Mitochondrial and potassium channel dysfunction in pulmonary arterial hypertension

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Declaration

I, Jeries Husam Jeries Abu-Hanna, confirm that the work presented in this thesis is my own, with the exception of the immunohistochemical staining, which was performed by Angelos Anastasakis under my supervision and as part of his summer research placement. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

Pulmonary arterial hypertension (PAH) is a rare, progressive and potentially fatal cardiopulmonary disorder, characterized by extensive remodeling and luminal narrowing of the small pulmonary arteries. Central to this remodeling is the proliferation of pulmonary arterial smooth muscle cells (PASMCs) within the medial layers of these arteries. Underlying the proliferative phenotype of PASMCs in PAH is mitochondrial dysfunction and the recently proposed metabolic theory of PAH posits that several, unrelated molecular abnormalities converge to either cause or promote this mitochondrial dysfunction. Moreover, loss of function of potassium (K⁺) channels, particularly TWIK-related acid-sensitive K⁺ channel 1 (TASK-1), is also thought to contribute to this abnormal PASMC phenotype and thereby the remodeling process in PAH. This thesis therefore aimed to explore this mitochondrial and K⁺ channel dysfunction in PASMCs from patients with PAH to aid in the identification of novel therapeutic targets for this disease.

In Chapter 3, excessive mitochondrial fragmentation was reported in PAH PASMCs. Increased protein expression and activating phosphorylation of the primary fission mediator dynamin-related protein 1 (DRP1) were found to underlie this increased mitochondrial fission in PAH PASMCs. Moreover, mitochondrial fission 1, which recruits DRP1 to sites of mitochondrial fission was upregulated in PAH PASMCs. Increased proteolytic cleavage and resultant inactivation of the inner mitochondrial fusion protein optic atrophy 1 was also observed in PAH PASMCs. Finally, IP or EP₂ prostanoid receptor agonism coupled with protein kinase A activation attenuated mitochondrial fission in PAH PASMCs by inhibiting the activating phosphorylation of DRP1 whilst inducing its inhibitory phosphorylation.

In Chapter 4, glucose flux through glycolysis was found to be markedly elevated in PAH PASMCs. Phosphofructokinase-1 (PFK1) catalyses the first rate-limiting step in glycolysis and is positively regulated by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3). Increased expression of the muscle (PFKM) and liver (PFKL) isoforms of PFK1 and PFKFB3 was found to underlie the increased glycolytic flux in PAH PASMCs. The step that generates the glycolysis end-product pyruvate is catalyzed by pyruvate kinase, namely the muscle isoform (PKM). Reduced PKM activity in PAH PASMCs was implied by an increase in the ratio of the less active splice variant PKM2 to the more active splice variant PKM1, allowing the accumulation of glycolysis intermediates and their spillover into biosynthetic pathways, predominantly the pentose phosphate shunt (PPS). Interestingly, increased protein expression of the PPS rate-
limiting enzyme glucose-6-phosphate dehydrogenase was reported in PAH PASMCs in this thesis, suggesting increased glucose shunting into the PPS.

In Chapter 5, mitochondrial respiration and ATP production were found to be increased in PAH PASMCs. This is contrary to previous findings and may reflect the metabolic heterogeneity among PAH patients. Underlying this increase in mitochondrial respiration was an increase in mitochondrial mass as a result of an increase in mitochondrial biogenesis coupled with a decrease in mitophagic flux. Accompanying this increase in mitochondrial respiration was an increase in the cellular production of reactive oxygen species (ROS) in PAH PASMCs. Reduced mitochondrial ROS scavenging as a result of the reduced protein expression of superoxide dismutase 2 was also reported in PAH PASMCs as well as an increase in the cytosolic ROS generator 5-lipoxygenase.

In Chapter 6, evidence was provided in support of TASK-1 channel dysfunction in PAH PASMCs and a role for this dysfunction in PAH pathogenesis. A trend towards a decrease in TASK-1 expression in PAH PASMCs was observed. In contrast to control PASMCs, TASK-1 channel blockade failed to promote the proliferation of PAH PASMCs. TASK-1 channel blockade was also found to inhibit mitochondrial respiration in control PASMCs, conferring a PAH metabolic phenotype on these cells. The anti-proliferative effects of different prostacyclin mimetics exhibited varying degrees of sensitivity to TASK-1 channel blockade. Finally, TASK-1 channel blockade promoted the apoptosis of pulmonary arterial endothelial cells, an early event in PAH pathogenesis.

In conclusion, this thesis provided evidence in support of alterations in mitochondrial and TASK-1 channel function in PAH.
Impact statement

Pulmonary arterial hypertension (PAH) is a rare, life-threatening and rapidly progressive disease, characterised by extensive remodelling and occlusive narrowing of the small pulmonary arteries. Despite advances in the management of this disease, prognosis remains poor and mortality high. The quest for a cure continues and there remains a dire need for the identification of novel therapeutic targets. Identifying a common denominator shared by the various biochemical and molecular abnormalities that underpin the vascular remodelling process in PAH constitutes a challenge. Recently, a metabolic theory was put forth for PAH, which posits that several, unrelated molecular abnormalities converge to either cause or promote mitochondrial dysfunction. Moreover, potassium channel dysfunction has long been implicated in the pathogenesis of PAH. This thesis therefore sought to understand this mitochondrial and potassium channel dysfunction in the hope of identifying targets that could one day be utilised in the treatment of this currently incurable disease.

Patient impact

PAH patients present with debilitating symptoms, which include, dyspnoea, fatigue, chest pain, syncope and peripheral oedema. Beyond clinical symptoms, PAH has a heavy burden on the physical, emotional and social well-being of patients. Patients with PAH reported severe restriction of their physical activities due to PAH and emphasised on the challenge of performing everyday tasks. PAH is also associated with a significant financial burden with patients concerned or frustrated about the impact of PAH on their employment. Moreover, patients described feelings of frustration, low self-esteem and anger, gaining very little pleasure from activities that they once found enjoyable. Drugs currently in clinical use fail to sufficiently alleviate symptoms and improve patients' lives. The pathomechanisms uncovered in this thesis could one day lead to the development of drugs with better clinical efficacy.

Societal impact

Pharmacotherapy of PAH is expensive and poses a heavy financial burden on the healthcare service. In the United States, for example, the average medication cost per patient is $80,000 (£61,129) per year. Developing cheaper drugs with better clinical efficacy would serve to relieve this financial burden and render treatment accessible to patients who cannot afford existing treatments.
Commercial and translational impact

This thesis advanced our understanding of mitochondrial and potassium channel dysfunction in PAH and unraveled several pathways that are impaired in the disease. The novel targets identified in this thesis could one day enable collaborations with pharmaceutical companies and clinicians to discover and develop drugs that may not only relieve patients of their symptoms but reverse the disease pathology.

