormal lung structures creating systemic hypoxia leading to ventricular hypertrophy and hypertension (Morani et al. 2006). It is likely then, that the ER pathway way contributes to sexual dimorphism in lung physiology and pathophysiology.

Despite the well documented female susceptibility of PAH in females the 'estrogen paradox' continues to impede experimental investigations into the gender disparity. Recent evidence however, suggests the action of estrogen in the lung during PH is ER-dependent. In males, estrogen-induced rescue of PH is

believed to be mainly mediated through ERß by reducing pulmonary fibrosis (Umar et.al. 2011a). In addition, Genistein, a natural soybean-derived phytoestrogen which shows much higher affinity for ERß than ERα attenuates development of monocrotaline-induced PH in male rats (Matori et.al. 2012). Furthermore, estrogen-induced protection against right heart failure in both male and female rats is reported to be directly mediated through ERß (Nadadur et.al. 2012). On the other hand, important protective effects of estrogen on haemodynamics and RVH in hypoxic PH are demonstrated to be mediated by ERα (Lahm et.al. 2012a). A role for both ERs in the lung during PH development is likely and further studies utilising intact females investigating *endogenous* and *exogenous* estrogen and ERs is required to complete the gender puzzle.

In clinical PAH, altered ER expression and signalling in the lung is reported. ESR1/ERα transcript is increased in lungs of female patients with PAH relative to non-PAH controls (Rajkumar et al. 2010) and polymorphisms in ESR1 have also been associated with an increased risk of developing portopulmonary hypertension (Roberts et al. 2009). Additionally, an evolutionary conserved estrogen receptor binding site has been identified in BMPR2, and estrogen signalling through ERα suppresses the BMPR2 signal (Austin et.al. 2009). In contrast, in animal studies, ERα protein expression in the lung is unchanged in rats with right heart failure compared to control rats, although ERß is significantly reduced in lungs from rats with right heart failure (Matori et.al. 2012). The loss of ERß expression is rescued with Genistein therapy. Alterations in ER signalling and/or tissue distribution during PAH may therefore contribute to the female predominance in PAH.

Very little is known about the role of ERs in the right ventricle. Expression of ERα and ERß has been confirmed in the right ventricle suggesting estrogen may mediate effects on cardiomyocytes in PAH (Matori et.al. 2012; Nadadur et.al. 2012). Indeed expression of ERß is significantly reduced in the RV from rats with right heart failure (Matori et.al. 2012). Moreover, estrogen exerts beneficial effects on RV function in monocrotaline- and hypoxia-induced PH (Lahm et.al. 2012a; Umar et.al. 2011a) where the mechanism of action is ER dependent. The loss of ERß also results in development of right ventricular hypertrophy (Morani

et.al. 2006). To date, there is no clinical data in humans investigating the role of ERs in the RV. However, together with the current animal studies, it is conceivable that estrogen does mediate a cardioprotective function through ERs given that RV function strongly correlates with estrogen levels (Ventetuolo et.al. 2011).

1.12 Estrogen Receptors as a Therapeutic Target in Pulmonary Hypertension

There is no doubt that estrogen has a fundamental role in the physiology and pathophysiology of various organs, including the lungs and cardiovascular system. Development of new therapies targeting estrogen receptors in pulmonary hypertension may provide novel treatment options considering the female preponderance.

Selective estrogen receptor modulators (SERMs) are an interesting class of compounds that act on estrogen receptors. They are characterised by their varying actions between tissues, thereby granting the possibility to selectively inhibit or stimulate estrogen-like action in target tissues. Such selectivity is made possible by the fact that the estrogen receptors of different target tissues vary in chemical structure. These differences allow estrogen-like drugs, such as SERMs, to act in different ways with the estrogen receptor of different tissues. For example, tamoxifen, the first SERM to be extensively researched for breast cancer, is an antagonist in the breast reducing cell proliferation, but in the uterus acts as an agonist inducing cell proliferation. On the other hand, raloxifene, is an antagonist at breast and uterine tissue, reducing breast cancer risk without stimulation of uterine cell division. However, raloxifene has estrogen agonistic properties toward the bone and cardiovascular system (Black et al. 1994; Leung et al. 2007); it may therefore be possible to develop a SERM therapy in PAH.

It has been reported that raloxifene improves endothelial function by increasing plasma nitric oxide and inhibition of endothelin-1 in healthy post-menopausal women (Saitta et al. 2001). In monocrotaline induced-PH, raloxifene attenuates progression of right ventricular hypertrophy and pulmonary arterial thickening (Nishida et al. 2009). Since raloxifene activates ERβ more effectively than ERα,

this supports previous studies which show estrogen mediated protection is ERß-dependent (Matori et.al. 2012; Umar et.al. 2011a). In addition, raloxifene enhances endothelial derived NO to promote vasodilation in isolated rat pulmonary arteries (Chan et al. 2007). Importantly, the pulmonary vasodilator response to raloxifene is sex dependent relaxing pulmonary blood vessels in male rats more effectively than female rats (Chan et al. 2005) although it is unknown whether similar sex-related effects occur *in vivo* in the pulmonary circulation. The effect of tamoxifen in the pulmonary circulation and experimental PH is unknown.

1.13 Testosterone

1.13.1 Synthesis and Metabolism

Testosterone is the main steroid hormone in the androgen group, although the androgen group comprises testosterone precursors and metabolites. Testosterone is primarily biosynthesized in the testes of males and in the ovaries of females, although small amounts are also secreted from the adrenal cortex, skin and adipose tissue (Channer 2011). In men, testosterone plays a central role in the development of male reproductive tissues as well as promoting secondary sex characteristics. Other roles for testosterone have also been described, such as in the prevention of osteoporosis (Tuck & Francis 2009) and as a risk factor in cardiovascular disease (Nettleship et al. 2009) and prostate cancer (Hyde et al. 2012).

Androstenedione is a 19-carbon steroid hormone produced as an intermediate in the biochemical pathway that produces both the androgen testosterone and the estrogens, estrone and estradiol. Androstenedione originates either from conversion of dehydroepiandrosterone (DHEA) or from 17α-hydroxyprogesterone by the enzymes CYP17A1 and 3ß-hydroxysteroid dehydrogenease-1 (3ß-HSD1), respectively (Figure 1-19). These two metabolites share a common precursor-17α-hydroxypregnenolone, a product of oxidative metabolism of cholesterol. DHEA can be further metabolised to androstendiol by 17ß-HSD2 whilst 17α-hydroxyprogesterone is converted to androstenedione by CYP17A1. Formation of testosterone from androstendiol and androstenedione involves 3ß-HSD isoforms, 3ß-HSD1 and 3ß-HSD-2 and -3, respectively. Testosterone can then be

bioconverted into 17ß-estradiol via aromatase, or into its immediate 5-alpha reduced dihydro-metabolites: 5α-DHT, via the enzyme 5α-reductase and 5ß-DHT via the enzyme 5ß-reductase. Subsequently, these dihydro-androgens undergo a 3α- or 3ß-hydroxylation via the enzymes 3α- or 3ß-HSD to produce biologically inactive tetrahydro-androgens which are excreted. It is important to note that the dihydro-and tetrahydro-androgens are non-aromatisable, thus they cannot be bioconverted into estrogens.

Like other steroid hormones, the synthesis of testosterone is regulated by the HPG-axis. LH secreted from the pituitary stimulates Leydig cells in the testes to produce testosterone and testosterone negative feedback to the pituitary blocks the actions of gonadotropin releasing hormone (GnRH) from the hypothalamus.

The physiology of testosterone is complex as the amount of biologically active testosterone is determined by sex hormone binding globulin (SHBG) and albumin. Approximately only 1-2% of testosterone is "bioavailable" or "free", whereas the majority circulates bound to SHBG (50-60%) and serum albumin (40—50%) (Dunn et al. 1981).

1.13.2 Androgen Receptor: Activation and Mechanism of Action

Testosterone exerts its effect in cells by binding to the androgen receptor (AR). The AR belongs to the superfamily of nuclear receptors responsible for mediating transcriptional events in cells and the AR gene is located on the X chromosome at position 12. Testosterone can activate AR directly, or following its conversion to DHT by the enzyme 5α-reductase. In fact, DHT is 10-times more potent in activation of AR than testosterone itself (Liu et al. 2003). .

Generally, activated AR upon ligand binding undergoes conformational changes to form a homodimer after dissociation from heat shock proteins and translocates to the nucleus to interact with androgen response elements (AREs) in the promoters of target genes. In the AR protein, the LBD is involved in dimerisation and ligand binding, whilst the DBD region allows AR to bind to DNA in cells. No membrane bound AR has been characterised, however as well as genomic transcriptional events, testosterone can mediate non-genomic rapid

signalling events involving second messenger cascades including cytosolic calcium, and activation of protein kinase A, protein kinase C and MAPK (Kousteni et al. 2001).

The actions of testosterone are mainly mediated by binding to AR. Given that testosterone exerts broad biological effects on many target organs, expression of AR is also found in non-reproductive tissues. AR is known to be expressed in other tissues such as skeletal muscle (Snochowski et al. 1980), bone (Riggs et al. 2002) and the brain (McGill, Jr. et al. 1980). Importantly, AR is also found in cardiomyocytes (Marsh et al, 1998), endothelial cells, vascular smooth muscle cells and fibroblasts (Lin et al. 1982). AR is also important in the developing lung and during lung cancer (Mikkonen et al. 2010). Therefore, testosterone and DHT effects are implicated in the heart and vasculature.

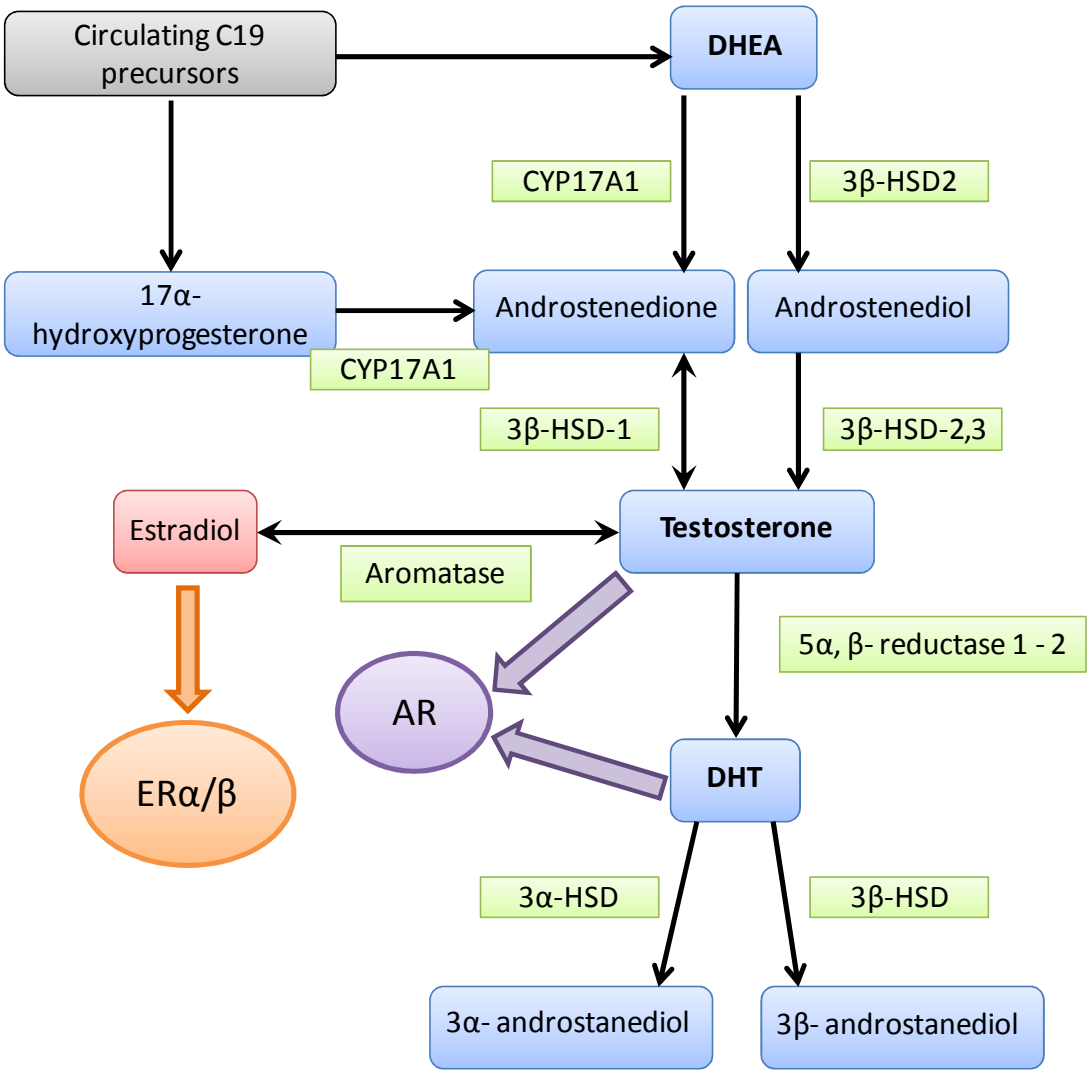


Figure 1-19: Androgen biosynthesis and metabolism.
Circulating carbon-19 precursors are converted to intermediates dehydroepiandrosterone (DHEA) and 17α-hydroxyprogesterone. Further metabolism of these produces testosterone via the hydroxysteroid dehydrogenase enzymes. Testosterone metabolism by aromatase produces estradiol, or by 5α-reductase enzymes produces the primary metabolite dihydrotestosterone (DHT). DHT is inactivated by conversion to 3α-and 3β-androstanediol which are excreted in urine.

1.14 Androgen Effects in Systemic Circulation

1.14.1 Testosterone and DHT

As male sex is regarded as a major risk factor for the development of cardiovascular disease, it is thought that androgens, including testosterone, promote detrimental effects on the heart and vasculature. In fact, androgen replacement is associated with cardiovascular-related adverse events in men of all ages (Basaria 2010; Fernandez-Balsells et al. 2010). Moreover, high testosterone levels are associated with higher mortality rates due to cardiovascular disease and diabetes (Araujo et al. 2011; Grossman & Messerli 2011; Stanworth & Jones 2009).

Several lines of evidence implicate a negative effect of testosterone in vasculature. Both testosterone and DHT stimulate proliferation of rat vascular smooth muscle cells (Fujimoto et al. 1994) and DHT increases macrophage and foam cell and plaque formation in atherosclerosis (McCrohon et al. 1999; McCrohon et al. 2000) suggesting a promitogenic and proinflammatory effect of these androgens in cardiac vasculature. Testosterone is also reported to increase synthesis of angiotensin-II, a potent vasoconstrictor and mitogen known to induce hypertension and atherosclerosis (Reckelhoff et al. 2000) and lowers HDL and raises LDL in vascular smooth muscle cells (Goh et al. 1995). In contrast, in cholesterol fed rabbits, testosterone appears anti-atherosclerotic independent of lipids (Alexandersen et al. 1999). On the other hand, in clinical data, men with an aromatase deficiency exhibit accelerated atherogenesis (Reckelhoff 2001). Perhaps the disparity between studies reflects local metabolism of testosterone to estrogen which exerts protective effects on the endothelium (Dubey et.al. 2002).

In addition, in rat models of hypertension, males exhibit higher blood pressure than age-matched females, which is prevented by castration and reproduced following testosterone treatment in spontaneously hypertensive (SHR) and Dahl salt-sensitive rats (Dubey et.al. 2002; Reckelhoff 2001). Epidemiological studies also show an inverse correlation between testosterone and blood pressure in men (Khaw & Barrett-Connor 1988). There is strong evidence that both testosterone and DHT cause structural and morphological changes in the human

heart (Achar et al. 2010) and act through the AR to initiate cardiac hypertrophy (Hayward et al. 2000). Altered metabolism of estrogen is predicted to play a pivotal role in testosterone-induced cardiac hypertrophy as males with LVH have increased expression of 5α-reductase and AR in the heart (Thum & Borlak 2002). Moreover, treatment with an AR antagonist significantly improves LVH in patients (Baltatu et al. 2003). The effect of *endogenous* and *exogenous* androgens on cardiac remodelling and function remains controversial, however, and is further complicated by aromatisation to estrogen.

1.14.2 DHEA

Extensive evidence implies a beneficial effect of DHEA on cardiovascular function. Numerous epidemiological studies have indicated that low plasma levels of DHEA and its sulphated form, DHEAS, are associated with elevated cardiovascular risk (Abbasi et al. 1998; Shono et al. 1996), cardiovascular morbidity (Alexandersen et al. 1996; Trivedi & Khaw 2001), coronary artery disease (Herrington et al. 1990; Ishihara et al. 1992; Mitchell et al. 1994) or atherosclerotic vascular diseases (Bernini et al. 1999; Bernini et al. 2001) irrespective of gender.

In animal studies, administration of DHEA attenuates development of atherosclerosis and plaque progression (Aragno et al. 2000; Ayhan et al. 2003; Eich et al. 1993; Gordon et al. 1988) and protects against ischemic-reperfusion injury and kidney injury following hypertension (Aragno et.al. 2000; Ayhan et.al. 2003). In addition, DHEA is suggested to have a protective effect on the endothelium, increasing endothelial proliferation and preventing endothelial cell apoptosis independent of AR and ERs (Liu et al. 2007; Williams et al. 2002). Antioxidant properties of DHEA have also been described in the heart and vasculature (Savineau et al. 2013).

1.15 Androgen Effects in Pulmonary Circulation

1.15.1 Testosterone and DHT

There is very limited data on the role of androgens in the pulmonary circulation and in PAH. In the few studies to date, testosterone has been identified as a potent vasodilator in isolated human pulmonary vasculature (Rowell et al. 2009;

Smith et al. 2008). It is in fact a more potent vasodilator than estrogen in this vascular bed (English et al. 2001). The effect of testosterone is proposed to be a rapid, non-genomic effect independent of AR and the endothelium and NO (Jones et al. 2002; Yue et al. 1995). Instead, the mechanism of vasodilation appears to involve inhibition of Ca^{2+} entry via voltage gated calcium channels (Hall et al. 2006; Scragg et al. 2004). Moreover, the action of testosterone is direct and not dependent on conversion to estrogen by aromatase as both aromatase inhibition and ER antagonism fail to prevent the vasodilatory response (Deenadayalu et al. 2001; Teoh et al. 2000; Tep-areenan et al. 2002)

In vivo, a correlation between the RV and testosterone is proposed. The degree of RVH in rats exposed to high altitude is greater in castrated males treated with testosterone (Vander et al. 1978) and the effects of hypoxia and testosterone appear additive. Recently, the first evidence for a role of testosterone in PH was reported. Hemnes et al (2012) showed that testosterone induced right ventricular fibrosis and increased cardiac myocyte size in the pulmonary artery banding (PAB) model of PH. The effects of testosterone in the RV were observed in the absence of any effects on haemodynamic properties, for example, right ventricular systolic pressure and cardiac output were unaffected by testosterone manipulation. This suggests, at least in PH, that the effects of testosterone are primarily involved in dysfunctional RVH and during RV stress and increased afterload in PAH drive the differences in survival rates between males and females (Hemnes et al. 2012).

1.15.2 DHEA

The effect of DHEA on PAH has been investigated in humans and animal models. It has been described as having a multifunctional protective role in PAH. In rats, DHEA treatment prevents and reverses chronic hypoxia induced-PH involving a decrease in pulmonary artery remodelling, especially PASMC proliferation and prevention of RVH (Hampl et al, 2003; Bonnet et al, 2003). Indeed, in patients with PH associated with chronic obstructive pulmonary disease (COPD), chronic DHEA treatment improves pulmonary haemodynamics (Dumas de la Roque et al, 2012).

In isolated PASMCs, DHEA directly inhibits Ca^{2+} influx and decreases KCl-induced contraction in isolated pulmonary artery (Bonnet et al, 2003). Vasodilator properties of DHEA have also been demonstrated in chronic hypoxic male rats and monocrotaline models associated with both opening of voltage gated potassium channels (Farrukh et al, 1998; Gupte et a, 2002) and increased expression and function of pulmonary artery Ca^{2+}-activated K^{+} channels (Bonnet et al, 2003; Hampl et al, 2003). Additionally, an antioxidant property of DHEA in human PASMCs decreases proliferation and resistance to apoptosis by modulating mitochondrial functions (Dumas de la Roque et al, 2010). In line with this, DHEA reverses pulmonary arterial remodelling by various pathways such as normalising RhoA/ROCK activity in hypoxia (Homma et al, 2007), decreasing Src/STAT3 activation with resorted BMPR2 (Paulin et al, 2011) and decreased accumulation of HIF-1α in PASMCs during hypoxia (Dessouroux et al, 2008). DHEA may therefore provide a promising therapeutic option in PAH therapy.

1.16 Aims

The principal aim of this research was to investigate the role of classical estrogen receptors ERα and ERß, and the novel G-protein coupled receptor, GPER in PAH in males and females. We also wished to determine the role of testosterone in development of PAH in males. These aims were investigated using the following approaches:

- Determine localisation of estrogen receptors lung and pulmonary artery smooth muscle cells in experimental and translational PH (Chapter 3)

- Characterise ERα in two in vivo models of PH: chronic hypoxia and $SERT^{+}$ mice (Chapter 3)

- Determine a mechanism for estrogen and estrogen receptor signalling in experimental and translational PH (Chapter 3)

- Characterise the role of GPER in $GPER^{-/-}$ male and female mice in the chronic-hypoxic PH model (Chapter 4)

- Identify the influence of testosterone in PH development in vitro and in vivo by testosterone manipulation (castration) (Chapter 5).

Chapter 2.

Materials and Methods

2.1 Materials

All chemicals and reagents obtained were of the highest grade and quality and supplied by the following companies: Innovative Research of America (Florida, USA), Sigma Aldrich (Poole, UK); Tocris Bioscience (Bristol, UK); Invitrogen (Paisley, UK), Fisher Scientific (Loughborough, UK) and Applied Biosystems/Life Technologies (Paisley, UK). All cell culture reagents were provided by Sigma Aldrich (Poole, UK) or Gibco (Paisley, UK), unless otherwise stated. Fetal Bovie Serum was supplied by Sera Laboratories International (West Sussex, UK).

2.2 Ethical Information

All experimental animal procedures conform with the United Kingdom Animal Procedures Act (1986) and with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and ethical approval was also granted by the University Ethics Committee.

Experimental procedures utilising human pulmonary artery smooth muscle cells (hPASMCs) conformed with the principles outlines in the Declaration of Helsinki and were approved by Cambridgeshire 1 Research Ethics committee (REC reference: 08/H0304/56).

2.3 Methods

2.3.1 **Animal Models**

2.3.1.1 **Wildtype Mice**

Wild-type inbred C57BL/6JOlaHsd male and female mice were obtained from Harlan Laboratories, Carshalton, UK. Mice were shipped at 6 weeks of age and were housed in the Central Research Facility in the University of Glasgow for one week to acclimatise before exposure to any surgical procedures.

2.3.1.2 **SERT^{+} Mice**

Mice over-expressing the human serotonin transporter gene transcript were generated and supplied by Professor Tony Harmer, University of Edinburgh, UK. SERT^{+} mice were generated using the C57BL/6 x CBA wild-type strain. The transgene was introduced using a 500-kb yeast artificial chromosome (YAC35D8) containing the human SERT gene flanked by 150 kb of 5' and 300kb of 3' sequence, with the "short" allele of the *SERTLPR* in the promoter region in the 10-repeat allele of the variable number tandem repeat in intron 2. In situ hybridisation analysis has previously shown that the expression of the human SERT gene closely resembles the pattern of the endogenous mouse SERT gene. Genotyping was performed by PCR on tail biopsies to confirm the expression of the human SERT transgene. C57BL/6 x CBA littermate mice were studied as controls for SERT^{+} mice.

2.3.1.3 **GPER$^{(-/-)}$ Mice**

GPER$^{(-/-)}$ mice were supplied by Frederick Leeb-Lundberg (Lund University, Sweden). The mouse model was developed with a completely disrupted GPER gene locus. This was achieved using a targeting vector containing a lox-flanked mGPER ORF and a lox-flanked TkNeo cassette assembled from a murine 129/SvJ BAC-clone. To provide negative selection, the long arm was flanked with a PGKDt-a expression cassette. The GPER gene was deleted by transient expression of cAMP response element- recombinase. This targeting strategy resulted in deletion of the whole GPER open-reading frame. The chosen three clones were injected into C57BL/6 blastocysts which were implanted into pseudopregnant females and the deletion was backcrossed six generations into the C57BL/6 genetic background. C57BL/6 mice were therefore studied as wild type controls for GPER$^{(-/-)}$ mice.

All mice were housed with littermates in the Central Biological Research Facility at the University of Glasgow. In all mouse studies, mice were exposed to a continuous 12 hour light/dark cycle with access to food and water *ad libitum*.

2.3.2 **MPP Dihydrochloride Administration**

Under general anaesthesia, 1%-3% (v/v) isoflurane supplemented with O_2, pellets containing either MPP Dihydrochloride [chemical name- 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride] (MPP) (0.56mg/14 day pellet, Innovative Research of America, Florida, USA) or vehicle pellets were inserted subcutaneously into the dorsal neck using a sterile 12-gauge hypodermic needle prior to hypoxic exposure for 14 days. The unique engineering of the Matrix Driven Delivery (MDD) pellet system effectively and continuously allowed the diffusion of MPP into the animal at a concentration of $2mgkg^{-1}day^{-1}$. Vehicle pellets are composed and designed to perform exactly as active pellets, but contain no active product. The unique engineering of the Matrix Driven Delivery (MDD) pellet system effectively and continuously allowed the diffusion of MPP into the animal at a concentration of $2mgkg^{-1}day^{-1}$. Vehicle pellets are composed and designed to perform exactly as active pellets, but contain no active product. This is the first study to administer MPP in pellet form, although, previously this pellet delivery system has been shown to work effectively at releasing 17ß-estradiol in mice (White et al, 2011) and we therefore consider this an effective, consistent and reliable method of drug delivery. Limitations of these pellets however, include the inability to compensate for varying body weights over the experiment duration. 48 hours after insertion of MPP/vehicle pellets, mice were exposed to 14 days chronic hypoxia.

2.3.3 **Chronic Hypoxia**

The development of hypoxia induced-pulmonary hypertension (PH) was achieved using a hypobaric hypoxic chamber. Mice were subjected to 14 days of an atmospheric pressure of 550mbar. The gradual depressurisation from ~1000mbar (ambient room pressure) to 550mbar reduces the oxygen availability from ~21% O_2 to ~10% O_2 and results in sustained hypoxic pulmonary vasoconstriction and the development of PH. During this time, room temperature was maintained at 21˚C-23˚C with a relative humidity of 30 – 50% and mice were re-housed with clean bedding and food/water every 5–7 days.

2.3.4 **Bilateral Orchidectomy**

For pre-operative care, male mice were administered the analgesic buprenorphine ($0.1mgkg^{-1}$) and $4mlkg^{-1}$ sterile saline via intra-peritoneal injection. Bilateral orchidectomy, (removal of both testicles) was performed under general anaesthesia (1%-3% (v/v) isoflurane supplemented with O_2) in 6 week old male C57BL/6 wild-type mice. This technique removes the sex organs responsible for the production of the male sex hormone, testosterone. Briefly, a small incision was made in the scrotum, each testicle was then pushed down through the incision and removed via cauterisation through the vas dererens. 1-2 surgical staples were used to close the ventral incision in the scrotum. Surgical staples were used for accuracy and consistency, and moreover to reduce the chances of infection as a complication during suturing. For post-operative care, a non-steroidal anti-inflammatory drug, carprofen ($2.5mgkg^{-1}$) and $4mlkg^{-1}$ sterile saline were administered via intra-peritoneal injection. Staples were removed one day following surgery and mice were given a further 4 weeks to recover from surgical procedure as well as allowing complete depletion of circulating testosterone levels. An interval of 14 days post orchidectomy surgery has previously demonstrated the absence of any detectable plasma testosterone levels (Wichmann et al. 1996). Sham- operated mice received a lateral incision in the scrotum although the testes and vas deferens remained intact. At 10 weeks, mice were placed in 14 days of hypobaric hypoxia and the assessment of PAH was performed on removal at 12 weeks.

One week following surgery, mice were monitored on a daily basis, and thereafter on a weekly basis, to ensure full recovery from surgery with an absence of any infections at both the wound site and overall general health.

Experimental design for each *in vivo* study described above is shown in Figure 2-1. In all studies, C57Bl/6 mice were used at 6-12 weeks of age; however $SERT^+$ mice and wildtype counterparts were required to be used at 20 weeks (~5-6 months) as it is at this age only that female mice exhibit the PH phenotype. One limitation of using mice of varying ages, and of interest in this study, is the concentration of circulating estrogen in these mice may fluctuate at different ages.

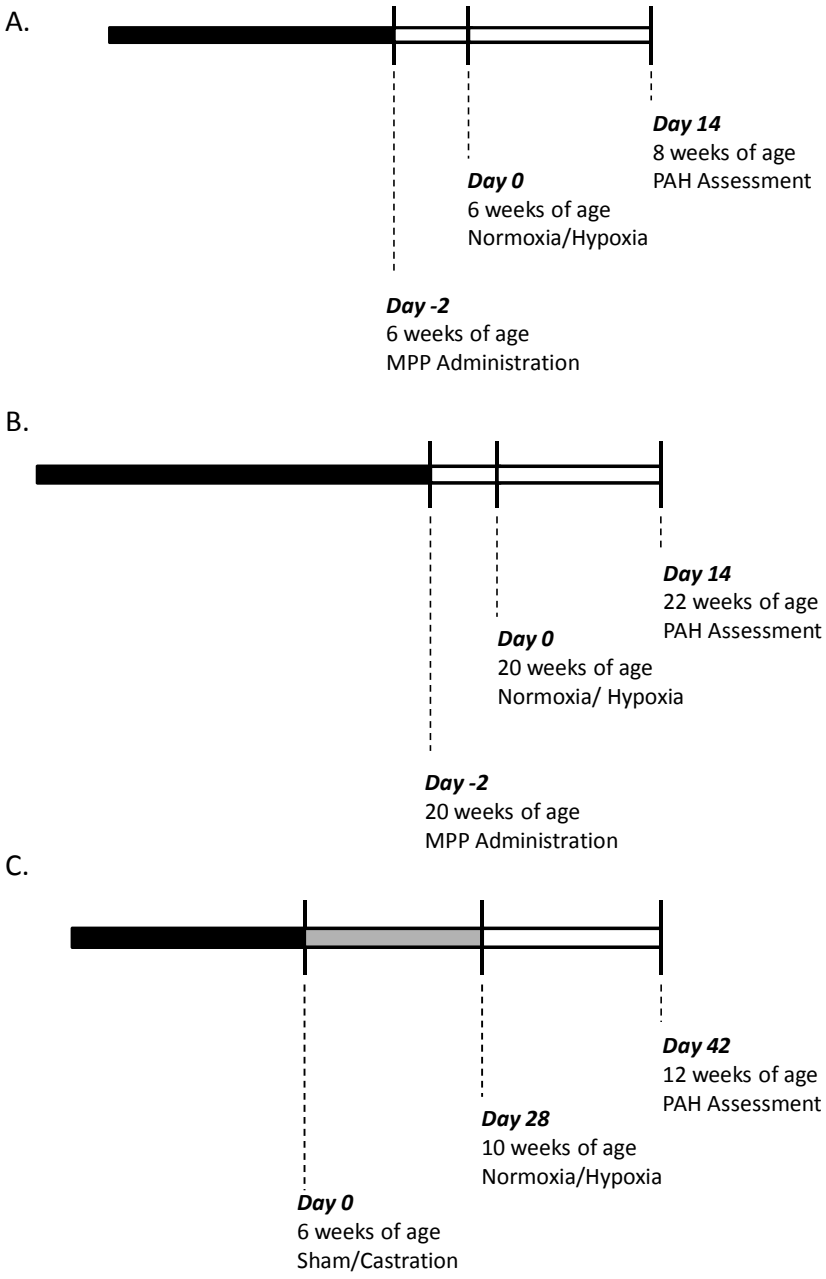


Figure 2-1: In vivo study design.
(A). C57Bl/6 male and female mice were dosed with MPP/vehicle for 2 weeks in the presence/absence of hypoxia. In vivo haemodynamics were assessed in these mice at 8 weeks of age. (B). SERT+ female mice were dosed with MPP/vehicle from 20 weeks of age for 2 weeks in the presence/absence of hypoxia and haemodynamics assessed at 22 weeks. (C). Male mice underwent sham/orchidectomy surgery at 6 weeks of age. Following 4 weeks recovery, mice were placed in normoxic/hypoxic conditions for 2 weeks and haemodynamics were assessed at 12 weeks.

2.4 Assessment of PAH

2.4.1 Haemodynamic Measurements

The induction of general anaesthesia was supplied via exposure to 3% (v/v) isoflurane supplemented with O_2. Acceptable anaesthesia at this point was observed by shallow abdominal respiration prior to removal and weight measurements. Mice were then placed in a facemask and continuously received 1-2% (v/v) isoflurane to maintain adequate general anaesthesia. Before initiation of surgical procedure, general anaesthesia was confirmed by assessing the absence of hind limb and tail reflex. These reflexes were also routinely assessed throughout the procedure.

2.4.1.1 Mechanism of fluid filled catheter system

Haemodynamic pressures were measured using a fluid filled catheter system implanted transdiaphragmatically into the right ventricle or directly into the carotid artery for measurement of right ventricular systolic pressure and systemic arterial pressure, respectively. Fluctuations of vascular pressure caused a pulsation of the saline column within the catheter leading to changes in the resistance. These changes in pressure were converted to an electrical signal and the output measured using a digital recorder (BIOPAC Systems, Inc, USA). The sampling rate was recorded as 200 samples/second.

2.4.1.2 Calibrating the system

The catheter was calibrated using a sphygmomanometer to a baseline of 0mmHg or atmospheric pressure (open to air) and a maximum of 150mmHg. In addition, pressure tubing was assessed to ensure they were free of air bubbles. A wave test was also applied to check for oscillations as an indicator of harmonic characteristics of the system (one oscillation was observed before wave returned to baseline).

2.4.1.3 Accuracy of system

Although use of the fluid filled catheter system is advantageous in that it provides direct and continuous measurements of arterial pressure, this system is not suited for continuous prolonged recording. Common sources of error

associated with the fluid filled catheter system include clotting or kinking in the cannula and bubbles in the cathter-transducer system resulting in decreased resonant frequency. Continuous flushing of the catheter system with heparin to prevent clotting of the catheter may result in volume overload in the mouse. Moreover, there is associated morbidity and mortality with use of this surgical system. Use of the Millar catheter with a transducer mounted on the catheter tip would minimise these inaccuracies and the pressure-volume loop (PV loop) system would provide a more thorough analysis of the heart functionality, including stroke volume and end-systolic and –diastolic volumes in future studies.

2.4.2 Right Ventricular Systolic Pressure

Right ventricular systolic pressure (RVSP) was measured by a transdiaphragmatic approach. Briefly, a small portion of skin was removed from the ventral chest to expose the anterior sternum. A 25mm gauge heparinised needle was then advanced into the mid-portion of the abdomen using a micromanipulator. A negative pressure reading was indicative of entry into the diaphragm and used as a marker for gauging to the right ventricle. The right ventricle was punctured through the right ventricular free wall and the pressure was confirmed by a characteristic waveform such as in Figure 2-2. From this recording, a measurement of RVSP was deduced and used as an indice of PAH. Following a five-to-eight minute trace, a control was performed by advancing the needle further into the heart to obtain left ventricular (LV) pressure and a typical LV waveform. At necropsy validation of right ventricular pressure was also assessed microscopically by confirming the presence of a puncture wound in the right ventricle free wall. The continuous measurement of RVSP was achieved using a calibrated 25mm gauge heparinised saline filled needle attached to an Elcomatic E751A pressure transducer connected to a MP100 data acquisition system (BIOPAC Systems Inc, Santa Barbra, USA). Specifically, mean RVSP, systolic and diastolic RVSP were measured at three independent areas of the trace which were steady. In each instance, the same peak following the reduction in transmural pressure was recorded.

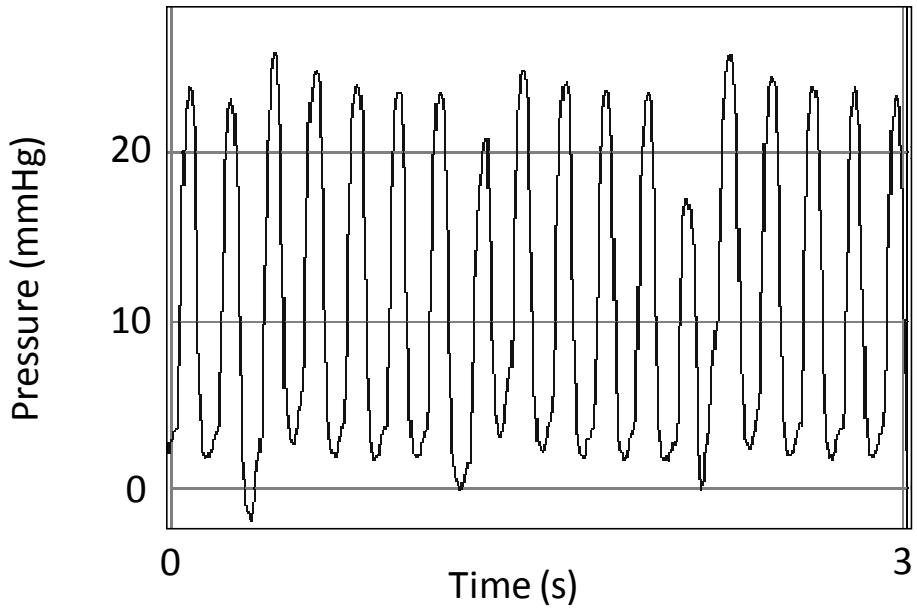


Figure 2-2: Representative image of right ventricular pressure.
Three second representative recording of right ventricular pressure in a normoxic wild type mouse. Approximate heart rate= 360.0 beats per minute (bpm). Low heart rates indicated could be a result of prolonged anaesthesia (normal adult mouse heart rate= ~500-600bpm).

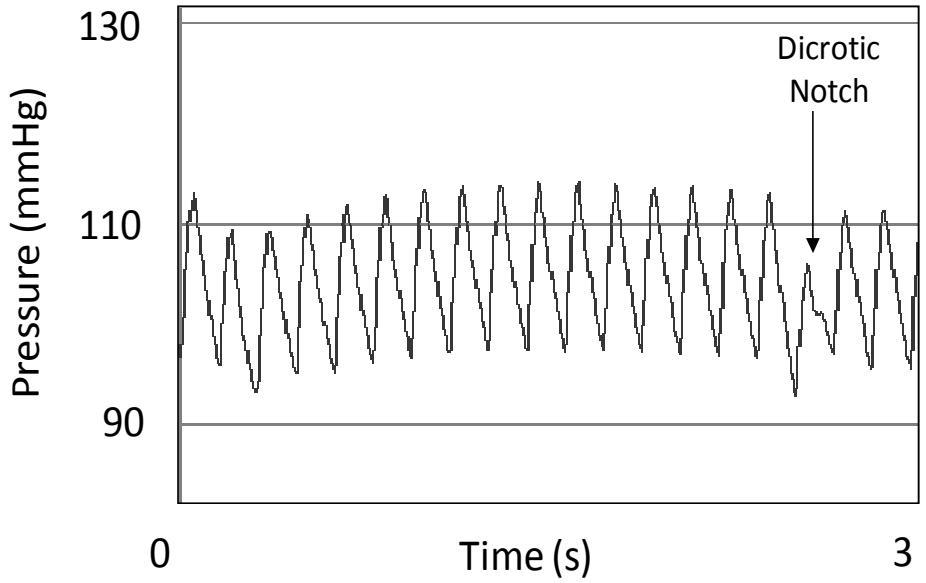


Figure 2-3: Representative image of systemic arterial pressure.
Three second representative recording of systemic arterial pressure in a normoxic wild type mouse. Approximate heart rate= 300.0 beats per minute (bpm). Closure of the aortic valve is indicated by the dicrotic notch. Low heart rates indicated could be a result of prolonged anaesthesia (normal adult mouse heart rate= ~500-600bpm).

2.4.3 **Systemic Arterial Pressure**

Systemic arterial pressure (SAP) was obtained by cannulation of the left common carotid artery. The carotid artery was exposed by an incision in the ventral neck and located by blunt dissection down the muscle lying inferior to the trachea. The left common carotid artery is typically positioned ~2mm lateral (left) and less than ~1mm posterior to the trachea. Precision dissection was used to isolate the carotid artery from the vagus nerve running parallel to the artery and without severing the nerve. To tie the artery distally to the heart a suture (5.0 silk non-braided) was used and an arterial clip (Harvard Apparatus, Boston, USA) was placed on the artery to occlude blood flow through the lumen proximal to the heart. Following an incision through the arterial wall, a heparinised saline-filled polypropylene cannula (Harvard Apparatus, Boston USA) was advanced 4-5mm into the artery and secured in place using a suture and the arterial clip removed. Continuous measurement of SAP was obtained using the same technique as for RVSP with an Elcomatic E751A pressure transducer and an MP100 data acquisition system (BIOPAC Systems Inc, Santa Barbra, USA). A typical SAP waveform is shown in Figure 2-3.

Following measurement of RVSP and SAP, mice were killed by cervical dislocation. The heart and lungs were then dissected out immediately *en* bloc and placed in ice cold physiological saline solution (PSS; pH 7.4; mmol/L, NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$2.5, $C_6H_{12}O_6$ 11.1). Liver, spleen, kidneys and uterus/testis were also dissected out and snap frozen in liquid nitrogen for storage in -80˚C conditions until further use.

2.4.4 **Right Ventricular Hypertrophy**

The right ventricle (RV) and the left ventricle plus septum (LV+S) were dissected free from surrounding pericardial fat and large blood vessels at necropsy. The RV was dissected from the LV+S and both were dry blotted. Right ventricular hypertrophy (RVH) was measured as a ratio of the right ventricular free wall (RV) over the LV+S and the ratio (RV/LV+S) was used in PAH assessment.

2.4.5 Pulmonary Vascular Remodelling

3µm sagittal sections of lung were stained with α- smooth muscle actin (α-SMA) and microscopically examined in a blinded fashion. Pulmonary arteries assessed were <80µm external diameter and were not associated with an airway. The arteries were considered muscularised by the presence of a distinct think vascular wall visible for at least half the diameter of the vessel. Four to six lung sections were analysed per group and approximately 100 arteries were counted per sagittal lung section. The percentage of remodelling was expressed as a percentage from the number of muscularised vessels/total number of vessels x 100.

2.5 Histology

2.5.1 Fixation

Human lung sections were supplied by Prof. Nicholas Morrell (Papworth Hospital, Cambridge, UK). Information regarding the part of lung removed from the patients at the hospital is unknown. Patient information for lung sections is depicted in Table 2-1. Low patient numbers are due to the rare nature of the PAH and are described as a limitation in experiments.

Following death, the superior and inferior lobe from the right lung were dissected free and harvested in 10% (v/v) neutral buffered formalin (NBF: 90% distilled H_2O, 10% formalin, 33mmol/L NaH_2PO_4, 45mmol/L Na_2HPO_4) for 24 hours under gentle agitation. Formalin fixed lungs were then paraffin-embedded and 3µm sagittal sections cut and mounted onto salinised glass microscopic slides.

2.5.2 Immunolocalisation of Estrogen Receptors in the Human Lung

3µm human lung sections were de-parrafinised in xylene and rehydrated through 100% ethanol to 70% ethanol and deionised water (100% xylene > 100% ethanol > 90% ethanol > 70% ethanol > deionised water; 10 minutes each step). Following rehydration, heat-induced epitope retrieval was performed for 20 minutes in 10molL^{-1} citric acid buffer (pH6.0) and then cooled to room temperature. Lung

sections were rinsed in deionised water for 10 minutes and then phosphobuffered saline (TBS). Endogenous peroxidise activity was then blocked via incubation in methanol containing 3% (v/v) hydrogen peroxide (H_2O_2) for 20 minutes at room temperature (Sigma Aldrich, Poole, UK). Non-specific blocking using Normal Horse Serum (2.5%) (Vector Laboratories, Peterborough, UK) was performed for 1 hour at room temperature before addition of primary antibody or IgG control overnight at 4^{o}C in diluent (15% (v/v) primary antiserum, 10% (w/v) BSA in PBS). See Table 2.2 for antibody concentrations. A negative control was also utilised by incubating a slide with antibody diluent only and appropriate IgG controls were used. Subsequently, sections were washed in TBS for 2 x 10 minutes at room temperature. Secondary incubation using anti-rabbit horseradish peroxidise polymer (Vector Laboratories, Peterborough, UK) was performed for 1 hour at room temperature followed by 2 x 10 minute washes in PBS. Following secondary incubation, protein visualisation was achieved under the microscope with the DAB substrate kit (3,3- diaminobenzidine, hydrogen peroxide and nickel solution; Vector Laboratories, Peterborough, UK) until a dark-brown staining was apparent which was typically 3-5 minutes. To stop the immunoperoxidase reaction sections were placed in deionised water. Sections were counterstained in haematoxylin and allowed to develop a blue/purple background before sections were dehydrated in an increasing ethanol gradient to histoclear (70% ethanol > 90% ethanol > 100% ethanol > 100% xylene; 10 minutes each step). Finally, glass coverslips were mounted onto each slide using Tissue-Mount (Sakura Finetek, Alphen aan den Rijnm, Netherlands).

2.5.3 **Alpha Smooth Muscle Actin and Von Willebrand Staining**

α-SMA staining was used to confirm pulmonary vascular remodelling and muscularisation in mouse models and smooth muscle staining in human lung sections. Von Willebrand staining (vWF) was utilised to confirm endothelial cells staining in human lung sections. 3μm mouse/human lung sections were de-parrafinised in xylene for 30 minutes and rehydrated through an alcohol gradient to water (100% ethanol (x2), 90% ethanol (x1) and 70% ethanol (x1)). Heat-induced epitope retrieval was carried out in 10mmol^{-1} citric acid buffer (pH6.0) for 20 minutes followed by a period to cool. Non-specific blocking using Normal Horse Serum (2.5%) (Vector Laboratories, Peterborough, UK) was performed for 1 hour at room temperature before addition of primary antibody or IgG control

overnight at 4°C in diluent (15% (v/v) primary antiserum, 10% (w/v) BSA in PBS).α-SMA/vWF primary antibodies were incubated over night at 4°C in diluent (15% (v/v) primary antiserum, 10% (w/v) BSA in PBS). Endogenous peroxidise activity was then blocked via incubation in methanol containing 3% (v/v) hydrogen peroxide (H_2O_2) for 20 minutes at room temperature (Sigma Aldrich, Poole, UK). Incubation with anti-rabbit horseradish peroxidise polymer conjugated secondary antibody (ImmPress anti-rabbit IgG peroxidise, Vector Laboratories, Peterborough, UK) was performed for 1 hour at room temperature followed by 2 x 10 minute washes in PBS. α-SMA/vWF staining was then visualised using DAB substrate kit producing a dark-brown colour within 3-5 minutes. Sections were counterstained with Harris haemotoxylin to develop a blue/purple background for clearer visualisation. Sections were then dehydrated in increasing ethanol concentrations to histoclear before mounting. Concentrations of antibodies used in immunohistochemistry are depicted in Table 2-2.