Scientific impact

This thesis has many scientific impacts that will expand our knowledge of how metabolic changes underlie the phenotypic switch of vascular smooth muscle cells, particularly those of the pulmonary artery, from contractile and quiescent to proliferative and synthetic. This phenotypic switch occurs not only in PAH but also in other diseases characterised by vascular remodeling. This thesis also presented evidence that challenges the Warburgian metabolism previously described in pulmonary vascular cells derived from PAH patients.
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**Common abbreviations**

Acetyl-CoA: acetyl-coenzyme A  
AMP: adenosine monophosphate  
ASD: atrial septal defect  
ATP: adenosine triphosphate  
BMP: bone morphogenetic protein  
BMPR2: bone morphogenetic protein receptor 2  
BOEC: blood outgrowth endothelial cell  
Ca$^{2+}$: calcium  
cAMP: cyclic adenosine monophosphate  
CDK: cyclin-dependent kinase  
cGMP: cyclic guanosine monophosphate  
COX: cyclooxygenase  
DAG: diacylglycerol  
DRP1: dynamin-related protein 1  
ECAR: extracellular acidification rate  
ET-1: endothelin-1  
ETC: electron transport chain  
F1,6BP: fructose-1,6-bisphosphate  
F2,6BP: fructose-2,6-bisphosphate  
F6P: fructose-6-phosphate  
FBS: foetal bovine serum  
FIS1: fission 1  
G6P: glucose-6-phosphate  
G6PD: glucose-6-phosphate dehydrogenase  
GLUT: glucose transporter  
HPAH: heritable pulmonary arterial hypertension  
IMM: inner mitochondrial membrane  
IPAH: idiopathic pulmonary arterial hypertension  
KCNK3: K$^+$ channel subfamily K member 3  
kDa: kilodalton  
LDH: lactate dehydrogenase  
LO: lipoxygenase  
MCT: monocarboxylate transporter  
MFF: mitochondrial fission factor  
MFN: mitofusin  
MiD49: mitochondrial dynamics protein of 49 kDa
MiD51: mitochondrial dynamics protein of 51 kDa
mPAP: mean pulmonary arterial pressure
MPC: mitochondrial pyruvate carrier
mtDNA: mitochondrial DNA
MTCO2: mitochondrially encoded cytochrome C oxidase II
n: number
NAD⁺: nicotinamide adenine dinucleotide
NADH: reduced nicotinamide adenine dinucleotide
NADP⁺: nicotinamide adenine dinucleotide phosphate
NADPH: reduced nicotinamide adenine dinucleotide phosphate
NDUFV: NADH dehydrogenase ubiquinone flavoprotein
NO: nitric oxide
NOS: nitric oxide synthase
NRF: nuclear respiratory factor
OCR: oxygen consumption rate
OMM: outer mitochondrial membrane
OPA1: optic atrophy 1
PAEC: pulmonary arterial endothelial cell
PAF: pulmonary adventitial fibroblast
PAH: pulmonary arterial hypertension
PASMC: pulmonary arterial smooth muscle
PBS: phosphate buffered saline
PDGF: platelet-derived growth factor
PDH: pyruvate dehydrogenase
PDK: pyruvate dehydrogenase kinase
PFK: phosphofructokinase
PFKFB: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PGC1α: peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
PGI₂: prostacyclin
PH: pulmonary hypertension
PKA: protein kinase A
PKC: protein kinase C
PKG: protein kinase G
PKM1: pyruvate kinase muscle 1
PKM2: pyruvate kinase muscle 2
PPARγ: peroxisome proliferator-activated receptor gamma
PPP: pentose phosphate pathway
PPS: pentose phosphate shunt
PVR: pulmonary vascular resistance
R5P: ribose-5-phosphate
RT-qPCR: reverse transcription-quantitative polymerase chain reaction
S: serine
SDH: succinate dehydrogenase
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM: standard error of the mean
SOD: superoxide dismutase
T: threonine
TASK-1: TWIK-related acid-sensitive K⁺ channel 1 (TASK-1)
TFAM: mitochondrial transcription factor A
TGFβ: transforming growth factor beta
TOM20: translocase of outer membrane 20
TXA₂: thromboxane A₂
VDAC1: voltage-dependent anion channel 1
Y: tyrosine
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1. Introduction

1.1 Pulmonary arterial hypertension

Pulmonary hypertension (PH) is a heterogeneous group of disorders, clinically defined by a sustained elevation of mean pulmonary arterial pressure (mPAP) to more than 25 mmHg at rest or to more than 30 mmHg during physical activity (Schermuly et al., 2010). Interestingly, however, the definition of PH has been revisited, suggesting that an mPAP > 20 mmHg and a pulmonary vascular resistance (PVR) ≥ 30 WU are required to define pre-capillary PH (Simmonneau et al., 2018). The World Health Organisation (WHO) classification of PH, recently updated at the 5th world symposium on PH (WSPH) in Nice, France (2013), classifies the disease into five groups, based primarily on aetiology and clinical characteristics of the condition (Table 1.1; Simmonneau et al., 2013). Pulmonary arterial hypertension is a complex, progressive and often fatal cardiopulmonary disorder, which constitutes group 1 of PH. Central to the pathophysiology of PAH is extensive remodelling and occlusive narrowing of the vessels within the pulmonary vasculature, particularly the small pulmonary arteries (< 500 µm in diameter; Rabinovitch, 2012). This results in increased PVR, increased mPAP and right heart afterload, which, in turn, lead to right ventricular hypertrophy, heart failure and ultimately death (Clapp and Gurung, 2015). PAH is difficult to diagnose and patients present with non-specific symptoms, which include breathlessness, fatigue, weakness, angina and syncope (Schermuly et al., 2010). Prior to the advent of modern therapies, the median life expectancy from the time of diagnosis for adults with PAH was fewer than 3 years (2.8 years), whereas the median life expectancy for children with PAH was fewer than 10 months (Clapp and Gurung, 2015). Despite the advances in PAH therapy, prognosis remains poor and morbidity and mortality high (Schermuly et al., 2010). There remains a dire need for the identification of novel therapeutic targets.
### Table 1.1: WHO classification of pulmonary hypertension as agreed upon at the fifth WSPH in 2013 in Nice, France (Simmonneau et al., 2013) and updated recently by the sixth WSPH Task Force (Simmonneau et al., 2018).

#### Classification of PH

1. Pulmonary arterial hypertension (PAH)
   - 1.1 Idiopathic PAH
   - 1.2 Heritable PAH
     - 1.2.1 BMPR2 mutations
     - 1.2.2 Other mutations
   - 1.3 Drug or toxin-induced
     - 1.4 Associated with
       - 1.4.1 Connective tissue diseases
       - 1.4.2 HIV infection
       - 1.4.3 Portal hypertension
       - 1.4.4 Congenital heart disease (e.g. atrial septal defect)
       - 1.4.5 Schistosomiasis

1’ Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis

1'' Persistent pulmonary hypertension of the newborn

2. Pulmonary hypertension due to left heart disease
   - 2.1 Left ventricular systolic dysfunction
   - 2.2 Left ventricular diastolic dysfunction
   - 2.3 Valvular heart disease
   - 2.4 Congenital/acquired left heart inflow/outflow obstruction and congenital cardiomyopathies

3. Pulmonary hypertension due to lung disease and/or hypoxia
   - 3.1 Chronic obstructive pulmonary disease
   - 3.2 Interstitial lung disease
   - 3.3 Other pulmonary disease with mixed restrictive and obstructive pattern
   - 3.4 Sleep-disordered breathing
   - 3.5 Alveolar hypoventilation disorders
   - 3.6 Chronic exposure to high altitude
   - 3.7 Developmental lung disease

4. Chronic thromboembolic pulmonary hypertension and other pulmonary artery obstructions
   - 4.1 Chronic thromboembolic pulmonary hypertension
   - 4.2 Other pulmonary artery obstructions

5. Pulmonary hypertension with unclear multifactorial mechanisms
   - 5.1 Haematologic disorders
   - 5.2 Systemic and metabolic disorders
   - 5.3 Others
   - 5.4 Complex congenital heart disease
1.2 Epidemiology of PAH

PAH is a rare disease, with a prevalence estimated at 15-50 individuals per million (Hoper and Gibbs, 2014; Lau et al., 2017). The disease affects people of all ages and is more prevalent in women than in men, with an estimated female:male ratio of 4:1, rendering sex a risk factor for PAH. Paradoxically, however, PAH is associated with worse prognosis in males (Mair et al., 2014). According to the WHO classification of PH (see Table 1), PAH can be idiopathic (IPAH), heritable (HPAH), drug or toxin-induced or associated with other diseases, such as connective tissue disease (CTD), congenital heart disease (CHD), portal hypertension, HIV infection or schistosomiasis (Lau et al., 2017). IPAH is the most common form of PAH, accounting for 39-61% of all PAH cases. In the western world, this is closely followed by PAH, which occurs secondary to CTD (e.g. systemic sclerosis; 15-30%), CHD-associated PAH (10-23%), portopulmonary PAH (5-10%), in which portal and pulmonary hypertension coexist, and HPAH (6%; Lau et al., 2017). The relative proportion of different PAH subtypes differs in non-western countries. In China for example, CHD-associated PAH was reported as the most common subtype, accounting for 43% of all PAH cases (Lau et al., 2017). In Brasil, where schistosomiasis is an endemic, schistosomiasis-associated PAH accounts for 20% of PAH cases and is the third most common form of PAH after IPAH and CTD-associated PAH (Lau et al., 2017).

1.3 Diagnosis of PAH

PAH is difficult to diagnose and patients present with non-specific symptoms, which include breathlessness, fatigue, weakness, angina and syncope, making early detection of PAH a clinical challenge (Frost et al., 2019). Symptoms only manifest after extensive pulmonary vascular obliteration and there is a delay of > 2 years between onset of symptoms and diagnosis (Frost et al., 2019). PAH diagnosis can only be accurately made by right heart catheterisation (RHC; Frost et al., 2019). This is performed by advancing a Swan-Ganz catheter through the right side of the heart (right atrium and ventricle) and into the pulmonary artery (Rosenkranz and Preston, 2015). RHC records pressures in the right atrium, right ventricle, main pulmonary artery and pulmonary artery wedge position. Mean pulmonary arterial pressure > 25 mmHg and pulmonary arterial wedge pressure < 15 mmHg are diagnostic of PAH (Rosenkranz and Preston, 2015). Despite RHC being the diagnostic gold standard for PAH, other less invasive tools are also used in the diagnosis of PAH, including electrocardiography, blood tests (e.g. levels of brain natriuretic peptide to assess right ventricular function), cardiopulmonary exercise
testing, transthoracic echocardiography and chest computed tomography (Frost et al., 2019).

1.4 Pathology of PAH

Current understanding of the histopathology of PAH derives from explanted lung tissues removed at the time of either transplantation or autopsy. In PAH, vascular lesions occur predominantly in small and medium-sized pulmonary arteries (Guignabert et al., 2013; Rabinovitch, 2012). As demonstrated in Figure 1.1, lesions within the lungs of patients with PAH include abnormal muscularisation of distal (normally non-muscular) pulmonary arteries, loss of small precapillary arteries or vascular pruning, medial hypertrophy of more proximal intra-acinar and pre-acinar muscular pulmonary arteries with concentric or eccentric laminar lesions, neointimal formation (particularly occlusive in arteries with diameters less than 500 μm) and formation of complex plexiform lesions with capillary-like (sinusoidal) channels, thought to be the consequence of disordered angiogenesis (Rabinovitch, 2012).
Figure 1.1: Pathological vascular changes in a PAH lung (Rabinovitch et al., 2012). This schematic illustrates the abnormalities throughout the pulmonary vasculature in a PAH lung. In PAH, normally non-muscular distal pulmonary arteries become muscularised. This muscularisation is associated with the differentiation of pericytes into SMCs followed by proliferation as well as the distal migration of SMCs from more proximal muscular pulmonary arteries. Small pre-capillary arterioles are lost or atrophied, a process known as vascular pruning, likely as a consequence of increased EC and pericyte apoptosis. The walls of more proximal intra-acinar and pre-acinar muscular arteries thicken as a result of increased proliferation of fibroblasts and SMCs within the adventitial and medial layers, respectively. Inflammatory cells infiltrate the walls of pulmonary arteries, where they release a soup of pro-inflammatory mediators that contribute to remodelling. SMCs, normally resident within the medial layers, proliferate and migrate inwards to encroach on the lumen, resulting in neointimal formation and
luminal obliteration. In advanced PAH, plexiform lesions form within the lumens of pulmonary arteries possibly as a result of the clonal expansion of apoptosis-resistant ECs and/or the recruitment of circulating endothelial progenitor cells to sites of endothelial denudation or injury. Aberrant capillary-like sinusoidal channels form within the plexiform lesions in an attempt to recanalise the otherwise obliterated lumen.