Table 2-1: Clinical characteristics for human lung sections. All available information for patients from Papworth Hospital, Cambridge is depicted in table. Duration of disease length and treatment course was unavailable. HPAH=heritable PAH; IPAH=idiopathic PAH.

	Available Patient Information	BMPR2 Mutation
Control		
1.	Female; No information available	
PAH		
1.	Female; HPAH; aged 38 years; transplant; mean PAP 56mmHg; IV epoprostinil treatment	No
2.	Female; HPAH; aged 30 years; transplant; mean PAP 46mmHg; IV iloprost and sildenafil	Yes
3.	Female; IPAH; aged 44; transplant; mean PAP 86mmHg; IV epoprostinil	No

Table 2-2: Antibodies used for Immunohistochemistry. α-SMA=alpha smooth muscle actin; BMPR-II=bone morphogenetic protein receptor-II; ERα=estrogen receptor alpha; ERβ=estrogen receptor beta; GPER= G-protein coupled estrogen receptor; vWF=Von Willebrand Factor.

Antibody	Type (Clone)	Source (Catalogue number)	Immunohistochemistry Dilution used
a-SMA	*Rabbit polyclonal*	*Abcam (ab5694)*	*1:500 (0.2μgml^{-1})*
ERa	*Rabbit polyclonal*	*Santa Cruz (sc-7207)*	*1:200 (1μgml^{-1})*
ERβ	*Rabbit polyclonal*	*Abcam (ab-3577)*	*1:200 (5μgml^{-1})*
GPER	*Rabbit polyclonal*	*Abcam, (ab-39742)*	*1:100 (5μgml^{-1})*
vWF	*Rabbit polyclonal*	*Dako (A0082)*	*1:1000 (0.1μgml^{-1})*

2.6 Vascular Reactivity

2.6.1 Small Vessel Wire Myography

Intralobar pulmonary artery was used for determination of pulmonary vascular reactivity. Briefly, the intralobar pulmonary artery (~250μm diameter) was dissected from the left lung located posterior to the hilum. Once isolated, the surrounding parenchyma and airway smooth muscle was gently removed from the pulmonary artery and the artery was placed in ice cold PSS until use.

Small vessel wire myography was used to assess the vascular reactivity in mice. A 4-channel wire myograph (DMT Multi Wire Myograph, Denmark) organ bath was prepared with 5ml of PSS, heated to 37^{o}C ± 0.5^{o}C and continuously bubbled with 16% O_2, 5% CO_2 and 79% N_2, designed to replicate gas composition and conditions observed in the lung *in vivo*. Following dissection from the lung, intra-lobar artery was divided into 2mm segments and two sections of stainless steel wire (40μm diameter, 3cm length) were passed through the vessel lumen and tied to their respective mounting jaws on the myograph and the vessel was securely tightened (Figure 2-4). Once mounted the vessels were allowed to equilibrate for ~30 minutes under resting/zero tension.

2.6.2 Application of Tension

The pulmonary circulation is a low pressure system with pulmonary arterial pressures between 9-18mmHg. To replicate in vivo pressures, control vessels from normoxic mice were set up with a tension to achieve a normal transmural pressure from 12-16mmHg representative of a normoxic mouse pressure in vivo. On the other hand, vessels from hypoxic mice were set up at tensions equivalent to their elevated in vivo mean pressures observed in PAH (25-30mmHg). These pressures were achieved by altering the force transducer in millinewtons (mN) and the pressure was deduced from the force of the tension using the equation in Figure 2-5. For these, resting/zero tension when no force is applied (X_o), active tension when force is applied and wires are stretched (X_i) and passive force (F) values were required. In addition, 2 (mm) was used as the constant for vessel length (L). The internal circumference with wires just touching before

application of tension is equal to (2+π) x wire diameter (40μm)= 205.6 μm. Tension was applied incrementally until the appropriate pressure was achieved.

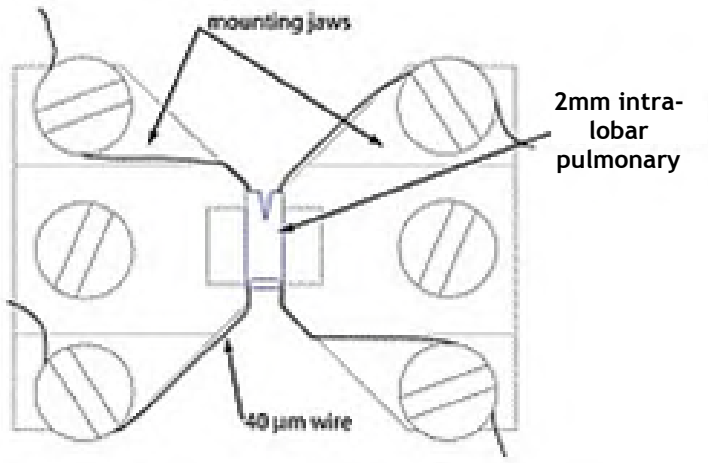


Figure 2-4: Wire myograph set-up.
Used to study small resistance vessels, vascular reactivity in the lung was assessed in 2mm sections of pulmonary intralobar artery.

Potassium chloride (KCl) was the contractile agent used to initiate depolarisation and contraction in smooth muscle cells. KCl-induced smooth muscle cell contraction is due to membrane depolarisation causing Ca^{2+} entry through voltage-operated Ca^{2+} channels and a reduction in K^{+} efflux (Ganitkevich et al. 1991). After a 30 minute equilibration, the response to 50mmolL^{-1} KCl was determined for a 30 minute period. The maximum KCl response produced from two consecutive readings was used to normalise the contraction in these vessels. A contractile response of ≥ 1 millinewton (mN) was considered sufficient to perform a cumulative concentration response curve.

2.7 Serotonin Cumulative Concentration Response Curve

A baseline tension was established in vessels prior to commencing the cumulative response curve to the potent pulmonary vasoconstrictor serotonin (Sigma Aldrich, Poole, UK). Curves were initiated at the lowest concentration of $1x10^{-9}$ molL^{-1} and increased in 0.5 log increments to a final organ bath concentration of $1x10^{-4}$ molL^{-1}. For consistency between vessels and experiments, serotonin was added once the vascular response had reached a plateau which was typically following ~3 minutes. Data was analysed using Myodaq/Myodata 2.01 M610+ software and GraphPad Prism 5.0© was used to derive E_{max} and EC_{50} values.

$$P_i = \frac{(2\pi) \times F}{2 \times L\,(205.6 + 2(X_i - X_o)) / 1000}$$

Figure 2-5: Equation to calculate transmural pressure.
The calculated Pi value must be divided by 0.1333 in order for successful conversion to mmHg. F=force (mN); L=length of vessel (2mm); X_i=active tension; X_o=resting/zero tension. 205.6µm is the calculated internal circumference of the wires when using 40µm diameter wires.

2.8 Tissue Culture

All tissue culture procedures were carried out in sterile conditions using a Biological Safety Class II vertical laminar flow cabinet. Human pulmonary artery smooth muscle cells (PASMCs) were the cell model utilised, provided by Nicholas. W. Morrell, University of Cambridge, UK. Cells were stored at 37°C and maintained in 5% CO_2, 95% air.

2.8.1 Human Pulmonary Artery Smooth Muscle Cells

Briefly, PASMCs utilised were derived from pulmonary arteries (1-3mm arterial diameter) from non-heritable idiopathic PAH patient origin or were obtained from non-PAH donors undergoing lung biopsies and studied as controls. Patient information for cell lines utilised is depicted in Table 2-3. PASMCs were grown in a 75cm^2 culture flask and media was replaced every 48 hours. Cells were grown in Dulbeccos's Modified Eagle Medium (DMEM; Gibco, Paisly, UK) supplement with 10% (v/v) fetal bovine serum (Sera Laboratories International, West Sussex, UK), 2mmolL^{-1} glutamine, and sterile filtered antibiotic antimycotic solution (contains 10,000 unitsml^{-1} penicillin G, 10mgml^{-1} streptomycin sulphate and 25µgml^{-1} amphotericin B; Sigma Aldrich, Poole, UK). Cells were passaged when the monolayer approached 90% confluency to prevent cell growth arrest by cell contact inhibition. For passaging, cells were rinsed with two washes of 0.06% (v/v) trypsin-ethylenediamine tetra-acetic acid (EDTA) (trypsin-EDTA; Gibco, Paisly, UK) and placed at 37°C to encourage the monolayer to detach from the flask surface. Once 95% cells were detached, which was typically following less than 5 minutes incubation, 10ml 10% (v/v) FBS DMEM was added to the flask to neutralise the trypsinisation reaction. For plating of cells, cell density was assessed using a haemocytomoeter.

2.8.2 **Proliferation Assays Using [^{3}H]-Thymidine Incorporation**

The [^{3}H] thymidine incorporation assay is a reliable assay used to measure proliferation by directly measuring DNA synthesis. It utilises a strategy wherein radioactive labelled [^{3}H]-thymidine is incorporated into new strands of chromosomal DNA during mitosis. PASMCs (passage 3-5) were seeded in 24 well plates (growth area 2.0cm^2) at a density of 10,000 cells per well and grown to 60% confluency in 10% (v/v) FBS DMEM. Cells were then quiesced in 0.2% (v/v) FBS phenyl-red free DMEM for a period of 24 hours prior to addition of agonist for 72 hours. After 48 hours, where appropriate, all antagonists were added 30 minutes before addition of agonist. Drugs applied were replaced every 48 hours and for the last 24 hours, 0.1µCi [^{3}H]-thymidine was added to each well. To stop the experiment following 72 hours incubation, cells were washed twice in PBS. Protein was precipitated by three washes in 5% (w/v) trichloroacetic acid followed by addition of 0.3molL^{-1} NaOH for 30 minutes to promote cell lysis. The total volume of each well (500µl) was then transferred to a 1.5ml eppendorf and 1ml Eicoscint A scintillation fluid (Eicoscint, Atlanta, USA) was added to each tube. The radioactivity level of [3H]-thymidine was measured as an index of DNA synthesis in a Wallac Scinitillation counter (PerkinElmer, Cambridge, UK). Data are expressed as fold change compared to 2.5% FBS control. Proliferation data are expressed as fold change compared to 2.5% FBS control.

Estrogen receptor agonists: PPT (chemical name: 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol); DPN (chemical name: 2,3-bis(4-Hydroxyphenyl)-propionitrile) and G1 (chemical name: (±)-1-[(3aR*,4S*,9bS*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone) were used at a concentration range between 0.001-10 nmolL^{-1}. All estrogen receptor antagonists: MPP (MPP dihydrochloride); PHTPP (chemical name: 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol) and G15 (chemical name: (3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinoline) were used at a concentration of 1µmolL^{-1}.

Table 2-3: Clinical characteristics and patient information for human PASMCs. All available patient information received from University of Cambridge is depicted in table below. HPAH= heritable PAH; IPAH= idiopathic PAH.

	Available Patient Information	BMPR2 Mutation
Control		
	Female	
1.	58 year old	No
2.	64 year old	No
3.	59 year old	No
4.	64 year old	No
5.	72 year old	No
	Male	
1.	62 year old with emphysema	No
2.	72 year old	No
3.	76 year old	No
PAH		
	Female	
1.	24 year old; IPAH	
2.	30 year old; HPAH	Yes (R899X)
3.	39 year old, HPAH	Yes (N903S)
	Male	
1.	23 year old, HPAH	Yes
2.	43 year old, IPAH	No
3.	56 year old, IPAH	No

2.9 Western Blotting

2.9.1 Human PASMCs

Human PASMCs were seeded (passage 3-5) in 6 well plates (growth area 9.6cm^2) at a density of 25,000 cells per well and grown to 80% confluency in 10% (v/v) FBS DMEM. Cells were then quiesced for 24 hours in 0.2% (v/v) FBS DMEM followed by addition of agonist and/or antagonist for experiment dependent required times. The experiment was terminated by placing the 6-well plates on ice and the media was immediately aspirated and cells were subsequently washed in ice cold PBS three times. Ice cold radioimmunoprecipitation assay (RIPA)-buffer (64mM HEPES, pH7.5, 192mM NaCl, 1.28% (v/v) Triton-X-100, 0.64 (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.5molL^{-1} sodium fluoride, 5mmolL^{-1} EDTA, 0.1molL^{-1} sodium phosphate, 1µgml^{-1} soyabean trypsin inhibitor, 1µgml^{-1} benzamidine, 0.01mmolL^{-1} PMSF) was then added to each well for 15 minutes under gentle agitation. Following this, cell lysates were collected by scraping and collected samples were stored at -80^{o}C until further use.

2.9.2 Mouse Pulmonary Arteries

Main left and right mouse pulmonary arteries and pulmonary trunk were dissected out at necropsy as these are the smallest that can be practically dissected from the mouse and provide a valid representation of the pulmonary circulation. Immediately following death, the pulmonary arteries were snap frozen in liquid nitrogen and transferred to -80^{o}C until required. Pulmonary arteries from one mouse were placed in 150µl of RIPA-buffer with three 5mm stainless steel balls (QIAGEN®, Manchester, UK). Pulmonary arteries were homogenised using a QIAGEN® TissueLyser II for 2 minutes (4 x 30 second intervals). Protein samples were then placed on ice to rest for 30 minutes prior to being centrifuged at full speed for 15 minutes at 4^{o}C. Supernatant was removed and placed in a fresh tube and then stored at -80^{o}C until use for Western Blot protein analysis.

2.9.3 SDS-PAGE

Protein was separated using SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), a technique that separates the proteins according to their

molecular weight. Briefly, protein samples were loaded at 10-20μgml^{-1} as assessed using a BCA (bicinchoninic acid) Protein Assay (Thermo Scientific) and standard curve analysis. Protein samples were subjected to reducing conditions in the presence of NuPAGE® LDS (lithium dodecyl sulphate) Sample Buffer (4X) and NuPAGE® Reducing Agent (10X) (10mmolL^{-1} dithiothreitol) and heated to 70^{o}C for 10 minutes. Samples were loaded into NuPAGE® Novex® 4-12% Bis-Tris Precast Gels and subjected to 150V constant in the presence of NUpAGE® MES or MOPS SDS Running Buffer. SeeBlue Plus2 pre-strained size standard (1kb) (Invitrogen, Paisley, UK) was used as a basis for protein molecular weight comparison. Once the samples had been fractionated they were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, County Durham, UK) for a 30V constant for 2 hours to allow high protein absorption. The protein-loaded PVDF membrane was then washed for 15 minutes three times in Tris-buffered saline (20mmolL^{-1} Tris pH7.5, 150mmolL^{-1} NaCl) containing 0.1% (v/v) Tween-20 (TBST; Sigma Aldrich, Poole, UK).

2.9.4 **Immunoblotting**

Immunoblotting was the analytical technique used to detect proteins. Initially, membranes were blocked in 5% (w/v) non-fat dry milk suspended in TBST at room temperature under gentle agitation to prevent interactions between the membrane and primary antibody used for detection of target protein. Following this, membranes were washed as before in TBST and were exposed to primary antibody diluted in 5% (w/v) bovine serum albumin (BSA; Sigma Aldrich, Poole, UK) in TBST overnight at 4^{o}C. See Table 2-4 for optimised antibody concentrations. Subsequently, membranes were washed in TBST before addition of horse-radish peroxidise (HRP) conjugated secondary antibody diluted in 5% (w/v) non-fat dry milk-TBST for 1 hour at room temperature. Again membranes were thoroughly washed in TBST to limit background activity. Protein was visualised using the enhanced chemiluminescent detection system (ECL-detection system; Amersham Bioscience UK Ltd. Buckingham, UK). Briefly, membranes were exposed for 1 minute to a 1:1 dilution of ECL solution mix, dry blotted and placed in a light sensitive cassette. General purpose Kodak X-ray photographic film was used to develop and visualise proteins.

2.9.5 **Quantitative Analysis of Protein**

α-tubulin was used as a loading control where the molecular weight did not overlap with the size of previously analysed proteins. Equal loading was assessed by densitometrical analysis and performed using TotalLab TL100 software via calculation of protein:α-tubulin ratio.

Table 2-4: Table of antibodies used for immunoblotting.

Primary antibodies were applied to membrane overnight at 4oC in 5% (w/v) BSA-TBST. Secondary antibodies were applied for 1 hour at room temperature in 5% (w/v) non-fat dry milk-TBST

Reactive Protein	Molecular weight	Primary antibody dilution	Supplier (Catalogue Number)	Antibody Origin	Secondary antibody dilution
BMPR2	*115kDa*	*1:500*	*BD Transduction Laboratories*	*Mouse monoclonal*	*1:5000*
ERa	*66kDa*	*1:250*	*Santa Cruz (sc-7207)*	*Mouse monoclonal*	*1:5000*
ERβ	*63kDa*	*1:1000*	*Cell Signalling Technology #5513 (discontinued)*	*Rabbit polyclonal*	*1:5000*
ERβ	*55kDa*	*1:1000*	*Abcam (ab3577)*	*Rabbit monoclonal*	*1:5000*
GPER	*55kDa*	*1:250*	*Abcam (ab39742)*	*Rabbit polyclonal*	*1:5000*
a-tubulin	*50kDa*	*1:5000*	*Abcam (ab7291)*	*Mouse monoclonal*	*1:10,000*

2.10 mRNA Expression

2.10.1 Mouse Lungs

One lobe dissected from the right lung was lysed in 700µl QIAzol Lysis Reagent (QIAGEN®, Manchester, UK). Briefly the lung lobe was placed in an RNase free 2ml tube with one 5mm stainless steel ball and homogenised using a QIAGEN® TissueLyser II for 2 minutes (4 x 30 second intervals).

2.10.2 Human PASMCs

Human PASMCs (passage 3-5) were seeded in 6 well plates at a density of 25,000 cells per well and grown to 90% confluency in 10% (v/v) FBS DMEM. Cells were washed three times with cold PBS to terminate experiment and 700µl QIAzol Lysis Reagent was then added directly to the cells for lysis. Cells in QIAzol were transferred to RNase free tubes for storage at -80^{o}C until further use.

2.10.3 RNA Extraction

RNA was extracted from mouse lungs and human PASMCs using the QIAGEN® RNeasy Mini Kit (QIAGEN®, Manchester, UK). Tubes were brought to room temperature allowing dissociation of nucleoprotein complexes. Chloroform was then added directly to the QIAzol and shaken vigorously to encourage phase separation in the subsequent step. Samples were then centrifuged for 15 minutes at 10,000rpm at 4^{o}C to separate RNA from DNA, and organic proteins and lipids. The upper colourless phase containing the RNA was transferred into a clean RNase free tube and 1.5 volumes of 100% ethanol were added to clean the RNA by gentle pipette mixing. Samples were then transferred to a spin column and centrifuged at room temperature for 15 seconds at 10,000rpm. No RNA extraction is completely sufficient at eliminating DNA therefore a DNase digestion was performed for a more complete DNA removal. Samples were washed in RWT Buffer and then treated with DNase I in RDD Buffer (QIAGEN®, Manchester, UK) for 30 minutes. Another RWT Buffer wash was performed before the RNA extraction was continued. Samples were washed in RPE Buffer twice at room temperature and to eliminate carryover of any buffer the spin column was then transferred to a 1.5ml tube. 30µl of RNase free water was

added directly onto the spin column and samples were then centrifuged at 10,000rpm for 1 minute at room temperature. RNA integrity and quantification was then assessed immediately using a NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies, Delaware, USA). Absorbance of RNA was quantified at 260nm and 280nm, and the 260/280 ratio was calculated. A 260/280 ratio of ≥2.0 was achieved in all samples and was indicative of RNA purity.

2.10.4 cDNA Synthesis

High Capacity cDNA Reverse Transcription Kits (Life Technologies, Paisley, UK) were used for reverse transcription (RT) of total RNA to single stranded cDNA. A 20µl reaction was used with 500ng of RNA. A master-mix containing dNTPs, random hexamers and RNase inhibitor, supplied in the kit, was added to the RNA in a 96 well plate and the following cycling conditions were used for amplification of cDNA: 10 minutes at 25^{o}C, 30 minutes at 48^{o}C, 5 minutes at 95^{o}C and 12^{o}C forever.

2.10.5 Quantitative Real Time-PCR

Quantitative real time-PCR (qRT-PCR) was used to validate mRNA expression using TaqMan® Gene Expression probes (Applied Biosystems, Paisley, UK). See Table 2-5 for Assay IDs. mRNA & long non-coding RNA probes (Life Technologies, Paisley, UK) consisted of unlabelled PCR primers and a TaqMan® probe with a FAM® or VIC® dye label on the 5' end and a nonfluorescent quencher on the 3' end. Samples were loaded at 1ug cDNA in triplicate using a 384-well plate format. To assess for reagent and reaction contamination a no template control (NTC) was used for each gene tested in triplicate per experiment. A Viia 7 machine (Life Technologies, Paisley, UK) was used to assess gene expression using the following cycling conditions: 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, then 50 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 minute. Gene expression was defined by the cycle threshold (Ct value) inversely proportional to the amount of target nucleic acid in the sample. Ct values were accepted ≤ 36 given technical replicate efficiency and were indicative of positive reactions. The data is represented as the relative quantification (RQ; $2^{-\Delta\Delta Ct}$) or fold change compared to the calibrator.

Table 2-5: TaqMan gene expression assay IDs supplied by Applied Biosystems.
AR=androgen receptor; BMPR2= bone morphogenetic protein receptor-II; ESR1=estrogen receptor alpha; ESR2= estrogen receptor beta; Id=Inhibitor of DNA binding; Srd5a1=5α-reductase type I.

Gene	Species	Assay ID
AR	*Mouse*	*Mm00442688_m1*
BMPR2	*Mouse*	*Mn00432134_m1*
ESR1	*Mouse* *Human*	*Mm00433149_m1* *Hs00174860_m1*
ESR2	*Mouse* *Human*	*Mm00599821_m1* *Hs01100353_m1*
GPER	*Mouse* *Human*	*Mm02620446_m1* *Hs01922715_s1*
GAPDH	*Mouse* *Human*	*Mm99999915_g1* *Hs03929097_g1*
Id1	*Mouse*	*Mm00775963_g1*
Id3	*Mouse*	*Mm01188138_g1*
Smad1	*Mouse*	*Mm00484723_m1*
Srd5a1	*Mouse*	*Mm00614213_m1*

2.11 Plasma Testosterone Levels in Mice

2.11.1 Preparation of Mouse Plasma Samples

Immediately following necropsy, approximately 1ml of whole blood was removed from the open chest of each mouse and stored in a 1.5ml tube. Whole blood was left undisturbed at 4°C for 30 minutes. Samples were then centrifuged for 15 minutes at 4000rpm in a refrigerated centrifuge at 4°C, the resulting supernatant is designated plasma. Plasma samples were immediately transferred to a clean tube and stored at -80°C until required.

2.11.2 ELISA

A testosterone ELISA (R&D Systems™, Abingdon, UK) was performed in mouse plasma samples following castration to confirm the successful depletion of circulating testosterone levels. The assay is based on a competitive binding technique using a monoclonal antibody specific for testosterone. Plasma samples were diluted 10-fold using calibrator diluent provided. Reconstituted testosterone standard was utilised at concentrations between 10ngml^{-1} to 0.041ngml^{-1} (3-fold dilutions) with 10ngml^{-1} standard serving as the high standard and the calibrator diluent alone serving as the zero standard (B_0) (0ngml^{-1}). Briefly, primary antibody solution was added to each well of the microplate for 1 hour at room temperature under gentle agitation. For non-specific binding (NSB) confirmation, 4 wells received no antibody incubation. Primary antibody was then aspirated and all wells of the microplate received a thorough wash four times using wash buffer provided. 100µl standard, control (R&D Systems™, Abingdon, UK) or sample was added to each well (in triplicate) and 100µl calibrator diluent was then added to NSB and to the zero standard (B_0) wells. 50µl testosterone conjugate was added to each well and incubated for 3 hours at room temperature under agitation at 500rpm on a horizontal orbital microplate shaker. Following incubation, all wells were washed 4 times with wash buffer and substrate solution was added to each well. At this stage the microplate was concealed and protected from light for 30 minutes at room temperature. Stop solution was subsequently applied and the plate was promptly analysed using a microplate reader set to 450nm.

2.11.3 Standard Curve Analysis

Triplicate readings for each standard, control and sample were averaged and the NSB optical density was subtracted. A standard curve was created by plotting mean absorbance for each standard against the concentration on a logarithmic x-axis and a best-fit curve was obtained. $\%B/B_0$ was calculated by dividing corrected optical density for each standard or sample by the corrected B_0 optical density and multiplying by 100.

2.12 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 5.0 Inc© software.

Unpaired student's t-tests were used for comparing two treatment groups. One-Way ANOVA was employed when two or more independent groups were compared, followed by Bonferroni's or Tukey's post hoc-test where appropriate. Two-Way ANOVAs were used to analyse all in vivo experiments where there was more than one independent variable and multiple observations for each variable, for example, normoxic/hypoxic and vehicle/MPP treatment. Two-Way ANOVAs were followed by a Bonferroni's post hoc-test. All data are expressed as mean ± standard error of mean (SEM).

Chapter 3.

Development of Pulmonary Arterial Hypertension in Females is Estrogen Receptor Alpha Dependent

3.1 Introduction

The incidence of pulmonary arterial hypertension (PAH) is higher in females (Badesch et.al. 2010; Humbert et.al. 2006; Ling et.al. 2012). For example, the female to male ratio is currently reported in the largest REVEAL Registry as approximately 4.1:1 in idiopathic pulmonary arterial hypertension (IPAH) and 3.8:1 in associated PAH (APAH) (Badesch et.al. 2010). However, despite an increased prevalence in females, females with PAH have better right ventricular function and survival compared to males (Benza et.al. 2012; Humbert et.al. 2010a; Shapiro et al. 2012). Reasons for these gender differences remain unclear. A role for gender-associated factors, such as sex hormones, and in particular estrogens, has been proposed to play a pivotal role in PAH pathogenesis.

Until recently, animal models have offered limited insight into gender differences in PAH owing to the 'estrogen paradox' whereby female rodents exhibit less severe hypoxic and monocrotaline PAH compared to males (Benza et.al. 2012; Humbert et.al. 2010a; McMurtry et al. 1973; Rabinovitch et.al. 1981; Resta et.al. 2001; Shapiro et.al. 2012). In addition, estrogen or estrogen metabolites can protect male or ovariectomised female rats against monocrotaline and hypoxia induced PAH (Benza et.al. 2012; Farhat et al. 1993; Lahm et.al. 2012a; Shapiro et.al. 2012; Yuan et.al. 2013). However, there is converging clinical evidence suggesting that the estrogen pathway is a major risk factor in females with PAH (Morse et al. 1999; Pugh & Hemnes 2010). Polymorphisms in aromatase (CYP19A1) are associated with higher estrogen levels and an increased risk of PAH development in female patients with advanced liver disease (Roberts et al. 2009b). In line with this, physiological concentrations of estrogen mediate proliferation of human pulmonary artery smooth muscle cells (PASMCs) (White et.al. 2011). Experimentally, in three serotonin-dependent models of pulmonary hypertension (PH) there is a female susceptibility related to endogenous circulating estrogen (Dempsie et.al. 2011; Dempsie et.al. 2013; White et.al. 2011). In addition, conversion of estrogen to active metabolites via cytochrome P450 1B1 (CYP1B1) mediates ER-independent effects of estrogen. Altered estrogen metabolism is implicated in PAH

development and CYP1B1 is expressed at elevated levels in lungs from IPAH and heritable (HPAH) female patients (White et al. 2012). Furthermore, CYP1B1 has been identified as a modifier gene in bone morphogenetic protein receptor type 2 (BMPR2) affected female HPAH patients inferring an increased disease risk (Austin et.al. 2009; West et.al. 2008).

Dysfunctional BMPR2 signalling is recognised to play a pivotal role in the development of PAH as mutations in BMPR2 are responsible for ~80% of HPAH cases (Machado et.al. 2009). Penetrance for this gene is incomplete however; as only 20% of BMPR2 mutation carriers manifest PAH (Newman et.al. 2004). Therefore it is assumed additional risk factors, genetic or environmental, are involved. Female gender increases the penetrance of BMPR2 mutations in HPAH (Machado et al. 2009) and consistent with this BMPR2 gene expression is higher among control males compared to females (Austin et al. 2012). BMPR2 may be a target of ERα (Rajkumar et.al. 2010) and it has been shown that BMPR2 expression is suppressed via this receptor (Austin et al. 2012) giving a functional role for estrogen and ERα in PAH.

The effects of estrogen are primarily mediated by activation of estrogen receptor ERα and ERß, and these effects can be both genomic and non-genomic. Very rapid, non-genomic effects of estrogen have also been described through activation of the G-protein coupled receptor, GPER. ERα and ERß antagonists have been shown to protect against hypoxic PH and monocrotaline PH in male rats (Lahm et al. 2012b; Umar et al. 2011b) and indicate both receptors play a functional role in the lung during PH. The potential roles that ERs play in the development of PH in ovary intact females, however, have yet to be established.

The aims of this study were to evaluate gender differences in estrogen receptor expression and to characterise the influence of endogenous estrogen activity at ERα and ERß in experimental models of PH. We hypothesised an ER dependent mechanism for estrogen mediated PH in females and suggest that this is a result of altered ER expression and signalling. Moreover we identify novel gender-specific BMPR2 pathways by which ERα mediates the development of chronic hypoxic PH in female mice and spontaneous PH in the $SERT^{+}$ female mouse model. In addition, we hypothesise that our results are therapeutically relevant by using ovary intact females.

3.2 Results

3.2.1 ERα and ERβ are Located in Human Pulmonary Vasculature and Expression is Altered in PAH

ER localisation was investigated in human lung from non-PAH control and PAH patients by immunohistochemistry (*see Methods: 2.5.2 Immunolocalisation of estrogen receptors in human lung*). In females, both ERα and ERβ expression was prominent in pulmonary arteries in both non-PAH control and PAH patients (Figure 3-1). ERα was localised to adventitia, smooth muscle cells and endothelial cells. Whilst some ERβ expression was observed in the adventitia and smooth muscle cells, expression was largely endothelial. Smooth muscle cell and endothelial cell localisation was confirmed by staining consecutive sections with alpha smooth muscle actin (α-SMA) and von Willebrand Factor (vWF), respectively.

Given the expression of both ERs in smooth muscle cells in human lung, we investigated the ER expression (both protein and mRNA) in isolated human PASMCs from female idiopathic (IPAH) and heritable (HPAH) PAH patients. ERα was significantly elevated in human PASMCs from HPAH patients relative to non-PAH controls (Figure 3-2A and B). Additionally, ERβ levels at protein and transcript levels were unchanged in control versus both IPAH and HPAH patients (Figures 3-2C and D).

3.2.2 Males and Females Express Different Levels of Estrogen Receptors in PASMCs

We thought it important to also investigate levels of ERs in females compared to males in isolated human PASMCs from non-PAH control and PAH patients. Levels of both ERα and ERβ were unchanged between male and female non-PAH controls (Figure 3-3A&B). Interestingly, females expressed significantly higher levels of ERα relative to males in PAH (Figure 3-4A). Conversely, ERβ was significantly less in females PAH PASMCs compared to males (Figure 3-4B).

3.2.3 ERα and ERβ are Expressed in Mouse Lung and Pulmonary Arteries

Following the observation of altered ER expression in human lung in non-PAH control and PAH patients, ER expression was investigated in the female and male hypoxic mouse model. In pulmonary arteries from female hypoxic mice there is a significant increase in the protein levels of ERα relative to pulmonary arteries from normoxic control mice (Figure 3-5A). mRNA transcript levels for ESR1, the gene encoding ERα, were unchanged between normoxia and hypoxia (Figure 3-5B). In contrast, ERβ protein was significantly reduced in pulmonary artery in hypoxia although ESR2 transcript levels were unchanged in whole lung (Figure 3-5C&D). Both ERα and ERβ protein expression was unchanged in the pulmonary arteries from hypoxic male mice (Figure 3.6A&B).

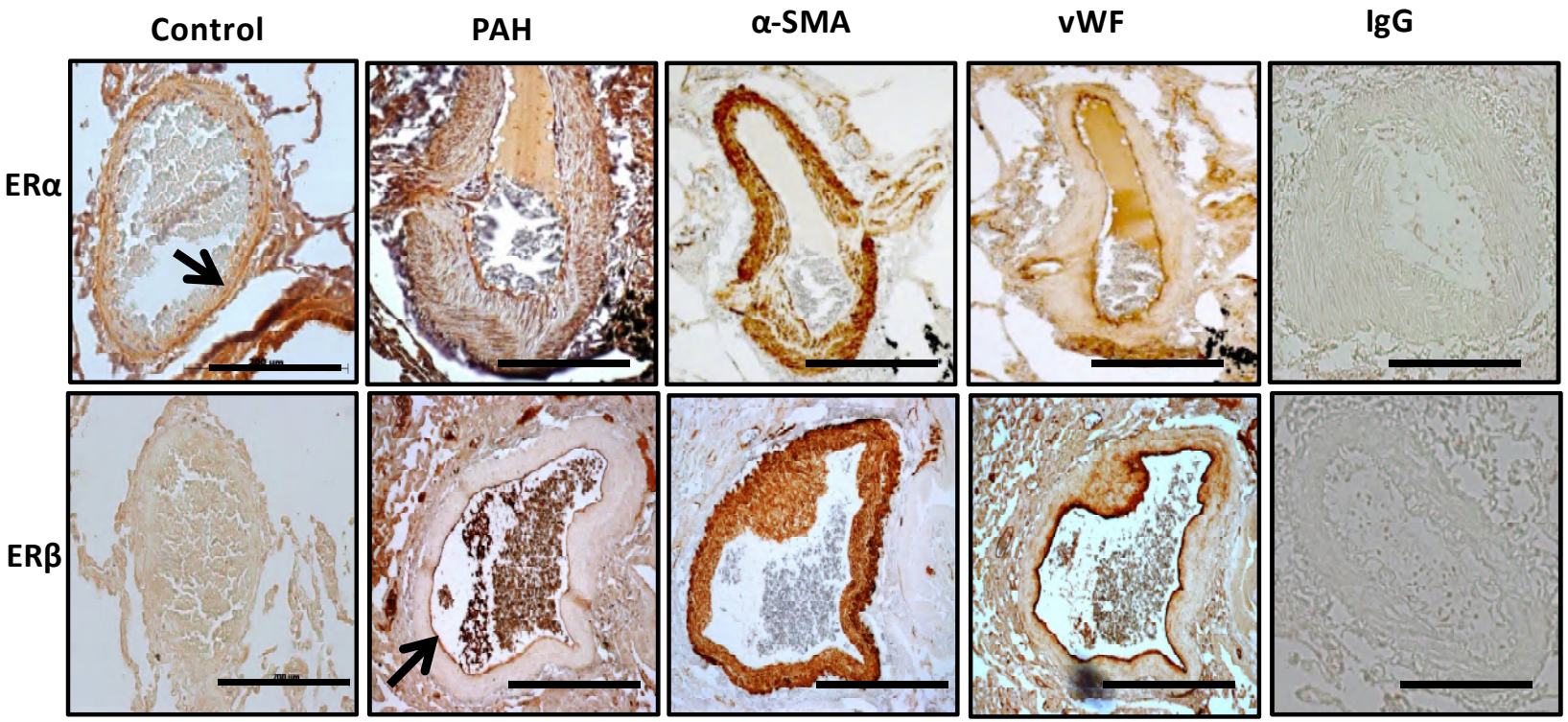


Figure 3-1: Immunolocalisation of ERα and ERβ in human non-PAH controls and PAH patient lung sections.
ERα and ERβ stained dark brown. Consecutive sections stained with α-SMA and vWF depicts smooth muscle and endothelial cell staining, respectively. α-SMA= alpha smooth muscle actin; vWF= Von Willebrand Factor. Arrows indicate smooth muscle and endothelial cell localisation. Patient information for controls and PAH patients are shown in *Materials and Methods section Table 2.1.* Scale bar=200μm.

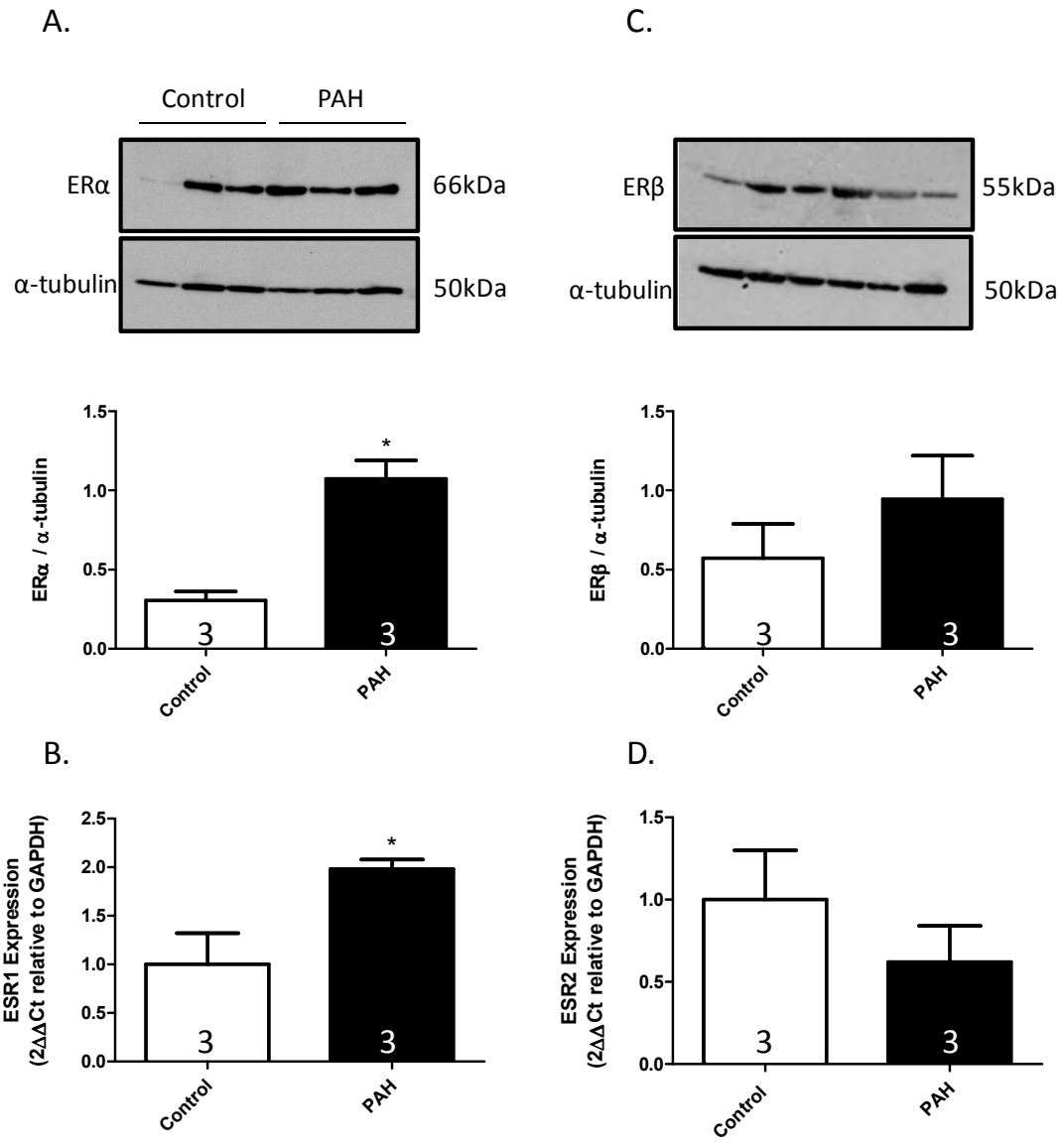


Figure 3-2: Estrogen receptor -α and –β expression in control human female PASMCs compared to PAH human female PASMCs.
ERα protein (A) and mRNA transcript (B) was significantly up-regulated in PAH human PASMCs compared to controls whereas ERβ protein (C) and mRNA transcript (D) was unaffected. Representative blots are shown for ERα and ERβ. Quantitative data are shown as ± SEM and analysed using an unpaired t-test. *$p<0.05$ vs. control. Patient information for three individual controls (controls 1-3), and three individual PAH patients (patients 1-3) are shown in *Materials and Methods section: Table* 2-3 n=3 individual patients/controls in each blot and the whole blot repeated in triplicate. n of patients is indicated in each individual bar.

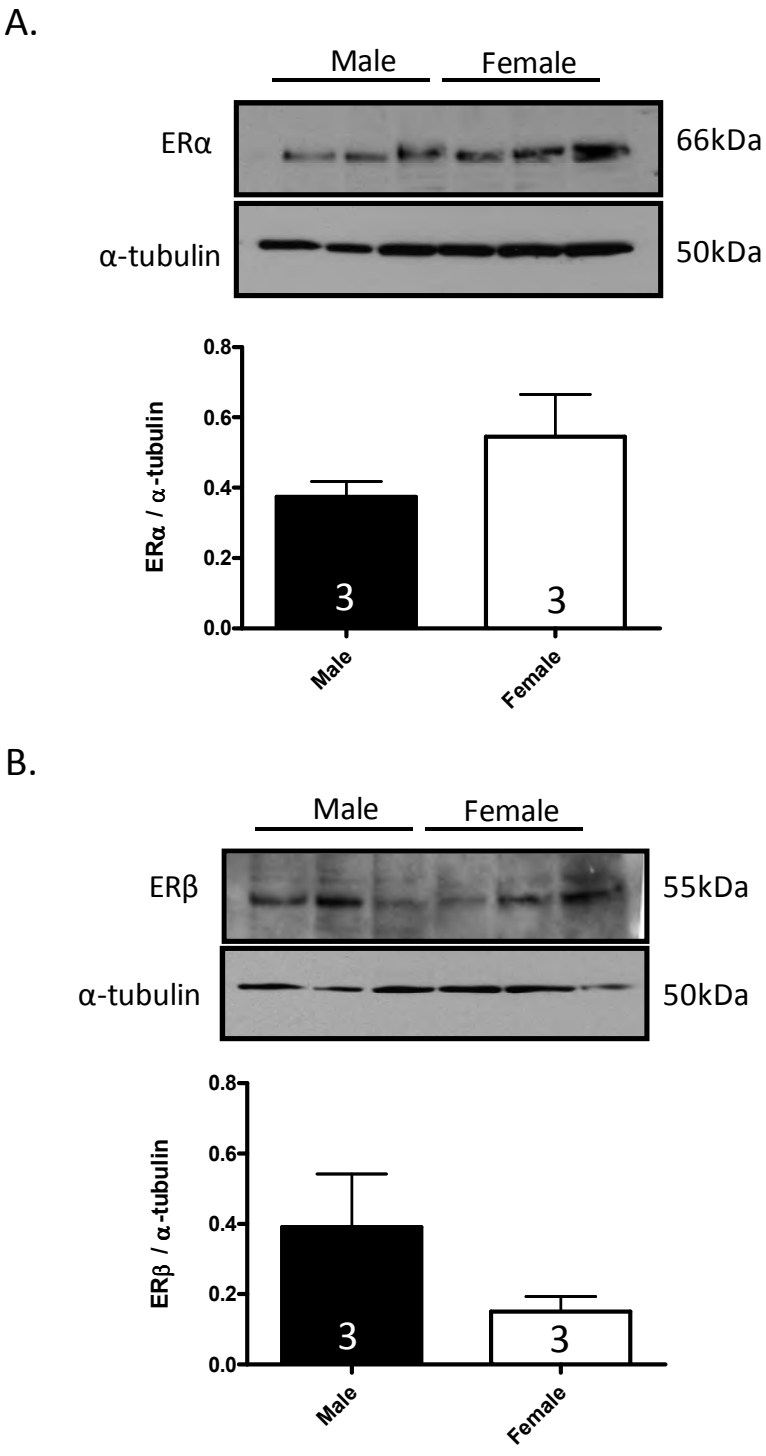


Figure 3-3: Comparison between ER expression in male and female human PASMCs from control non-PAH patients.
ERα protein (A) and ERβ protein (B) were unchanged between female human PASMCs compared to males. Representative blots are shown for ERα and ERβ. Quantitative data are shown as ± SEM and analysed using an unpaired t-test. Patient information for females (control 1-3), and male non-PAH controls (controls 1-3), are shown in *Materials and Methods section: Table* 2.3. n=3 individual patients repeated in triplicate. n is indicated in each individual bar.

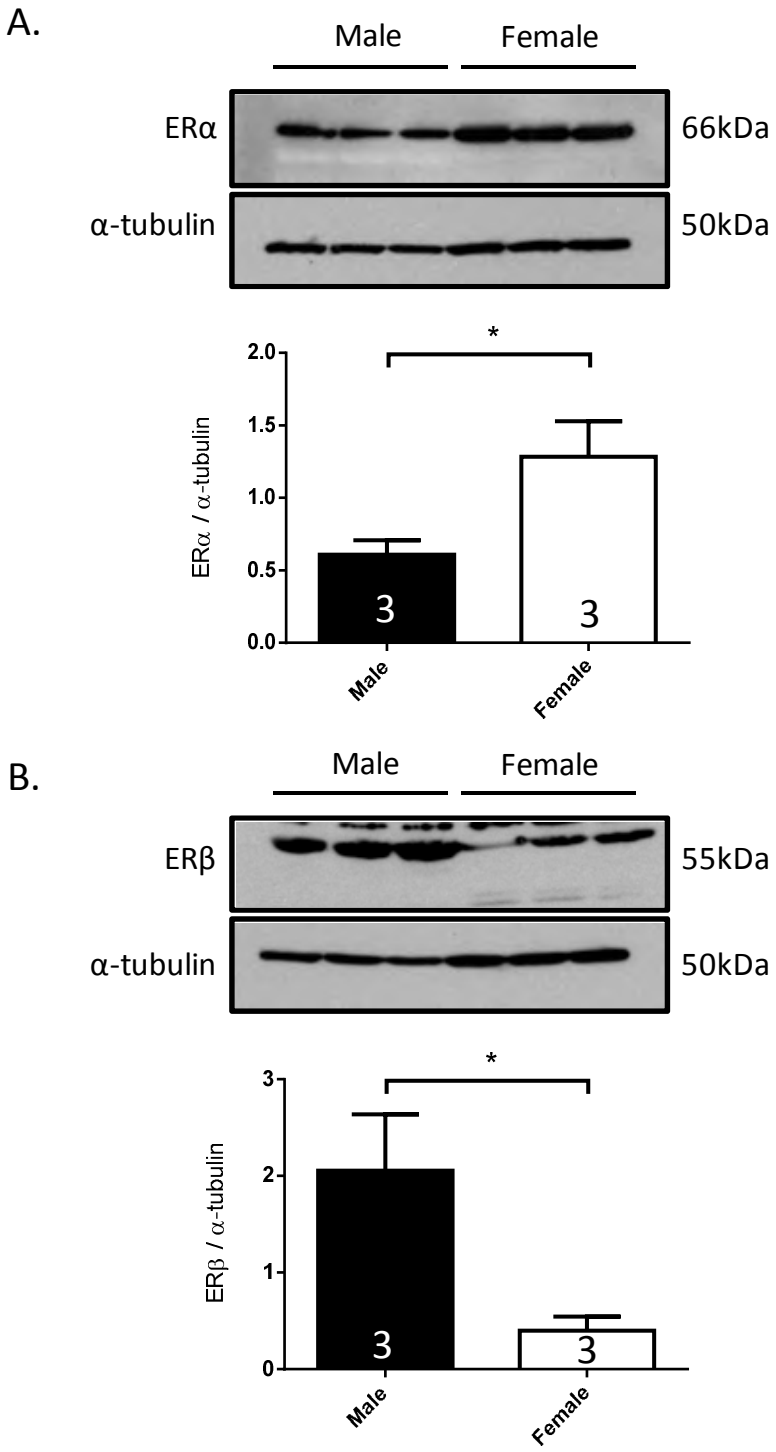


Figure 3-4: Comparison between ER expression in male and female human PASMCs from PAH patients.
ERα protein (A) was significantly higher in female human PASMCs compared to males. ERβ protein (B) was significantly reduced in female human PASMCs compared to males. Representative blots are shown for ERα and ERβ. Quantitative data are shown as ± SEM and analysed using an unpaired t-test. *p<0.05 vs. control. Patient information for females (patients 1-3), and male patients (patients 1-3), are shown in *Materials and Methods section: Table* 2.3. Females: n=3 individual patients repeated in triplicate. n is indicated in each individual bar.