1.5 Pathogenesis of PAH

The pathobiology of PAH is complex and poorly understood. Several causal pathomechanisms have been proposed, involving different biochemical pathways and cell types. Endothelial damage, originating from a combination of environmental (e.g. hypoxia) and genetic (e.g. BMPR2 mutations) factors, is thought to initiate and drive the disease (Ranchoux et al., 2018). A dysfunctional endothelium upsets the balance between endogenous vasoconstrictors and vasodilators, mitogens and growth inhibitors, and pro-thrombotic and anti-thrombotic determinants, tipping it in favour of vasoconstriction, remodelling and thrombosis in the hypertensive lung (Ranchoux et al., 2018). Endothelial dysfunction is also thought to lead to increased pulmonary endothelial permeability in the disease, allowing pro-remodelling factors circulating in the bloodstream to permeate the arterial wall and promote the proliferation of pulmonary vascular cells (Ranchoux et al., 2018; Zhou et al., 2018). Although they may differ in their underlying causes, all PAH subtypes are characterised by phenotypic changes and exaggerated proliferation of cells within the three concentric layers of the vascular wall (the intima, media and adventitia; Schermuly et al., 2011). In the intimal layer, PAECs, initially dysfunctional and pro-apoptotic, proliferate and give rise to plexiform lesions. Plexiform lesions are complex and believed to be derived from the monoclonal expansion of PAECs and/or the accumulation of circulating endothelial progenitor cells at sites of vascular injury (Toshner et al., 2009). PASMCs of the medial layer, normally quiescent and contractile, acquire a hyper-proliferative, synthetic phenotype and become resistant to apoptosis (Rabinovitch, 2012). Fibroblasts, resident within the adventitia, proliferate and transdifferentiate into myofibroblasts, acquiring a more contractile and migratory phenotype and depositing copious amounts of extracellular matrix (ECM) proteins (e.g. collagen, elastin, fibronectin; Schermuly et al., 2011).

1.5.1 Vasoactive mediators in PAH pathogenesis

There is an imbalance between vasoconstrictors and vasodilators in patients with PAH, with the generation of the former increased and that of the latter reduced. Potent pulmonary vasodilators include nitric oxide (NO) and prostacyclin (PGI$_2$). NO is generated within the pulmonary endothelium by the action of endothelial nitric oxide
synthase (eNOS) on L-arginine (Klinger, 2007). It then diffuses to the underlying smooth muscle cells, where it binds to and activates soluble guanylate cyclase (sGC), which in turn produces the second messenger cyclic guanosine monophosphate (cGMP; Klinger, 2007). A rise in intracellular cGMP in PASMCs causes pulmonary arterial vasodilation and inhibits proliferation. In PASMCs, NO/cGMP signalling is terminated by phosphodiesterase type 5 (PDE5), which preferentially breaks down cGMP to GMP (Klinger, 2007). Reduced NO bioavailability has been observed in patients with PAH and is believed to contribute to the excessive pulmonary vasoconstriction and pulmonary vascular remodelling (Ozkan et al., 2001). Several mechanisms have been shown to underlie this decreased NO production in PAH. For example, reduced eNOS expression, was reported in PAECs derived from the lungs of patients with PAH (Giaid and Saleh et al., 1995; Xue and Johns, 1995). Moreover, increased levels of asymmetric dimethylarginine, an endogenous inhibitor of eNOS, were reported in patients with PAH and associated with unfavourable pulmonary haemodynamics and poor outcomes in these patients (Kielstein et al., 2005).

PGI₂ is the major product of the cyclooxygenase (COX) pathway in PAECs. PGI₂ synthesis in PAECs requires the liberation of arachidonic acid (AA) from membrane phospholipids by the enzyme phospholipase A₂, followed by the oxygenation of AA by COX to yield the prostaglandin intermediate PGH₂. PGH₂ is then converted to PGI₂ by the enzyme PGI₂ synthase (PGI₂S). Reduced PGI₂ production in PAH has been shown to occur secondary to diminished PGI₂S expression in PAECs (Tuder et al., 1999). In PASMCs, PGI₂ signals primarily via the PGI₂ (IP) receptor to stimulate vasodilation and inhibit proliferation (Clapp and Gurung, 2015). IP receptor couples via Gs to activate membrane-bound adenylate cyclase (mAC), which generates the second messenger cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). cAMP then activates protein kinase A (PKA; Clapp and Gurung, 2015). In addition to reduced PGI₂ production, the IP receptor, through which PGI₂ predominantly signals in PASMCs, has been shown to be downregulated in PAH, implicating depressed PGI₂ signalling in PAH pathogenesis (Falcetti et al., 2010).

In sharp contrast to vasodilatory mediators, levels of potent vasoconstrictors, such as endothelin-1 (ET-1) and thromboxane A₂ (TXA₂), are increased in PAH patients. ET-1 is a 21-amino acid peptide that is synthesised in the endothelium from a larger peptide called preproendothelin-1 (Davenport et al., 2016). Preproendothelin-1 is cleaved into big-ET-1 by the enzyme furin convertase (Davenport et al., 2016). Mature ET-1 is then generated from big-ET-1 by the action of an additional enzyme, endothelin converting enzyme (Davenport et al., 2016). ET-1 is also produced by PASMCs and macrophages
ET-1 produces its vascular effects by acting on two endothelin receptor subtypes: \( \text{ET}_A \) and \( \text{ET}_B \) receptors (Pepke-Zaba and Morrell, 2005). \( \text{ET}_A \) receptors are predominantly expressed in PASMCs, where they mediate vasoconstriction, proliferation, migration and fibrosis (Davie et al., 2002; Pepke-Zaba and Morrell, 2005). \( \text{ET}_B \) receptors, on the other hand, are present in both PASMCs and PAECs. In PASMCs, \( \text{ET}_B \) receptor activation has similar effects to those mediated via the \( \text{ET}_A \) receptor (Davie et al., 2002; Pepke-Zaba and Morrell, 2005). In PAECs, \( \text{ET}_B \) receptors stimulate the production of NO and \( \text{PGI}_2 \), both of which exert vasodilatory and anti-proliferative effects on the underlying PASMCs (Pepke-Zaba and Morrell, 2005). Additionally, \( \text{ET}_B \) receptors are believed to promote the clearance of ET-1 from the circulation by receptor-mediated endocytosis of ET-1 (Fukuroda et al., 1994). Davie et al. (2002) demonstrated a two-fold increase in ET-1 binding sites in proximal pulmonary arteries and lung parenchyma in PAH patients compared to control subjects. There was no difference, however, in the relative proportion of \( \text{ET}_A \) and \( \text{ET}_B \) receptor subtypes between control subjects and PAH patients, suggesting that both the \( \text{ET}_A \) and \( \text{ET}_B \) receptors are upregulated in PAH and that ET receptor signalling is augmented in this disease (Davie et al., 2002).

Like \( \text{PGI}_2 \), \( \text{TXA}_2 \) belongs to the prostanoid family of lipid mediators. It is produced in the endothelium by \( \text{TXA}_2 \) synthase, which converts the prostaglandin intermediate \( \text{PGH}_2 \) to \( \text{TXA}_2 \). \( \text{TXA}_2 \) is a potent pulmonary vasoconstrictor and platelet activator. \( \text{TXA}_2 \) was reported to be elevated in patients with PAH, as indicated by increased urinary excretion of its stable metabolite \( \text{TXB}_2 \) (Christman et al., 1992). Signalling via the prostanoid receptors \( \text{TP}_\alpha \) and \( \text{TP}_\beta \), which arise through differential mRNA splicing, \( \text{TXA}_2 \) causes pulmonary vasoconstriction and PASMC proliferation (Cogolludo et al., 2003; Smyth, 2010). Increased \( \text{TXA}_2 \) production in PAH patients is therefore likely to contribute to the excessive pulmonary vasoconstriction and vascular remodelling that are hallmark features of the disease. In addition to mediating vasoconstriction and SMC proliferation, \( \text{TXA}_2 \) is a potent activator of platelets and its increased levels in PAH may also underlie the thrombotic lesions that occlude the pulmonary arteries in the disease (Lannan et al., 2014; Paul et al., 1999).

In addition to the vasoconstrictors ET-1 and \( \text{TXA}_2 \), serotonin or 5-hydroxytryptamine (5-HT) has also been shown to contribute to the exaggerated vasoconstriction and PASMC proliferation that is characteristic of PAH (Rabinovitch, 2012). 5-HT is synthesised within the pulmonary endothelium from L-tryptophan by the sequential actions of tryptophan hydroxylase 1 (\( \text{THP1} \)) and 5-hydroxytryptophan decarboxylase. 5-HT then acts in a paracrine fashion on the underlying PASMCs, either entering the PASMCs via serotonin
transporters (SERTs) or activating the cell-surface 5-HT receptor 5-HT_{1B} to induce PASMC contraction and proliferation (MacLean, 2018). TPH1 expression has been found to be increased in PAECs from patients with PAH, leading to increased 5-HT production and signalling in the neighbouring PASMCs (MacLean, 2018). THP1 inhibition was also found to attenuate PH in two rat models (Aiello et al., 2017). Furthermore, increased expression of the 5-HT_{1B} receptor has been reported in PASMCs from female patients with PAH, also suggesting increased 5-HT signalling in PASMCs in the disease. Consistently, 5-HT_{1B} receptor antagonism attenuated PH in mice subjected to chronic hypoxia and those overexpressing the human SERT gene (Hood et al., 2017).

1.5.2 Growth factors in PAH pathogenesis

Several growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ), epidermal growth factor (EGF) and fibroblast growth factor (FGF), are elevated in patients with PAH (Clapp and Gurung, 2015). They are important in driving the vascular remodelling that is characteristic of PAH. PDGF is produced by many different cell types, including SMCs, ECs and macrophages, and, in PASMCs, it signals via the receptor tyrosine kinases PDGFRα and PDGFRβ to induce proliferation and migration (Hassoun et al., 2009; Perros et al., 2008). Both PDGFRα and PDGFRβ and their ligands PDGFA and PDGFB were found to be upregulated in small pulmonary arteries from patients with severe IPAH, describing a pathogenic role for increased PDGF signalling in PAH (Hassoun et al., 2009; Perros et al., 2008). Moreover, the PDGFR inhibitor imatinib, which inhibits PDGF-induced proliferation and chemotaxis of PASMCs, was demonstrated to attenuate experimental PH (monocrotaline- and hypoxia-induced) and was consequently trialled in PAH patients, demonstrating efficacy concordant with the preclinical findings (Antoniu, 2012; Schermuly et al., 2005).

VEGF is a potent pro-angiogenic factor that is abundantly expressed in the lung (Voelkel and Gomez-Arrovo, 2014). VEGFA, the predominant circulating VEGF isoform, signals via two receptor tyrosine kinases VEGFR1 and VEGFR2 (Voelkel and Gomez-Arrovo, 2014). The mitogenic, proangiogenic and permeability-enhancing effects of VEGFA on the pulmonary endothelium are primarily mediated by VEGFR2, whereas VEGFR1 is thought to act as a decoy receptor, preventing VEGFA from activating VEGFR2 (Voelkel and Gomez-Arrovo, 2014). VEGF and its receptor VEGFR2 are robustly expressed in the complex plexiform lesions in the lungs of patients, suggesting a critical role for VEGF signalling in the formation of these lumen-obliterating intravascular lesions in end-stage PAH (Tuder et al., 2001; Voelkel and Gomez-Arrovo, 2014). The increased expression
of VEGF in PAH has been suggested to be secondary to increased expression of the transcription factors HIF1α and HIF1β, both of which regulate VEGF expression (Tuder et al., 2001; Voelkel and Gomez-Arrovo, 2014). Paradoxically, treatment of rodents with an antiangiogenic VEGFR inhibitor (sugen 5416) together with chronic hypoxia has been shown to induce angio-obliterrative PAH, suggesting that, in the early stages of PAH, reduced VEGF signalling together with a second hit, such as hypoxia, could constitute a key initiator and driver of pulmonary vascular remodelling (Taraseviciene-Stewart et al., 2001; Voelkel and Gomez-Arrovo, 2014).