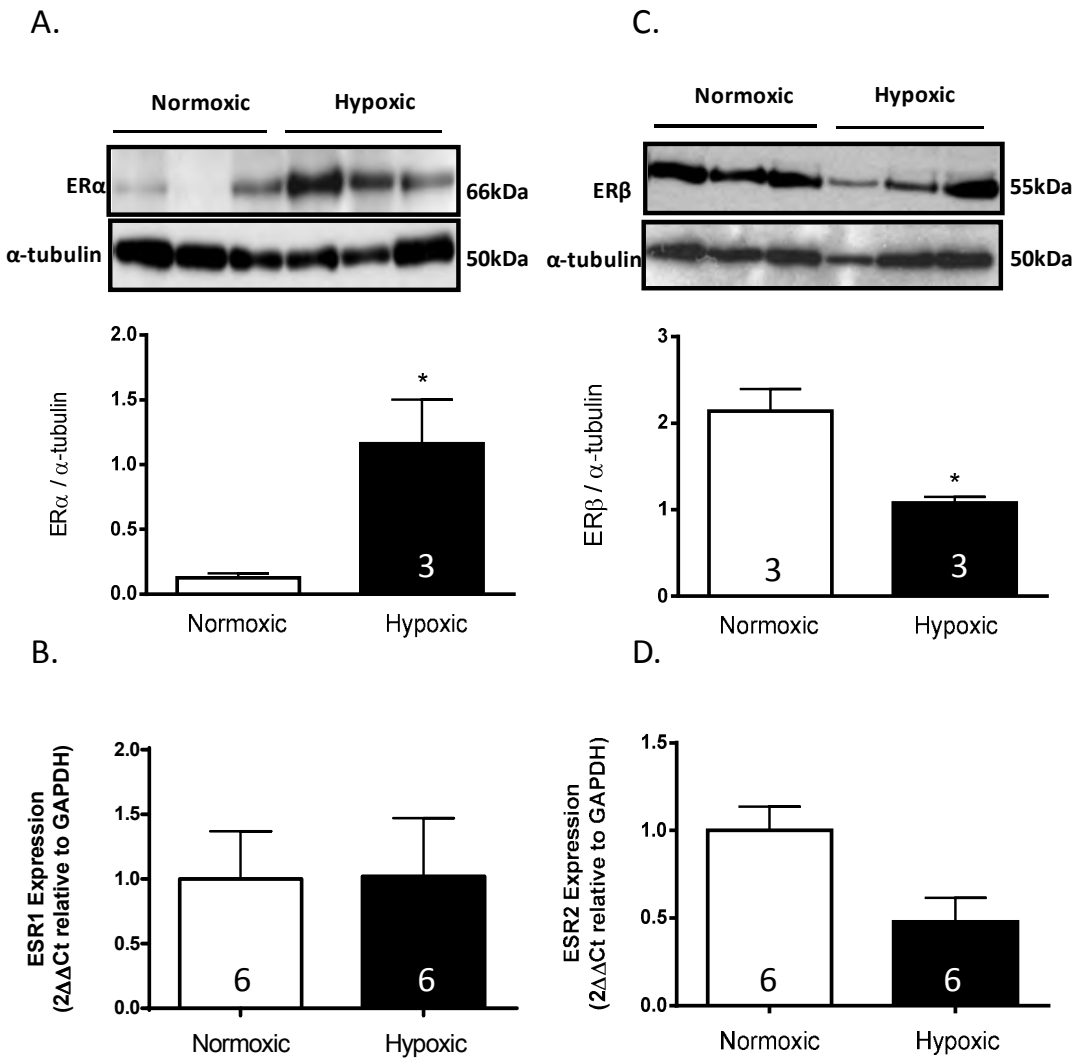


Figure 3-5: Estrogen receptor expression is altered in female mouse pulmonary artery in hypoxia.

ERα protein in pulmonary artery (A) and mRNA transcript in whole lung (B) was significantly up-regulated in hypoxia whereas ERβ protein in pulmonary artery (C) and mRNA transcript in whole lung (D) was significantly reduced. Representative blots are shown for ERα and ERβ. Quantitative data are shown as ± SEM and analysed using an unpaired t-test. *$p<0.05$ vs. control. n=3 pulmonary artery repeated in triplicate for each Western experiment; qRT-PCR, n=6 mouse whole lung samples repeated in triplicate. n of pulmonary artery/lung is indicated in each individual bar.

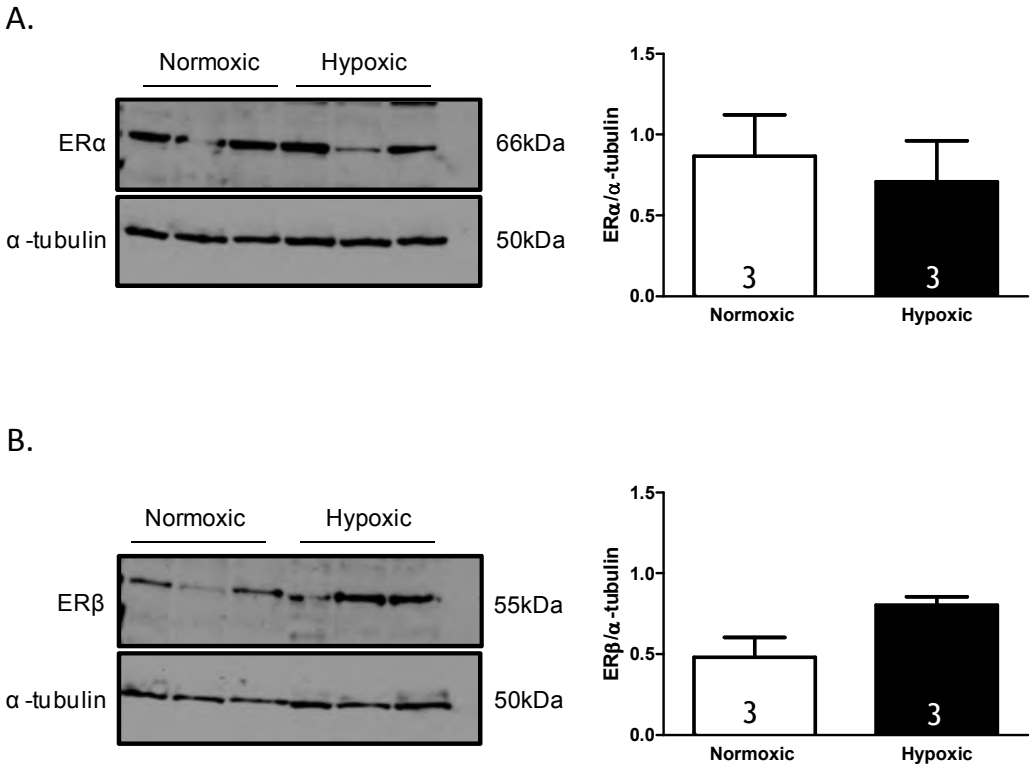


Figure 3-6: Estrogen receptor expression is unchanged in male mouse pulmonary artery in hypoxia.
ERα protein in pulmonary artery (A) and ERβ protein expression (B) unchanged in hypoxia in pulmonary arteries from hypoxic male mice. Representative blots are shown for ERα and ERβ. Quantitative data are shown as ± SEM and analysed using an unpaired t-test. n=3 pulmonary artery repeated in triplicate for each Western experiment.

3.2.4 **Administration of an ERα Selective Antagonist Attenuates Development of Hypoxia-Induced PH in Female Mice**

As expression of ERα was increased in pulmonary arteries from female PAH patients and hypoxic female mice, this suggests ERα may play a role in the development of PAH specific to females. To investigate this experimentally, both male and ovary intact female mice were dosed with the ERα antagonist MPP Dihydrochloride (MPP; $2mgkg^{-1}day^{-1}$) or vehicle containing pellets during exposure to normoxia or 14 days of chronic hypoxia. At the end of this 14 day period, RVSP, pulmonary vascular remodelling and RVH were assessed (*see Methods: 2.3.3 Chronic Hypoxia*). Female mice developed hypoxia induced PH; RVSP, RVH and pulmonary vascular remodelling were all increased (Figure 3-7A-C). MPP attenuated the development of hypoxic PH by reducing elevations in RVSP (Figure 3-7A) whilst there was no effect on RVH (Figure 3-7B). Representative images of RVSP traces in females are shown in Figure 3-8. Furthermore, pulmonary vascular remodelling in response to hypoxia was significantly reduced by MPP treatment (Figure 3-7C) and muscularisation of distal pulmonary arteries was noticeably reduced (Figure 3-9). The administration of MPP had no effect on the systemic circulation as indicated by no changes in mean systemic arterial pressure (mSAP) (Figure 3-10A) and heart rate (HR) (Figure 3-10B). In this instance, hypoxia resulted in an increase in HR in vehicle treated mice.

3.2.5 **Administration of an ERα Selective Antagonist has no Effect of the Development of Hypoxia Induced PAH in Male Mice**

The effect of MPP was then assessed in male mice to determine any gender differences in the response to the ERα antagonist. In contrast to the results observed in ovary intact females, MPP had no effect on the development of PH in males. MPP has no effect on RVSP, RVH and pulmonary vascular remodelling (Figure 3-11A-C). Representative images of RVSP traces in males are shown in Figure 3-12. MPP treatment also had no effect on pulmonary arterial

muscularisation in male mice (Figure 3-13). In addition, neither hypoxia nor MPP treatment had an effect on mSAP and HR in males (Figure 3-14A&B). Absolute values for haemodynamics are depicted in Table 3-1.

3.2.6 Body Weight and Uterine Weight were Unaffected by ERα Selective Antagonism

Body weight in both females and males were unaffected by hypoxia and administration of MPP (Figure 3-15A&B). Uterine weight at necropsy was also investigated as ERα knockout mice are observed to have abnormal reproductive function (Lubahn et al. 1993). However, we observed no difference in uterine weight (Figure 3-16A) or in uterine weight to body weight ratio (Figure 3-16B) between vehicle treated and MPP treated groups. In addition, hypoxia had no effect on uterine weight. Absolute values for body weight and uterine weight are shown in Table 3-2.

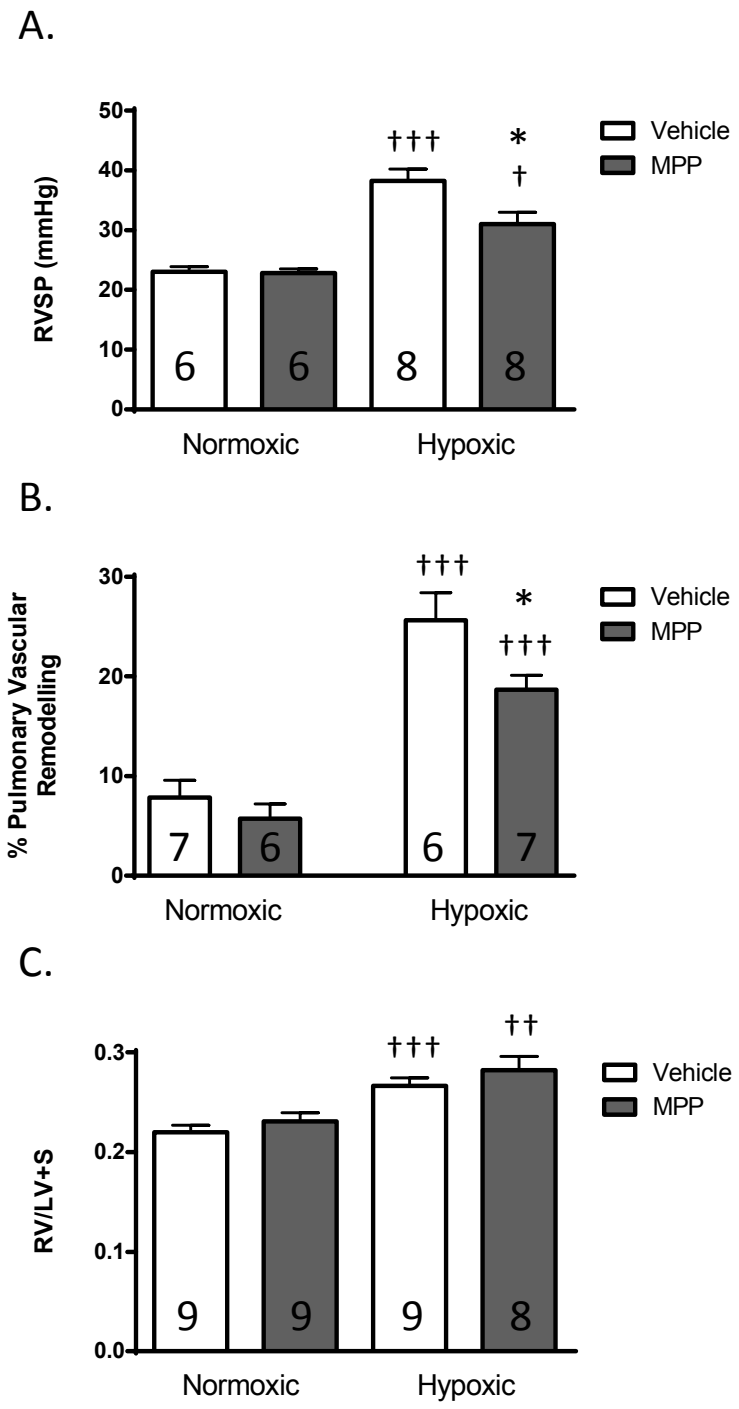


Figure 3-7: MPP (2mgkg^{-1}day^{-1}) attenuates the development of hypoxic pulmonary hypertension in female mice.
Right ventricular systolic pressure (RVSP) (A), pulmonary vascular remodelling (B) and right ventricular hypertrophy (C) assessment in female mice. Data are expressed as ± SEM analysed by Two-Way ANOVA followed by a Bonferroni's post-hoc test. † $p<0.05$; † † $p<0.01$; † † † $p<0.001$ vs. normoxic; * $p<0.05$ vs. vehicle. n=6-9 per group; n for each group is indicated on bar.

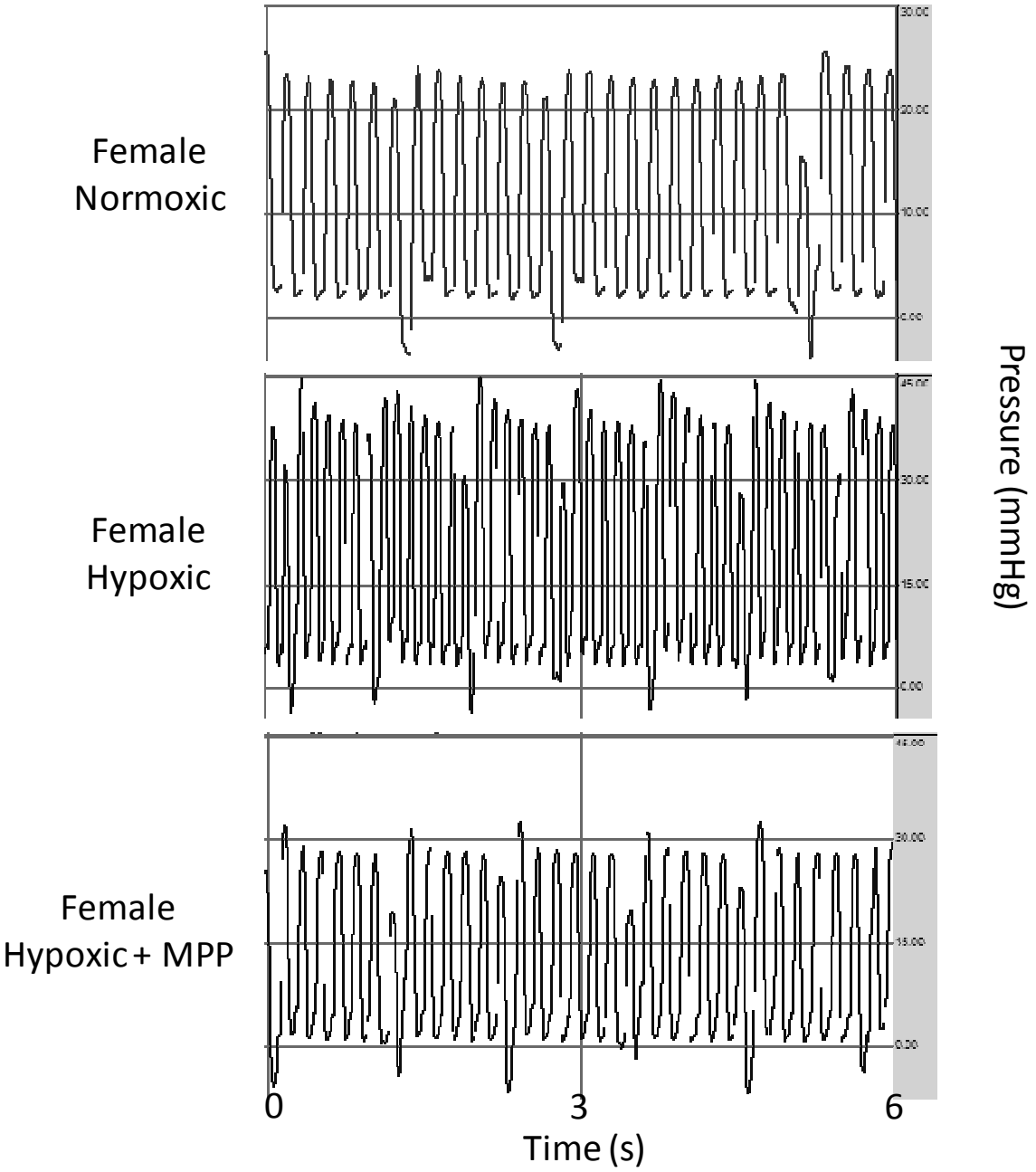


Figure 3-8: Representative RVSP traces from female mice.
Six second representative traces are shown for vehicle treated normoxic and hypoxic females, and a female mouse treated with 2mgkg^{-1}day^{-1} MPP.

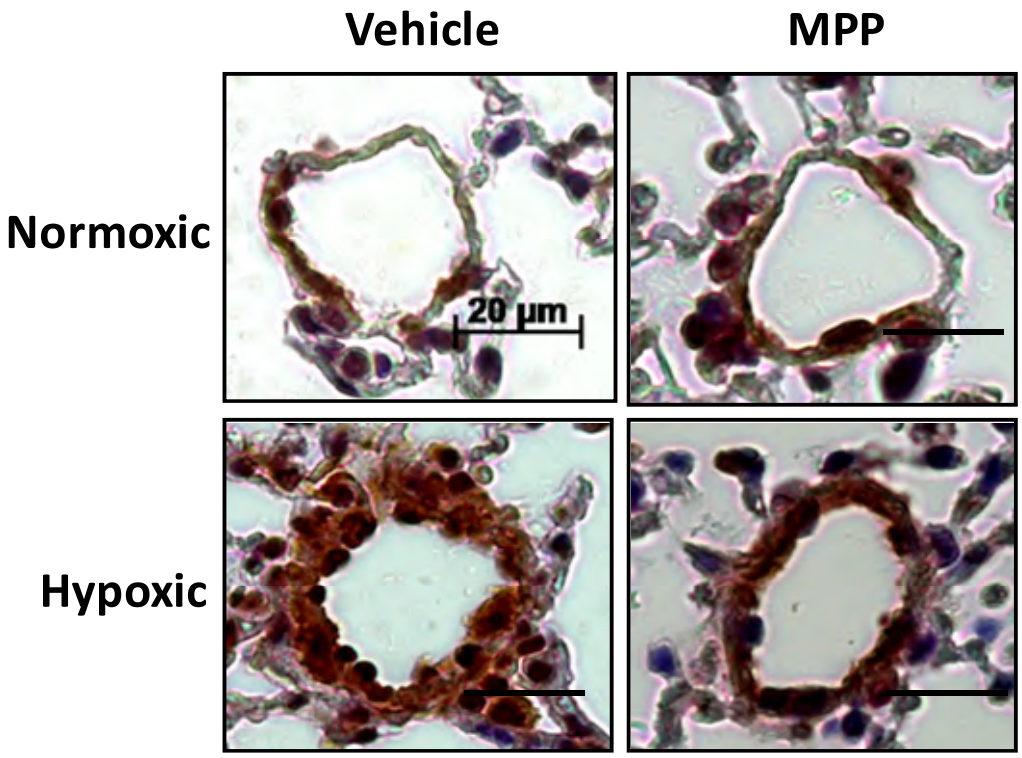


Figure 3-9: MPP (2mgkg^{-1}day^{-1}) attenuates the development of hypoxic-induced pulmonary vascular remodelling in females.
α-smooth muscle actin stains smooth muscle cells dark brown; counterstain is haematoxylin (purple/blue). Representative images are shown from each group. Scale bar = 20µm.

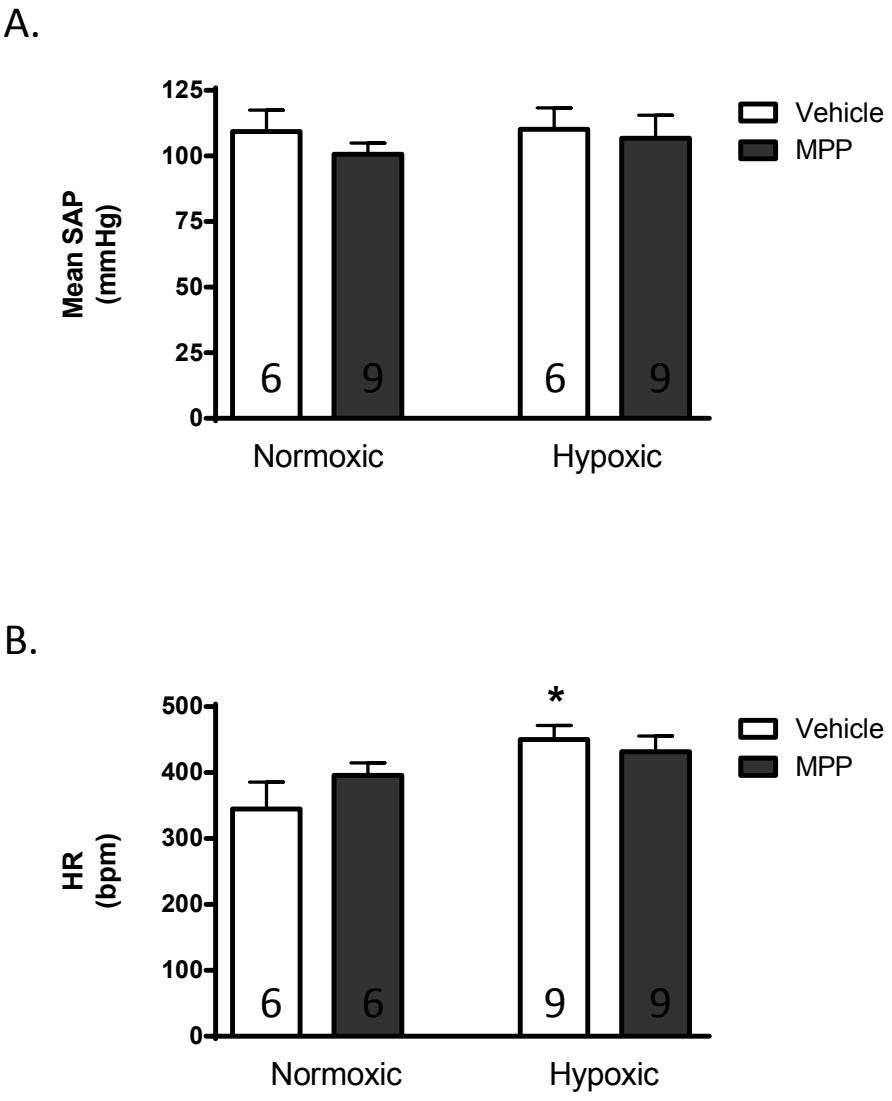


Figure 3-10: MPP (2mgkg^{-1}day^{-1}) treatment has no effect on systemic parameters in female mice.

Mean systemic arterial pressure (Mean SAP) (A) and heart rate (HR) (B) are unchanged. Data are expressed as ± SEM analysed by Two-Way ANOVA followed by a Bonferroni's post-hoc test. *p<0.05 vs. normoxic vehicle. n=6-9 per group; n of mice for each group is indicated on bar. Low heart rates observed (normal adult mouse ~500-600bpm) due to prolonged anaesthesia.

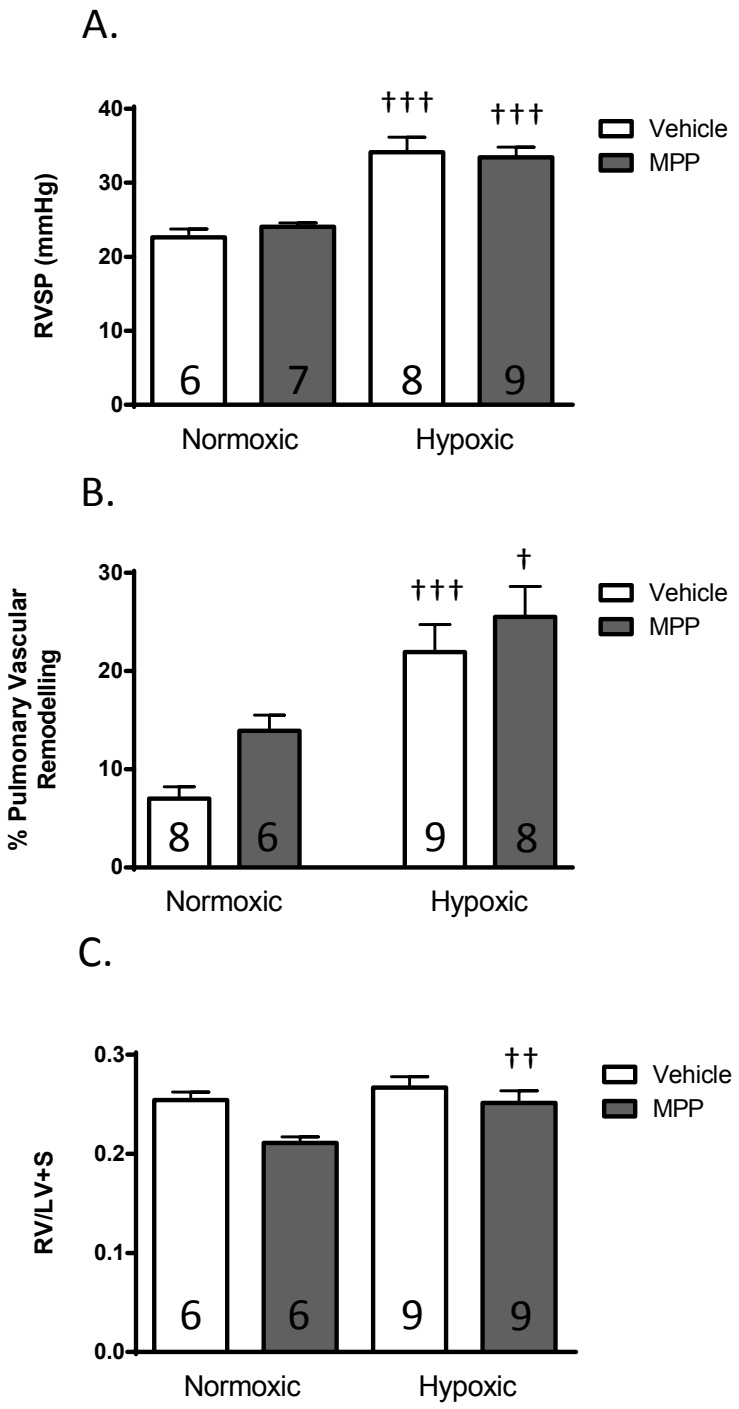


Figure 3-11: MPP (2mgkg^{-1}day^{-1}) has no effect on the development of hypoxic pulmonary hypertension in male mice.

Right ventricular systolic pressure (RVSP) (A), pulmonary vascular remodelling (B) and right ventricular hypertrophy (C) assessment in male mice. Data are expressed as ± SEM analysed by Two-Way ANOVA followed by a Bonferroni's post-hoc test. † $p<0.05$; † † $p<0.01$; † † † $p<0.001$ vs. normoxic. n=6-9 per group; n of mice for each group is indicated on bar.

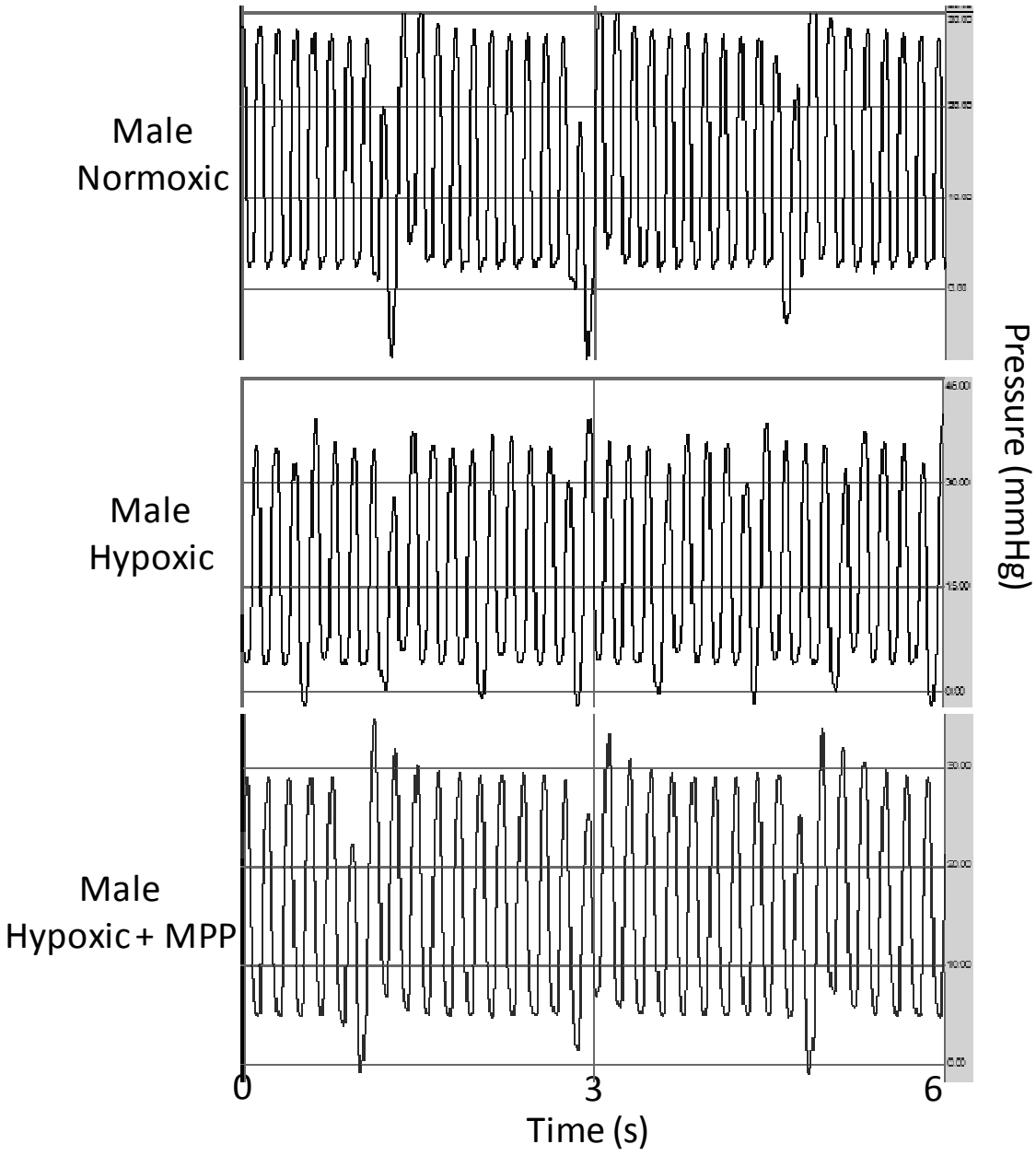


Figure 3-12: Representative RVSP traces from male mice.
Six second representative traces are shown for vehicle treated normoxic and hypoxic males, and a male mouse treated with 2mgkg^{-1}day^{-1} MPP.

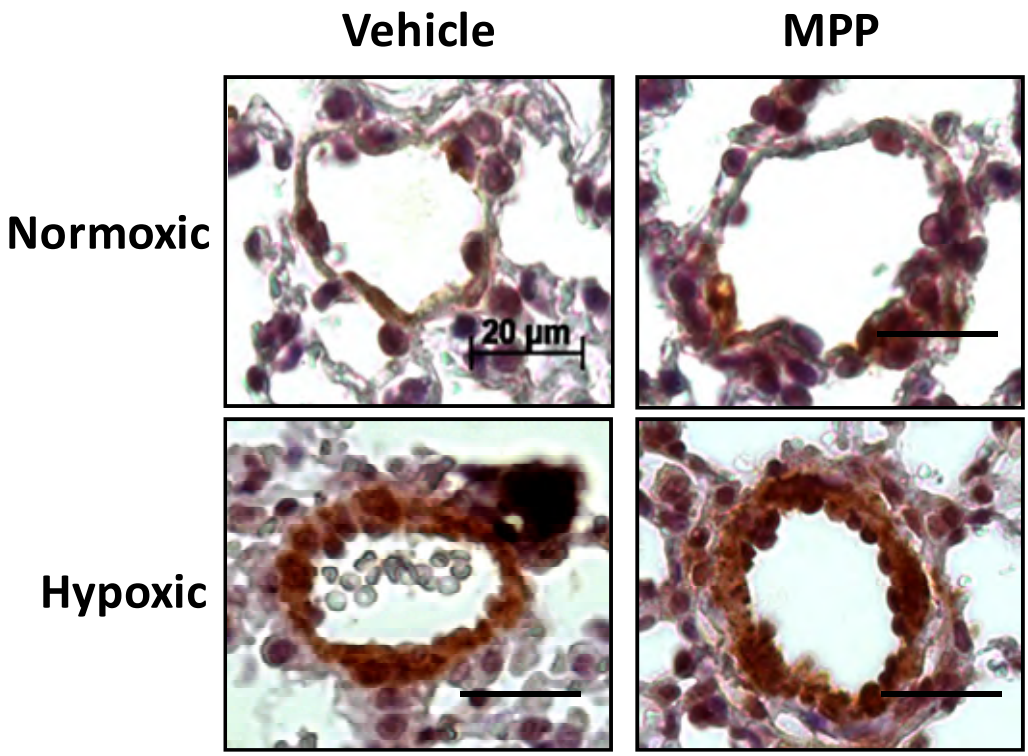
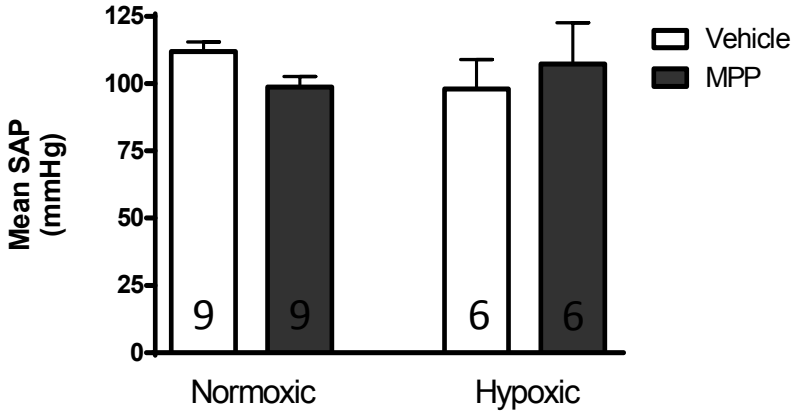


Figure 3-13: MPP (2mgkg^{-1}day^{-1}) has no effect on the development of hypoxic-induced pulmonary vascular remodelling in males.
α-smooth muscle actin stains smooth muscle cells dark brown; counterstain is haematoxylin (purple/blue). Representative images are shown from each group. Scale bar = 20µm.

A.



B.

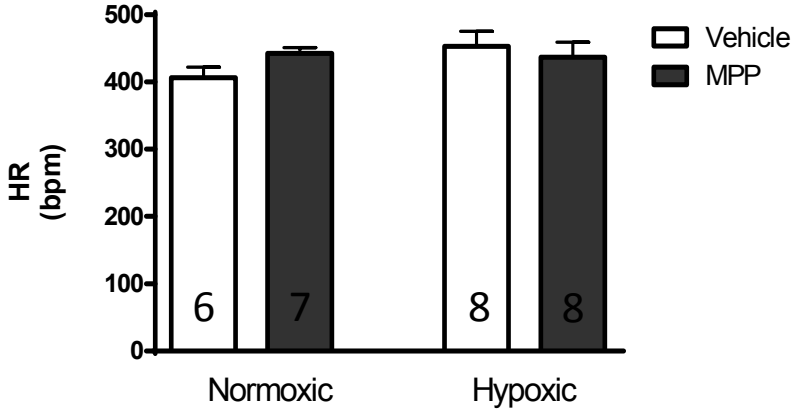
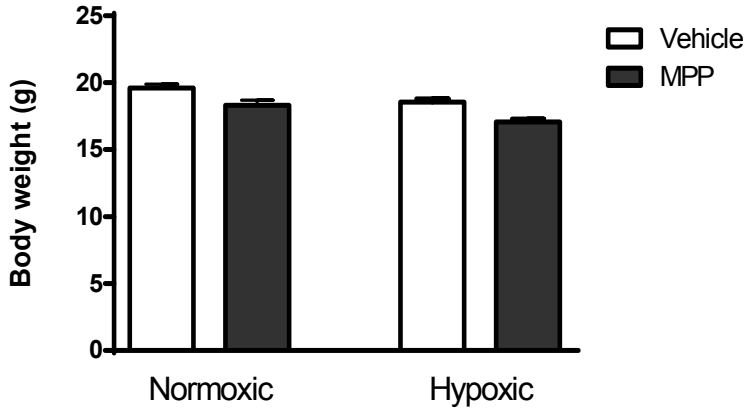


Figure 3-14: MPP (2mgkg^{-1}day^{-1}) treatment and hypoxia have no effect on systemic parameters in male mice.
Mean systemic arterial pressure (Mean SAP) (A) and heart rate (HR) (B) are unchanged. Data are expressed as ± SEM analysed by Two-Way ANOVA followed by a Bonferroni's post-hoc test. n=6-9 per group; n of mice for each group is indicated on bar.

A.



B.

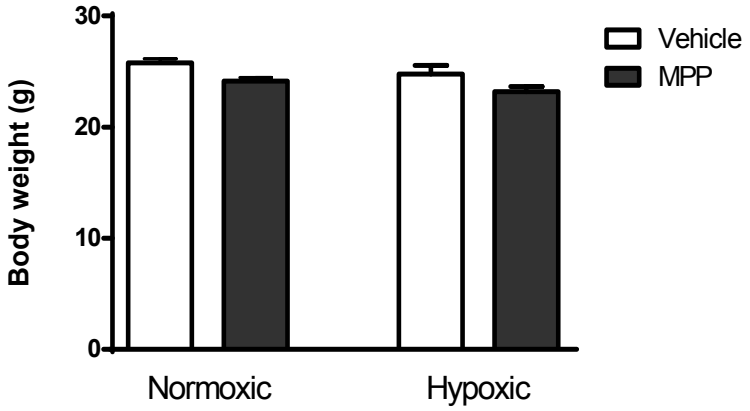


Figure 3-15: MPP (2mgkg^{-1}day^{-1}) treatment and hypoxia have no effect on body weight in female and male mice.
Body weight in female (A) and male (B) mice. Data are expressed as ± SEM analysed by Two-Way ANOVA followed by a Bonferroni's post-hoc test. n=10 mice per group.

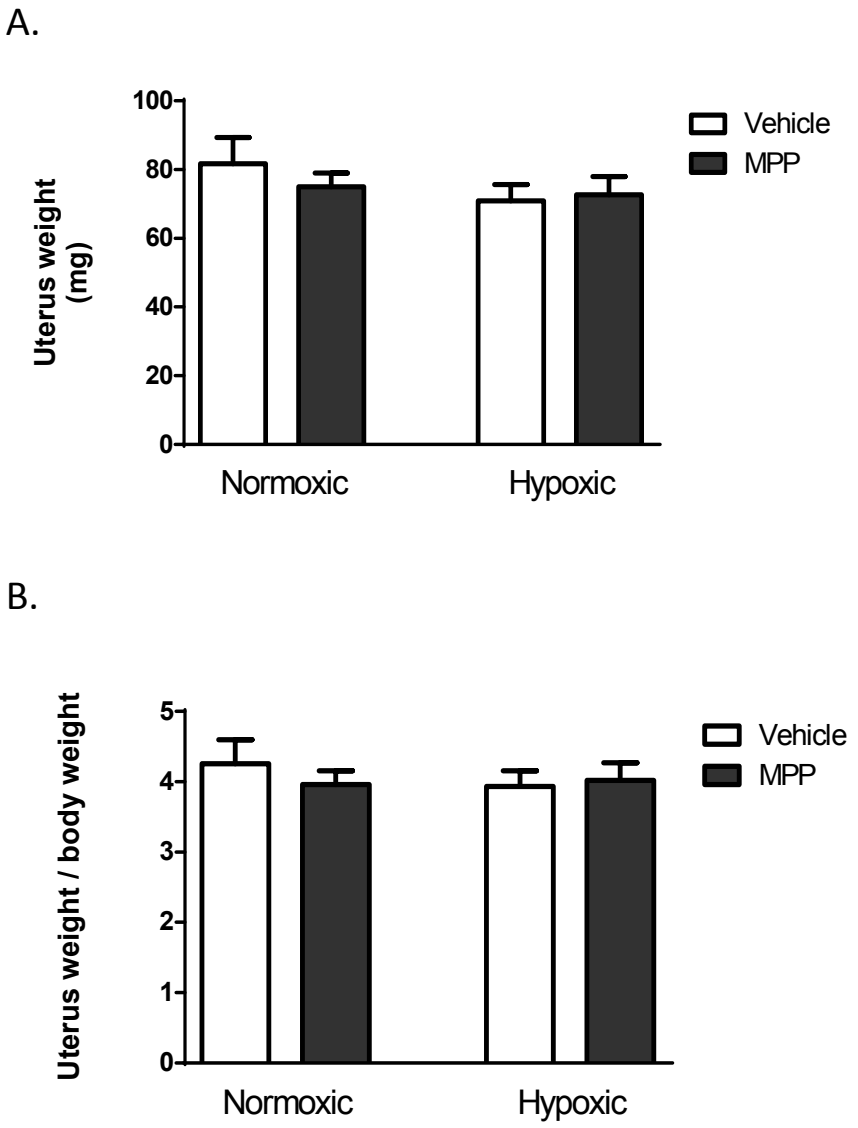


Figure 3-16: MPP (2mgkg^{-1}day^{-1}) treatment and hypoxia have no effect on uterine weight in females

Uterine weight in female mice (A) and uterus weight/body weight ratio (B) are unchanged by hypoxia and MPP treatment. Data are expressed as ± SEM analysed by Two-Way ANOVA followed by a Bonferroni's post-hoc test. n=10 mice per group.

Table 3-1: Haemodynamic parameters in male and female mice dosed with MPP (2mgkg^{-1}day^{-1}).

Right ventricular systolic pressure (RVSP); Mean right ventricular pressure (MRVP); right ventricular diastolic pressure (RVDP); systolic systemic arterial pressure (sSAP); mean systemic arterial pressure (mSAP); diastolic systemic arterial pressure (dSAP); Heart rate (HR). Data expressed as ± SEM analysed by a Two-way ANOVA followed by a Bonferroni's post hoc test. *p<0.05, **p <0.01, ***p<0.001 vs. normoxic mice. † p <0.05, †† p<0.01, ††† p<0.01 vs. vehicle dosed mice. n = 7-14 mice per group.

Parameter	Male Vehicle	Male MPP	Female Vehicle	Female MPP
Normoxic				
RVSP, mmHg	22.6 ± 1.1	24.0 ± 0.5	23.1 ± 0.8	22.8 ± 0.7
MRVP, mmHg	14.8 ± 0.6	15.3 ± 0.5	13.7 ± 0.6	13.7 ± 0.4
RVDP, mmHg	4.1 ± 0.34	4.4 ± 0.4	3.6 ± 0.5	3.7 ± 0.27
sSAP, mmHg	119.9 ± 3.6	115.6 ± 2.6	109.9 ± 10.1	108.4 ± 5.7
mSAP, mmHg	111.9 ± 3.5	98.7 ± 3.9	98.3 ± 12.9	100.7 ± 4.3
dSAP, mmHg	102.2 ± 3.5	85.5 ± 5.3	83.1 ± 14.7	86.9 ± 4.2
HR, bpm	406.6 ± 15.7	442.1 ± 8.9	344.7 ± 40.8	396.2 ± 18.5
Hypoxic				
RVSP, mmHg	34.1 ± 2.1 ***	33.5 ± 1.9 ***	38.3 ± 1.9 ***	31.0 ± 1.9 *†
MRVP, mmHg	16.1 ± 0.9	16.1 ± 0.6	18.7 ± 1.8 *	15.7 ± 1.4
RVDP, mmHg	3.4 ± 0.8	2.8 ± 0.5	5.5 ± 0.6 *	2.9 ± 0.5 †††
sSAP, mmHg	113.0 ± 11.6	144.4 ± 15.9* †	131.3 ± 8.2	118.2 ± 8.5
mSAP, mmHg	97.9 ± 107	107.4 ± 15.2	110.1 ± 8.3	106.7 ± 8.9
dSAP, mmHg	84.7 ± 12.5	136.0 ± 20.23 **††	92.1 ± 7.5	96.9 ± 10.5
HR, bpm	452.9 ± 22.3	436.7 ± 22.7	450.4 ± 20.9 *	431.9 ± 23.9

Table 3-2: Ventricle, body weight and uterus weight in male and female mice WT mice dosed with MPP ($2mgkg^{-1}day^{-1}$). Right ventricle (RV); left ventricle plus septum (LV + S); RV/LV+S ratio, body weight and uterus weight. Data expressed as ± SEM analysed by a Two-way ANOVA followed by a Bonferroni's post hoc test. * p<0.05 ** p <0.01, *** p<0.001 vs. normoxic mice. † p <0.05, †† p<0.01 vs. vehicle dosed mice. n = 5-14 mice per group.