TGFβ plays a key role in the initiation and progression of PAH. In most cells, TGFβ signals by binding to TGFβ receptor type II (TβRII), which complexes with TβRI or ALK5 (Rol et al., 2018). Canonical TGFβ signalling involves phosphorylation of the R-SMADs, SMAD2 and SMAD3, followed by complexing with the Co-SMAD, SMAD4, and nuclear translocation to regulate transcription of target genes (Rol et al., 2018). Aberrant TGFβ signalling and growth responses were reported in PAH PASMCs (Morrell et al., 2001; Richter et al., 2004). Small pulmonary arteries from PAH patients exhibited heightened SMAD2 phosphorylation, particularly in the endothelium, indicative of increased canonical TGFβ signalling in the disease. In contrast to normal PASMCs, upon which TGFβ1 has growth suppressive effects, TGFβ1 was found to promote the proliferation of PAH PASMCs, likely a consequence of reduced BMPR2 signalling (Morrell et al., 2001).

TGFβ has also been implicated in PAH-associated endothelial-to-mesenchymal transition, a phenomenon whereby PAECs lose their endothelial cell markers (e.g. CD31, vWF) and barrier function and gain mesenchymal markers (e.g. αSMA, calponin; Ranchoux et al., 2015). Inhibition of TGFβ with either a neutralising antibody or a TGFβ1/3 ligand trap attenuated experimental PAH both in rats and mice (Megalou et al., 2010; Yung et al., 2016).

EGF and FGF, which signal via the receptor tyrosine kinases EGFR and FGFR, respectively, have also been implicated in PAH pathogenesis. EGF and FGF have both been shown to promote proliferation and migration of PASMCs (Dahal et al., 2010; Merklinger et al., 2005). Three clinically approved EGFR antagonists were found to be ineffective, however, at attenuating pathology in mice with chronic hypoxia-induced PH (Dahal et al., 2010). Moreover, EGFR expression in the lungs of PAH patients was similar to that in the lungs of control subjects (Dahal et al., 2010). These findings suggest that EGF signalling through EGFR may not play a significant role in the pathogenesis of PAH as once thought. Inhibiting FGFR has been reported to ameliorate monocrotaline-induced PH in rats and to rescue defective BMPR2 signalling. The FGFR receptor subtype FGFR1 and its ligand FGF2 were both also shown to be upregulated in the
pulmonary arteries of patients with PAH (Zheng et al., 2015). In contrast to EGFR signalling, exuberant FGF signalling through FGFR seems to play a role in mediating PAH.

**1.5.3 Inflammation in PAH pathogenesis**

Inflammation is also a key feature of PAH, as suggested by extensive infiltration of T and B lymphocytes, monocytes, dendritic and mast cells in the remodelled arteries and elevated levels of pro-inflammatory (e.g. interleukin-1β (IL-1β), IL-6, tumour necrosis factor α (TNFα)) and chemotactic (e.g. monocyte chemotactic protein-1 (MCP-1), IL-8) cytokines in the sera of PAH patients (Rabinovitch et al., 2014). Classically, endothelial injury and concomitant upregulation of adhesion molecules (e.g. intercellular adhesion molecular 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1)) on the intimal surface are thought to promote the transmigration of these inflammatory cells into the vascular wall – the so-called inside-out theory (Clapp and Gurung, 2015). This is being increasingly contested, however, by the outside-in hypothesis, which posits that inflammation may be initiated in the adventitial and perivascular layers and progress inwards towards the intima (Stenmark et al., 2011; Stenmark et al., 2012). Within the adventitia of injured arteries, activated fibroblasts are thought to create a microenvironment permissive for the recruitment of circulating leukocytes through the production and retention of soluble factors, such as chemokines, cytokines and growth factors (Stenmark et al., 2012).
Figure 1.2: Infiltration of a wide range of adaptive and innate immune cells into the pulmonary arterial wall promotes vascular remodelling in PAH (Rabinovitch et al., 2014). In the top panel is a representative histopathology of a pulmonary artery, showing a single layer of endothelial cells, eccentric neointimal formation with inflammatory cell infiltrates and poorly differentiated SMCs, thickening of the medial and adventitial layers and increased inflammatory cell infiltration into the perivascular region. The bottom panel illustrates the various types of adaptive (e.g. B cells, T cells) and innate (e.g. neutrophils, mast cells, macrophages, dendritic cells) immune cells that infiltrate the pulmonary arterial wall in PAH. Immune cells are recruited to the vessel wall either from the lumen via diapedesis or from the perivascular region into the adventitia. The vessel wall is also decorated with complement and autoantibodies, which promote further inflammation.
1.5.4 Thrombosis in PAH pathogenesis

Thrombotic arteriopathy is another, often overlooked, pathological feature of PAH pathogenesis, with PAH patients having a hypercoagulable phenotype, as demonstrated by Tournier et al. (2010) using calibrated automated thrombography. The dysfunctional endothelium in PAH is thought to present a pro-thrombotic surface, upon which platelet activation and coagulation can occur, leading to the formation of intraluminal thrombi.

Tissue factor (TF), for example, which is normally expressed at low levels in the pulmonary vessel wall and is a key component of the coagulation cascade, was found to be elevated in vascular lesions of PAH patients (Lannan et al., 2014; White et al., 2007). Increased numbers of TF-expressing endothelial cell microparticles were also detected in the pulmonary circulation of PAH patients (Bakouboula et al., 2008). Increased thrombin activity, downstream of TF, was reported in PAH patients, as indicated by elevated levels of fibrinopeptide-A, which is a product of fibrinogen cleavage by thrombin (Eisenberg et al., 1990). Fibrin clots are degraded by plasmin, which is generated from its inactive zymogen plasminogen, in a process termed fibrinolysis (Lannan et al., 2014). Plasminogen conversion to plasmin has been shown to be depressed in PAH patients as a consequence of increased levels of plasminogen activator inhibitor-1 (PAI-1; Welsh et al., 1996). Moreover, increased levels of circulating von Willebrand factor (vWF) were reported in PAH patients and associated with decreased survival (Kawut et al., 2005). vWF is a glycoprotein that resides within Weibel-Palade bodies in endothelial cells and is released upon endothelial cell activation to mediate the interaction between endothelial cells and platelets (Lannan et al., 2014). It is also found in the alpha granules of platelets, from which it is released following platelet activation (Lannan et al., 2014). Platelet hyperactivity as a result of increased levels of platelet activators (e.g. serotonin, TXA$_2$) coupled with reduced levels of platelet inhibitors (e.g. NO, PGI$_2$) has been proposed to also contribute to the formation of thrombotic lesions in PAH (Lannan et al., 2014).

1.5.5 Ion channels in PAH pathogenesis

PASMCs derived from the explanted lungs of patients with PAH or animal models of PAH were found to exhibit a significantly depolarised resting membrane potential (RMP), caused by reduced expression and activity of K$^+$ channels, including voltage-gated (e.g. Kv1.1, Kv1.2, Kv1.5, Kv4.3), Ca$^{2+}$-activated (e.g. BK$_{Ca}$) and two-pore domain (e.g. TASK-1) K$^+$ channels (Yuan et al., 1998; Lambert et al., 2018). The loss of K$^+$ channel function in PAH is thought to confer upon PASMCs a proliferative and apoptosis-resistant phenotype (Burg et al., 2008). Apoptosis-inducing agents, such as staurosporine, have been shown to induce PASMC apoptosis by augmenting voltage-dependent K$^+$ currents. Consistently, rescuing the expression of Kv1.5 in PAH PASMCs has been shown to
promote the apoptosis of these cells (Bonnet et al., 2007). Interestingly, BMP2 has been reported to upregulate the mRNA expression of a number of Kv channel subunits, including Kv1.5, to induce PASMC apoptosis (Fantozzi et al., 2006). Moreover, siRNA-mediated knockdown of TASK-1 has been shown to promote the proliferation of PASMCs (Lambert et al., 2019).

The depolarised RMP in PAH PASMCs is accompanied by an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), which, in PASMCs, constitutes a major trigger for vasoconstriction, proliferation and dedifferentiation (Lambert et al., 2018). Several Ca\(^{2+}\) entry pathways were reported to be upregulated in PAH PASMCs. These include store-operated Ca\(^{2+}\) entry, which is activated upon depletion of intracellular Ca\(^{2+}\) stores, receptor-operated Ca\(^{2+}\) entry, which requires receptor ligation and coupling, and voltage-operated Ca\(^{2+}\) entry, which occurs upon membrane depolarisation (Lambert et al., 2018). The two predominant voltage-operated calcium currents in PASMCs are those of the L-type, mediated by Cav1.2 channels, and the T-type, carried by Cav3.1 and Cav3.2 channels (Lambert et al., 2018). Of the two T-type calcium channels shown to be expressed in PASMCs, Cav3.1 has been reported to be upregulated in PASMCs from patients with PAH, mediating calcium currents that are redirected towards the activation of pro-proliferative pathways (e.g. Akt1) and the inhibition of pro-apoptotic mediators (e.g. FoxO3A; Sankhe et al., 2017).

Store-operated calcium entry occurs when the sarcoplasmic reticulum (SR) in PASMCs is depleted of calcium. The drop in SR luminal calcium concentration is sensed by the SR membrane-spanning proteins stromal-induced molecules 1 (STIM1) and 2 (STIM2), which then aggregate to form punctae. The STIM punctae then translocate close to the sarcolemma, where they interact with and activate calcium entry through transient receptor potential (TRP) channels, particularly the canonical subtype (TRPC), and Orai, of which there are three isoforms (Orai1-3; Lambert et al., 2018). Store-operated calcium entry has been shown to be enhanced in PASMCs from patients with PAH and to underpin their increased proliferative capacity and their transition from a contractile to a synthetic phenotype (Lambert et al., 2018; Rode et al., 2018). This has been suggested to be due to the upregulation of the SR calcium sensor STIM2 and the store-operated calcium channels Orai2, TRPC3 and TRPC6 (Fernandez et al., 2015; Song et al., 2018; Yu et al., 2004). Finally, receptor-operated calcium influx is activated by receptors that couple via G\(_q\) to activate PLC\(\beta\), which hydrolyses the membrane phospholipid PIP\(_2\) to liberate IP\(_3\) and DAG, and mediated mainly by TRPC channels. DAG can either activate TRPC channels directly or indirectly by activating PKC, which phosphorylates TRPC (Lambert et al., 2018). Given the increase in the expression of TRPC3 and TRPC6 and
the increased $G_{q}$-coupled receptor signalling in PAH PASMCs, enhanced receptor-operated calcium entry is also thought to contribute to the rise in intracellular calcium levels in these cells.

In addition to the augmented calcium influx in PAH PASMCs, evidence derived from animal models of PH suggests that remodelling or reorganisation of intracellular calcium stores may also occur in human PAH, particularly in PASMCs, contributing to the rise in intracellular calcium (Gilbert et al., 2014). There are two SR compartments in freshly isolated, normal PASMCs (Gilbert et al., 2014). The SR compartment that is beneath the plasmalemma (subplasmalemmal SR) is highly enriched in the calcium release channel ryanodine receptor 1 (RyR1) and the calcium pump sarcoplasmic/endooplasmic reticulum calcium ATPase 2b (SERCA2b; Gilbert et al., 2014). The calcium depletion of this compartment is thought to activate store-operated calcium entry (Gilbert et al., 2014). The other SR compartment that is in close proximity to the nucleus (perinuclear SR) is enriched in SERCA2a and RyR3 (Gilbert et al., 2014). Calcium release from this compartment is thought to activate gene transcription via the calcineurin/NFAT pathway (Gilbert et al., 2014). In PASMCs, derived from chronically hypoxic rats, the appearance of a new subplasmalemmal SR compartment containing RyR3 and SERCA2a alongside the RyR1/SECA2b SR compartment was observed, suggesting loss of the functional SR segregation that is seen in freshly isolated, normal PASMCs (Gilbert et al., 2014). This loss of SR compartmentalisation is analogous to that observed in cultured PASMCs, suggesting that it may underlie the phenotypic switch of PASMCs from contractile to synthetic in PAH (Gilbert et al., 2014).