Group	RV (mg)	LV + S (mg)	RV/LV+S	Body weight (g)	Uterus weight (mg)
Normoxic					
Male					
Vehicle	29.6 ± 1.1	111.4 ± 2.3	0.27 ± 0.01	25.8 ± 0.8	
MPP	23.2 ± 0.7 †††	103.9 ± 2.2	0.22 ± 0.01	24.1 ± 0.3	
Female					
Vehicle	19.6 ± 0.7	87.8 ± 1.6	0.22 ± 0.01	19.6 ± 0.3	85.9 ± 5.7
MPP	23.1 ± 2.0	79.2 ± 2.7 †	0.26 ± 0.01 †	18.3 ± 0.4	74.9 ± 6.4
Hypoxic					
Male					
Vehicle	25.0 ± 0.9 **	94.6 ± 3.8 ***	0.27 ± 0.01	24.8 ± 0.8	
MPP	20.9 ± 1.2	85.9 ± 2.1 ***	0.24 ± 0.01 **	23.2 ± 0.5	
Female					
Vehicle	22.38 ± 0.9	84.61 ± 4.4	0.27 ± 0.01 ***	18.6 ± 0.2	70.9 ± 4.8
MPP	18.6 ± 0.9 †	70.9 ± 1.4 ††	0.26 ± 0.02 **	17.1 ± 0.2	72.7 ± 5.3

3.2.7 ERα and ERβ Expression is Unchanged in Pulmonary Arteries from Female SERT$^+$ Mice

We wished to investigate the role of ERs in another model of PH which did not involve hypoxia. ER expression was therefore examined in the pulmonary arteries from the female mice over-expressing the serotonin transporter (SERT$^+$) that are susceptible to PH (MacLean et.al. 2004; White et.al. 2011). Basal levels of ERs were investigated at 2 months of age prior to the onset of spontaneous PH. It was observed that both ERα protein and ESR1 gene transcript were unchanged in SERT$^+$ female mice relative to wildtype controls (Figure 3-17A&B). On the other hand, ERß protein was down-regulated in female SERT$^+$ pulmonary artery (Figure 3-17C) whereas ESR2 gene transcript was unchanged in whole lung relative to wildtype controls (Figure 3-17D).

3.2.8 Spontaneous PH and Exaggerated Hypoxia-Induced PAH in Female SERT$^+$ Mice is Reversed by ERα Selective Antagonism

We wished to investigate if the effect of MPP were consistent in another model of PH that did not require chronic hypoxia. We have previously shown that normoxic female SERT$^+$ mice develop spontaneous PH at 5 months of age in an estrogen-dependent manner whilst male mice do not (White et al. 2011). We hypothesised that the predominant circulating female hormone estrogen mediates the development and progression of PH in this model through activity at ERα. We confirmed that vehicle treated female normoxic SERT$^+$ mice demonstrated elevated RVSP and pulmonary vascular remodelling relative to vehicle-treated wildtype controls. This was observed in the absence of RVH in normoxic conditions (Figure 3-18A-C) as previously described (White et al. 2011). The increase in RVSP and pulmonary vascular remodelling was abolished by MPP 2mgkg^{-1}day^{-1}.

We also exposed these mice to hypoxia as we previously showed that the PH phenotype was greatly enhanced in SERT$^+$ hypoxic mice. As before, the hypoxic

wildtype mice developed PH demonstrating increased RVSP and this was reduced by MPP (Figure 3-18A). Pulmonary vascular remodelling and RVH were also elevated in wildtype mice, although MPP did not influence these. This data supports previous results from this study using the C57Bl/6 wildtype background strain of wildtype mouse (Figure 3-7A-C). Increased RVSP in hypoxic $SERT^+$ vehicle-treated mice was reversed by MPP administration (Figure 3-18A). In vehicle-treated $SERT^+$ mice, hypoxia caused enhanced pulmonary vascular remodelling relative to hypoxic wildtype vehicle treated mice and this was markedly reduced by MPP (Figure 3-18B). Representative images of RVSP traces in $SERT^+$ females are shown in Figure 3-19. Additionally, pulmonary vascular muscularisation, as observed by alpha smooth muscle actin staining was notably attenuated in normoxic and hypoxic $SERT^+$ mice following MPP treatment (Figure 3-20).

Mean systemic arterial pressure was unaffected by hypoxic and MPP treatment in both wildtype and $SERT^+$ mice (Figure 3-21A). MPP treatment did however, noticeably reduce heart rate in normoxic $SERT^+$ mice. Further in hypoxia, wildtype mice had a reduced heart rate compared to normoxic wildtype mice (Figure 3-21B). Absolute values for haemodynamics are shown in Table 3-3.

3.2.9 **Uterine Weight is Reduced Following MPP Administration**

ERα, but not ERß knockout mice have abnormal reproductive function (Lubahn et al, 1993) suggesting that ERα may be central to a role in uterine development. In line with this we show in both WT and $SERT^+$ mice that MPP significantly attenuates uterine weight (Figure 3-22A). This is also evident when expressed as a ratio over body weight (Figure 3-22B). Absolute values for body weight and uterine weight are depicted in Table 3-4.

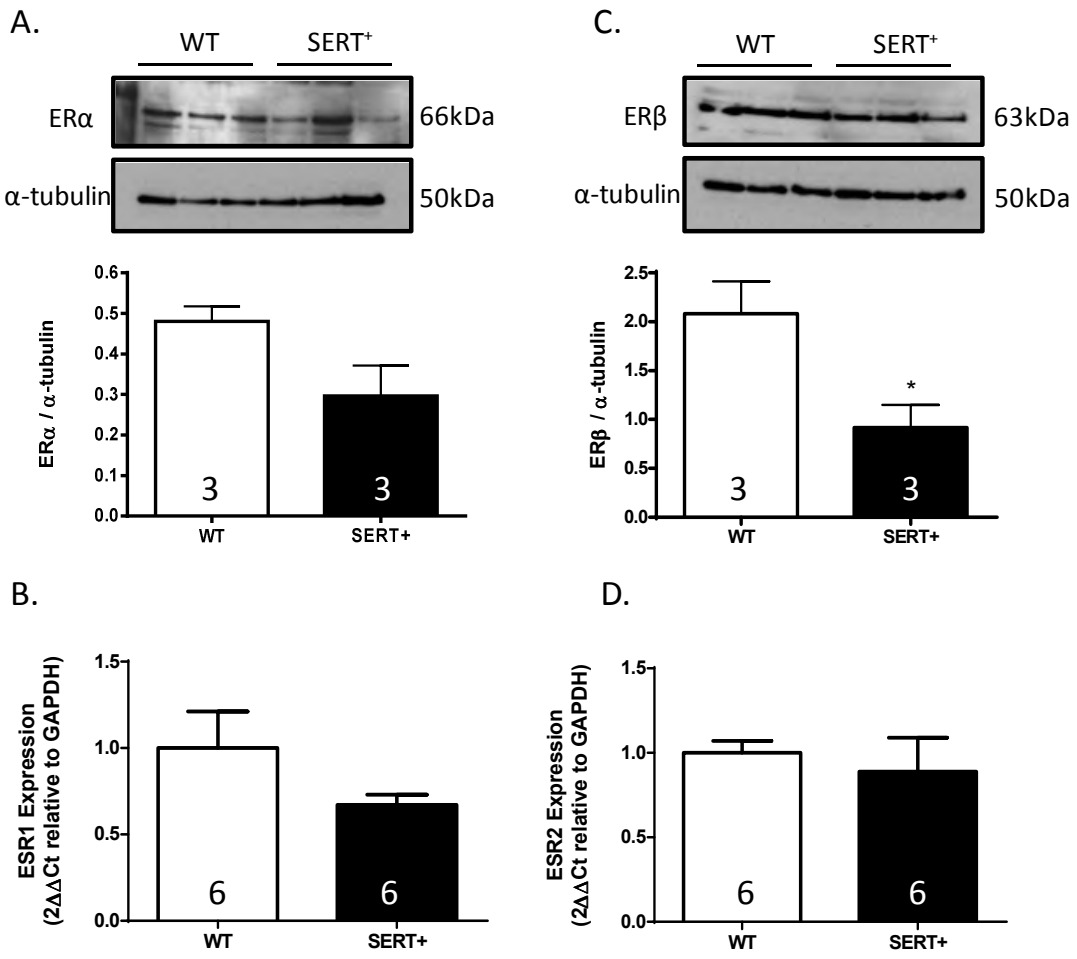


Figure 3-17: Estrogen receptor expression is unchanged in 2 month female SERT$^+$ mouse pulmonary artery.
ERα protein in pulmonary artery (A) and mRNA expression in lung (B) is unchanged in female SERT$^+$ mice. ERβ protein in pulmonary artery (C) is decreased in SERT$^+$ mice and mRNA expression (D) in lung is unchanged. Representative blots are shown for ERα and ERβ. Quantitative data are shown as ± SEM and analysed using an unpaired t-test. *p<0.05 vs. control. n=3 pulmonary artery repeated in triplicate for each Western experiment; qRT-PCR, n=6. n of pulmonary arteries/lung for each group is indicated on bar in graph. WT= wildtype.

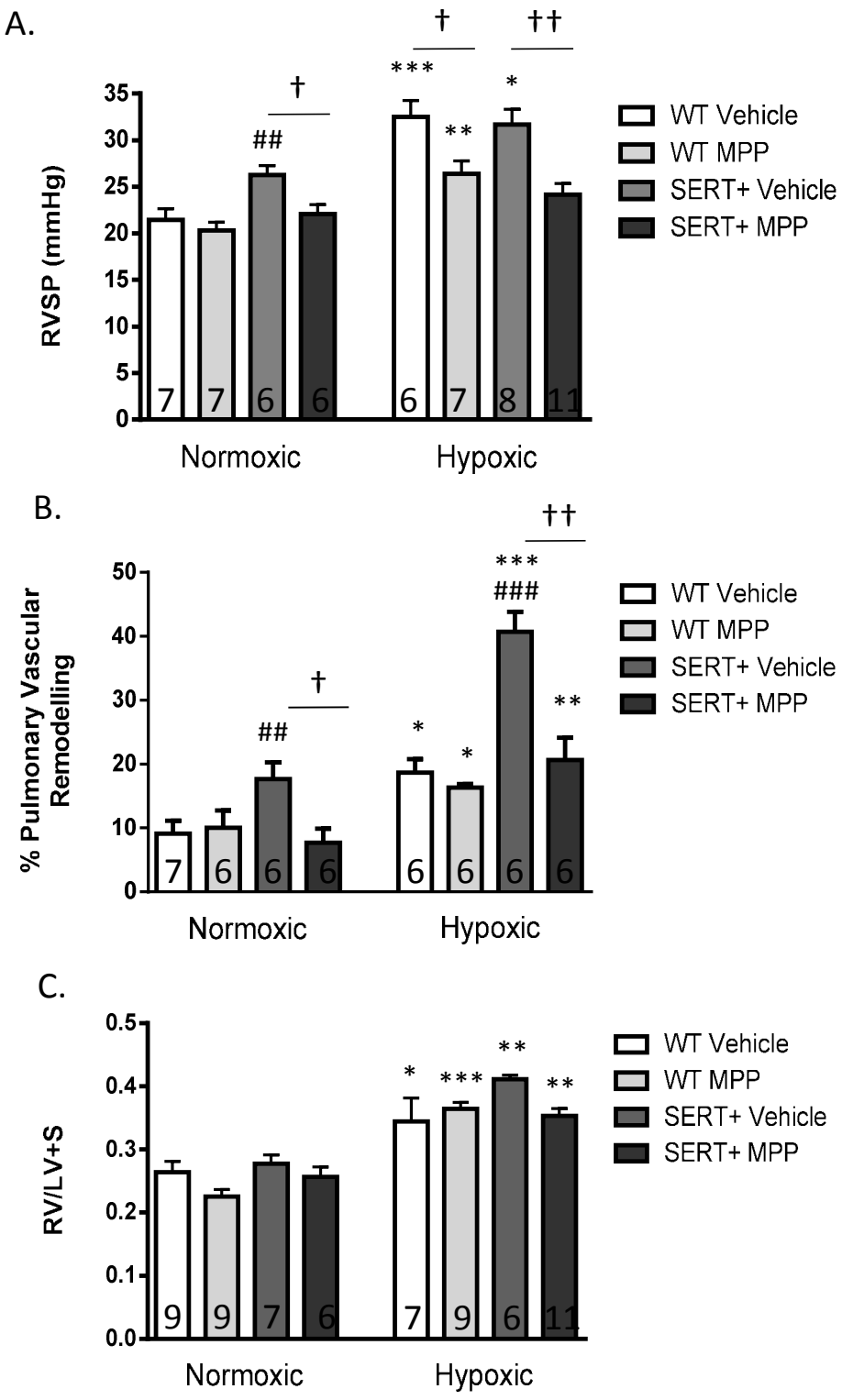


Figure 3-18: MPP (2mgkg^{-1}day^{-1}) attenuates the development of pulmonary hypertension in female SERT^{+} mice.

Right ventricular systolic pressure (RVSP) (A), pulmonary vascular remodelling (B) and right ventricular hypertrophy assessment (C). Data are expressed as ± SEM and analysed using a Two-Way ANOVA followed by a Bonferroni's post-hoc t-test. *p<0.05, ** p<0.01, *** p<0.001 vs. normoxic mice. † p <0.05, †† p<0.01 vs. vehicle dosed mice. ## p<0.01, ###p<0.001 vs. wildtype mice. n=6-11 mice per group; n of mice for each group is indicated in bar of graph. WT=wildtype.

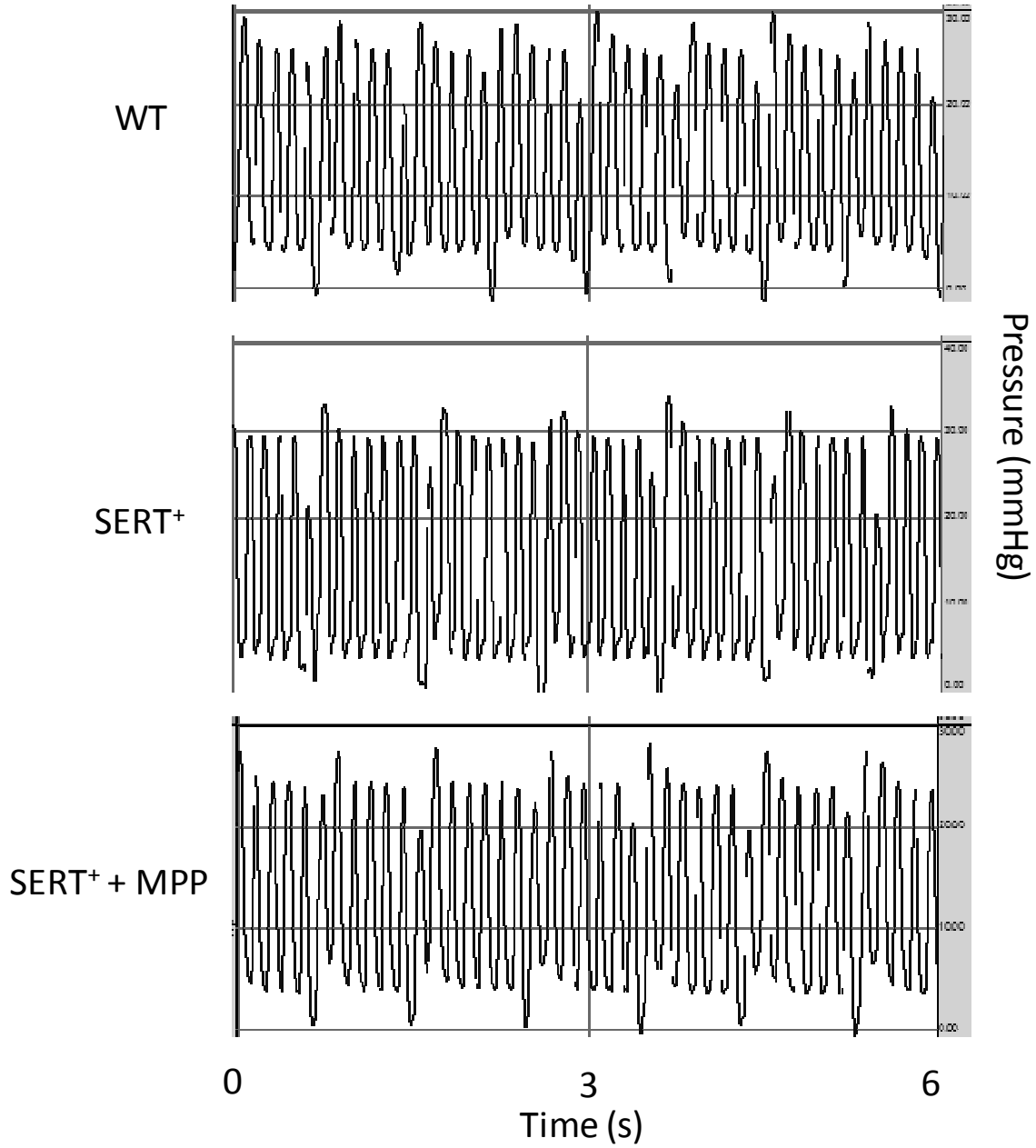


Figure 3-19: Representative RVSP traces from normoxic female SERT$^+$ mice.
Six second representative traces are shown for vehicle treated WT and SERT$^+$ females, and a female SERT$^+$ mouse treated with 2mgkg^{-1}day^{-1} MPP. WT=wildtype.

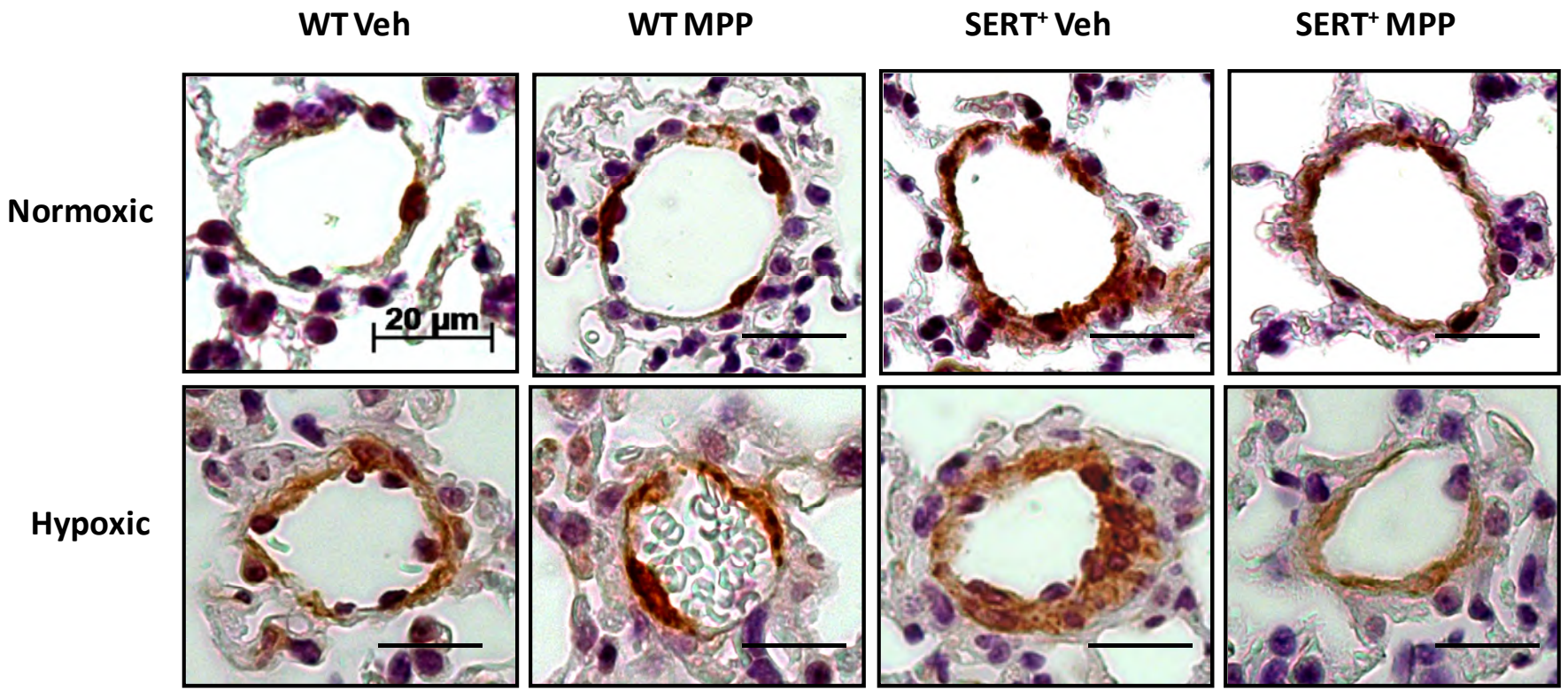


Figure 3-20: MPP (2mgkg^{-1}day^{-1}) attenuates pulmonary vascular remodelling female SERT^{+} mice.
α-smooth muscle actin stains smooth muscle cells dark brown; counterstained with haematoxylin. Representative images are shown from each group stained with alpha-smooth muscle actin. Scale bar=20µm.

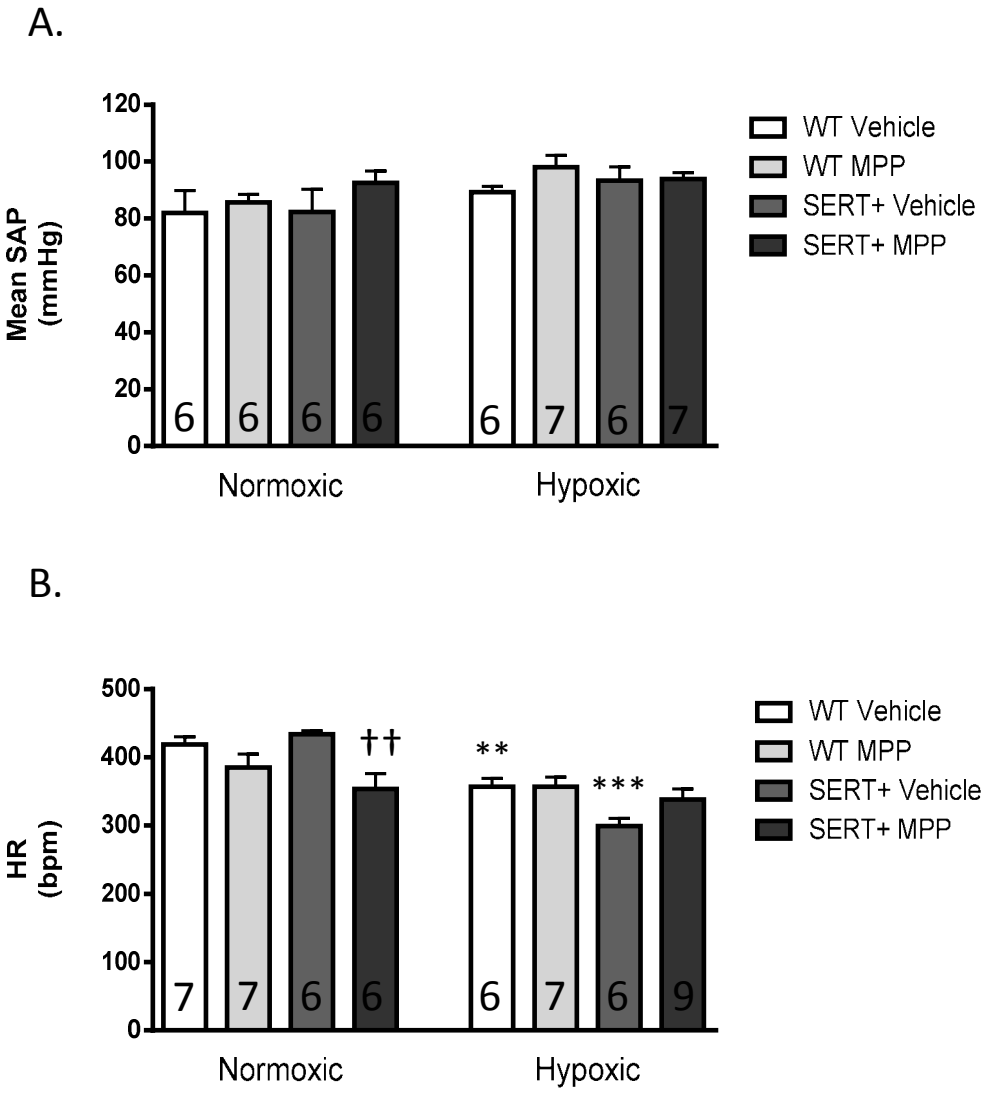


Figure 3-21: Systemic parameters in SERT^{+} female mice following hypoxia and MPP (2mgkg^{-1}day^{-1}) treatment.
Mean systemic arterial pressure (Mean SAP) (A) and HR (B) in female SERT^{+} mice. Data are expressed as ± SEM and analysed using a Two-Way ANOVA followed by a Bonferroni's post hoc t-test. **p <0.01, ***p<0.001 vs. normoxic mice; †† p<0.01 vs. vehicle dosed mice n=6-9 mice per group; n of mice for each group is indicated in bar of graph. WT= wildtype.

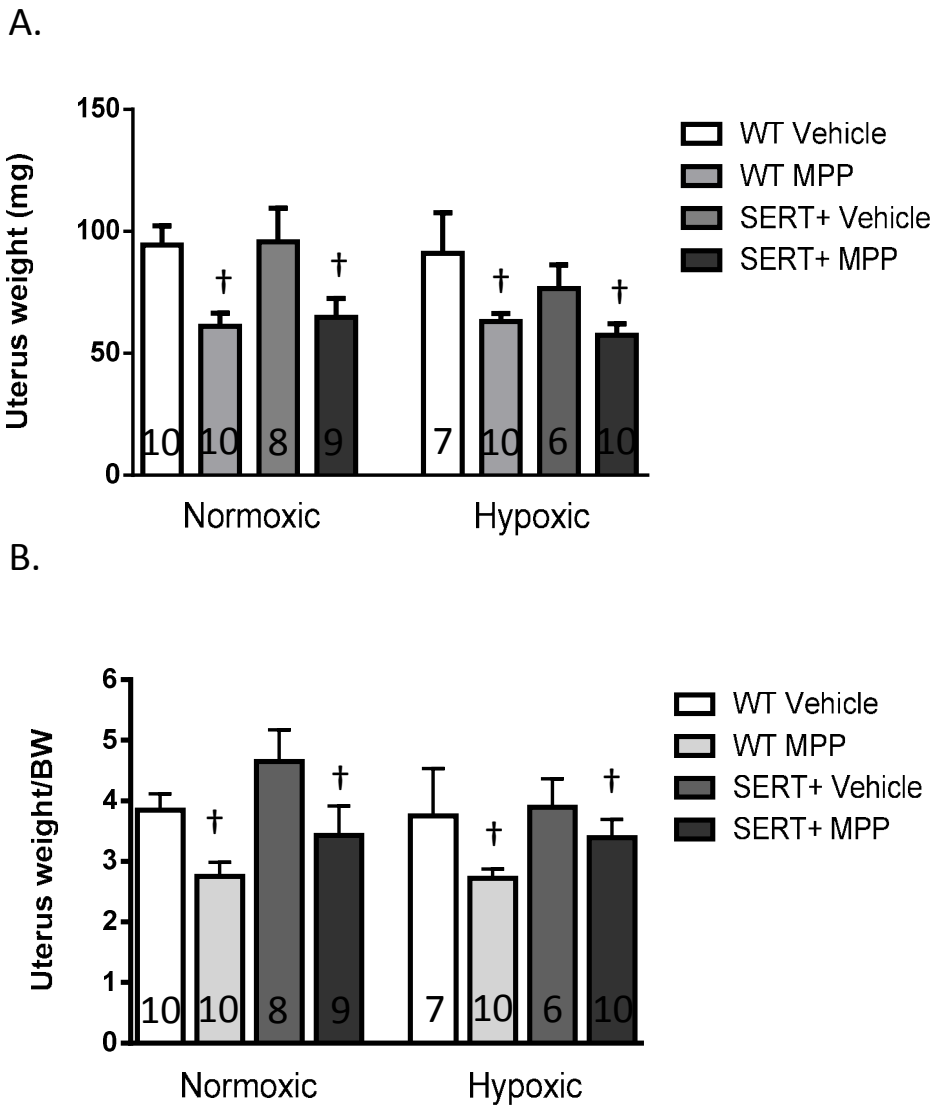


Figure 3-22: MPP (2mgkg^{-1}day^{-1}) treated WT and SERT^{+} mice exhibit reductions in uterine weight.
Uterine weight in WT and SERT^{+} mice (A) and uterus weight/body weight ratio (B) are reduced in by MPP treatment. Data are expressed as ± SEM analysed by Two-Way ANOVA followed by a Bonferroni's post-hoc test. † $p<0.05$ vs. vehicle. n=6-10 mice per group; n of mice for each group is indicated in bar of graph. WT= wildtype.

Table 3-3: Haemodynamic parameters in WT and SERT+ mice dosed with MPP (2mgkg^{-1}day^{-1}).

Right ventricular systolic pressure (RVSP); Mean right ventricular pressure (MRVP); right ventricular diastolic pressure (RVDP); systolic systemic arterial pressure (sSAP); mean systemic arterial pressure (mSAP); diastolic systemic arterial pressure (dSAP); Heart rate (HR). Data expressed as ± SEM analysed by a Two-way ANOVA followed by a Bonferroni's post hoc test. *p<0.05, ** p<0.01, *** p<0.001 vs. normoxic mice. † p <0.05, †† p<0.01 vs. vehicle dosed mice. ## p<0.01 vs. wildtype mice. n = 6-10 mice. WT= wildtype.

Parameter	WT Vehicle	WT MPP	SERT$^+$ Vehicle	SERT$^+$ MPP
Normoxic				
RVSP, mmHg	21.4 ± 1.2	20.3 ± 0.8	26.3 ± 1.0 ##	22.1 ± 1.0 †
MRVP, mmHg	14.13 ± 0.4	12.7 ± 1.0	14.8 ± 0.2	15.8 ± 0.5 #
RVDP, mmHg	1.6 ± 0.2	0.8 ± 0.3	1.3 ± 0.3	2.8 ± 0.6 ## †
sSAP, mmHg	84.2 ± 7.7	92.7 ± 4.9	88.8 ± 7.7	99.9 ± 3.6
mSAP, mmHg	81.9 ± 7.8	85.7 ± 2.8	82.3 ± 8.0	92.6 ± 4.2
dSAP, mmHg	76.6 ± 7.3	78.2 ± 3.4	72.8 ± 8.9	83.8 ± 5.7
HR, bpm	419.0 ± 10.9	385.2 ± 19.5	433.8 ± 5.0	354.1 ± 22.3 ††
Hypoxic				
RVSP, mmHg	32.5 ± 1.8 ***	26.4 ± 1.4 ** †	31.7 ± 1.6 *	24.2 ± 1.2 ††
MRVP, mmHg	18.9 ± 0.8 ***	15.2 ± 1.1	15.7 ± 0.8 †	12.4 ± 0.9 * †
RVDP, mmHg	1.5 ± 0.1	2.6 ± 0.5 *	1.7 ± 0.6	1.5 ± 0.4
sSAP, mmHg	99.4 ± 3.1	114.6 ± 3.9 ** †	103.8 ± 4.9	106.6 ± 2.1
mSAP, mmHg	89.3 ± 2.1	98.1 ± 4.2	93.4 ± 4.2	93.9 ± 2.6
dSAP, mmHg	78.0 ± 3.7	80.9 ± 4.5	80.89 ± 6.2	75.6 ± 3.6
HR, bpm	357.3 ± 12.1 **	357.2 ± 13.7	299.3 ± 11.3 ***	338.5 ± 15.3

Table 3-4: Ventricle, body weight and uterus weight in WT and SERT+ mice dosed with MPP ($2mgkg^{-1}day^{-1}$). Right ventricle (RV); left ventricle plus septum (LV + S); RV/LV+S ratio, body weight and uterus weight. Data expressed as ± SEM analysed by a Two-way ANOVA followed by a Bonferroni's post hoc test. * p<0.05, ** p <0.01, *** p<0.001 vs. normoxic mice. † p <0.05 vs. vehicle dosed mice. ### p<0.001 vs. wildtype mice. n = 6-12 mice per group.

Group	RV (mg)	LV + S (mg)	RV/LV+S	Body weight (g)	Uterus weight (mg)
Normoxic					
Wildtype					
Vehicle	21.3 ± 1.4	80.9 ± 3.2	0.26 ± 0.02	24.5 ± 0.9	94.5 ± 7.9
MPP	19.9 ± 2.2	81.7 ± 3.1	0.23 ± 0.01	22.6 ± 1.4	61.1 ± 5.4 †
$SERT^+$					
Vehicle	21.6 ± 1.2	72.3 ± 2.9	0.28 ± 0.01	18.4 ± 0.6 ###	95.7 ± 13.7
MPP	23.5 ± 2.0	74.6 ± 3.3	0.26 ± 0.02	19.4 ± 0.8	64.79 ± 7.7 †
Hypoxic					
Wildtype					
Vehicle	26.1 ± 3.2	76.1 ± 4.1	0.34 ± 0.04 *	24.9 ± 1.2	90.99 ± 16.7
MPP	26.3 ± 0.9 *	69.9 ± 1.2 ** †	0.36 ± 0.01 ***	23.4 ± 0.3	63.1 ± 3.3 †
$SERT^+$					
Vehicle	27.3 ± 1.3	66.1 ± 3.2 #	0.41 ± 0.01 **	18.6 ± 1.4 ###	76.62 ± 9.6
MPP	22.7 ± 0.8	64.4 ± 1.9 *	0.35 ± 0.01 **	17.2 ± 0.5 ###	57.5 ± 4.7 †

3.2.10 Effects of MPP Administration on Serotonin Induced Pulmonary Vascular Reactivity

To determine if pulmonary vascular reactivity is affected in mice following administration of MPP 2mgkg^{-1}day^{-1}, we tested isolated pulmonary arteries by wire-myography. Serotonin is a known potent pulmonary vasoconstrictor, therefore serotonin was used to pre-constrict the arteries and measure the contractile response. In addition, estrogen has been shown to relax pre-constricted pulmonary arteries in an ER-dependent manner (Lahm et al. 2007).

Pulmonary arteries from female hypoxic vehicle treated mice exhibited an increased contractile response to serotonin (Figure 3-23A) and showed higher area under the curve (AUC) (Figure 3-23B) values than normoxic vehicle pulmonary arteries. AUC analysis integrated the entire curve and was used as an overall measure of the cumulative contractile response for each individual treatment (i.e. normoxic or hypoxic conditions ± MPP). In female mice, serotonin-induced vasoconstriction was unaffected by administration of MPP in normoxic and hypoxic conditions although there was a trend toward a decreased contractile response (Figure 3-23). MPP administration in male mice showed a similar effect on serotonin induced vasoreactivity and hypoxia elevated the contractile response to serotonin (Figure 3-24). As previously reported, the potency of serotonin in SERT^{+} mice is reduced and we observe a rightward shift of the dose-response curve and reduced pEC_{50} in normoxic SERT^{+} vehicle treated mice and a reduction in the maximal contraction (Figure 3-25A). This is in line with a reduced AUC in SERT^{+} vehicle mice relative to wildtype vehicle mice (Figure 3-25B). This effect was not further influenced by MPP 2mgkg^{-1}day^{-1} administration in normoxic conditions. Additionally, MPP administration in normoxic SERT^{+} mice appears to increase the contractile response to serotonin compared to SERT^{+} vehicle mice as the maximal contraction (E_{max}) was significantly increased in normoxic SERT^{+} mice treated with MPP compared to vehicle controls. In contrast, in the pulmonary arteries of chronically hypoxic female SERT^{+} mice, MPP administration has no effect on serotonin- induced vasoconstriction (Figure 3-26A) or AUC (Figure 3-26B). In both normoxic and hypoxic conditions, the half maximal effective concentration (EC_{50}) was reduced

in $SERT^+$ vehicle control mice (Table 3-6). Values for EC_{50} and maximal responses (E_{max}) to serotonin are shown in Table 3-5 and 3-6.

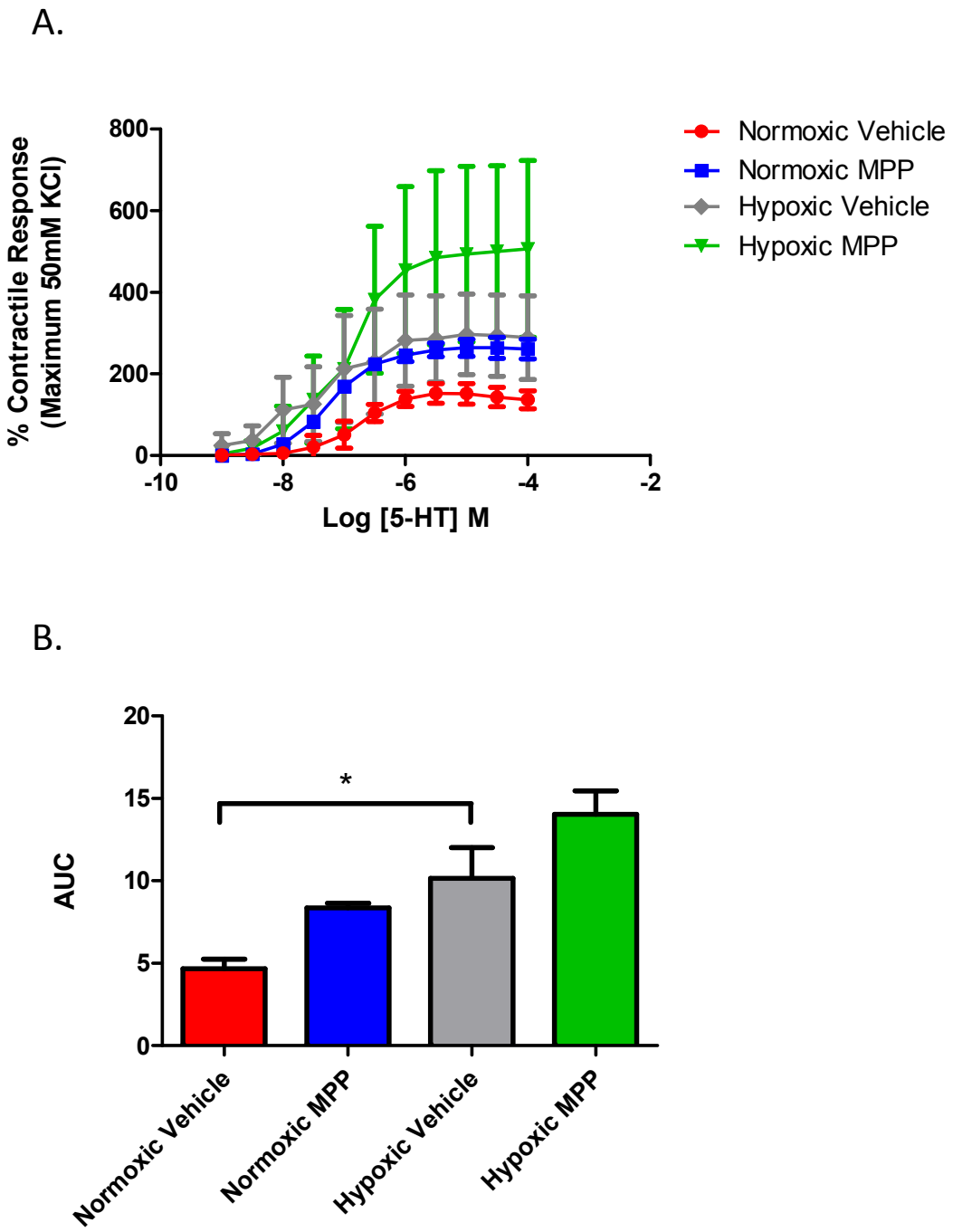


Figure 3-23: Serotonin- induced pulmonary artery vasoconstriction is unaffected by MPP ($2mgkg^{-1}day^{-1}$) administration in female mice.
(A) Percentage contractile response to 50mmol/l KCl are unchanged in response to MPP. (B) Area under the curve (AUC) is increased in hypoxic vehicle relative to normoxic vehicle. Data are represented as ± SEM and (A) analysed by a Two-Way ANOVA followed by a Bonferroni's post-hoc t-test and (B) analysed by a One-Way ANOVA followed by a Bonferroni's post hoc test. *p<0.05 vs. Normoxic Vehicle. n=6-8 mice per group. Values for E_{max} and EC_{50} shown in Table 3.5.

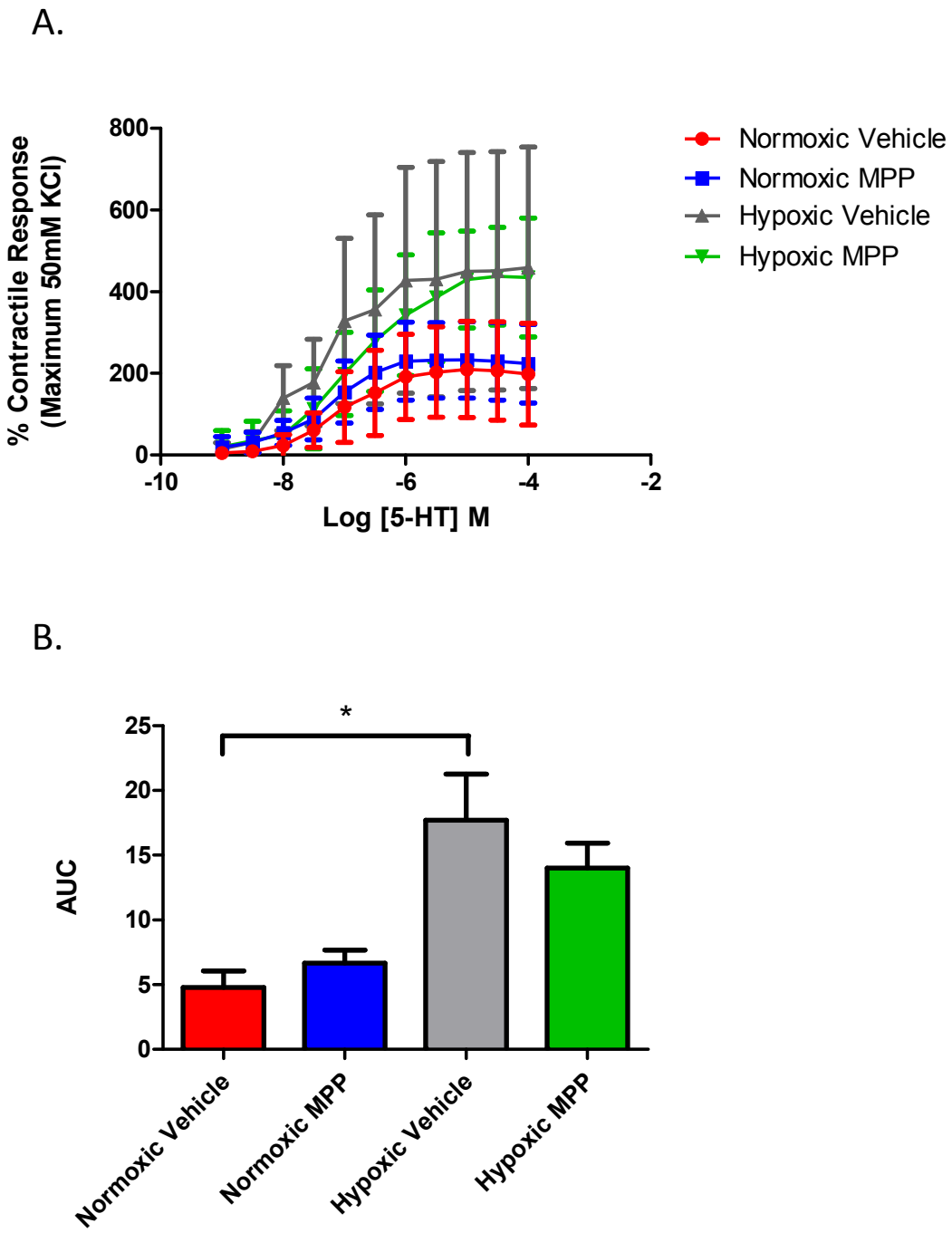


Figure 3-24: Serotonin- induced pulmonary artery vasoconstriction is unaffected by MPP ($2mgkg^{-1}day^{-1}$) administration in male mice.
(A) Percentage contractile response to 50mmol/l KCl are unchanged in response to MPP. (B) Area under the curve (AUC) is increased in hypoxic vehicle relative to normoxic vehicle. Data are represented as ± SEM and (A) analysed by a Two-Way ANOVA followed by a Bonferroni's post-hoc t-test and (B) AUC analysed by a One-way ANOVA followed by a Bonferroni's post hoc test. *$p<0.05$ vs. Normoxic Vehicle. n=6-8 mice per group. Values for E_{max} and EC_{50} shown in Table 3.5.

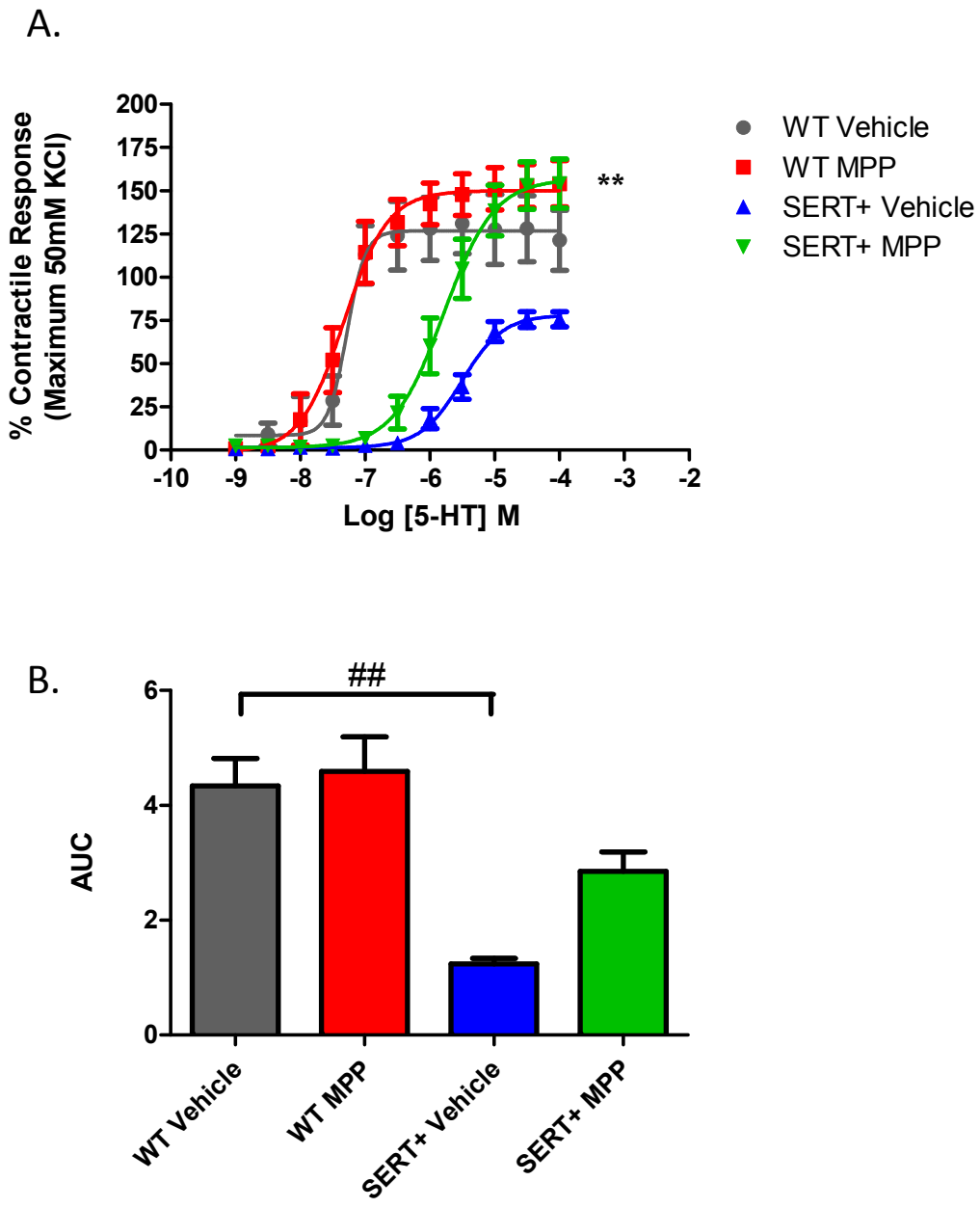


Figure 3-25: Serotonin- induced pulmonary artery vasoconstriction in normoxic female SERT$^+$ mice is augmented by MPP (2mgkg^{-1}day^{-1}) administration.
(A) Percentage contractile response to 50mmol/l KCl are unchanged in response to MPP. (B) Area under the curve (AUC) is decreased in SERT$^+$ vehicle relative to WT vehicle. Data are represented as ± SEM and (A) analysed by a Two-Way ANOVA followed by a Bonferroni's post-hoc t-test and (B) by a one-way ANOVA followed by a Bonferroni's post hoc test. **p<0.01 vs. vehicle. n=6-8 mice per group. WT= wildtype. Values for E_{max} and EC_{50} shown in Table 3.6.

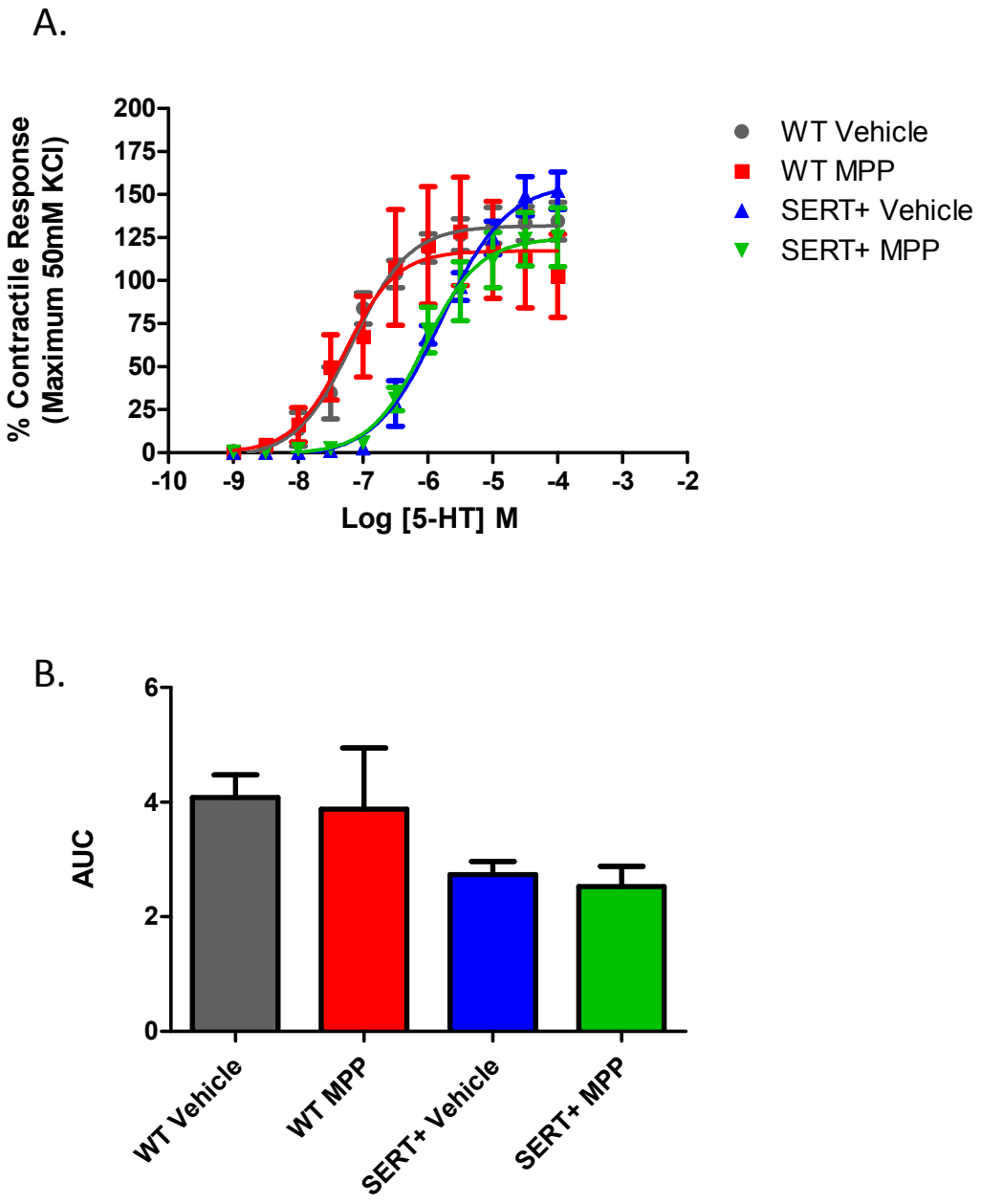


Figure 3-26: Serotonin- induced pulmonary artery vasoconstriction in hypoxic female SERT^{+} mice is unaffected by MPP (2mgkg^{-1}day^{-1}).
(A) Percentage contractile response to 50mmol/l KCl and (B) area under the curve (AUC) are unchanged in response to MPP. Data are represented as ± SEM and (A) analysed by a Two-Way ANOVA followed by a Bonferroni's post-hoc t-test and (B) by a one-way ANOVA followed by a Bonferroni's post hoc test. n=6-8 per group. WT=wildtype. Values for E_{max} and EC_{50} shown in Table 3.6.

Table 3-5: pEC50 and Emax values in female and male mice administered with MPP (2mgkg^{-1}day^{-1}).
pEC50, half maximal effective dose; Emax, maximal effective dose. Data are expressed as ± SEM and analysed by a One-Way ANOVA followed by a Bonferroni's post hoc test. n=6-8 mice per group. *p<0.05 vs. normoxic vehicle.

	Female Vehicle	Female MPP	Male Vehicle	Male MPP
Normoxic				
pEC50	6.85 ± 0.1	7.24 ± 0.09	7.06 ± 0.3	7.23 ± 0.1
Emax	161.8 ± 14.1	264.0 ± 16.3	206.0 ± 58.9	233.5 ± 42.8
n	7	6	6	6
Hypoxic				
pEC50	7.51 ± 0.3	7.05 ± 0.1	7.42 ± 0.6	6.69 ± 0.3
Emax	292.4 ± 41.2*	470.0 ± 75.2	446.8 ± 117.9	502.9 ± 48.6
n	6	8	6	6

Table 3-6: pEC50 and Emax values in female SERT^{+} administered with MPP (2mgkg^{-1}day^{-1}).
pEC50, half maximal effective dose; Emax, maximal effective dose. Data are expressed as ± SEM and analysed by a One-Way ANOVA followed by a Bonferroni's post hoc test. ###p<0.001 vs. WT; **p<0.01 vs. vehicle. n=6-7 mice per group. WT= wildtype.

	WT Vehicle	WT MPP	SERT+ Vehicle	SERT+ MPP
Normoxic				
pEC50	7.52 ± 0.3	7.37 ± 0.1	5.54 ± 0.1 ###	5.80 ± 0.1
Emax	127.6 ± 17.7	137.3 ± 15.4	77.2 ± 4.8	181.0 ± 27.6 ### **
n	6	7	6	6
Hypoxic				
pEC50	7.26 ± 0.2	7.04 ± 0.3	5.82 ± 0.06 ###	5.98 ± 0.1
Emax	131.1 ± 11.0	120.6 ± 27.4	155.8 ± 12.6	126.5 ± 13.1
n	6	6	6	6

3.2.11 Effects of Estrogen, PPT and DPN on Human Pulmonary Artery Smooth Muscle Cell Proliferation

To summarise our *in vivo* studies we demonstrated that development of PH in hypoxia and in a female susceptible $SERT^+$ model of PH is ERα dependent. In addition, ERα selective antagonism has no effect on development of hypoxia-induced PH in male mice. As we also show that there is elevated expression of ERα in the PASMCs from PAH females and in the female hypoxic mouse pulmonary arteries, we hypothesised that ERα may be promoting PH specifically in females. To determine potential mechanisms by which ERα activation could be facilitating PH we examined the role of estrogen and its receptors in cultured female human PASMCs using ERα and ERβ selective agonists and antagonists.

Estrogen was examined at physiological concentrations (0.1-1nmolL^{-1}) and a supraphysiological concentration of 10nmolL^{-1}. At 1nmol/l estrogen induced proliferation (Figure 3-27A). The ERα selective agonist, PPT, also caused proliferation of PASMCs at 0.01-0.1nmolL^{-1} (Figure 3-27B) whereas diarylpropionitrile (DPN), the ERβ selective agonist, had no effect on PASMCs (Figure 3-27C). Estrogen induced proliferation at 1nmolL^{-1} was inhibited by ERα selective antagonism using MPP (1μmolL^{-1}) whilst ERβ selective antagonism with PHTPP (1μmolL^{-1}) had no effect on PASMC proliferation induced by estrogen. ICI 182, 780 (1μmolL^{-1}), a non- selective ERα/β antagonist also had no effect on 17β-estradiol induced proliferation (Figure 3-28).

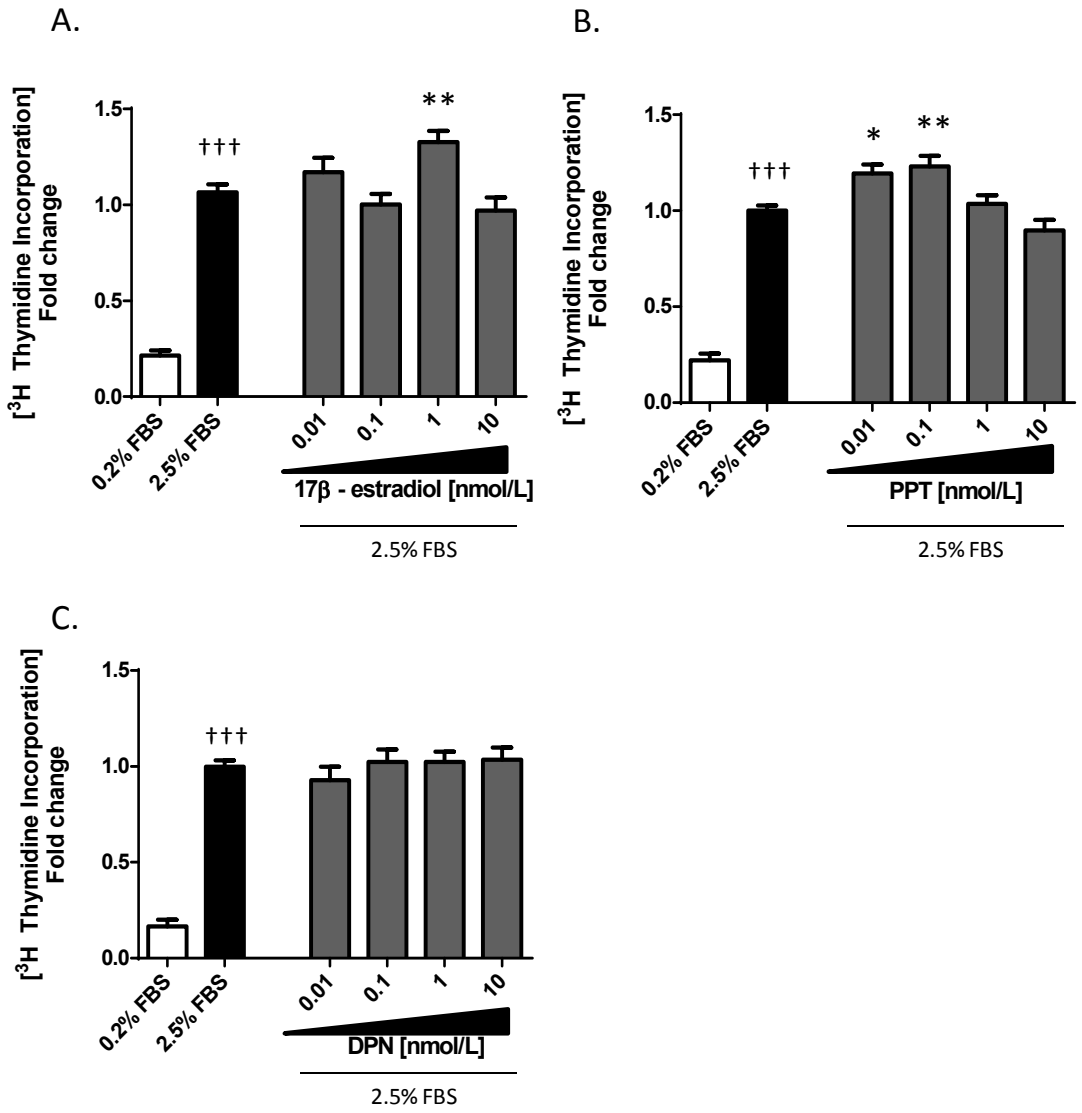


Figure 3-27: 17β-estradiol induces proliferation of female pulmonary artery smooth muscle cells through ERα.

Estrogen (A) and PPT, an ERα agonist (B) induce proliferation at physiological concentrations whilst DPN, an ERβ agonist (C) has no effect on proliferation of hPASMCs. Data are expressed as mean ± SEM analysed using a One-Way ANOVA followed by a Tukey's post hoc test. ††† $p<0.001$ vs. 0.2% FBS; * $p<0.05$, ** $p<0.01$ vs. 2.5% FBS. Patient information for controls (1, 2, 3 and 5), are shown in *Materials and Methods section: Table* 2.3 (passage 3-5). n=4 per experiment and performed in triplicate in separate female control cell lines.

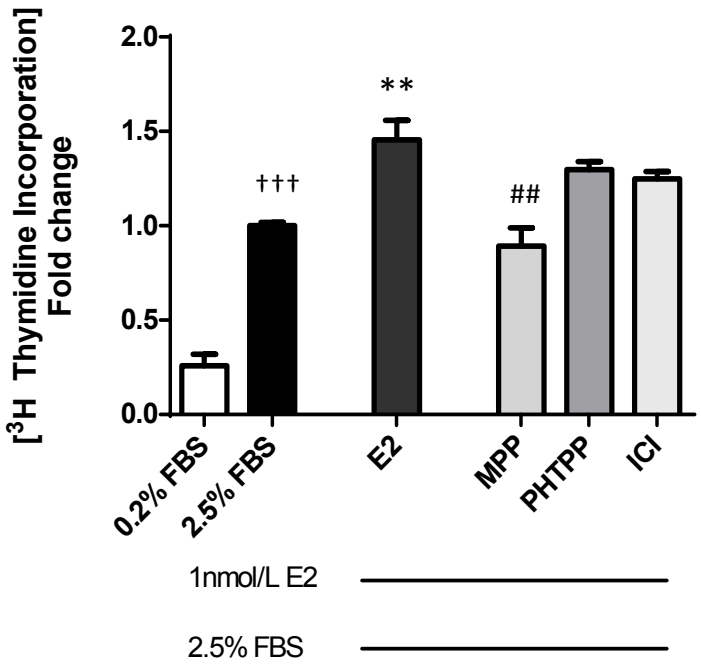


Figure 3-28: 17β-estradiol induced proliferation is inhibited by an ERα antagonist.
Proliferation of human PASMCs to 1nmol/l of estrogen was inhibited in the presence of the ERα selective antagonist, MPP. PHTPP, an ERβ selective antagonist, and ICI 182, 780 (ICI), a non-selective ERα/β antagonist has no effect on estrogen induced proliferation. Data are expressed as mean ± SEM and analysed using a One-Way ANOVA followed by a Tukey's post hoc test. ††† $p<0.001$ vs. 0.2% FBS; **$p<0.01$ vs. 2.5%; # $p<0.05$; ## $p<0.01$ vs. 1nmol/l E_2. Patient information for controls (1, 2, 3 and 5) are shown in *Materials and Methods section: Table* 2.3 (passage 3-5). n=4 per experiment and performed in triplicate in separate female control cell lines.

3.2.12 Estrogen Requires Downstream MAPK and PI3K/Akt Signalling to Promote Human PASMC Proliferation via ERα

PI3K/Akt and MAPKs can phosphorylate and activate ER and their co-regulators to enhance nuclear effects on transcription and regulate cell survival and proliferation (Marino et al. 2006). We therefore examined the proliferative effects of estrogen and PPT on human PASMC proliferation in the presence on PI3K and ERK/MAPK inhibitors. Both estrogen and PPT induced proliferation were inhibited in the presence of U0126 (10μmolL^{-1}) and LY294002 (10μmolL^{-1}), a MEK inhibitor and a PI3K inhibitor, respectively (Figure 3-29).

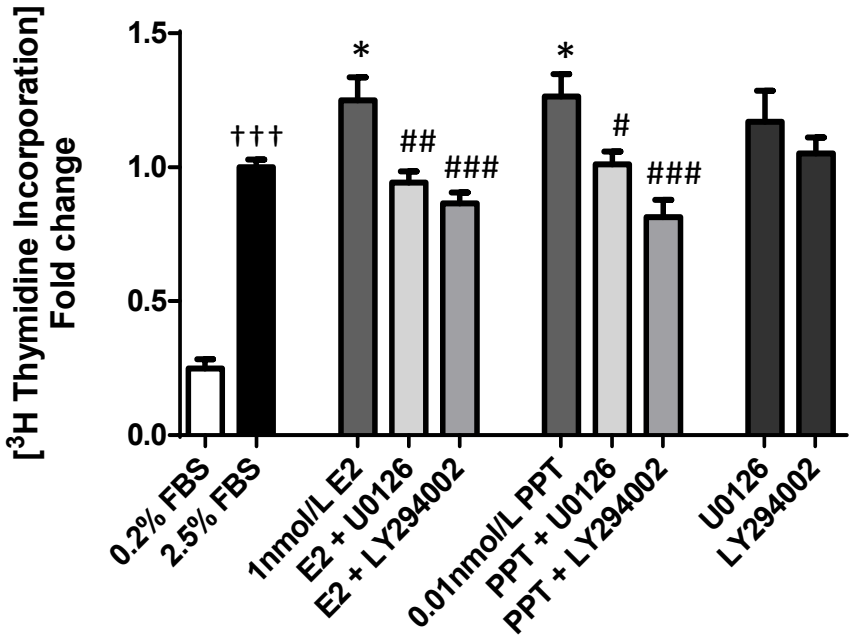


Figure 3-29: 17β-estradiol and PPT induced proliferation is inhibited by pERK and PI3K inhibitors.

Proliferation of human PASMCs to 1nmol/l of estrogen and 0.01nmol/l PPT was inhibited in the presence of the U0126 (10μmol/l) and LY294002 (10μmol/l), pERK and PI3K inhibitors, respectively. Data are expressed as mean ± SEM and analysed using a One-Way ANOVA followed by a Tukey's post hoc test.. ††† $p<0.001$ vs. 0.2% FBS; *$p<0.05$ vs. 2.5%; # $p<0.05$; ## $p<0.01$; ### $p<0.001$ vs. 1nmol/l E_2 or 0.01nmol/l PPT. Patient information for controls (1, 2, 3 and 5) are shown in *Materials and Methods section: Table* 2.3 (passage 3-5). n=4 per experiment and performed in triplicate in separate female control cell lines.

3.2.13 Estrogen Suppresses BMPR2 Expression and Downstream Smad and Id Expression in the Lung via ERα

To determine the mechanism by which estrogen and ERα interplay and promote PH pathogenesis in females we investigated the BMPR2 pathway and downstream signalling molecules as dysregulation of this pathway is involved in aberrant smooth muscle cell proliferation. We examined levels of BMPR2, total Smad-1, Id1 and Id3 in lung samples from mice dosed with the ERα selective antagonist, MPP.

In chronic hypoxic female mice BMPR2 expression levels (both protein and mRNA) are suppressed. This loss of BMPR2 expression in pulmonary arteries was restored in mice treated with MPP (Figure 3-30A). Additionally we observed rescue of the BMPR2 mRNA transcript in whole lung samples from mice treated with MPP (Figure 3-30B). The BMPR2 protein levels in $SERT^+$ female mice were unchanged compared to female WT counterparts (Figure 3-30C) although we did observe a down-regulation of the mRNA transcript (Figure 3-30D). In both WT and $SERT^+$ mice, treatment with MPP resulted in an increase in BMPR2 protein levels (Figure 3-30C). In $SERT^+$ mice mRNA levels were also increased by MPP (Figure 3-30D).

We also wished to examine further downstream BMPR2 signalling in lungs from both hypoxic and $SERT^+$ female mice treated with MPP. We decided to investigate expression levels of total Smad1, Id1 and Id3 as these downstream signalling components have also been shown to be decreased in vascular lesions and PASMCs from PAH patients (Yang et al. 2008; Yang et.al. 2005). In normoxic female mice, MPP had no effect on expression levels of Smad1, Id1 or Id3 (Figure 3-31A-C). Although hypoxia in vehicle treated mice had no effect on either Smad1 levels or Id3 levels, Id1 transcript expression was significantly reduced (Figure 3-31B). Consistent with BMPR2, the expression of Id1 mRNA transcript was restored in lungs treated with MPP. Smad1 levels were also increased following MPP treatment in female hypoxic lung (Figure 3-31A).

In whole lung from female $SERT^+$ mice, Smad1 and Id1 levels were unchanged between WT vehicle and $SERT^+$ vehicle mice. Additionally, MPP had no effect on

the expression levels of Smad1 and Id1 mRNA transcript compared to vehicle treated mice (Figure 3-32A&B). On the other hand, we observed a significant down-regulation of Id3 in $SERT^{+}$ vehicle mice compared to WT vehicle mice (Figure 3-32C). The loss of Id3 expression was rescued by MPP treatment.

Interestingly, in male mice, expression levels of BMPR2 and downstream signalling mediators Smad1, Id1 and Id3, were unaffected by hypoxia and/or MPP treatment (Figure 3-33A-D).

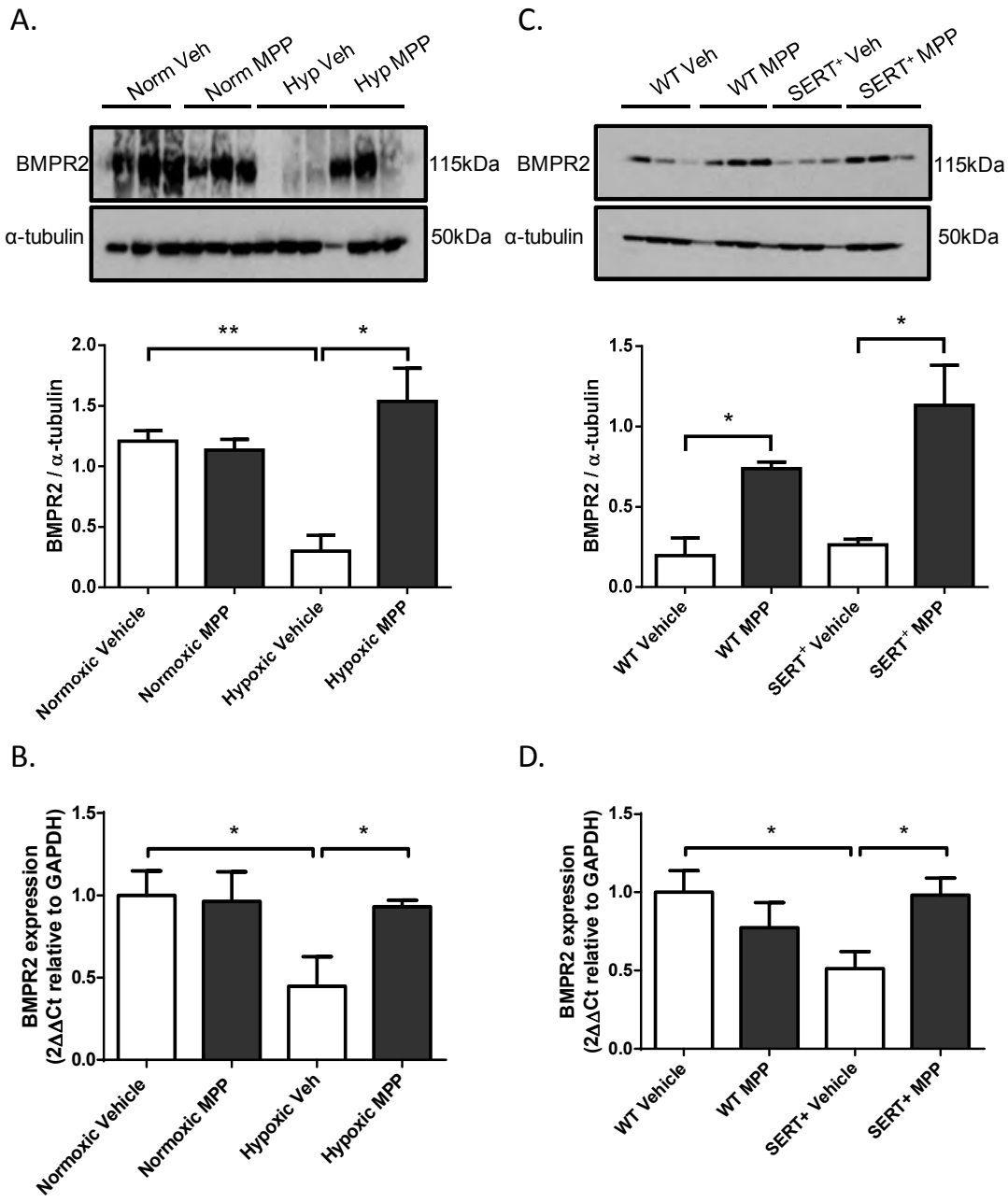


Figure 3-30: Regulation of the BMPR2 pathway by MPP (2mgkg^{-1}day^{-1}) and chronic hypoxia in female mouse pulmonary artery and lung.
BMPR2 expression levels in pulmonary artery (A) and mRNA transcript in whole lung (B) is regulated by estrogen and ERα in chronic hypoxia-induced PH. BMPR2 protein (C) and mRNA transcript (D) is also regulated by ERα in SERT^{+} female mouse lung. Quantitative data are expressed as ± SEM and analysed by a one-way ANOVA followed by a Tukey's post hoc-test. *p<0.05, **p<0.01. n=6 pulmonary artery and whole lung performed in triplicate.

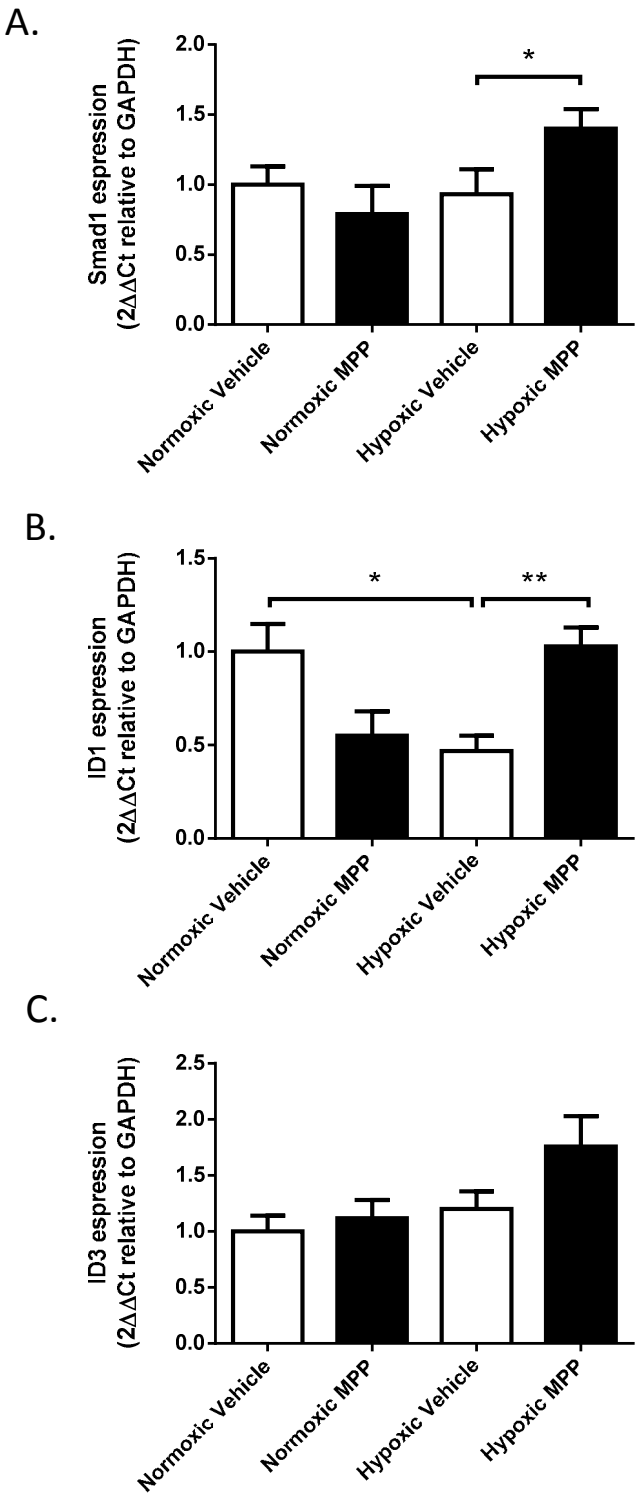


Figure 3-31: Effects of MPP (2mgkg^{-1}day^{-1}) and chronic hypoxia on regulation of the mRNA transcript levels and components of the BMPR2 pathway female mouse lung.
Figure 4.26: (A) Smad-1 (B) Id1 and (C) Id3 expression levels in female mouse lung. Data are expressed as ± SEM and analysed by a one-way ANOVA followed by a Tukey's post hoc-test. *p<0.05, **p<0.01. n=6 whole lung performed in triplicate.

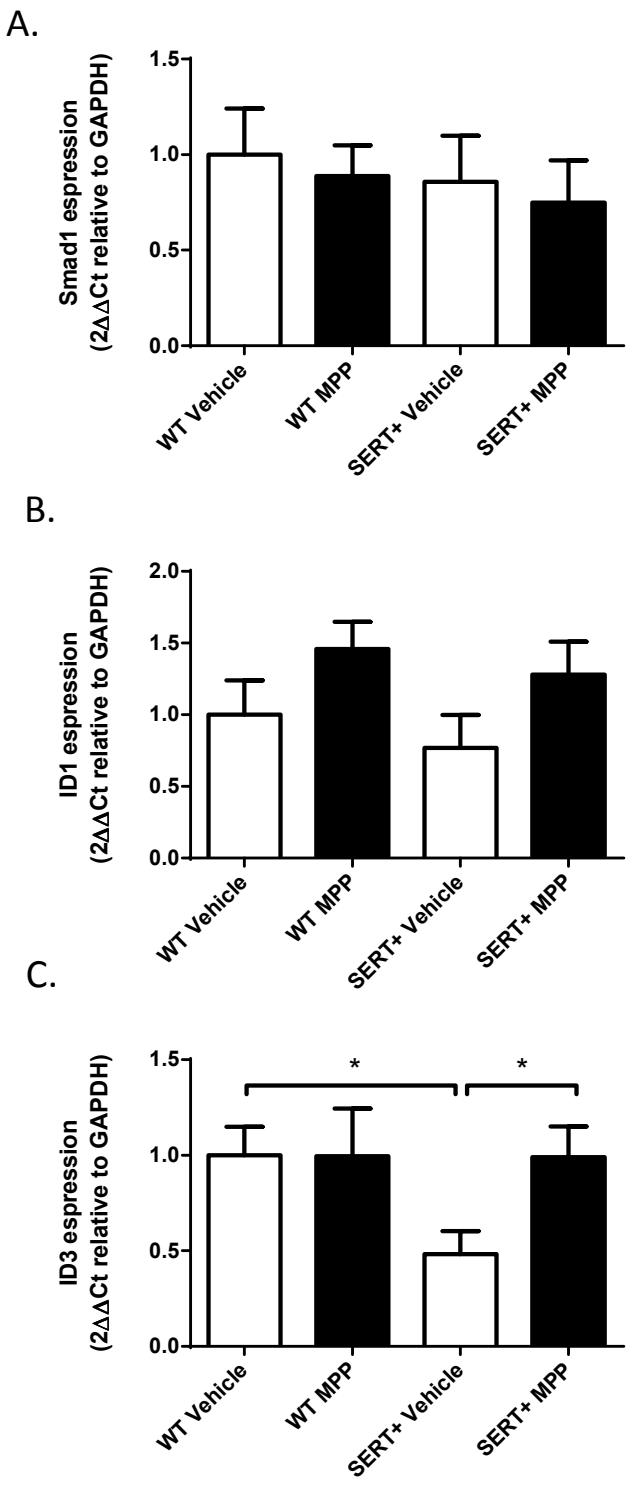


Figure 3-32: Effects of MPP (2mgkg^{-1}day^{-1}) on regulation of the mRNA transcript levels and components of the BMPR2 pathway in SERT^{+} female mouse lung.
(A)Smad-1 (B) Id1 and (C) Id3 expression levels in female SERT+ mouse lung. Data are expressed as ± SEM and analysed by a one-way ANOVA followed by a Tukey's post hoc-test. *p<0.05. n=6 whole lung performed in triplicate.

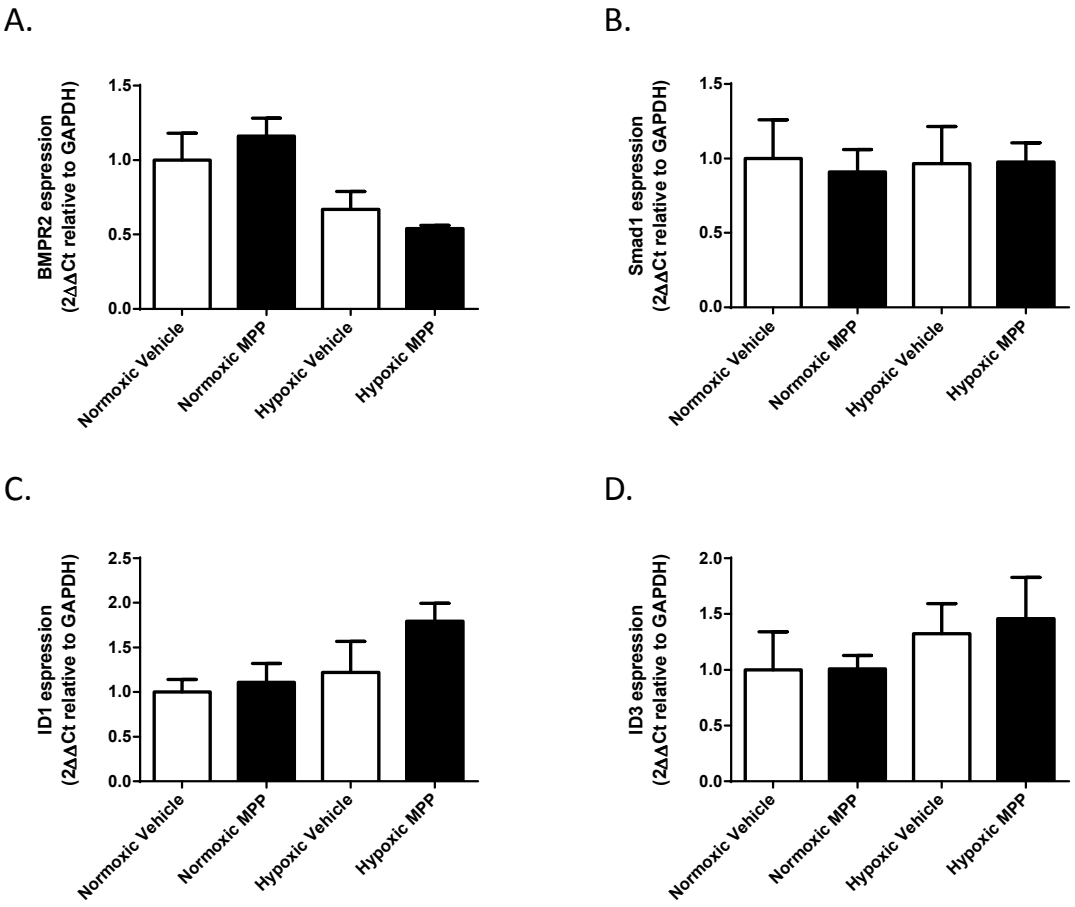


Figure 3-33: Effects of MPP (2mgkg^{-1}day^{-1}) and chronic hypoxia on regulation of the mRNA transcript levels and components of the BMPR2 pathway in male mouse lung
BMPR2 (A); Smad-1 (B); Id1 (C) and Id3 (D) mRNA transcript levels are unchanged in male mouse lung. Data are expressed as ± SEM and analysed by a one-way ANOVA followed by a Tukey's post hoc-test. n=6 mice lungs performed in triplicate.

3.2.14 Estrogen Receptor Expression in Human PASMCs is Regulated by Serotonin

We have previously demonstrated that estrogen can increase the expression of tryptophan hydroxylase 1 (TPH1), SERT and 5-HT_{1B} (White et al. 2011). Here we wished to examine the effects of serotonin on ER expression in human PASMCs.

In female human PASMCs we demonstrated that expression of ERα protein was significantly increased (Figure 3-34A) whilst expression of ERβ was significantly decreased (Figure 3-34B) by 1μmol/l serotonin.

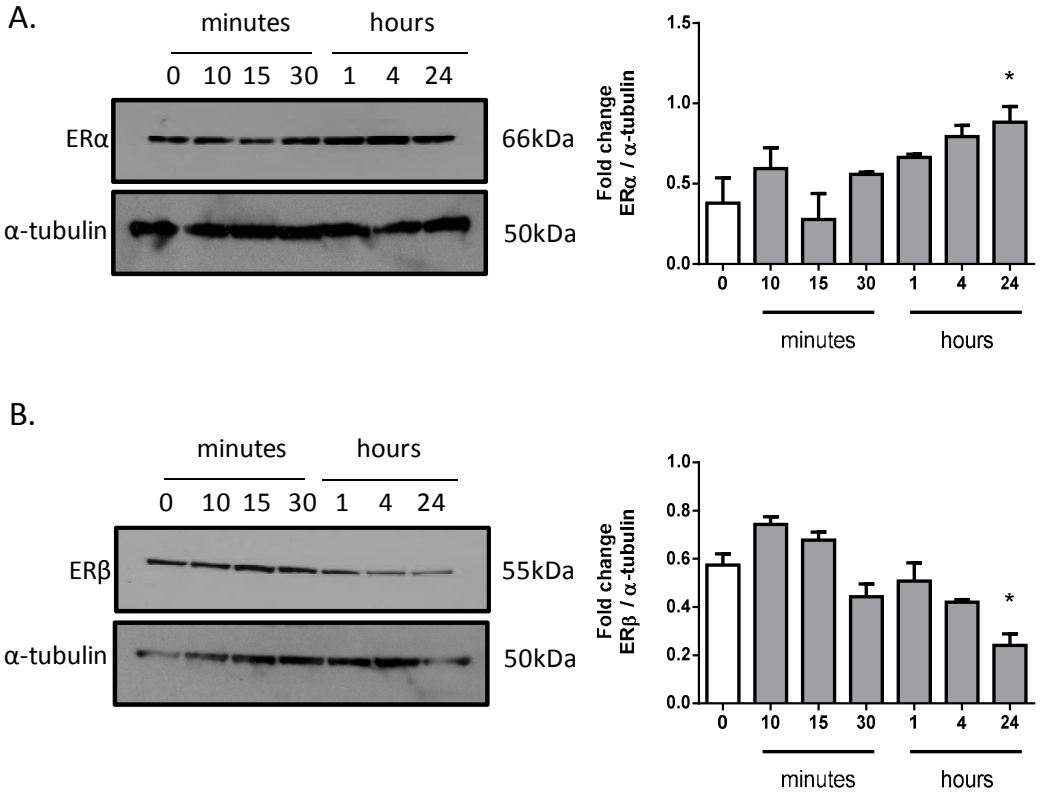


Figure 3-34: Estrogen receptor expression in female control human PASMCs stimulated with 5-HT.
ERα (A), ERβ (B). Data are expressed as ± SEM and analysed by One-Way ANOVA followed by a Dunnet's post-hoc test. Representative blots are shown. n=3 performed in triplicate. *p<0.05 vs. 0, control.

3.3 Discussion

In the most recent worldwide registries, it is reported that women develop both idiopathic and heritable PAH up to four times as frequently as men (Badesch et.al. 2010; Humbert et.al. 2006; Peacock et al. 2007; Thenappan et al. 2007) Although women are more susceptible to PAH, paradoxically in animal models, male rodents demonstrate more severe hypoxia induced PH (Rabinovitch et.al. 1981) and estrogens appear to protect against PH in male animal models (Farhat et.al. 1993; Lahm et.al. 2012a). This is known as the 'estrogen paradox' of PAH and has been central in impeding our understanding of increased susceptibility to PAH in women.

This study aimed to investigate the role of estrogen receptors, in the development of PH by utilising translational *in vitro* and *in situ* studies and experimental *in vivo* models. We also wished to determine the influence of gender by comparing males and females. Our results demonstrate that activation of ERα plays a key role in the development of PH in two rodent models of PH, the hypoxic mouse model and $SERT^+$ mouse. This is only observed in female mice however. In addition we demonstrate that there is elevated expression of ERα in the lungs from female PAH patients and in female hypoxic mouse lung. Furthermore, ERα is noticeably increased in female human PASMCs compared to males. We demonstrate that ERα activation in female human PASMCs leads to proliferation driven by PI3K/Akt and ERK MAPK activation. We also suggest that ERα negatively regulates BMPR2 expression in females and contributes to the PH phenotype. Additionally, the pulmonary mitogen serotonin can up-regulate ERα expression in PASMCs.

Altered Expression of Estrogen Receptors in Translational and Experimental Pulmonary Hypertension

We investigated the classical ERs, ERα and ERß, which predominantly regulate gene transcription (Prossnitz et.al. 2008). We demonstrated expression of both ERα and ERß in adventitial, smooth muscle and endothelial cells in human pulmonary arteries. In humans, expression of ERα and ERß has been identified in endothelial cells and vascular smooth muscle cells of aortic and coronary

vasculature as well as in cardiomyocytes (Mendelsohn 2002; Meyer et.al. 2006). The presence of both ERα and ERβ mediate physiologically important effects of estrogen in human vasculature. In the lung, ERβ is thought to be the predominant ER (Nilsson et.al. 2001), however here we show an important role for ERα in PH and *o*ur results suggest that it is ERα that drives the PH phenotype in females.

Firstly, we show that expression of ERα (both protein and mRNA) is increased in PASMCs from female HPAH patients relative to normal female controls. This is consistent with gene expression data in PAH patients demonstrating an up-regulation of ESR1 in PAH subjects relative to controls (Rajkumar et.al. 2010). Polymorphisms in ESR1 have also been associated with an increased risk of developing portopulmonary hypertension independent of gender (Roberts et.al. 2009b). Uniquely we report here that there are higher levels of ERα expression in female PAH human PASMCs compared with male human PASMCs from PAH patients, whilst ERβ expression is greatest in male PAH PASMCs. One limitation of the expression data reported here is the small number of patient samples available due to the rare nature of PAH. However, gender differences in tissue localisation of ERs during development and disease is not an entirely novel concept and has been reported in other conditions. In aortic vasculature during aneurysm, a correlation between increased ERα expression in females but not males has been identified (Laser et al. 2013) and gender differences in vascular function has been attributed to differences in expression, distribution and/or activity of ERs in response to vasoconstrictors (Ma et al. 2010; Rubanyi et al. 1997). In human PAH however, this is the first direct comparison of ERs in females versus males.

In the lungs of female mice, both ERα and ERβ are required for the formation of full functional and morphological development of alveoli structures, although they have a much smaller effect on alveolar dimensions in male mice (Massaro & Massaro 2004; Massaro & Massaro 2006). It is likely then, that the ER pathway contributes more significantly to pathophysiology of female lung compared to male lung. We therefore investigated ER expression in the pulmonary artery from chronic hypoxic female and male mice. It has been identified previously in rat lung that ESR1 is a key regulatory component in response to intermittent hypoxia and further, is involved in regulation of ESR2 and androgen receptor,

which are also differentially expressed in hypoxia (Wu et al. 2008). We demonstrated expression of both receptors in the pulmonary vasculature of female mice in line with previous studies (Lahm et.al. 2012a; Umar et.al. 2011a). ERα protein expression was elevated by hypoxia in female mice, although expression of mRNA was unchanged suggesting post-transcriptional regulation of ERα in mouse pulmonary artery by hypoxia. Consistent with our observations that ERβ expression was reduced in human lung from PAH patients we show that ERβ expression is also reduced in the pulmonary artery of the female hypoxic mouse. This is also consistent with the down-regulation of ERβ observed in the lung and right heart in a right heart failure rat model (Matori et.al. 2012). It has previously been shown that loss of ERβ in female mice leads to abnormal lung structures and systemic hypoxia contributing to ventricular hypertrophy (Morani et.al. 2006). ERα may therefore be pathogenic in PH whilst ERβ may be protective (Nadadur et.al. 2012; Umar et.al. 2011a). The reduced expression of ERβ we observe in female human PASMCs and in the female model of PH may result in a loss of the protective effects of estrogen.

Estrogen Receptor-α Antagonist MPP, Attenuates RVSP and Pulmonary Vascular Remodeling in Chronic Hypoxic-PH and in the Female Susceptible SERT^{+} Model

We investigated the potential pathogenic role of ERα further by examining the effect of the ERα antagonist, MPP, on the development of PH in the chronic hypoxic mouse model. MPP attenuated the development of PH by reducing RVSP and pulmonary vascular remodelling in the ovary intact female chronic hypoxic mouse, but not in males. One explanation for this could be the elevated expression of ERα in females compared to males and suggests that endogenous estrogen is activating ERα receptors to facilitate the development of PH. In addition, estrogen receptor expression was unchanged in the pulmonary arteries of hypoxic male mice suggesting that ERα may play less of a pathogenic role in males.

Transcription of ERs is subject to regulation by hypoxia. Hypoxia response elements have been identified on the promoters of both ERα and ERβ (Wu et al. 2012) and in breast cancer cells, it has been demonstrated that hypoxia induces

ESR1 repression at the transcriptional level in a process dependent on hypoxia-inducible factor 1α (Ryu et al. 2011). Due to the effect hypoxia may have on transcriptional regulation of ERα, we also wished to examine the effects of ERα antagonism in a model of PH that did not require hypoxic exposure. We have previously demonstrated that PH develops in $SERT^+$ mice (White et.al. 2011), S100A4/Mts1 over-expressing mice (Dempsie et.al. 2011) and mice dosed with dexfenfluramine (Dempsie et.al. 2013). These models are all serotonin-dependent and PH only develops in ovary intact females. Further, we demonstrate that ovarian estrogen underpins the PH phenotype in these models. To investigate if ERα mediates this effect of endogenous estrogen in females, we investigated the effect of the MPP in the $SERT^+$ model in the absence and presence of hypoxia. We show that MPP reversed the PH and associated pulmonary vascular remodelling in these mice, both under normoxic and hypoxic conditions. This suggests a pivotal role for ERα in the causative effects of endogenous estrogen in the development of PH in this model and this was also observed independent of any effects of hypoxia. Previously we have identified an estrogen-dependent regulation of the serotonin system in human PASMCs whereby estrogen can up-regulate tryptophan hydroxylase-1 (TPH1), SERT and the $5\text{-}HT_{1B}$ receptor (White et.al. 2011). Here we now show that serotonin can indeed regulate the expression of both ERα and ERß expression in human PASMCs. Serotonin induced an up-regulation of ERα whereas the expression of ERß is decreased by serotonin. This up-regulation of ERα may contribute to the pathogenic effects of serotonin in females during PAH.

Previous studies have shown that exogenous administration of estrogen can protect male rodents from hypoxia-induced PH (Xu et al. 2013) and via activation of ERα and ERß (Lahm et al. 2012). Although these studies are valuable, we do not feel our results are directly comparable as we address the role of endogenous estrogen and not artificially elevated circulating estrogen, acting via ERs in males and females with intact ovaries. Given the gender specific effects of ERα antagonism, this study has highlighted the value of understanding the differential role of endogenous estrogen in the development of PH in males and females. Moreover, our results provide more therapeutically relevant data owing to the effect of MPP on naturally occurring estrogens. Males have lower circulating levels of estrogen, and as shown here, low ERα expression

compared to females, it would not be surprising therefore, if estrogen plays a lesser role in the development of PH in males. In line with this, we have shown that inhibition of estrogen synthesis is protective only in female rodent models of PH (Mair et al, unpublished). In addition in females, altered estrogen synthesis and/or metabolism (White et al. 2012) may contribute to PH development. Indeed, there is a putative estrogen receptor element in the promoter region of the estrogen metabolising enzyme, CYP1B1, suggesting metabolism may also be regulated by ERα (Han et al. 2005).

Despite the incidence of RVH in both our models of PH we did not detect an ERα dependent rescue of the RVH nor did ERα antagonism exacerbate RVH. Women are frequently reported to have an improved prognosis compared to men despite their predisposition to developing PAH (Benza et.al. 2012; Humbert et.al. 2010a). This has been attributed to an RV cardioprotective effect of estrogen. Indeed estrogen levels correlate with higher right ventricular ejection fraction and survival in females (Kawut et al. 2009; Ventetuolo et.al. 2011). Genuinely protective effects of exogenous estrogen in PH observed in other studies (Nadadur et.al. 2012; Umar et.al. 2011a) may therefore arise from a direct structural effect on the right ventricle and an influence on right ventricular function as opposed to an effect on vascular remodelling and pulmonary pressures. It is possible that the protective influence of estrogen on the right ventricle may be mediated predominantly by ERβ (Pedram et.al. 2008). Further, our results suggest that inhibition of ERα is not having a detrimental effect on the RV and therefore this may still be a feasible therapeutic strategy in females (Barrett-Connor & Bush 1991). One limitation of measuring RVH via the weight ratio of RV/LV+S is perhaps that development of RVH could be masked by alterations in LV weight. Future investigations should consider the weight of RV as a ratio to body weight or tibia length.

Estrogen has also been identified to have an influence on vascular reactivity in the systemic and pulmonary vasculature involving both ERα and ERβ. In systemic vasculature, estrogen promotes vasodilation via genomic and non-genomic mechanisms attributed to activation of endothelial nitric oxide synthase (eNOS) and release of nitric oxide (NO) in aortic and carotid arteries (Pare et.al. 2002; Zhu et.al. 2002). Further, in both normoxic and hypoxic conditions, estrogen is reported to attenuate pulmonary artery vasoconstriction (English et.al. 2001;

Lahm et.al. 2012a). In pulmonary vasculature, the onset of the ERα and ERß-mediated decrease in vasoconstriction is rapid and is therefore suggested to be a non-genomic effect dependent on NO release (Chambliss et.al. 2000; Lahm et.al. 2007). In this study we therefore wished to determine the effect ERα antagonism had in isolated pulmonary arteries treated with serotonin. In normoxic conditions in both male and female mice, MPP had no effect on serotonin induced vasoconstriction. Hypoxia augmented the response to serotonin compared to normoxic conditions, however, MPP had no further effect on serotonin induced vasoconstriction in hypoxic male and female mice. Certainly there is evidence implicating estrogen mediated-induction of both SERT and the 5-HT_{2A} receptor in the brain which is estrogen receptor dependent (Sumner et al. 2007) and the 5-HT_{2A} receptor is the predominant receptor involved in hypoxic pulmonary vasoconstriction in mice (MacLean & Dempsie 2010). Additionally, we investigated the effect of serotonin and ERα antagonism in isolated pulmonary arteries from female $SERT^{+}$ mice. In this model, the vasoconstricive response to serotonin was heightened in $SERT^{+}$ mice treated with MPP independent of hypoxia. This may be explained by a loss of re-uptake of serotonin by SERT contributing to higher concentrations of circulating levels of serotonin available to act on serotonin receptors. Here, in isolated pulmonary arteries, there were no sex-dependent effects of ERα antagonism.

Estrogen-Induced Proliferation is Mediated Through Estrogen Receptor-α and Involves Downstream ERK/MAPK and PI3K Signalling Mechanisms

Excessive smooth muscle cell proliferation is a main component of the pulmonary vascular remodelling and lesions observed in PAH. Estrogen is a pro-proliferative factor in human PASMCs (White et al. 2010; White et al. 2012), breast cancer cells (Pattarozzi et al. 2008), and has been shown to have pro-inflammatory properties in systemic vasculature (Mendelsohn 2002). This suggests that estrogen may mediate proliferation of human PASMCs and may contribute to PAH pathology. We investigated this further by examining ERα mediated proliferation in female human PASMCs. We demonstrate for the first time that estrogen can induce proliferation of human PASMCs via ERα activation, although the effect of estrogen and the ERα agonist, PPT are not dose

dependent. The absence of a dose dependent effect for estrogen is surprising given previous findings highlighted this effect (White et al, 2011), however our results are consistent with reporting estrogen-induced proliferation at $1nmolL^{-1}$. Levels of estrogen receptors between patient human PASMCs may vary and contribute to this anomaly for estrogen and PPT induced proliferation. In addition we show an ERβ agonist, DPN, has no effect on proliferation of human PASMCs suggesting that ERα is the receptor that mediates estrogen-induced proliferation in PASMCs. Moreover, we determine the pro-proliferative effects of estrogen and the ERα agonist, PPT, are dependent on activation of downstream PI3K/Akt and ERK/MAPK signalling. ERK and Akt signalling pathways are closely involved in cardiac hypertrophy and pulmonary vascular remodelling and estrogen has been shown to regulate activation of both pathways in right heart failure (Nadadur et.al. 2012; Zhou et al. 2011). Additionally, selective activation of ERα in endothelial cells in aorta increases ERK expression and ERK1/2 mediated cell proliferation and activation of PI3K/Akt in human endothelial cells is dependent on ERα but not ERβ (Chambliss et.al. 2000; Meyer et.al. 2006). These results provide some insight into the molecular mechanisms by which estrogen and its receptors may mediate pulmonary vascular remodelling during PAH and hence MPP attenuates pulmonary vascular remodelling in female mice.

Dysfunctional BMPR2 signalling plays a pivotal role in aberrant smooth muscle growth and endothelial cell proliferation and apoptosis in PAH, and mutations in BMPR2 are responsible for ~80% of HPAH cases (Machado et.al. 2009). Loss of BMPR2 function mediates proliferation by reducing induction of cell cycle inhibitors (Id proteins), particularly Id1 and Id3 in PASMCs (Yang et.al. 2008; Yang et.al. 2005). A gene-gender relationship for BMPR2 was proposed in a recent study where BMPR2 expression was shown to be decreased in lymphocytes and whole lung from female patients compared with males (Austin et.al. 2012). In line with this, BMPR2 is recognised as a gene target of ESR1 (Rajkumar et.al. 2010). A highly conserved functional estrogen response element has recently been identified in the BMPR2 promoter and estrogen exposure suppresses the BMPR2 signal through ERα (Austin et al. 2012). In the present study, BMPR2 protein and mRNA expression was decreased following hypoxia consistent with previous studies (Long et al. 2009; Takahashi et al. 2006). Lung BMPR2

expression was also decreased in SERT^{+} mice at mRNA levels but not protein. In both our chronic hypoxic female mouse model and our hypoxia-independent SERT^{+} mouse model, low levels of BMPR2 were rescued by the ERα antagonist. Hence in females we have demonstrated high ERα expression supporting human PASMC proliferation and decreased BMPR2 expression. Combined with high circulating endogenous estrogen levels in females this may explain the female susceptibility to develop PAH and/or the selective beneficial effects of the ERα antagonist in females. Previously, the growth inhibitory effects of BMPs have been shown to be Smad1 dependent (Yang et.al. 2005), therefore we also wished to determine the influence of estrogen and ERα on Smad-1 expression. Id1 and Id3 have also been reported to be major targets of BMP signalling in PASMCs where they are involved in growth suppression. Furthermore, induction of both Id1 and Id3 is dependent on intact BMPR2 (Yang et al. 2013). Indeed, we report here that Smad-1 and Id1 and Id3 are also subject to regulation by estrogen via ERα and suggest that therapeutic effects of MPP *in vivo* involve restoring the dysfunctional BMPR2 signalling axis in PASMCs.

Conclusion

In conclusion, we have determined an ERα dependent mechanism of PAH development in females. Our data supports a hypothesis whereby the higher prevalence of PAH in women may be a result of increased ERα distribution and signalling. We suggest that defective estrogen signalling in PAH drives a female susceptibility in concordance with defective estrogen metabolism (White et al. 2012b). Furthermore, we propose that in PAH, the genetic susceptibility which infers an increased risk in females to develop the condition is related to interplay between dysfunctional ERα and the BMPR2 signalling axis. In the setting of PH, gender represents an important modifier of disease and should be considered carefully in future research and in clinical implications.

Chapter 4.

Influence of the Novel G Protein-Coupled Estrogen Receptor, GPER in the Development of Experimental Pulmonary Hypertension

4.1 Introduction

The incidence of PAH is well reported to be more prevalent in women than in men (Badesch et.al. 2010; Humbert et.al. 2010a; Ling et.al. 2012). In fact, in the most recent registries, the female to male ratio is approximately 4.1:1 in idiopathic PAH (IPAH) and 3.8:1 in associated PAH (APAH) (Badesch et.al. 2010). Despite clear epidemiologic evidence demonstrating female susceptibility in many forms of PAH, the underlying reasons for this gender disparity remain unclear. This female predominance in PAH has led to numerous studies investigating the role of gender and sex hormones in the development of experimental PAH. In particular, estrogen has been proposed to play a pivotal role in PAH pathogenesis.

Classically, cellular signalling of estrogen is mediated through two estrogen receptors (ER), ERα and ERβ. These receptors belong to the super family of nuclear receptors. Activation of these receptors results in slow responses to estrogen, involving activation of estrogen response elements (EREs) in the promoters of target genes, and influencing gene transcription (Heldring et al. 2007b). However, in addition to the well studied transcriptional effects of estrogen, rapid non-genomic effects of estrogen occurring within seconds to minutes have been observed. These have been attributed to a third estrogen receptor, the seven transmembrane G-protein coupled receptor 30 (GPR30) or GPER; (Filardo & Thomas. 2007; (Prossnitz et.al. 2008).

In PAH, a role for both ERα and ERβ in physiology and pathophysiology has been described (Lahm et.al. 2012a; Umar et.al. 2011a). The identification and characterisation of GPER in the pulmonary circulation remains to be investigated. However, several promising cardiovascular responses to G1 suggest there may be potential for GPER mediated effects in the pulmonary circulation and during PAH. In a cardiovascular setting, estrogens are observed to exert protective effects through ERα whilst it is often presumed that ERβ has opposing effects to ERα. Slowly, evidence is building for a role of GPER in some physiological and pathophysiological cardiovascular and metabolic conditions in

addition to ERα and ERß. GPER has been identified in different vasculature including mesenteric and carotid artery (Haas et al, 2009; Martensson et al. 2009) and localised to the smooth muscle and endothelial cells of carotid and cerebral arteries (Lindsey et al. 2009). It is not surprising therefore that GPER is involved in vascular reactivity of estrogen. For example a GPER selective agonist, G1 initiates gender-independent relaxation of vascular smooth muscle cells (Broughton et al. 2010) although this is dependent on the vascular bed and agonist used for contraction as isolated arterial rings pre-contracted endothelin-1 are relaxed by G1 whilst those contracted with serotonin are unaffected by G1 (Haas et al. 2009). In pulmonary arteries, the potential role of estrogen action via GPER is unknown. In addition, G1 has been demonstrated to reduce blood pressure (Lindsey et al. 2009) and elicits an antiproliferative effect in endothelial cells (Holm et al. 2011).

Due to the severe lack of evidence investigating the role of GPER in PAH despite the well defined influence of estrogen in development of PAH in females, we wished to evaluate and characterise GPER. To our knowledge, this is the first study to characterise GPER in translational *in vitro* and *in situ* studies of PAH and in an experimental *in vivo* rodent model of PH. We use a GPER knockout mouse ($GPER^{-/-}$) to investigate GPER in chronic hypoxia induced-PH. The aims of this study were to evaluate the expression of GPER in human and mouse lungs in the development of PAH, and to determine any gender differences in activity of GPER.

4.2 Results

4.2.1 GPER Localisation in Human Lung and Pulmonary Artery

Initially we wished to investigate if GPER was expressed in human lung and pulmonary artery and then further determine if localisation was cell type specific. We utilised human lung sections from female non-PAH control and PAH patients. To our knowledge, GPER expression in PAH development and progression has never been investigated.

We observed GPER expression in both non-PAH control and PAH patient lungs. GPER was found to be localised to some, but not all, human PASMCs in non-PAH control and PAH patient lung sections. In addition, some GPER was also localised to endothelial cells in control and PAH patients. GPER expression was determined by positive dark brown staining (Figure 4-1). Consecutive sections stained for alpha smooth muscle actin and von Willebrand factor further identified smooth muscle and endothelial cell specific staining, respectively. Lung sections were also counterstained with haemotoxylin (purple/blue).

4.2.2 GPER Expression in Human Pulmonary Artery Smooth Muscle Cells

In isolated human PASMCs, we then examined if there was a difference in expression of GPER between non-PAH control and idiopathic PAH (IPAH) and heritable PAH (HPAH) patients. Levels of expression between non-PAH control and both IPAH and HPAH were similar at both protein (Figure 4-2A) and mRNA transcript levels (Figure 4-2B).

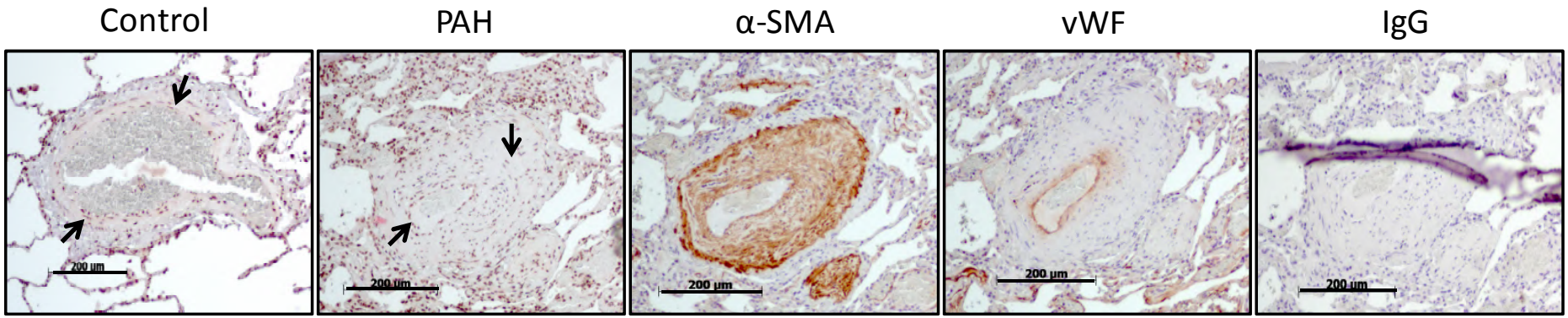


Figure 4-1: Immunolocalisation of GPER in human lung sections.

Expression of GPER (dark brown staining) is localised to the pulmonary artery smooth muscle cells and endothelial cells as indicated by the black arrow in control and PAH lung sections. Consecutive sections stained for alpha smooth muscle actin (α-SMA) and von Willebrand factor (vWF) are shown, and the IgG control is shown for PAH patient. *Patient information is available in Materials and Methods section Table 2.1.* Scale bar=200µm.

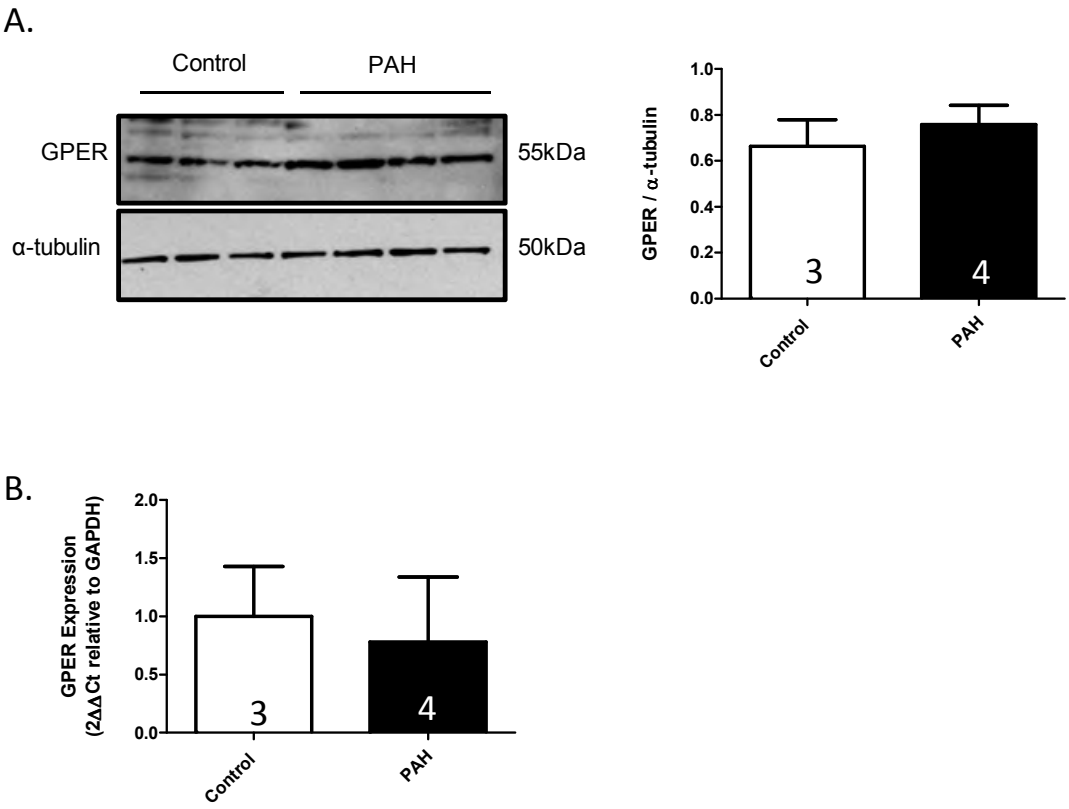


Figure 4-2: Expression of GPER in isolated female human PASMCs.

Expression of GPER protein (A) and mRNA transcript (B) is unchanged in IPAH and HPAH human PASMCs compared to non-PAH control. Representative blots are shown. Quantitative data are represented as ± SEM and analysed by an unpaired t-test. *Patient information is available Materials and Methods sections Table 2.3; control samples 1,3, 4 and 5 and patients samples 1-3.* n=3/4 individual control or patients per group repeated in triplicate; n for each group is indicated on bar in graph.

4.2.3 GPER Expression in Female Mouse Pulmonary Artery and Lung in Chronic Hypoxia Induced-PH

The expression of GPER in mouse pulmonary artery and lung during the development of chronic hypoxia induced-PH is unknown. Here we wished to firstly determine if GPER is expressed in female mouse pulmonary artery and lung, and secondly investigate if expression levels of GPER are altered during PH development.

Using female mouse pulmonary artery from wildtype mice we observed a significant decrease in expression of GPER protein in hypoxic pulmonary artery compared to normoxic controls (Figure 4-3A). On the other hand, expression of GPER mRNA transcript was unchanged in whole lung from hypoxic females (Figure 4-3B).

Interestingly, expression of GPER mRNA transcript was much lower in female mouse lung in normoxic and hypoxic conditions compared to ESR1, the gene encoding ERα (Figure 4-4A&B).

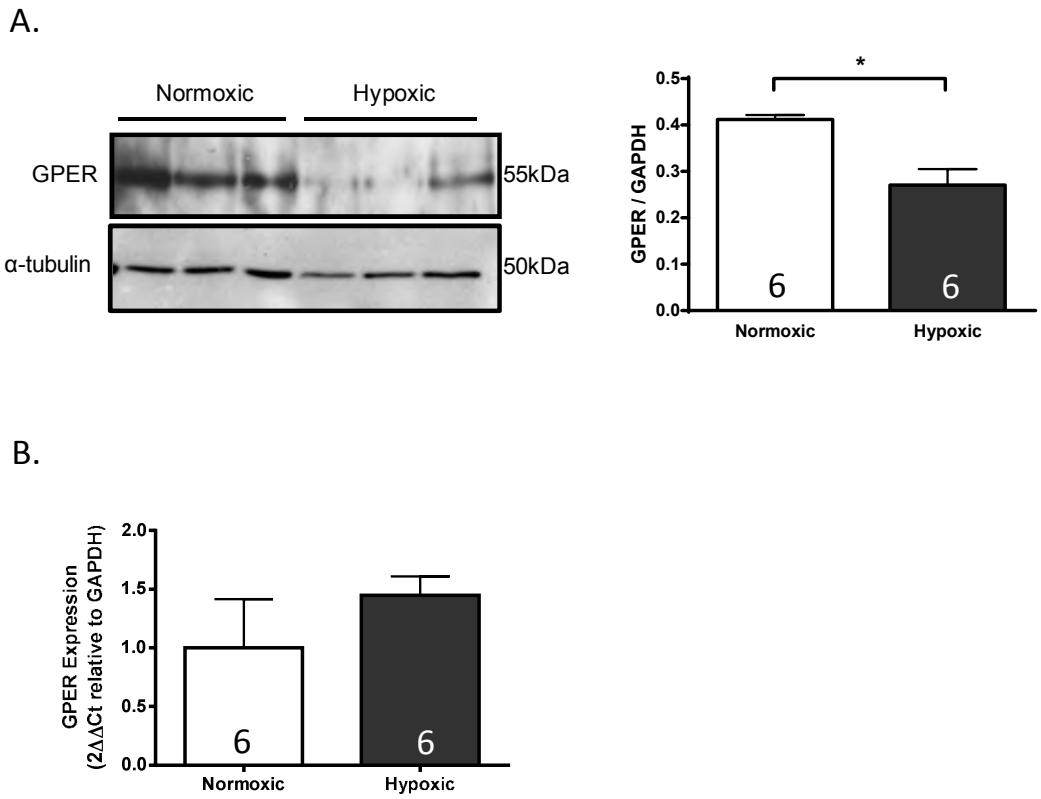
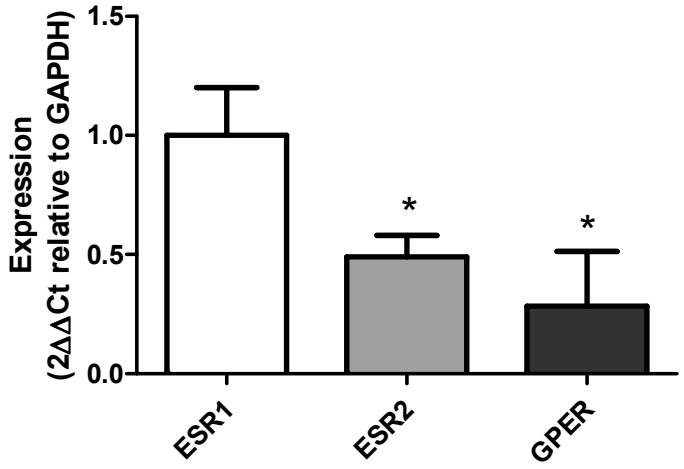


Figure 4-3: Expression of GPER in female mouse pulmonary artery and lung.

Expression of GPER in hypoxic pulmonary artery is decreased relative to normoxic control (A). GPER mRNA transcript expression is unchanged in whole lung in normoxic versus hypoxic (B). Representative blots are shown. Quantitative data are represented as ± SEM and analysed by an unpaired t-test. n=6 pulmonary artery/lung per group repeated in triplicate; n for each group is indictaed in bar on graph.

A.



B.

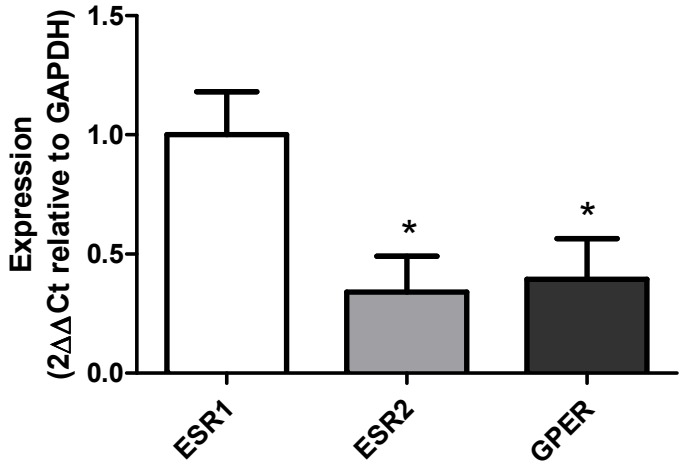


Figure 4-4: Estrogen receptor mRNA expression in whole lung from female mice.
Expression of estrogen receptors in (A) normoxic lung and (B) hypoxic lung. Data are expressed as ± SEM analysed by an unpaired t-test. *p<0.05 vs. ESR1. n=6 mice/lungs. ESR1=gene encoding ERα; ESR2=gene encoding ERβ

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4.2.4 Development of Chronic Hypoxia Induced-PH in Male and Female GPER$^{-/-}$ Mice

GPER is a novel G-protein coupled estrogen receptor and the role of GPER in the development of PH is unknown. To our knowledge, this is the first study investigating the role of GPER in an in vivo model of PH. Here we utilise a GPER knockout mouse (GPER$^{-/-}$) and the chronic hypoxic model of PH to determine the influence of GPER in males and females during PH development.

The development of chronic hypoxia induced-PH was examined by right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH) and percentage of pulmonary vascular remodelling and pulmonary artery muscularisation. In both males and females, development of chronic hypoxia induced-PH was confirmed by elevations in RVSP, RVH and pulmonary vascular remodelling (Figure 4-5A-C). In normoxic conditions, deletion of GPER had no effect on RVSP, RVH and pulmonary vascular remodelling. This was observed in both males and females. In hypoxia, the development of PH was unaffected in males and females by GPER deletion as RVSP (Figure 4-5A) and RVH (Figure 4-5B) were unchanged between wildtype (WT) and GPER$^{-/-}$ mice. On the other hand, pulmonary vascular remodelling was increased in GPER$^{-/-}$ females in hypoxia relative to hypoxic wildtype mice. This effect was not observed in male GPER$^{-/-}$ mice (Figure 4-5C). GPER deletion however, had no effect on pulmonary artery muscularisation in normoxic and hypoxic conditions in males and females (Figure 4-6). We observed no effects on systemic haemodynamics in GPER$^{-/-}$ mice relative to wildtype controls. Mean systemic arterial pressure (mSAP) (Figure 4-7A) and heart rate (HR) (Figure 4-7B) were unchanged in males and females in normoxia and hypoxia. Absolute haemodynamic values are shown in Table 4-1.

4.2.5 Effect of GPER on Body Weight and Uterus Weight in Chronic Hypoxia Induced-PH

Wildtype and GPER$^{-/-}$ males exhibited increased body weights relative to wildtype and GPER$^{-/-}$ females, respectively. However, in both males and females, body weight was unaffected by hypoxia. In males, deletion of GPER had no effect on body weight in normoxic and hypoxic conditions, although in

females, body weight was significantly increased in $GPER^{-/-}$ mice relative to wildtype controls (Figure 4-8A).

The role of GPER in regulating estrogen dependent uterine biology is unclear. Here we identified that GPER deletion in female mice has no effect on uterus weight. Uterus weight was expressed as a ratio corrected for body weight given the influence GPER had on overall body weight in females (Figure 4-8B). Absolute values for body weight and uterus weight are shown in Table 4-2.

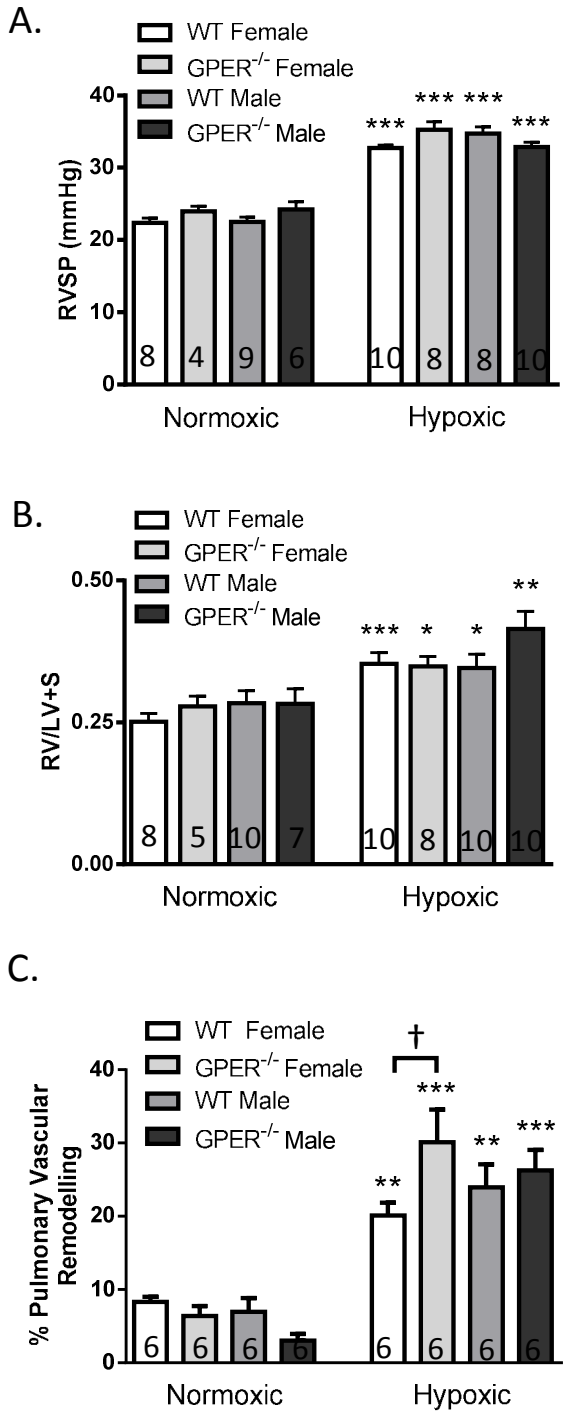


Figure 4-5: Development of chronic hypoxic PH in male and female GPER$^{-/-}$ mice.

In vivo, (A) RVSP, (B) RVH and (C) pulmonary vascular remodelling are unchanged in WT versus GPER$^{-/-}$ mice in both males and females. Data are expressed as ± SEM analysed by a Two-Way ANOVA followed by a Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 vs. normoxic; †p<0.05 vs. WT. n=6-9 mice per group; n per group is indicated in the bar in the graph..

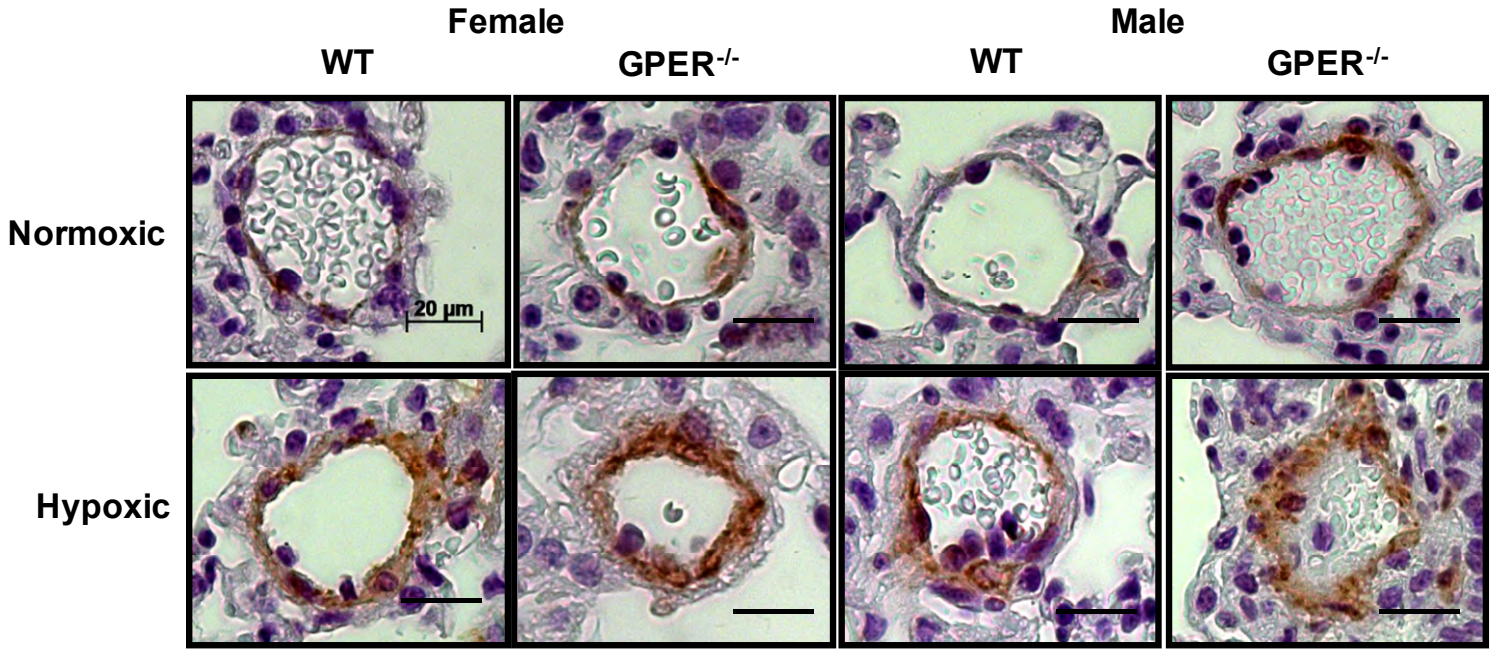
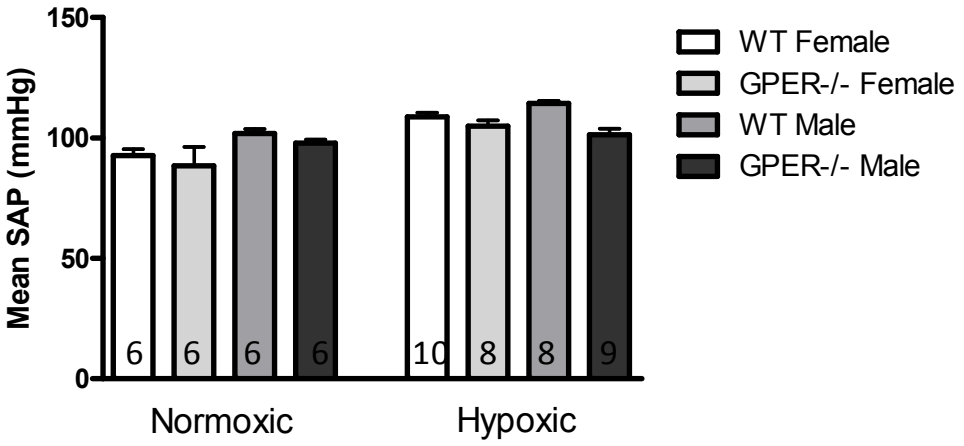


Figure 4-6: Pulmonary artery muscularisation in male and female GPER$^{-/-}$ mice.

Smooth muscle layer stains dark brown with alpha- smooth muscle actin. Representative images are shown from each group. Scale bar=20µm.

A.



B.

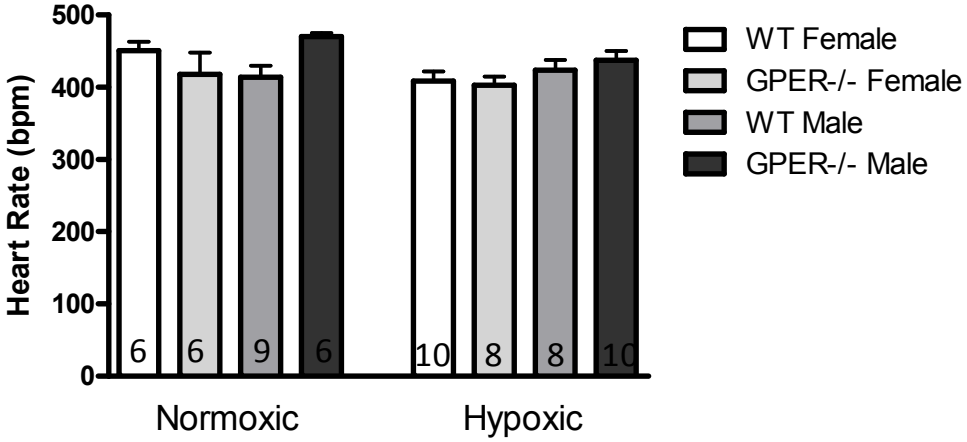
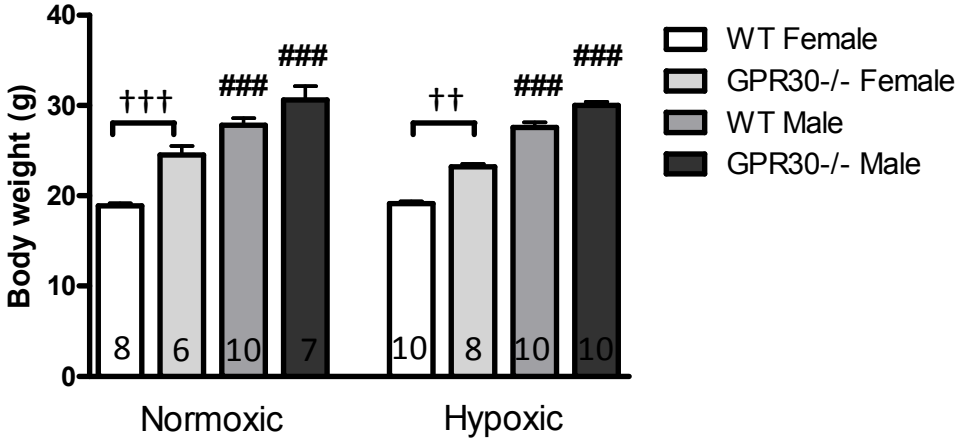


Figure 4-7: Systemic haemodynamic parameters in male and female GPER$^{-/-}$ mice.
Mean systemic arterial pressure (mSAP (A) and heart rate (HR) (B) are unchanged in male and female GPER$^{-/-}$ mice relative to WT controls. Data are expressed as ± SEM analysed by a Two-Way ANOVA followed by a Bonferroni's post hoc test. n=6-10 mice per group; n of mice per group is indicated in the bar in the graph.

A.



B.

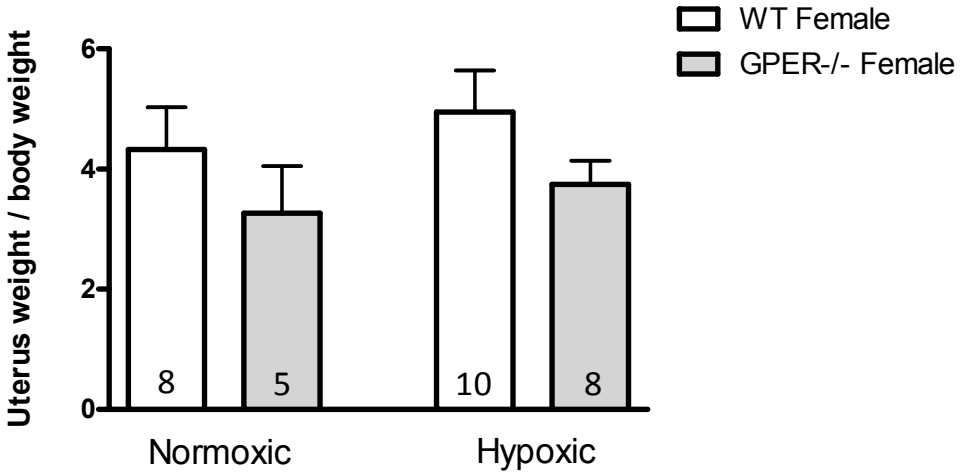


Figure 4-8: Body weight and uterus weight in male and female GPER$^{-/-}$ mice.

(A) Body weight is increased in female GPER$^{-/-}$ mice relative to WT controls whilst male GPER$^{-/-}$ body weight is unaffected. (B) Uterus weight/body weight ratio is unchanged in female GPER$^{-/-}$ mice. Data are expressed as ± SEM analysed by a Two-Way ANOVA followed by a Bonferroni's post hoc test. ††p<0.01, †††p<0.001 vs. WT; ###p<0.001 vs. female. n=5-10 mice per group; n per group is indicated in bar in graph.

Table 4-1: Haemodynamic parameters in male and female WT and GPER$^{-/-}$ mice in chronic hypoxia.

Right ventricular systolic pressure (RVSP); Mean right ventricular pressure (RMVP); right ventricular diastolic pressure (RVDP); systemic systolic arterial pressure (sSAP); mean systemic arterial pressure (mSAP); diastolic systemic arterial pressure (dSAP); Heart rate (HR). Data expressed as ± SEM analysed by a Two-Way ANOVA followed by a Bonferroni's post hoc test. *p<0.05, **p<0.01, ***p<0.001 vs. normoxic; †† p<0.01 vs. WT. n = 6-10 mice per group.

Parameter/Group	**Male WT**	**Male GPER$^{-/-}$**	**Female WT**	**Female GPER$^{-/-}$**
Normoxic				
RVSP, mmHg	22.52 ± 0.68	24.21 ± 1.07	22.38 ± 0.67	23.98 ± 0.69
RMVP, mmHg	13.61 ± 0.47	14.36 ± 0.77	14.77 ± 0.46	13.60 ± 1.27
RVDP, mmHg	1.40 ± 0.40	0.53 ± 0.26	2.74 ± 0.75	1.45 ± 0.67
sSAP, mmHg	116.6 ± 2.82	99.74 ± 3.38	96.11 ± 1.88	103.2 ± 5.91
mSAP, mmHg	101.8 ± 1.83	97.85 ± 1.43	92.63 ± 2.70	88.41 ± 7.94
dSAP, mmHg	83.75 ± 1.55	96.07 ± 3.56	83.59 ± 2.72	73.98 ± 5.74
HR, bpm	413.8 ± 16.06	470.2 ± 5.00	450.8 ± 12.37	418.2 ± 29.73
Hypoxic				
RVSP, mmHg	34.74 ± 0.90***	32.85 ± 0.66***	32.76 ± 0.40***	35.27 ± 1.07***
RMVP, mmHg	18.17 ± 0.74***	18.87 ± 0.62**	19.80 ± 0.68***	20.39 ± 0.71***
RVDP, mmHg	2.21 ± 0.48	2.63 ± 0.38*	2.66 ± 0.39	1.99 ± 0.44
sSAP, mmHg	123.8 ± 1.61	109.4 ± 3.29††	113.0 ± 2.09**	111.2 ± 2.83
mSAP, mmHg	114.3 ± 1.17	101.3 ± 2.54	108.8 ± 1.79	104.9 ± 2.41
dSAP, mmHg	101.4 ± 2.13***	89.83 ± 2.68††	102.8 ± 2.26***	94.12 ± 2.93**
HR, bpm	424.1 ± 13.60	437.2 ± 12.92	408.5 ± 13.26	403.1 ± 11.80

Table 4-2: Ventricle, body weight and uterus weight in male and female mice WT mice and GPER$^{-/-}$ mice

Right ventricle (RV); left ventricle plus septum (LV + S); RV/LV+S ratio, body weight and uterus weight. * p<0.05 ** p <0.01, *** p<0.001 vs. normoxic mice; †† p<0.01, ††† p<0.001 vs. GPER$^{-/-}$ mice; ###p<0.001 vs. female mice. Data are expressed as ± SEM analysed by a Two-Way ANOVA followed by a Bonferroni's post hoc test. n = 6-10 mice per group.

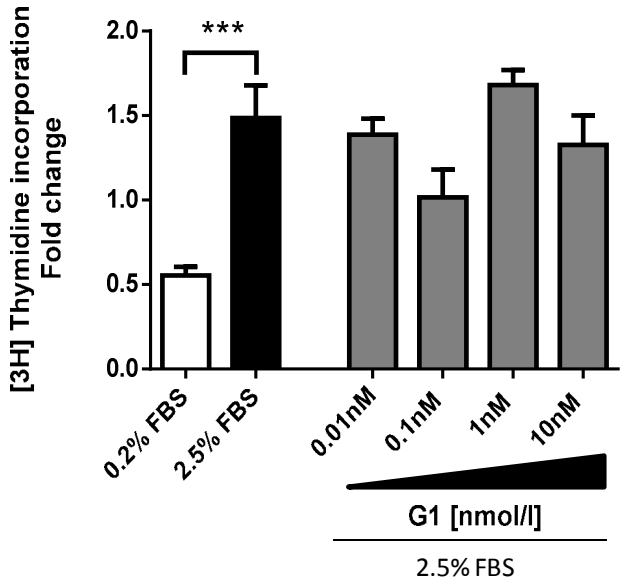
Group	**RV (mg)**	**LV + S (mg)**	**RV/LV+S**	**Body weight (g)**	**Uterus weight (mg)**
Normoxic					
Male					
WT	31.18 ± 1.58#	112.2 ± 4.74###	0.28 ± 0.02	27.82 ± 0.76###	
GPER$^{-/-}$	35.14 ± 2.05	128.3 ± 9.55###	0.28 ± 0.03	30.63 ± 1.52###	
Female					
WT	20.47 ± 1.26	81.76 ± 2.85	0.25 ± 0.01	18.90 ± 0.29	82.77 ± 14.22
GPER$^{-/-}$	25.79 ± 2.34	91.82 ± 3.88	0.28 ± 0.02	24.52 ± 1.00†††	78.96 ± 18.09
Hypoxic					
Male					
WT	37.81 ± 2.65#	109.7 ± 4.15###	0.35 ± 0.02*	27.55 ± 0.59###	
GPER$^{-/-}$	45.09 ± 3.16##	109.7 ± 5.09	0.42 ± 0.03**	30.00 ± 0.42###	
Female					
WT	28.02 ± 1.34	79.52 ± 1.78	0.35 ± 0.02***	19.14 ± 0.24	94.39 ± 11.91
GPER$^{-/-}$	32.19 ± 1.28	92.82 ± 2.84	0.35 ± 0.02*	23.20 ± 0.30††	86.92 ± 9.43

4.2.6 Effects of GPER on Human Pulmonary Artery Smooth Muscle Cell Proliferation

Smooth muscle cell proliferation is integral to the pulmonary vascular remodelling process. We therefore utilised human PASMCs *in vitro* to determine the role of GPER using a selective agonist and antagonist.

The GPER selective agonist, G1, had no effect on proliferation of PASMCs at the range of concentrations tested (0.01-10nmolL^{-1}) (Figure 4-9A). In previous studies we have shown estrogen induced proliferation of PASMCs at physiological concentrations of 1nmol/l (Dempsie et.al. 2013; White et.al. 2011). In this study we also demonstrate estrogen induced proliferation at 1nmolL^{-1}, however, the GPER selective antagonist, G15, has no effect on estrogen induced-human PASMC proliferation (Figure 4-9B).

A.



B.

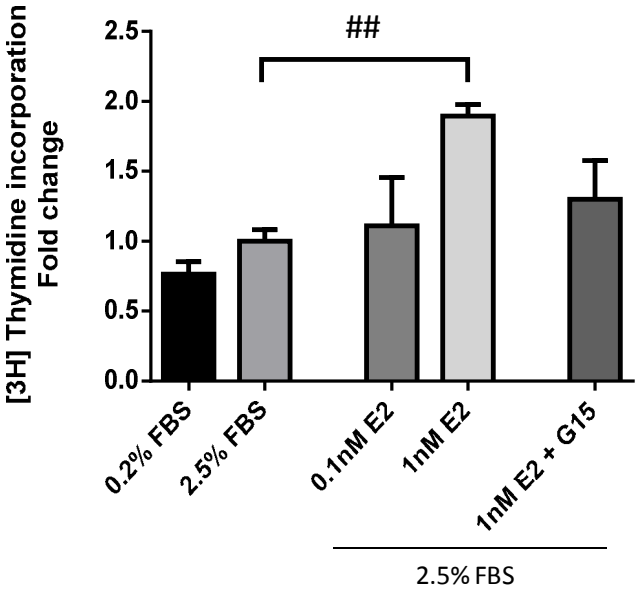


Figure 4-9: Proliferation in female human PASMCs is unaffected by a GPER agonist and antagonist.

(A) The GPER selective agonist, G1, has no effect on human PASMC proliferation. (B) The GPER selective antagonist, 1μmolL^{-1} G15, has no effect on estrogen (E_2) induced proliferation. Data are expressed as ± SEM analysed by a One-Way ANOVA followed by a Tukey's post hoc test. . n=3 female control cell lines repeated in triplicate (Controls 1, 4 and 5, *See Table 2-3 in Material and Methods*). ***p<0.001 vs. 2.5% FBS; ##p<0.01 vs. 1nmoll^{-1} E_2.

4.2.7 **Effect of Serotonin on GPER Expression in Human Pulmonary Artery Smooth Muscle Cells**

We have previously demonstrated that physiological estrogen (1nmolL^{-1}) can increase the expression of tryptophan hydroxylase 1 (TPH1), the serotonin transporter (SERT) and the 5-HT_{1B} receptor in human PASMCs (White et.al. 2011). Here we wished to examine the effects of serotonin on GPER expression in human PASMCs.

In female human PASMCs we show that the expression of GPER in human PASMCs is unchanged by 1µmolL^{-1} serotonin (Figure 4-10).

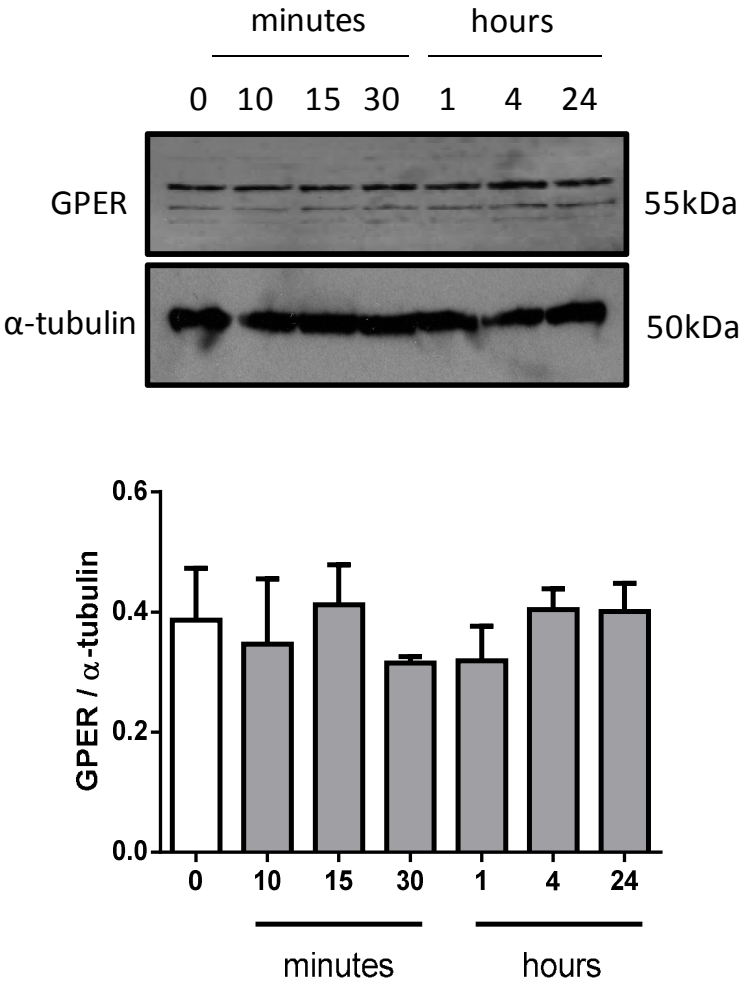


Figure 4-10: Serotonin has no effect on GPER expression in female human PASMCs. Representative blots are shown. Quantitative data is expressed as ± SEM analysed by a One-Way ANOVA followed by a Tukey's post hoc test. n=3 female control cell lines repeated in triplicate (Controls 1, 4 and 5, *See Table 2-3 in Material and Methods*).

4.3 Discussion

It is clear from the most recent epidemiologic data that there is a female predominance in many forms of PAH, including IPAH and HPAH (Badesch et.al. 2010; Humbert et.al. 2006; Peacock et.al. 2007; Thenappan et.al. 2007). The reasons underlying this gender disparity remain unresolved, although several lines of evidence suggest estrogen plays a pivotal role in PAH physiology and pathogenesis. The 'estrogen paradox' however, has hampered our understanding of estrogen in the development of PAH in women.

In this study, we aimed to investigate the role of the novel estrogen receptor, GPER, in the development of PH by utilising translational *in vitro* and *in situ* studies, and a $GPER^{-/-}$ mouse in the *in vivo* chronic hypoxic model of PH. Importantly we evaluated the role of GPER *in vivo* in both males and females to determine the influence of gender. Our results demonstrate that development of chronic hypoxia induced-PH is likely independent of estrogen mediated effects through GPER. From our translational human data, we also demonstrate that GPER is expressed in human lung and localised to smooth muscle cells and endothelial cells, however expression is unchanged in PASMCs in PAH. Furthermore, estrogen induced proliferation of human PASMCs is independent of GPER. Together our results indicate GPER is not crucial in estrogen mediated effects in PH development.

GPER mediates rapid and transcriptional events in response to estrogen in cells lacking classical nuclear ERα/ERß, through activation of diverse transduction cascades like the ERK1/2 pathway (Filardo et al. 2002; Revankar et al. 2005) and phosphatidylinositol-3-kinase (PI3K)/Akt (Thomas et al. 2005). GPER has previously been identified in human internal mammary arteries and saphenous veins at a level comparable to ERα, although 10-fold lower than ERß (Haas et al. 2007). Expression of GPER has also been detected in mouse mesenteric artery (Martensson et.al. 2009), carotid artery (Haas et.al. 2009) and in the cardiomyocytes of both the rodent and human heart (Deschamps & Murphy 2009; Filice et al. 2009; Patel et al. 2010). Furthermore, it is clearly evident that vascular GPER protein is localised to both endothelial and smooth muscle cells in rat aorta, carotid and cerebral arteries, although more intense staining for GPER

is demonstrated in endothelial cells (Broughton et.al. 2010). GPER is therefore believed to contribute to cardiovascular actions of estrogens. Characterisation of GPER in the lung however, is less well defined, although enhanced GPER expression has recently been attributed to a pathological role in lung cancer cells (Jala et.al. 2012; Siegfried et al. 2009). In particular, GPER expression and localisation in human and mouse lung and pulmonary artery in PH is unknown. In the present study we observed GPER expression in both human non-PAH control and PAH patient lung sections, although expression levels were low. Additionally, we show no difference in protein and mRNA expression levels of GPER between non-PAH controls and IPAH and HPAH patients in isolated PASMCs. In contrast, protein expression of GPER was significantly reduced in pulmonary arteries from hypoxic female mice relative to controls, although this was not observed at the mRNA level, suggesting a post-transcriptional regulation of GPER in hypoxia. This data conflicts previous evidence in cancer cells and cardiomyocytes where GPER was identified as a target gene of hypoxia inducible factor-1α (HIF-1α), leading to an increase in expression upon hypoxic conditions, compared to normoxia (Recchia et al. 2011). The up-regulation of GPER was associated with an adaptive response to stressful microenvironments in the rat heart during hypertension. Here, our results may differ due to tissue and species differences. Interestingly, we show in mouse lung that expression levels of GPER mRNA transcript are significantly less than ESR1 levels in both normoxic and hypoxic conditions, although comparable to ESR2 levels. Together this data suggests that the role of GPER in the lung during PH development may be less important than ERα.

Despite the low expression levels of GPER in the human and mouse lung, we wished to characterise the role of GPER *in vivo* as these represent novel findings in the development of PH. Moreover, we showed a reduction of GPER protein in hypoxic pulmonary arteries from females. We utilised male and female $GPER^{-/-}$ mice between 2-3 months of age due to the age dependent development of systemic arterial pressure observed in female $GPER^{-/-}$ mice (Martensson et.al. 2009). Chronic hypoxia was used to induce PH in $GPER^{-/-}$ mice as it produces potent vasoconstriction in pulmonary arteries (Pugh & Hemnes 2010). In normoxic conditions, deletion of GPER had no influence on RVSP, RVH or pulmonary vascular remodelling. Following exposure to chronic hypoxia, we

observed development of chronic hypoxia induced-PH in wildtype male and female mice as they exhibited elevations in RVSP, RVH and pulmonary vascular remodelling. However, in male and female GPER$^{-/-}$ mice, hypoxia did not attenuate, nor did it exaggerate PH development compared to wildtype counterparts. These findings indicate that GPER is not involved in elevated pulmonary pressures and cardiac remodelling in chronic hypoxic PH. Interestingly, female GPER$^{-/-}$ mice did demonstrate an increase in pulmonary vascular remodelling compared to hypoxic wildtype females, although this effect was absent in males. This may suggest that activation of GPER mediates a protective effect in hypoxia although the effects are not sufficient enough to completely reverse the PH phenotype. In addition, other ERs or environmental stimuli must be involved.

G1, a GPER selective agonist has been shown to reduce vascular smooth muscle (Haas et.al. 2009) and endothelial cell proliferation (Holm et al. 2011). Indeed, whilst the former provides a protective effect against remodelling, inhibition of endothelial cell proliferation may prevent re-endothelialisation in arteries following injury and contribute to loss of endothelial function. In contrast to vascular cells, in cancer cells, GPER has been demonstrated to mediate proliferative effects of estrogen in endometrial cancer cells and aggressive disease in breast cancer cells (Filardo et al. 2006). G1 induces proliferation via epidermal growth factor receptor (EGFR)/ERK1/2 mediated pathways leading to elevated cyclin D1 and c-fos expression (Maggiolini et.al. 2004; Sirianni et al. 2008). The role of GPER activation in pulmonary vasculature and proliferation is not clear. Here we identify that human PASMCs did not respond to G1 between 0.01–10nmolL^{-1} concentration range. This is in line with our data which shows that human PASMC proliferation induced by a physiological estrogen concentration (1nmolL^{-1}) is unaffected by the GPER selective antagonist, G15. Together, our evidence provides the first insight into the role of GPER in isolated human PASMCs and indicates that estrogen-induced proliferation in PAH is likely GPER independent. However, future studies in PASMCs using G1 should use a larger concentration range to ensure the effects at higher concentrations are investigated, for example, 1μmolL^{-1} has been shown recently to promote proliferation in breast cancer associated fibroblasts (Luo et al. 2014).

This we believe is the first study to characterise the role of GPER in the development of PAH. Much more evidence is available on the cardioprotective effects of GPER in humans and animal models. In several vascular beds, for example, in rat aorta and carotid artery (Broughton et.al. 2010; Lindsey et.al. 2011) and in porcine coronary artery (Meyer et al. 2010) it is confirmed that G1 initiates endothelium-dependent vascular relaxation. In female $GPER^{-/-}$ mice there is an age-dependent increase in mean arterial pressure at 9 months associated with an increase in vascular resistance as opposed to an increase in heart rate and cardiac output (Martensson et.al. 2009), further implicating protective effects on GPER in vasculature. Consistent with this, in our study using mice aged 2-3 months, we observed no difference in mean systemic arterial pressure (mSAP) and heart rate in our $GPER^{-/-}$ male and female mice. In addition, G1 has been shown to lower blood pressure in normotensive rats and relax rodent and human vessels (Haas et.al. 2009). In isolated hearts, G1 pre-treatment attenuates ischemia-reperfusion and infarct size potentially through a reduction in inflammation (Bopassa et al. 2010; Deschamps & Murphy 2009; Patel et.al. 2010). Although we show here that the potent vasoconstrictor serotonin (MacLean & Dempsie 2010) has no effect on GPER expression in PASMCs, further characterisation of GPER is required to decipher vasoactive properties which may contribute to pulmonary artery vasoconstriction and development of PH.

The localisation of GPER to human blood vessels clearly implies a role for this receptor in cardiovascular disease and there is confounding evidence to support this. Additionally, a role for GPER in some cancer cells, including breast cancer, endometrial cancer and lung cancer is proposed. In contrast, there is sparse evidence describing GPER localisation in pulmonary vasculature and this we believe is the first study to characterise GPER in this vascular bed. From this study, we conclude that expression of GPER in PASMCs has a limited influence on PH pathology independent of gender. This evidence builds on answering the female predominance observed in PAH.

Chapter 5.

Investigating the Influence of Testosterone in the Development of Experimental Pulmonary Hypertension

5.1 Introduction

Despite a gender bias in PAH with women up to four-fold more likely to present with disease compared to men (Badesch et.al. 2010; Walker et.al. 2006), male patients show poorer survival in established PAH, even following treatment intervention (Benza et.al. 2012; Humbert et.al. 2010a; Humbert et.al. 2010b). There is a paucity of studies in PAH investigating male hormones in pulmonary vasculature structure and function, but the exact role of androgens in physiology and the pathophysiology of PAH remains uncertain. Androgens may therefore play a crucial role in the progression of PAH in males.

The influence of testosterone on the pulmonary vasculature is a potential mechanism by which males may show shortened survival. However, right ventricular (RV) function is the most important prognostic factor and indicator of survival in PAH (D'Alonzo et al. 1991). In line with this, males show a propensity to RV dysfunction and lower right ventricular ejection fraction (RVEF) in PAH compared to females, (Kawut et.al. 2009) and estrogen levels are shown to strongly correlate with improved RV function (Ventetuolo et.al. 2011). Androgen receptors (AR) have been identified in both the RV and left ventricle (LV), although the latter has been more extensively studied. Both testosterone and the primary metabolite, dihydrotestosterone (DHT) mediate genomic effects through the AR, although DHT is 10-times more potent in activation of the AR than testosterone (Liu et.al. 2003). There is convincing evidence that testosterone can initiate concentric hypertrophy via AR dependent mechanisms (Achar et.al. 2010; Hayward et.al. 2000). In addition, structural and morphological changes in human hearts are associated with alterations in the metabolism of testosterone, with elevated levels of the DHT described in human left ventricular hypertrophy (Thum & Borlak 2002).

Recent evidence from the pulmonary artery banding (PAB) model of PAH which exhibits severe RVH and RV dysfunction in the absence of pulmonary vascular remodeling, describes a pro-fibrotic effect of testosterone in the RV (Hemnes et.al. 2012). Atrial natriuretic peptide (ANP), a marker of cardiac hypertrophy

was significantly attenuated following removal of testosterone by castration in PAB mice while myocyte size was augmented with chronic testosterone replacement. The MESA-Right Ventricle Study has also shown in men, that testosterone is associated with elevated RV mass and right ventricular end-diastolic and systolic volumes, whereas this was not observed in women (Ventetuolo et.al. 2011). A potentially negative effect of testosterone in the stressed RV may contribute towards dysfunctional and maladaptive RV hypertrophy in males.

On the other hand, testosterone is known to be a potent vasodilator in the isolated pulmonary vascular beds and has been extensively studied in isolated vessels from humans where the observed effect is independent of gender (Rowell et.al. 2009; Smith et.al. 2008). Importantly, this effect of testosterone is accepted to be a rapid, non-genomic effect and is therefore not mediated via AR (Jones et.al. 2002; Yue et.al. 1995) In addition, the effect is not due to conversion to estrogen as the vasodilatory response is unaffected by aromatase (CYP19A1) inhibition, the enzyme that converts testosterone to estrogen, or by estrogen receptor antagonism (Deenadayalu et.al. 2001; Teoh et.al. 2000; Tep-areenan et.al. 2002).

Given the high incidence of idiopathic, heritable and some associated forms of PAH in women (Benza et.al. 2012; Humbert et.al. 2010a), research addressing the gender bias has been focused on estrogen. However, the effects of testosterone may infer a contradiction in PAH with highlighted multiple actions in the RV and pulmonary vasculature. Since survival is worse in males and survival is closely linked to RV function, a negative effect of testosterone or its metabolites is proposed in the RV. Here we investigated the effects of testosterone *in vitro* and *in vivo* to elucidate the role of male hormones in PAH development and progression. We also determine vasoreactive properties in pulmonary arteries following testosterone manipulation.

5.2 Results

5.2.1 Castration Does Not Attenuate the Development of Chronic Hypoxia Induced-PH

To investigate the effects of testosterone on PH *in vivo*, we castrated wildtype C57Bl/6 male mice to deplete their endogenous testosterone. To assess PH development, right ventricular systolic pressure (RVSP), pulmonary vascular remodelling and right ventricular hypertrophy (RVH) were measured in normoxic and hypoxic conditions. Following 14 days exposure to chronic hypoxia, elevations in RVSP, vascular remodelling and RVH parameters were observed. Development of chronic hypoxia induced-PH was not attenuated by removal of endogenous testosterone by castration. There were no changes in RVSP, pulmonary vascular remodelling or RVH in castrated mice compared to SHAM control mice in both normoxic and hypoxic conditions (Figure 5-1A-C). Muscularisation of pulmonary arteries assessed by alpha smooth muscle actin, also showed no effect of castration in normoxic or hypoxic conditions (Figure 5-2).

Similarly, hypoxia and castration had no effect on systemic arterial pressure (Figure 5-3), although a reduction in the heart rate in hypoxic SHAM mice was reported, compared to normoxic SHAM controls (Figure 5-4). Absolute values for haemodynamic measurements are depicted in Table 5-1.

5.2.2 Testosterone levels were halved following castration

To confirm that castration in mice was effective in reducing testosterone levels, an ELISA (R&D Systems, UK) was performed in plasma samples to test remaining 'free' circulating testosterone. In normoxic SHAM mice we observed testosterone levels of 3.59 ± 0.07 $ngml^{-1}$ which were reduced following castration to 1.32 ± 0.009 $ngml^{-1}$. In hypoxia we also observed reduced plasma testosterone levels following castration from 4.03 ± 0.09 $ngml^{-1}$ in sham mice compared to 1.79 ± 0.02 $ngml^{-1}$ in hypoxic castrated mice (Figure 5-5).

We also wished to determine if exposure to chronic hypoxia had an effect on the levels of plasma testosterone *in vivo*. Interestingly, no differences were observed in testosterone levels between normoxic and hypoxic SHAM mice (Figure 5-5).

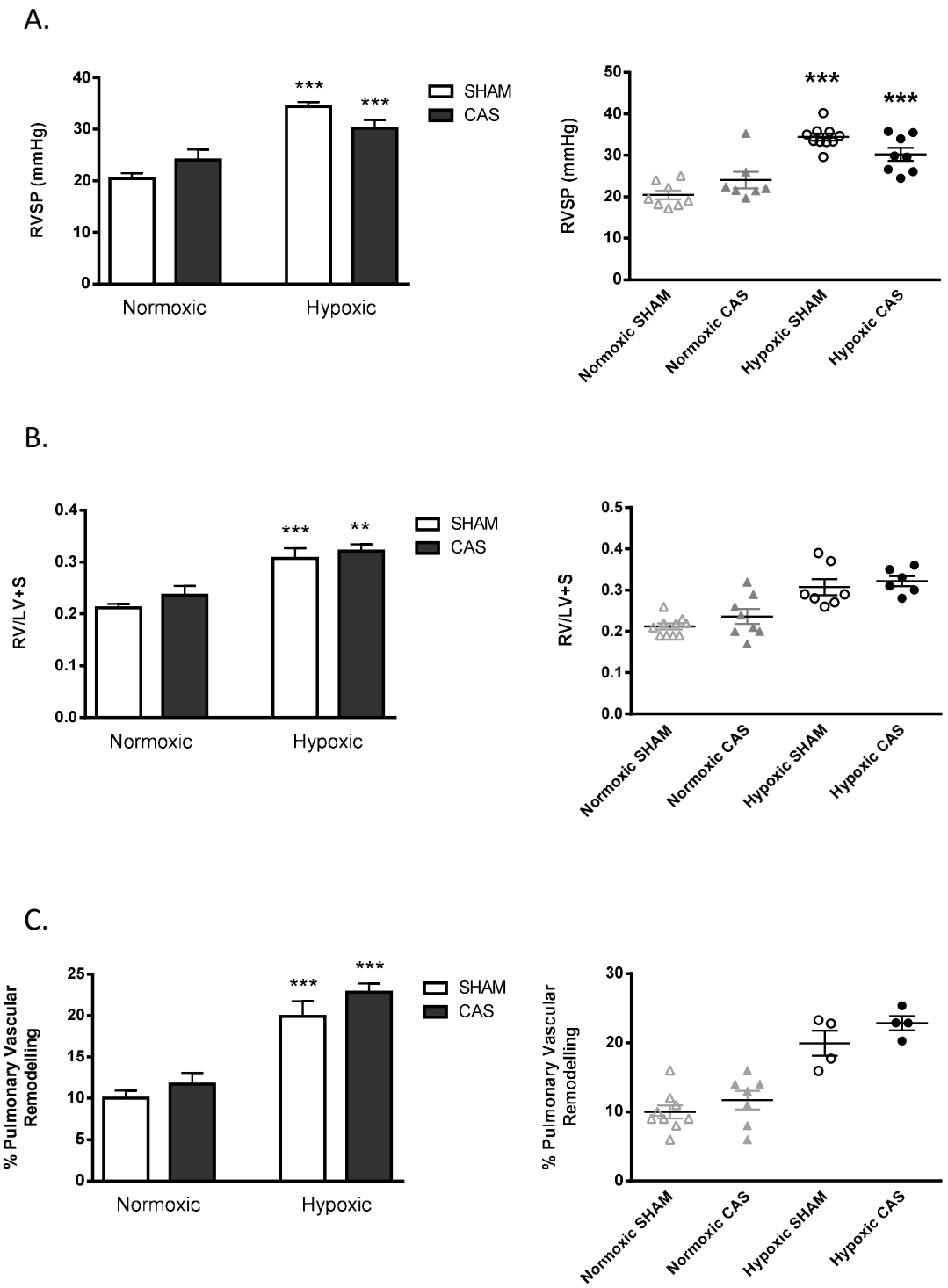


Figure 5-1: Castration has no effect on the development of chronic hypoxia induced- PAH *in vivo*.

Right ventricular systolic pressure (RVSP; A); pulmonary vascular remodelling (B) and right ventricular hypertrophy (RVH; C) were unchanged following castration in both normoxia and hypoxia. Data are expressed as mean ± SEM and analysed by a Two-way ANOVA followed by a Bonferroni's post-hoc test. Scatter plots were used to identify the spread of data. **p<0.01, ***p<0.001 vs. normoxic. n=4-10 mice per group.

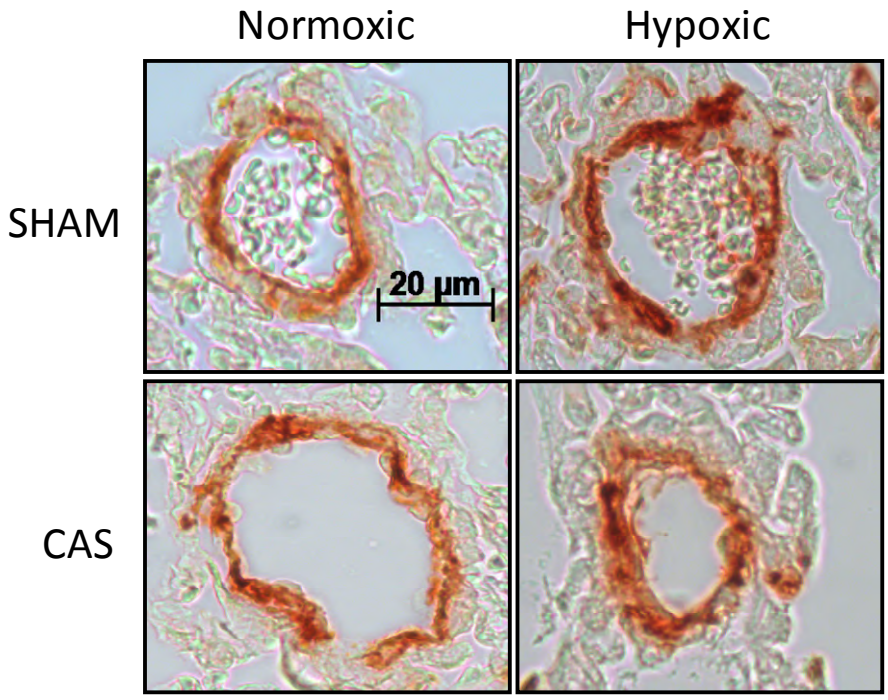


Figure 5-2: Castration has no effect on pulmonary vascular remodelling.
Representative images are shown stained with alpha smooth muscle actin (brown). Scale bar = 20µm.

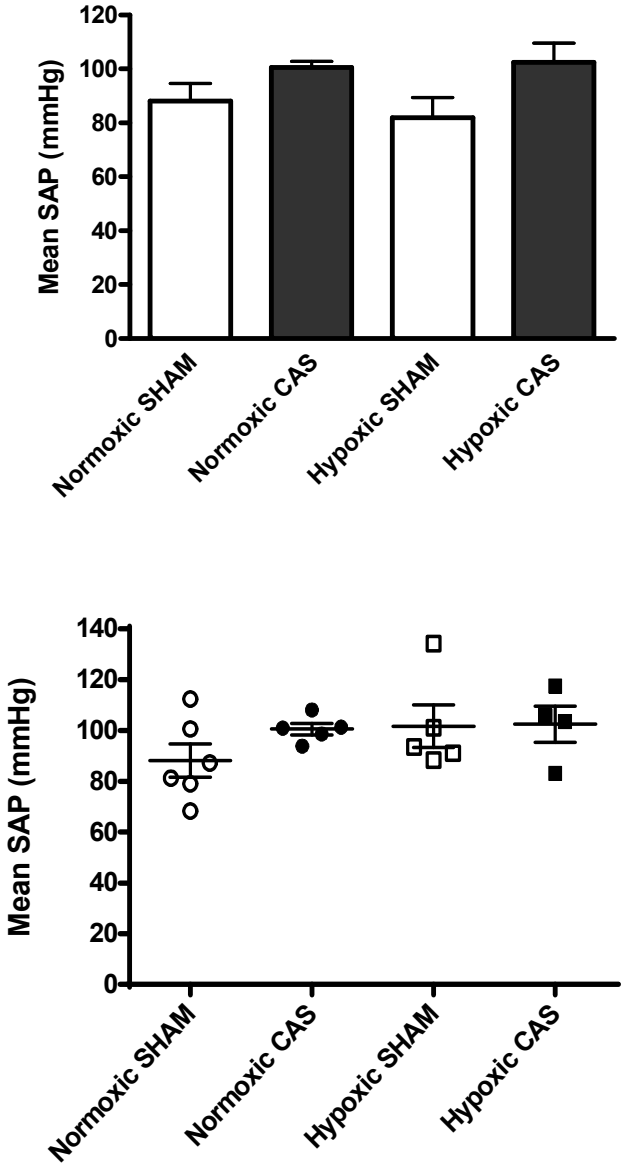


Figure 5-3: Castration has no effect on systemic arterial pressure in either normoxia or chronically hypoxic conditions.
Mean systemic arterial pressure (mSAP) is unaffected by removal of testosterone in both normoxia and hypoxia. Data is expressed as mean ± SEM and analysed by a Two-way ANOVA followed by a Bonferroni's post-hoc test. Scatter plots were used to identify the spread of data. n=4-6 mice per group.

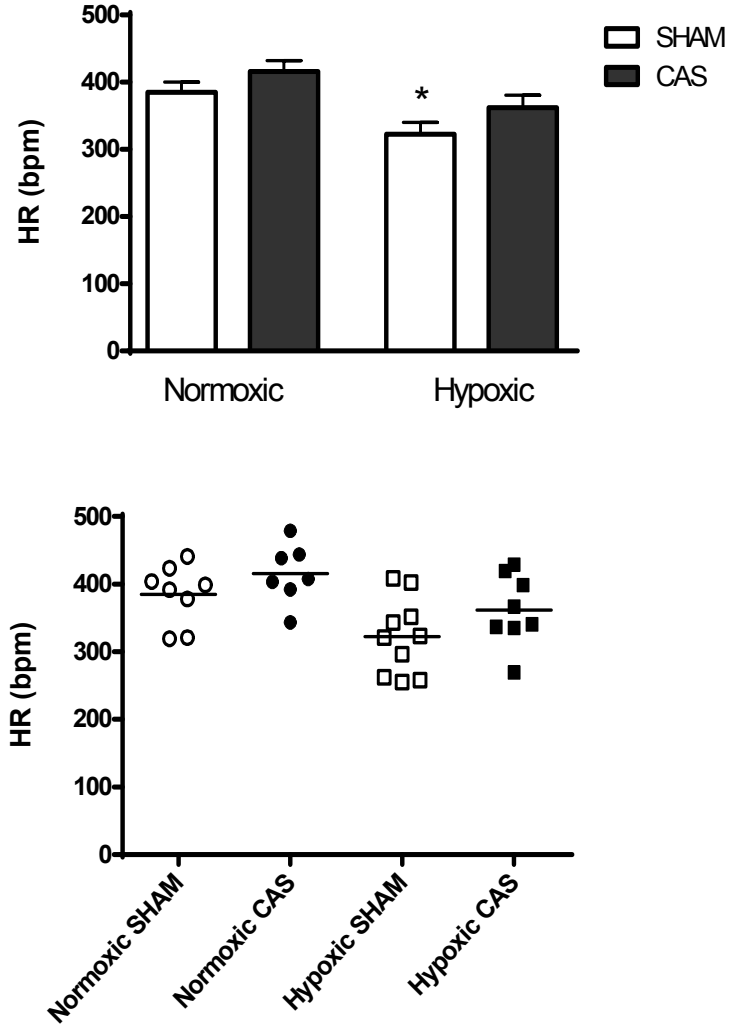


Figure 5-4: Castration has no effect on heart rate in either normoxia or chronically hypoxic conditions.
Castration had no effect on heart rate (HR) however HR was significantly reduced in SHAM mice in hypoxia. Data is expressed as mean ± SEM and analysed by a Two-way ANOVA followed by a Bonferroni's post-hoc test. Scatter plots were used to identify the spread of data. *$p<0.05$ vs. normoxic. n=7-10 mice per group.

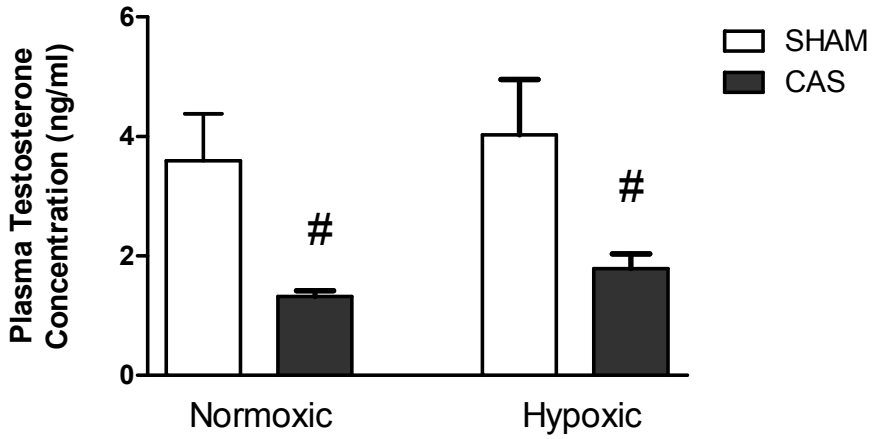


Figure 5-5: Plasma testosterone levels are reduced in mice following castration.
Plasma levels of testosterone in normoxic and hypoxic mice following castration. Data is expressed as mean ± SEM and was analysed using a non-fit standard curve corrected for the dilution factor followed by an unpaired t-test. #$p<0.05$ vs. SHAM. n=6 samples per group.

5.2.3 Body Weight and Heart Weight are Decreased Following Castration

Testosterone is an important androgen in regulation of growth and muscle mass (Humbert et.al. 2010a; Nunez et al. 1980) we therefore investigated the effect castration had on body weight in both normoxic and hypoxic conditions. In normoxic mice no difference in body weight was observed in castrated mice compared to SHAM controls. However, body weight was significantly decreased following castration in hypoxic mice compared to SHAM controls (Figure 5-6). In addition, hypoxia had no effect on body weight compared to normoxic mice.

Total heart weight was also investigated in SHAM controls and castrated mice in both normoxia and hypoxia. Chronic hypoxia had no effect on the total heart weight in both SHAM and CAS mice. Interestingly, in both normoxic and hypoxic conditions, castration resulted in a decrease in heart weight (Figure 5-7).

5.2.4 Left Ventricular Weight in the Heart is Subject to Regulation by Testosterone

Previous studies have identified an association between left ventricle (LV) mass and testosterone (Cavasin et al. 2003). LV weight was significantly reduced in male mice that underwent castration in both normoxic and hypoxic conditions. Chronic hypoxic treatment had no effect on LV weight in SHAM control mice (Figure 5-8). LV weight expressed as a ratio to body weight showed there were no effects of hypoxia on LV weight.

On the other hand, right ventricle (RV) weight was increased in both SHAM and castrated mice following exposure to chronic hypoxia. Castration in this instance had no effect on RV weight in either normoxia or hypoxia (Figure 5-9). RV weight expressed as a ratio to body weight was increased in hypoxia (5-10). Absolute values for heart weights and body weights are depicted in Table 5-2.

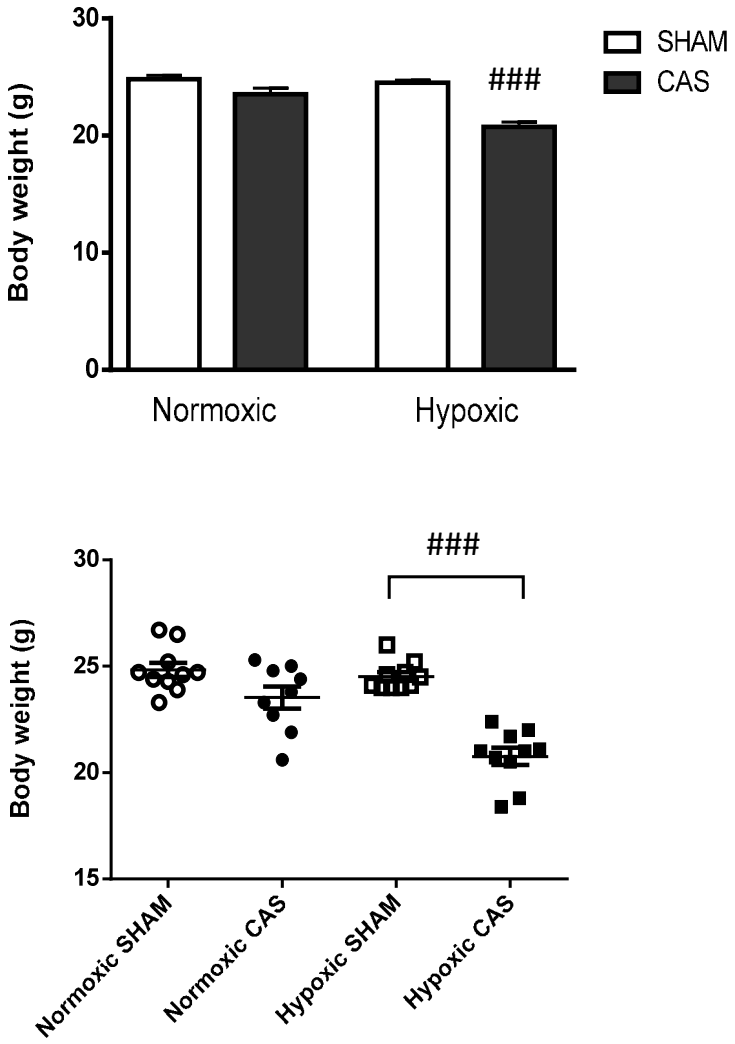


Figure 5-6: Body weight was decreased in hypoxic castrated male mice compared to SHAM control mice.
Heart weight is reduced following castration in hypoxia although remains unchanged in normoxia. Data are expressed as mean ± SEM and analysed by a Two-way ANOVA followed by a Bonferroni's post-hoc test. Scatter plots were used to identify the spread of data. ###p<0.001 vs. SHAM. n=9-10 mice per group.

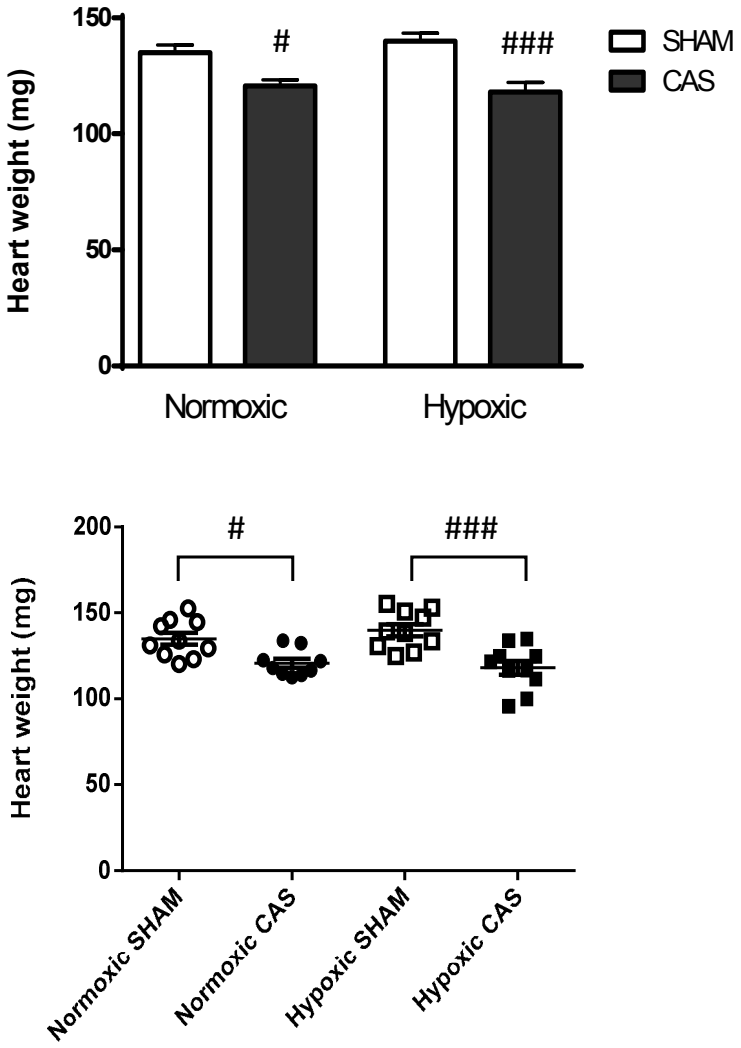


Figure 5-7: Heart weight was decreased in castrated male mice compared to SHAM control mice.

Heart weight is reduced following castration, however hypoxia has no effect on heart weight. Data are expressed as mean ± SEM and analysed by a Two-way ANOVA followed by a Bonferroni's post-hoc test. Scatter plots were used to identify the spread of data. #p<0.05; ###p<0.001 vs. SHAM. n=10 mice per group.

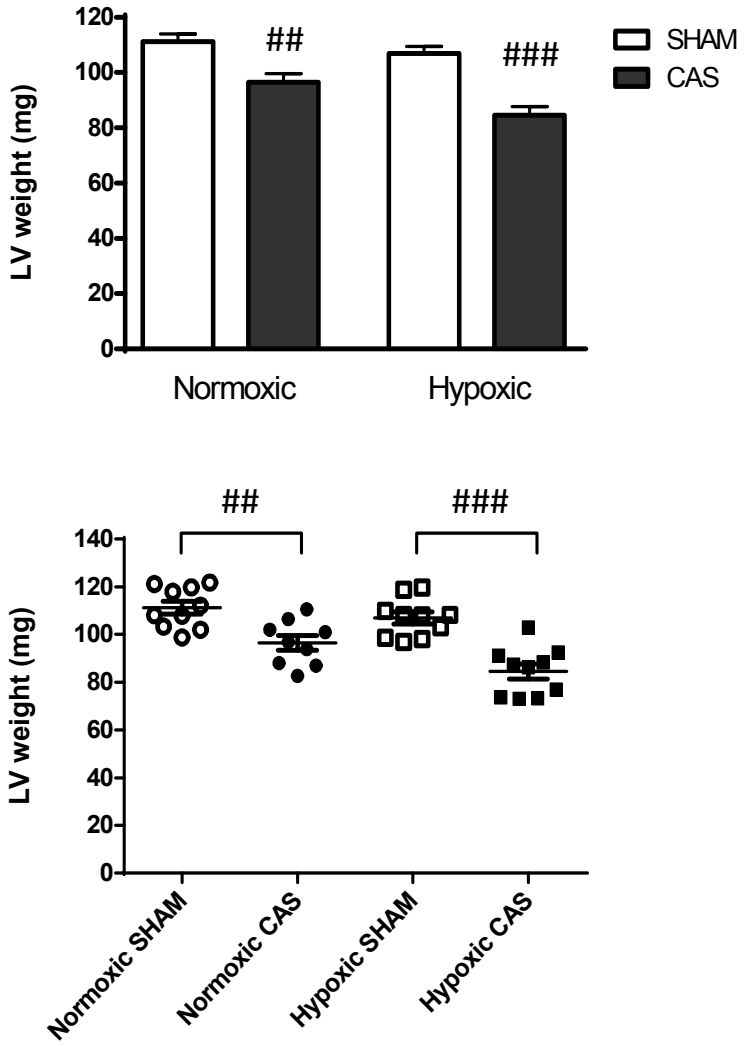


Figure 5-8: Left ventricular weight was decreased in castrated male mice compared to sham mice.

Left ventricular (LV) weight is reduced following castration, however hypoxia has no effect on LV weight. Data are expressed as mean ± SEM and analysed by a Two-way ANOVA followed by a Bonferroni's post-hoc test. Scatter plots were used to identify the spread of data. ##p<0.01; ###p<0.001 vs. SHAM. n=9-10 mice per group.

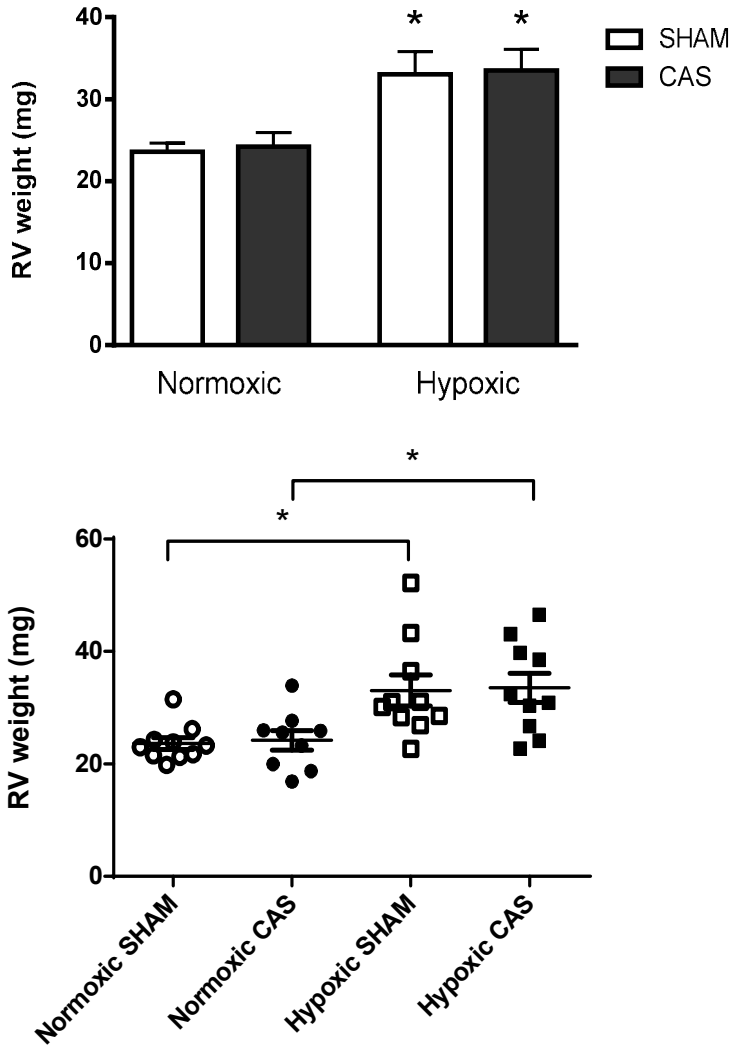


Figure 5-9: Right ventricular weight is unaffected following castration.
Castration has no effect on right ventricular (RV) weight in either normoxia or hypoxia, however RV weight is increased in both sham and castrated mice in hypoxia. Data are expressed as mean ± SEM and analysed by a Two-way ANOVA followed by a Bonferroni's post-hoc test. Scatter plots were used to identify the spread of data. *$p<0.05$ vs. normoxic. n=9-10 mice per group.

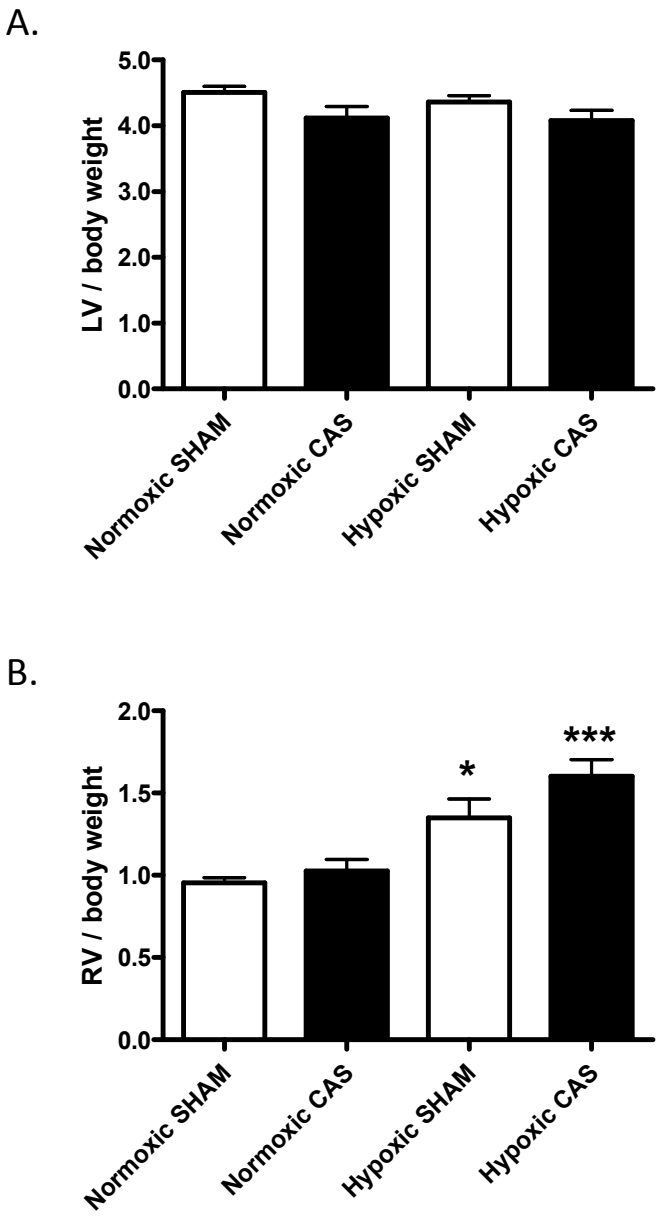


Figure 5-10: LV and RV weight expressed as a ratio to body weight.
(A) LV weight (mg)/body weight (g) ratio is unchanged in hypoxia whereas (B) RV weight (mg)/body weight (g) ratio is increased in hypoxia. This indicates an increase in RV weight relative to the body weight in hypoxia. Data are expressed as mean ± SEM and analysed by a Two-way ANOVA followed by a Bonferroni's post-hoc test. *p<0.05, **p<0.01 vs. normoxic.

Table 5-1: Haemodynamic parameters in SHAM and castrated mice exposed to chronic hypoxia.
RVSP=right ventricular systolic pressure; MRVP=mean right ventricular pressure; RVDP=right ventricular diastolic pressure; sSAP=systolic systemic arterial pressure; mSAP=mean systemic arterial pressure; dSAP=diastolic systemic arterial pressure; HR=heart rate; SHAM=control; CAS=castration. Data are expressed as ± SEM analysed by a Two-Way ANOVA followed by a Bonferroni's post hoc test.*p<0.05, ***p<0.001 vs. normoxic. n=4-10 shown in table for individual parameters.

Parameter	Normoxic SHAM	Normoxic CAS	Hypoxic SHAM	Hypoxic CAS
RVSP, mmHg	20.43 ± 1.05	22.18 ± 0.85	33.78 ± 0.63***	30.22 ± 1.56***
n	8	7	10	8
MRVP, mmHg	9.83 ± 0.53	12.26 ± 1.54	14.98 ± 0.39***	14.79 ± 0.66
n	8	7	10	8
RVDP, mmHg	1.15 ± 0.56	1.91 ± 0.58	3.25 ± 0.38*	3.38 ± 0.32
n	8	7	10	8
sSAP, mmHg	103.4 ± 2.93	114.3 ± 3.85	102.4 ± 8.38	108.9 ± 5.52
n	6	6	5	4
mSAP, mmHg	88.14 ± 6.5	100.6 ± 2.29	81.9 ± 7.6	102.5 ± 7.16
n	6	6	5	4
dSAP, mmHg	77.51 ± 8.01	88.71 ± 3.52	77.93 ± 11.22	95.04 ± 9.09
n	6	6	5	4
HR, bpm	384.7 ± 15.6	415.6 ± 16.4	322.3 ± 17.7*	362.0 ± 18.7
n	8	7	10	8

Table 5-2: Ventricle, heart and body weight in SHAM and castrated mice exposed to chronic hypoxia. RV= right ventricle; LV+S= left ventricle plus septum; SHAM= control; CAS=castration. Data are expressed as ± SEM analysed by a Two-Way ANOVA followed by a Bonferroni's post hoc test. *p<0.05, ***p<0.001 vs. normoxic; #p<0.05, ##p<0.01, ###p<0.001 vs. SHAM control. n=6-10 and shown for individual parameters in table.

Group	**RV (mg)**	**LV + S (mg)**	**RV/LV+S**	**Heart weight (mg)**	**Body weight (g)**
Normoxic					
SHAM	23.6 ± 1.0	111.3 ± 2.7	0.21 ± 0.007	134.9 ± 3.4	24.8 ± 0.34
n	10	10	9	10	10
CAS	24.2 ± 1.7	96.5 ± 3.1##	0.24 ± 0.01	120.7 ± 2.6#	23.5 ± 0.52
n	10	10	8	10	10
Hypoxic					
SHAM	33.1 ± 2.8*	106.9 ± 2.5	0.31 ± 0.02***	140.0 ± 3.5	24.5 ± 0.2
n	10	10	7	10	10
CAS	33.5 ± 2.7*	84.5 ± 3.2###	0.32 ± 0.01***	118.0 ± 4.1###	20.8 ± 0.4###
n	10	10	6	10	10

5.2.5 Effect of Castration on Serotonin Induced Pulmonary Artery Vasoconstriction

To determine if castration in male mice had any effect on hypoxic pulmonary vascular reactivity we assessed serotonin-induced vasoconstriction in pulmonary arteries. Serotonin was studied as it is a potent pulmonary vasoconstrictor (MacLean & Dempsie 2010). Pulmonary arteries from hypoxic SHAM mice had an increased vasoreactivity to serotonin compared to normoxic SHAM mice as evidenced by a significantly increased E_{max}. In addition, area under the curve analysis further highlights the elevated response to serotonin in hypoxic SHAM mice compared to normoxic SHAM mice (Figure 5-11A).

Serotonin induced-vasoconstriction was similar in both normoxic SHAM mice and normoxic castrated mice. Similarly, vasoconstriction was unaffected in arteries from hypoxic SHAM mice compared to hypoxic castrated mice (Figure 5-11A). Hypoxic castrated mice however, do not exhibit a heightened hypoxic response compared to normoxic castrated mice as E_{max} and AUC (Figure 5-11B) are not increased in hypoxia. Absolute values for pEC_{50} and E_{max} are shown in Table 5-3.

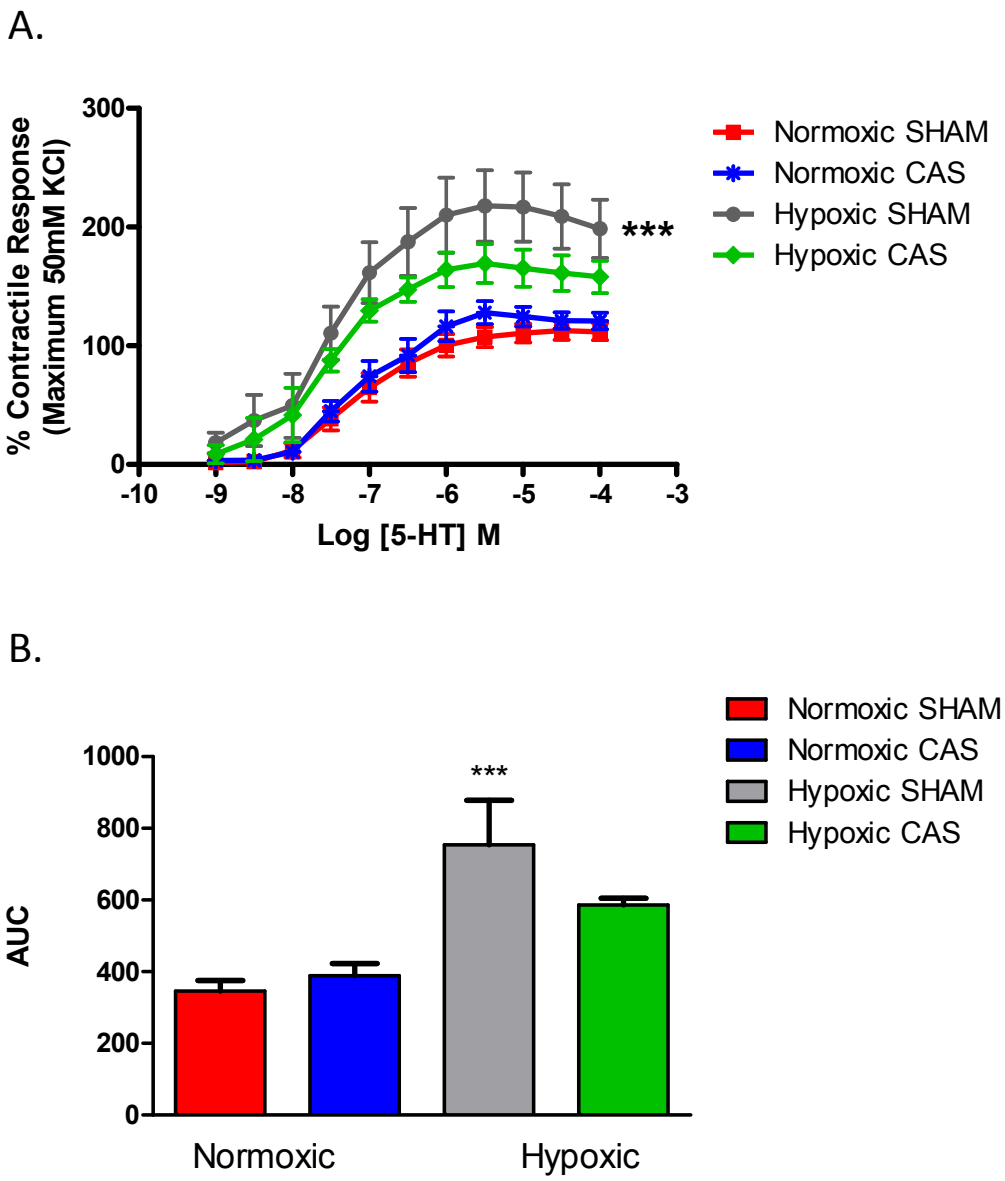


Figure 5-11: Serotonin- induced pulmonary arterial contraction is unaffected following castration in chronic hypoxic mice.
(A) Cumulative concentration response curve to serotonin shows no effect of castration on vascular activity, however, the response to serotonin in SHAM mice is elevated in hypoxia compared to normoxic SHAM mice. (B) Area under the curve (AUC) demonstrates an elevated response in hypoxic SHAM mice relative to normoxic SHAM mice. Data are expressed as mean ± SEM and (A) analysed using a Two-Way ANOVA followed by a Bonferroni's post hoc test and (B) by a one-way ANOVA followed by a Bonferroni's post hoc test. ***p<0.001 vs. normoxic. n=4-9.

Table 5-3: pEC50 and Emax values in male SHAM and castrated mice in normoxic and hypoxic conditions. pEC50, half maximal effective dose; Emax, maximal effective dose; CAS=castrated. Data are expressed as ± SEM and analysed by a One-Way ANOVA followed by a Bonferroni's post hoc test. n=4-9 mice per group.

	Normoxic SHAM	Normoxic CAS	Hypoxic SHAM	Hypoxic CAS
pEC50	7.14 ± 0.2	7.12 ± 0.2	7.52 ± 0.1	7.89 ± 0.5
Emax	111.9 ± 7.6	119.2 ± 7.25	210.6 ± 29.0***	163.7 ± 14.3
n	9	6	4	6

5.2.6 Effects of Testosterone and Dihydrotestosterone in Human Pulmonary Artery Smooth Muscle Cells

To further evaluate possible influences of testosterone we investigated proliferative responses to testosterone and its primary metabolite, dihydrotestosterone (DHT) in human pulmonary artery smooth muscle cells (PASMCs). Data on a role for androgens in pulmonary vasculature and specifically in remodelling of the vasculature is lacking. The effects of these androgens were examined within physiological circulating levels (8-30 $nmolL^{-1}$) (Smith et.al. 2008). Proliferation in human PASMCs was assessed by [^{3}H] thymidine incorporation. At all concentrations investigated, testosterone had no effect on proliferation (Figure 5-12A). On the other hand, DHT had a dose dependent effect on proliferation and at 3 $nmolL^{-1}$ DHT stimulated PASMC proliferation as assessed by [^{3}H] thymidine incorporation (Figure 5-12B).

5.2.7 Androgen Receptor Expression in Chronic Hypoxic Mouse Lung

Both testosterone and DHT mediate their effects through the androgen receptor (AR), although DHT is 10 times more potent at this receptor than testosterone itself (Liu et.al. 2003). We therefore wished to investigate the mRNA transcript expression of AR in chronic hypoxic mouse pulmonary artery and determine if hypoxia has any effect on the expression of this receptor.

Firstly, AR mRNA transcript was found to be expressed in the lungs of male mice. The levels of AR mRNA however, were unchanged between normoxic SHAM mice and hypoxic SHAM mice. Expression of AR was also unchanged between SHAM and CAS mice both in normoxic and hypoxic conditions (Figure 5-13).

5.2.8 5α-reductase Expression in Chronic Hypoxic Mouse Lung

Testosterone is metabolised by the cytochrome P450 enzyme aromatase (CYP19A1) to estrogen. Alternatively, some testosterone is also metabolised to

DHT by the enzyme 5α-reductase. As our results demonstrated that DHT caused proliferation of human PASMCs, we wished to determine if expression levels of 5α-reductase mRNA were altered during hypoxia-induced PH in mouse lung. We examined 5α-reductase type 1 as this has previously been identified in the lung (Kimura et al. 2003).

We found expression levels of SRD5A1, the gene encoding 5α-reductase type I were unchanged between normoxic SHAM mice and hypoxic SHAM mice. Additionally, CAS had no effect on the mRNA levels of SRD5A1 in both normoxia and hypoxia (Figure 5-14).

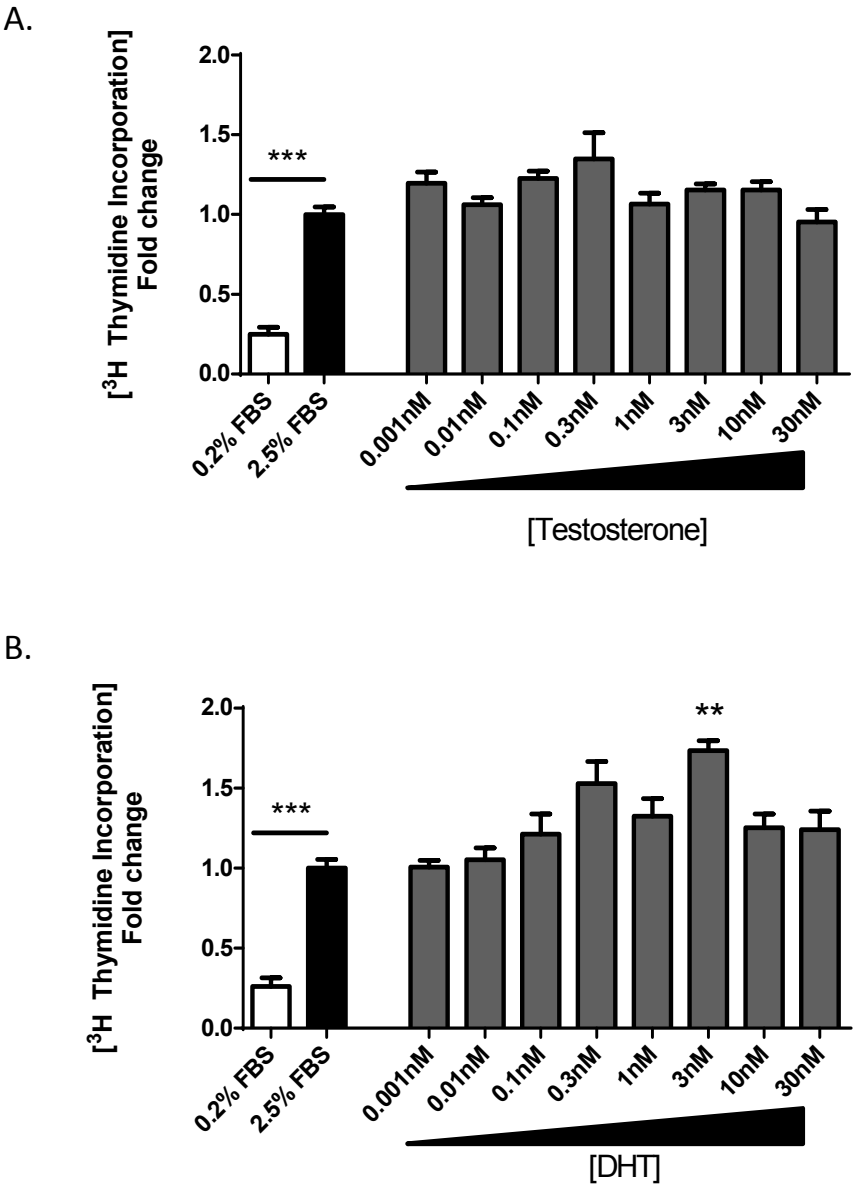


Figure 5-12: Dihydrotestosterone stimulates proliferation of control male human pulmonary artery smooth muscle cells.
Testosterone has no effect on proliferation (A) whereas DHT at 3nmol/l increases proliferation (B). ### p<0.01 vs. 0.2% FBS; **p<0.1 vs. 2.5% FBS. Data are expressed as mean ± SEM and analysed by a One-way ANOVA followed by a Bonferroni's post-hoc test. n=3 controls performed in triplicate (Control 1-3; *See Table 2-3 in Materials and Methods Section*). DHT=dihydrotestosterone.

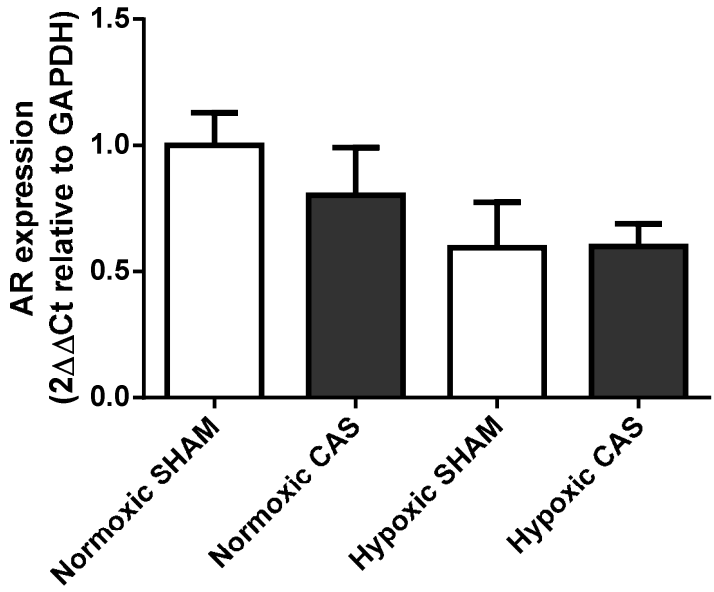


Figure 5-13: Androgen receptor expression is unchanged in hypoxia and following castration.
Data are expressed as mean ± SEM and analysed by a one-way ANOVA followed by a Tukey's post hoc test. n=6 lungs/group. AR= androgen receptor.

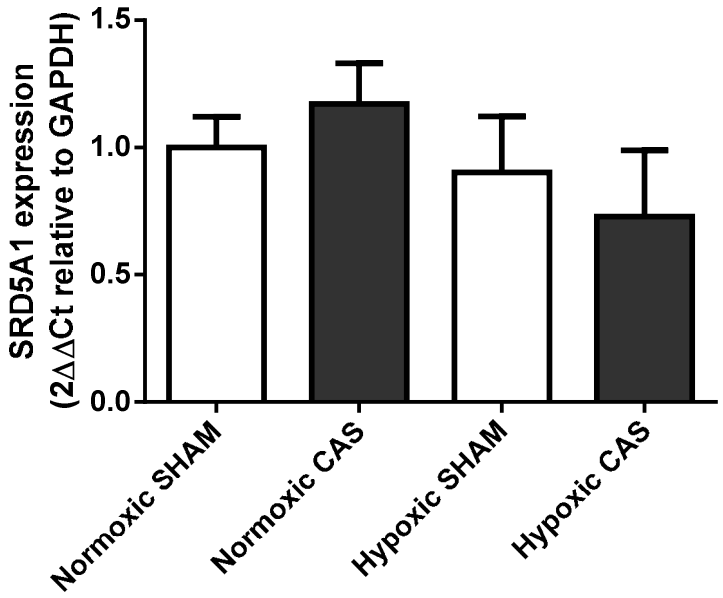


Figure 5-14: 5α-reductase type 1 expression is unchanged in hypoxia and following castration.
Data are expressed as mean ± SEM and analysed by a one-way ANOVA followed by a Tukey's post hoc test. n=6 lungs/group. SRD5A1= 5α-reductase type I.

5.3 Discussion

There is a paucity of data on male hormones in PAH development and the role they play in both physiology and pathophysiology in the lung. Here we investigated the primary circulating hormone in males *in vivo* by removal of testosterone via surgical castration to address the question "does testosterone contribute to development and progression of PAH in males?" We also wished to determine the influence of testosterone and its primary metabolite dihydrotestosterone (DHT), in translational studies *in vitro* in human PASMCs.

In this study we show that development of chronic hypoxia induced-PH is not dependent on testosterone as there were no significant alterations in haemodynamic measurements in response to castration. In line with this we observed no effect of hypoxia on plasma testosterone levels in our chronic hypoxic mouse model. Muscularisation of pulmonary arteries was also unaffected by testosterone manipulation although we found that DHT induced a proliferative response in isolated PASMCs. Our findings imply that testosterone may not be involved in regulation of pulmonary pressures although altered metabolism and increased DHT in PASMCs may contribute to the remodelling process.

Recent epidemiological evidence from the REVEAL registry supports female gender as a risk factor in development of PAH with a female to male ratio of 4.3:1 reported in the total PAH group (Walker et.al. 2006) and 4.1:1 within idiopathic PAH (IPAH) cases (Badesch et.al. 2010). Estrogen has since been regarded as an environmental risk factor in pulmonary artery smooth muscle cells (PASMCs) contributing to proliferation and pulmonary arterial remodelling (White et.al. 2011). Further, alterations in estrogen synthesis (Roberts et.al. 2009b); Mair et al. unpublished), metabolism (Austin et.al. 2009; West et.al. 2008; White et al. 2012c), and estrogen receptor expression (Rajkumar et.al. 2010) all contribute to PAH development. However, in the most established animal models, males exhibit more severe hypoxic and monocrotaline induced-PH compared to females (McMurtry et.al. 1973; Rabinovitch et.al. 1981; Resta et.al. 2001). In line with this, despite an increased prevalence of PAH in women than men, the estimated survival rate in men is consistently worse than in

women even with treatment intervention (Benza et.al. 2012; Humbert et.al. 2010a). The role of testosterone in PAH may therefore have been overlooked.

Right ventricular (RV) dysfunction is the most important prognostic factor and indicator of survival in PAH (D'Alonzo et.al. 1991). In males, poor survival rates are strongly associated with reduced RV function and cardiac output (Humbert et.al. 2010a; Kawut et.al. 2009). Therefore a correlation between testosterone and the RV is proposed. Higher testosterone levels have previously been associated with structural effects on the RV contributing to greater RV mass and larger RV volumes in men. On the other hand, estrogen infers protective functional effects whereby estrogen levels strongly correlate with increased right ventricular ejection fraction (Ventetuolo et.al. 2011). Indeed there is a lower risk of cardiovascular disease in ageing men with a high estrogen/low testosterone state (Arnlov et al. 2006; Tivesten et al. 2007). In a recent study by Hemnes et al (2012) lower testosterone levels were reported to be protective in mice with RV load stress associated with pathologic hypertrophy (Hemnes et.al. 2012). Additionally, changes in the morphological structure of the RV, including increased myocyte size and fibrosis, in relation to testosterone levels were found, although this had minimal effects on RV function (Hemnes et.al. 2012). Our data are congruent with this study showing that manipulation of testosterone via surgical castration had no effect on RVSP at baseline or following hypoxia. However, surprisingly in our study there was no effect of castration observed on the right ventricle and both SHAM and castrated mice developed RVH to a similar degree. Although this study does provide evidence that testosterone is involved in regulating myocyte size in the LV as castration results in decreased LV weight in both normoxic and hypoxic conditions. This perhaps reflects a difference in the PAB model which induces more substantial pressure overload in the RV and RV dysfunction compared to the chronic hypoxic model. RV dysfunction in chronic hypoxia may not be severe enough to evaluate the benefit of removing testosterone on the RV. Together, this evidence suggests that the effects of testosterone are primarily involved in dysfunctional RV hypertrophy. Following increased afterload inflicted by elevated pulmonary pressures during PAH, testosterone may be the underlying cause of the difference in survival rate between males and females.

The degree of RVH in rats exposed to high altitude is greater in castrated males treated with testosterone (Vander et.al. 1978) and the effects of testosterone and hypoxia appear additive. Testosterone biosynthesis also seems to be activated by hypoxia (Hwang et al. 2009). We therefore examined the plasma levels of testosterone in mice in the absence of, and following exposure to, chronic hypoxia. No statistically significant difference in 'free'-circulating testosterone levels were observed in normoxic or hypoxic male mice. However, testosterone has a high affinity for sex hormone binding globulin (SHBG) and circulates mainly bound to SHBG, with only 1-2% unbound or 'free' to exert its biological activity (Dunn et.al. 1981). In fact, as plasma testosterone levels increase and SHBG becomes saturated, albumin binding of testosterone increases. The total testosterone unbound changes relatively little over physiological concentration ranges (Dunn et.al. 1981). It may therefore be important to disseminate bound and unbound/'free' testosterone to confirm whether the absence or alteration in testosterone levels between normoxia and hypoxia relates to SHBG levels.

It could also be considered that local production of testosterone in either the lung or RV may confer a more important influence than total circulating testosterone. Certainly, elevated testosterone in the human heart causes structural and morphological changes (Achar et.al. 2010) and local metabolism of testosterone is changed in the hypertrophic heart with increased DHT levels described in left ventricular hypertrophy (Thum & Borlak 2002). Here we also show that local metabolism of testosterone is possible in the lung, as the lung expresses 5α-reductase. mRNA levels of 5α-reductase are however, unchanged between normoxic and hypoxic mice. Additionally, the presence of the AR also infers activity of either/both testosterone and DHT in the lung. Although we show AR transcriptional expression is unchanged in hypoxia consistent with previous findings (Khandrika et al. 2009), others have reported increased androgen ligand binding to AR in hypoxia (Mitani et al. 2011; Park et al. 2012). Together these findings may infer increased activity of testosterone in established hypoxic PH. Further investigation of the protein levels of both AR and 5α-reductase are required to confirm these observations.

In contrast to the detrimental effects of testosterone reported in the heart, testosterone-induced vasodilation has been identified in numerous vascular

beds, including isolated rabbit coronary arteries and aorta (Yue et.al. 1995), rat pulmonary arteries (English et.al. 2001; Jones et.al. 2002), and more recently isolated human pulmonary and mesenteric arteries (Jones et al. 2003). It is in fact, a more potent vasodilator than estrogen in pulmonary vasculature (English et.al. 2001) and this effect is independent of gender (Rowell et.al. 2009; Smith et.al. 2008). Importantly, the direct vasodilatory action of testosterone is accepted to be a rapid non-genomic effect independent of AR (Jones et.al. 2002; Yue et.al. 1995). The mechanism is believed to be through inhibition of Ca^{2+} entry via voltage gated calcium channels (Hall et.al. 2006; Scragg et.al. 2004) and independent of endothelium (Perusquia et al. 1996; Yue et.al. 1995) and nitric oxide release (Honda et al. 1999). However, these studies are carried out in the absence of hypoxia and assess vasodilation in pre-constricted isolated vessels. Therefore, we wished to investigate the effects of castration on pulmonary vascular reactivity and analyse the hypoxic pulmonary vasoconstrictive response. We examined the response of isolated pulmonary arteries to serotonin, a potent pulmonary vasoconstrictor (MacLean & Dempsie 2010). In normoxic conditions we observed no effect of castration on serotonin-induced vasoconstriction. SHAM mice exhibited an elevated vasoconstrictive response to serotonin in hypoxia, however, this response was absent in hypoxic castrated mice. This suggests that hypoxic pulmonary vasoconstriction in males may involve an interaction between serotonin and testosterone. Indeed, testosterone has been shown to increase levels of 5-HT_{2A} receptor in male rat brain, although this is likely due to its enzymatic conversion to estrogen via aromatase (Sumner & Fink 1998). 5-HT_{2A} is the predominant receptor involved in serotonin-induced pulmonary vasoconstriction in hypoxia in rodents (MacLean & Dempsie 2010). Thus, reduced testosterone in castrated mice may result in lower levels of 5-HT_{2A} in pulmonary arteries and hence attenuate the hypoxic vasoconstrictive response to serotonin.

These findings suggest that although testosterone may infer protective vascular effects at baseline, providing rationale for a lower incidence of PAH in men, as PH progresses and the environment becomes more hypoxic due to remodelling, protective effects of testosterone may be compromised. Additionally, disease progression in men may become more exaggerated due to negative effects of testosterone on the right ventricle, leading to right ventricular failure. Hence,

multiple actions of testosterone in the lung and RV, and in normal and diseased states complicate the gender paradox in PAH.

One limitation of this study is the high remaining circulating testosterone in the plasma. This could be due to length of time after surgery in which haemodynamic measurements were assessed which may not be adequate to completely deplete testosterone levels. Alternatively, the high testosterone could be a result of conversion of DHEA released from the adrenal glands to testosterone. To eliminate these complications in future studies, the testosterone levels should be monitored throughout the study to ensure that haemodynamic parameters are measured when testosterone levels are low.

Our results indicate that elevated pulmonary pressures and pulmonary arterial muscularisation develop independent of testosterone in chronic hypoxia induced-PH. In hypoxic PH, testosterone levels are unchanged, however we suggest altered testosterone metabolism in the lung and pulmonary arteries may promote enhanced PASMC proliferation via DHT, leading to remodelling as PH progresses. In conclusion we suggest that testosterone may not be involved in initiating disease development but rather facilitates disease progression in established PAH.

Chapter 6.

General Discussion

6.0 General Discussion

The impact of sex hormones in the development of PAH is complex and incompletely understood owing to the 'estrogen paradox'. Both estrogen and androgens are vasoactive in the pulmonary vasculature and PASMCs. The effects of estrogen are mediated either through conversion to estrogen metabolites, or directly via two classical estrogen receptors, ERα and ERβ. In the lung, ERα and ERβ are expressed in PAECs (Venkov et al. 1996), PASMCs (Karas et al. 1994) and the airway (Hamidi et al. 2011). In general, however, the role and expression of ERα and ERβ in physiology and pathophysiology of the pulmonary vasculature is not as well described as in the systemic vasculature.

Increased female susceptibility in human PAH is well described. Recent epidemiological studies highlight this gender bias and the Registry to Evaluate Early and Long-term PAH Disease Management (REVEAL) reports a strong gender bias whereby 79.5% of prevalent adult PAH patients were female. This female predominance was observed across the majority of PAH subtypes (Badesch et.al. 2010; Benza et.al. 2012). In addition, incident cases of PAH in UK and Ireland support a female susceptibility with 69.9% PAH cases reported in females (Ling et.al. 2012). Together, this emerging evidence with regard to a gender bias has implicated an influence of sex hormones on the pulmonary vasculature and right heart. In particular, estrogen and estrogen metabolites have been attributed to PAH development and progression. Indeed, polymorphisms in aromatase, the estrogen synthesising enzyme, have been linked to elevated estrogen levels in the lungs of female patients with portopulmonary hypertension (Roberts et.al. 2009b). Moreover, altered estrogen metabolism arising from polymorphisms in the estrogen metabolising enzyme, CYP1B1, have also been associated with PAH development in women (West et.al. 2008). Currently, no animal model has recapitulated this female susceptibility. Additionally, *exogenous* administration of estrogen appears protective in male rodents (Lahm et.al. 2012a; Resta et.al. 2001) and paradoxically, male rodents exhibit a susceptibility to both hypoxic- and moncrotaline-induced PH and exhibit more severe PH compared to females

(Rabinovitch et.al. 1981). Male rodents are therefore more commonly utilised in PAH research although this has offered limited insight into the female gender bias observed in human PAH. For this reason, we investigated the effects of *endogenous* estrogen and estrogen antagonism in both males and females. We hypothesised that there may be a disparity in the effects of an ERα antagonist between males and females.

Here, we provide evidence that development of PAH is ERα dependent in females only (Chapter 3). Dysregulation of the BMPR2 pathway via ERα appears central to this gender specific effect. We observed that expression of ERα transcript and protein was increased in PASMCs from female PAH patients compared to normal female controls whilst ERβ was down-regulated in PASMCs from female PAH patients. This is consistent with gene expression data that implicates up-regulation of ESR1 in PAH pathogenesis (Rajkumar et.al. 2010) and polymorphisms in ESR1 associated with higher expression and an increased risk of developing portopulmonary hypertension (Roberts et.al. 2009b). Uniquely we report that there are higher levels of ERα in female PAH PASMCs compared with male PASMCs from PAH patients. We suggest it is likely then, that the ERα pathway contributes more significantly to pathophysiology of the female lung during PAH compared to the male lung. To translate functional and clinical relevance to our findings we investigated the effects of ER agonists and antagonists *in vitro* in isolated human PASMCs. Consistent with previous findings (White et.al. 2011; White et.al. 2012c) we demonstrate that estrogen stimulates proliferation of PASMCs at physiological concentrations. Excessive PASMC proliferation is a main component of pulmonary vascular remodelling and lesions, and is currently considered irreversible in PAH (Humbert et.al. 2004). Indeed we show for the first time that estrogen can induce proliferation in PASMCs via ERα activation, whilst an ERβ agonist and antagonist have no effect on proliferation of PASMCs. Moreover, we determine the pro-proliferative effects of estrogen and the ERα agonist, PPT, are dependent on activation of downstream PI3K/Akt and ERK MAPK signalling (Figure 6-1). These pathways have both been implicated previously in cardiac hypertrophy and pulmonary vascular remodelling in right heart failure during PAH (Nadadur et.al. 2012; Zhou et.al. 2011). Pro-proliferative effects of estrogen in PASMCs may therefore be essential to the observed female susceptibility in PAH by initiating vascular

remodelling involving activation of ERs and transcription of proliferative genes, for example cyclin D1 and *c-fos*. Of further interest, evaluation of the effect of estrogen and ERs in PAECs involved in development of plexiform lesions observed in severe PAH may offer some insight into PAH progression in females.

We investigated ERα *in vivo* in two models of PAH. In chronic hypoxia-induced PH, the ERα antagonist, MPP attenuated the development of PH in female mice, but not in males. Given the elevated expression of ERα we observed in pulmonary arteries from female mice in hypoxia we propose that *endogenou*s estrogen activates ERα to facilitate the development of PH in females. On the other hand, ERα expression was unchanged in the pulmonary arteries of hypoxic male mice suggesting that ERα may not be involved in pathogenesis of PH in males in chronic hypoxia. Furthermore, we examined the effects of MPP in $SERT^+$ mice which demonstrate a female susceptibility dependent on circulating estrogen and serotonin. We show that MPP reversed PH in $SERT^+$ females under both normoxic and hypoxic conditions. Here, we suggest a pivotal role for ERα in the causative effects of *endogenous* estrogen in the development of PH independent of hypoxia. Interactions between the estrogen and the serotonin system have been described previously whereby estrogen can up-regulate TPH-1, SERT and $5\text{-}HT_{1B}$ (White et.al. 2011). We now show that serotonin can also up-regulate the expression of ERα and therefore we propose that synergistically these mediators may contribute to the pathogenesis of PAH in females. In addition, we identify an important interaction between ERα and BMPR2 in these *in vivo* models specific to females. Dysfunctional BMPR2 signalling plays a pivotal role in aberrant smooth muscle growth and endothelial cell proliferation and apoptosis in PAH. Loss of BMPR2 function mediates proliferation by reducing cell cycle inhibitors, Id1 and Id3 in PASMCs (Yang et.al. 2008; Yang et.al. 2005) and is responsible for ~80% of HPAH cases (Machado et.al. 2009). In the present study, we demonstrate decreased BMPR2 expression in the lung (Figure 6-1), associated with loss of downstream Smad 1 and Id signalling, following hypoxia and in $SERT^+$ female mice. Interestingly, this loss of expression was restored in female mice treated with MPP although male mice remained unaffected. This is consistent with a previous report in which a highly conserved functional estrogen response element with a co-repressor function was observed in the BMPR2 promoter whereby estrogen exposure resulted in suppression of the BMPR2 signal

through ERα (Austin et.al. 2009). In conclusion of our findings, we describe a mechanism of female susceptibility in PAH which combines elevated *endogenous* estrogen levels in females during PAH, with increased ERα expression and defective signalling, resulting in dysfunctional BMPR2 expression and function (Figure 6-1). Hence in females we propose that the selective effects of the ERα antagonist are likely through restoration of the dysfunctional BMPR2 signalling axis. Future studies investigating ERα should examine if the effects of estrogen are via membrane bound ERα or an effect on the transcription of genes in the nucleus. In addition, more studies are required to highlight the interactions between ERα and the serotonin system, for example, SERT, 5-HT receptors and THP-1.

Given the diverse effects of estrogen on the pulmonary vasculature, we also wished to investigate the novel G-protein coupled estrogen receptor, GPER which mediates rapid, non-genomic effects of estrogen (Chapter 4). The influence of GPER in pulmonary vasculature and during PAH development and progression is unknown. To our knowledge, this is the first study to determine immunolocalisation of GPER to smooth muscle cells in human non-PAH control and PAH patient lung. Immunoblotting and qRT-PCR revealed that expression of GPER was however unchanged between female non-PAH control and PAH patients. In contrast, protein expression of GPER was significantly reduced in pulmonary arteries from hypoxic female mice relative to controls. In response to hypoxia in cancer cells and cardiomyocytes, it has previously been shown that GPER expression is increased as an adaptive response to stressful microenvironments (Recchia et.al. 2011). Here, our results may differ due to tissue and species differences. Regardless, the relevance of this observation of a down-regulation of GPER expression to the pathogenesis of experimental PH is unclear; therefore we characterised GPER *in vivo* using $GPER^{-/-}$ mice. Due to age-dependent development of systemic arterial hypertension in female $GPER^{-/-}$ mice we investigated chronic hypoxia induced-PH in both male and female aged 2-3 months (Martensson et.al. 2009). In both normoxic conditions in the absence of PH, and following development of chronic hypoxic-PH we observed no effect of GPER deletion in male mice. In female mice, hypoxic $GPER^{-/-}$ mice exhibited exaggerated pulmonary vascular remodelling compared to wildtype hypoxic mice although in the absence of any elevations in RVSP and RVH. We therefore

suggest that activation of GPER may mediate a protective effect in hypoxia, although the effect is not sufficient enough to reverse completely the hypoxic PH phenotype. However, the implications of this remains unresolved as the GPER selective agonist, G1 had no effect on PASMC proliferation. Moreover, the G15, a GPER selective antagonist, did not influence estrogen induced proliferation. In conclusion, our data suggests that activation of GPER has a limited effect on PH pathology. However, further characterisation of GPER is required to decipher vasoactive properties in the pulmonary vasculature. Certainly, G1 has been shown to lower blood pressure in isolated human aorta (Haas et al, 2009) and reduce inflammation in the heart during ischemia-reperfusion (Deschamps & Murphy 2009).

Despite clear epidemiologic evidence demonstrating a female predominance in the development of multiple forms of PAH, PAH is often more severe in men and they show worse survival rates compared to women (Shapiro et.al. 2012). Here we investigated testosterone, the main circulating sex hormone in men, to address if testosterone is the key to the gender paradox observed in translational and experimental PH (Chapter 5). Males are demonstrated to exhibit more severe hypoxia induced-PH compared to females, however in this study we show that elevated RVSP, pulmonary vascular remodelling and RVH in hypoxia are not dependent on testosterone. Our data are congruent with a recent study showing that manipulation of testosterone via surgical castration has no effect on pulmonary haemodynamics (Hemnes et.al. 2012). In addition, we show that plasma testosterone levels are unaffected in hypoxia. It would be of interest however, to investigate local testosterone production in the lung and RV as poor survival rates in males are strongly associated with reduced RV function and cardiac output (Humbert et.al. 2010a; Kawut et.al. 2009). For example, high testosterone levels have previously been associated with structural effects on the RV contributing to greater RV mass and larger RV volumes in men (Ventetuolo et.al. 2011). Indeed, testosterone has recently been shown to increase RV myocyte size and fibrosis in male mice with RV load stress (Hemnes et.al. 2012). Moreover, altered testosterone metabolism and elevated DHT levels, the primary metabolite of testosterone, are described in left ventricular hypertrophy (Thum & Borlak 2002). Here, we also demonstrate that DHT stimulates proliferation of human PASMCs. Although we determined

that expression of 5α-reductase, the testosterone metabolising enzyme, and androgen receptor are unchanged in whole lung from male mice, expression in the RV may be of more importance. Certainly RV dysfunction is the most important prognostic factor and indicator of survival in PAH (D'Alonzo et.al. 1991).

From this evidence we suggest that the absence of testosterone in females is therefore unlikely to drive the female predominance. We propose that testosterone may not be involved in protection against PAH nor is it involved in initiating disease development in males. Instead we suggest that altered testosterone levels and/or metabolism of testosterone facilitate disease progression in established PAH by multiple actions on RV structure and function.

In summary, through a translational approach we have identified key concepts that have improved our understanding of the female susceptibility in PAH. This includes the up-regulation of ERα in PASMCs which results in dysfunctional BMPR2 signalling specific to females. For future perspective, the estrogen pathway and particularly targeting the estrogen receptors provides a novel therapeutic strategy and hold promise for treating PAH in females.

Future Perspectives

Therapeutic targeting of the estrogen pathway is a promising candidate for PAH therapy. Moreover, this provides the potential for a personalised medicine approach to treatment with regards to women who exhibit estrogen responsive PAH. For example, SERMs are currently a successful treatment in women with ER positive breast cancer by preventing proliferation of breast cancer cells (Park & Jordan 2002; Swaby et al. 2007). Indeed, raloxifene has already been implicated as a beneficial treatment in monocroaline-induced PH with effects on vascular and cardiac remodelling (Nishida et.al. 2009). Future studies are required to evaluate the influence of raloxifene and tamoxifen in isolated human PASMCs and in additional experimental PH models which better recapitulate the human phenotype such as the Sugen-hypoxic model.

The value of understanding the downstream pathways activated by ERs during PAH will also highlight further routes of interest for therapy. In particular, pro-

proliferative genes activated by genomic ERs may offer novel targets which could be crucial in attenuating or reversing, the pulmonary vascular remodelling process which leads to these plexiform lesions, and which obliterate arteries in severe/end-stage PAH.

Targeting the estrogen pathway, estrogens biosynthesis, metabolism or activation of ERs, will provide novel therapeutic targets in treatment of PAH treating females selectively. Indeed, antioxidant therapy appears more beneficial in treatment of coronary artery disease in females compared to men. Gender specific treatments may therefore be a novel approach in treating PAH.

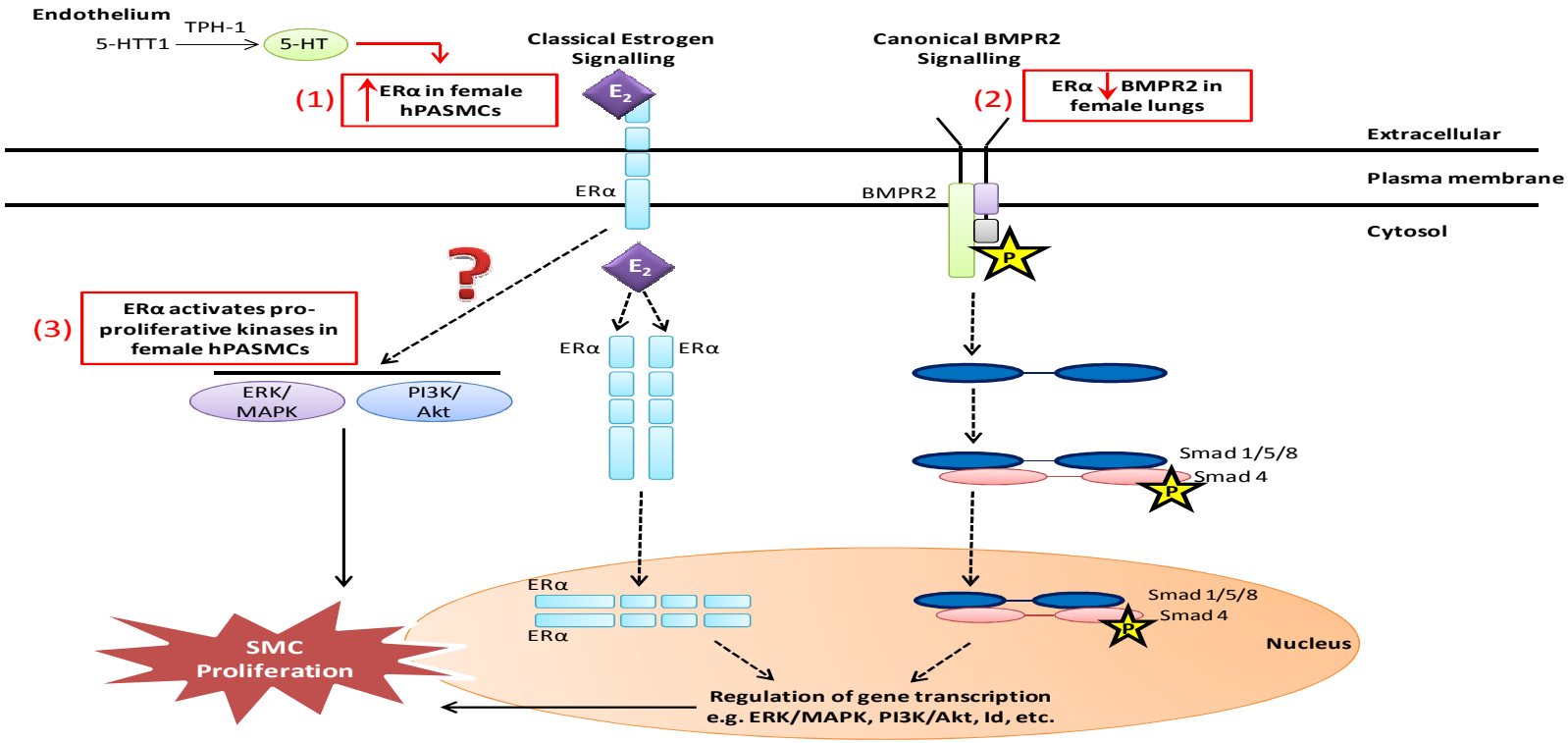


Figure 6-1: Key findings and conclusions of estrogen signalling via ERα in PAH (Chapter 3).
In Chapter 3 of this study we demonstrate (1) ERα is elevated in female human PASMCs, possibly via 5-HT; (2) expression of BMPR2 is reduced in lungs via ERα, and (3) in isolated female human PASMCs, estrogen, via ERα, activates pro-proliferative kinases in PASMCs. It is unclear from this study if the effects on ERK/MAPK and BMPR2 are via membrane bound ERα or via an effect on transcription in the nucleus. Overall, we suggest these mechanisms contribute to SMC proliferation and pulmonary vascular remodeling in vivo. Furthermore, an ERα antagonist, MPP, effectively attenuates development of PH in chronic hypoxic PH and PH in $SERT^{+}$ female mice.

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