1.6 Genetics of PAH

Loss-of-function mutations in the gene encoding bone morphogenetic protein receptor 2 (BMPR2), a member of the TGFβ superfamily, were the first to be associated with PAH and now account for the majority of HPAH cases (82%) and 17% of IPAH cases (Ghataorhe et al., 2017; Girerd et al., 2017). BMPR2 mutations are inherited in an autosomal dominant fashion and exhibit incomplete penetrance, with only 24% of carriers developing PAH (Rabinovitch et al., 2012). This suggests that other genetic, epigenetic and/or environmental factors are required for the disease to manifest in carriers of BMPR2 mutations. Thus far, more than 300 disease-causing mutations have been described throughout the BMPR2 gene, including critical regions such as the kinase domain, and cause loss-of-function and reduced signalling downstream of the receptor (Evans et al., 2016). BMPR2 is a transmembrane serine/threonine kinase, which serves as a receptor for bone morphogenetic proteins (BMPs; Morrell et al., 2016).
BMPR2 is expressed on the surface of a wide range of cell types, including PAECs and PASMCs (Morrell et al., 2016). In association with a co-receptor, BMPR2 can signal through multiple pathways, including the canonical SMAD1/5/8-dependent pathway (Morrell et al., 2016; Rabinovitch, 2012). In normal PASMCs, ligation and subsequent activation of BMPR2 inhibits proliferation and promotes apoptosis (Upton et al., 2013; Fantoozi et al., 2006). PAH PASMCs harbouring BMPR2 mutations are resistant to the growth suppressive effects of BMPs, namely BMP2 and BMP4. Loss of responsiveness to the growth suppressive effects of BMPs has also been reported in PASMCs derived from PAH patients lacking BMPR2 mutations, primarily as a consequence of downregulated BMPR2 expression or post-translational BMPR2 cleavage (Hurst et al., 2017). In PAECs, the BMPR2/ALK1 receptor complex signals selectively in response to the circulating ligands BMP9 and BMP10. BMP9 has been demonstrated to attenuate apoptosis in PAECs and to strengthen the barrier function of the pulmonary endothelial monolayer (Long et al., 2015). These effects are lost in PAECs isolated from PAH patients with BMPR2 mutations (Long et al., 2015).

Less common mutations have also been reported in other members of the TGFβ superfamily, including activin receptor-like kinase 1 (ALK1), endoglin (ENG), SMAD1, SMAD4, SMAD8 and BMP9, further implicating the BMP signalling pathway in PAH pathogenesis. Like BMPR2 mutations, these mutations exhibit incomplete penetrance and are inherited in an autosomal dominant fashion. ALK1 is a type 1 TGFβ receptor that complexes with BMPR2 to form a functional receptor. ALK1 mutations were found to be more common among children with PAH (10% of paediatric PAH cases) compared to adults with PAH. SMAD1 and SMAD8 are receptor-regulated SMADs or R-SMADs that function as signal transducers and transcriptional modulators downstream of BMPR2 (Morrell et al., 2016). Upon activation, BMPR2 phosphorylates SMAD1 and SMAD8, causing them to translocate to the nucleus, where they regulate the transcription of a number of genes, including those that encode inhibitor of DNA binding (ID) proteins (Morrell et al., 2016). ID proteins are therefore major downstream transcriptional targets of BMPR2 signalling and have been shown to underlie the anti-proliferative effects of BMPs on PASMCs (Yang et al., 2013). SMAD4 is a common SMAD (Co-SMAD), which complexes with phosphorylated R-SMADs to drive their nuclear translocation. As mentioned above, BMP9 preferentially activates BMPR2 complexed with ALK1 in the pulmonary endothelium to maintain pulmonary vascular homeostasis and quiescence (Long et al., 2015). Loss of BMP9 is thought to cause endothelium dysfunction and subsequent pulmonary vascular remodelling (Long et al., 2015).
In 2013, Ma et al. identified novel heterozygous mutations in KCNK3, the gene encoding the two-pore domain potassium channel TASK-1, in patients with IPAH and HPAH, describing the first channelopathy in PAH. Although inherited in a similar manner to BMPR2 mutations, KCNK3 mutations appear to display a much higher penetrance than those which occur in BMPR2, suggesting a key role for the channel in the disease pathogenesis and rendering it desirable as a therapeutic target (Ma et al., 2013). Moreover, carriers of heterozygous KCNK3 mutations were found to develop a severe form of PAH, as indicated by haemodynamic measurements and histopathological analysis (Ma et al., 2013). Patients had severely raised mean pulmonary arterial pressures at right heart catheterisation and failed to respond to an acute vasodilator challenge (Ma et al., 2013). Also, pulmonary arteries from carriers showed medial hypertrophy and eccentric arterial dilatation or aneurysm with complex plexiform lesions occluding the lumen (Ma et al., 2013). A homozygous mutation in KCNK3 was very recently reported in a patient, diagnosed with an aggressive, early-onset form of the disease at the age of 2 months. The patient’s mother also developed PAH a year after giving birth. Rare variants in other K⁺ channels or accessory K⁺ channel subunits are also associated with PAH. Bohnen et al. (2018) reported a loss-of-function missense variant in the ABCC8 gene in a child with IPAH. ABCC8 encodes sulphonylurea receptor 1, which associates with ATP-sensitive K⁺ channels and, as its name suggests, confers upon them sensitivity to sulphonylureas and ATP (Bohnen et al., 2018). Moreover, single nucleotide polymorphisms (SNPs) were identified in the promoter and translated regions of KCNA5, the gene encoding the voltage-gated K⁺ channel subunit Kv1.5, in patients with IPAH (Remillard et al., 2007). These SNPs were suggested to underlie the reduced expression and/or activity of Kv1.5 in PASMCs from IPAH patients (Remillard et al., 2007).

Complicating the disease further, mutations have been identified in genes other than those encoding members of the TGFβ superfamily and K⁺ channels. These genes include CAV1, SOX17, AQP1, ATP13A3, EIF2AK4 and TBX4 (Morrell et al., 2018). The frequency of these mutations among PAH patients are summarised in Table 1.2. The roles of the protein products of these genes, which are also summarised in Table 1.2, in the pathogenesis of PAH remain poorly understood.
Table 1.2: Mutations associated with PAH. All mutations are inherited in an autosomal dominant fashion with the exception of *EIF2AK4* mutations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein product</th>
<th>Frequency among PAH patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMPR2</td>
<td>BMPR2; type 2 TGFβ superfamily receptor for BMPs</td>
<td>82% of HPAH cases; 17% of IPAH cases; 12.5% of paediatric PAH cases</td>
</tr>
<tr>
<td>ALK1</td>
<td>ALK1; type 1 TGFβ superfamily receptor</td>
<td>10% of paediatric PAH cases; 0.9% of adult patients with IPAH, HPAH and anorexigen-associated PAH (Morrell et al., 2018)</td>
</tr>
<tr>
<td>ENG</td>
<td>Endoglin; accessory receptor for TGFβ</td>
<td>0.6% of IPAH, FPAH and anorexigen-associated PAH patients (Morrell et al., 2018)</td>
</tr>
<tr>
<td>SMAD1</td>
<td>SMAD1; downstream mediator of canonical BMP signalling</td>
<td>Very rare</td>
</tr>
<tr>
<td>SMAD4</td>
<td>SMAD4; downstream mediator of canonical BMP and TGFβ signalling</td>
<td>Very rare</td>
</tr>
<tr>
<td>SMAD9</td>
<td>SMAD8; downstream mediator of canonical BMP signalling</td>
<td>0.4% of IPAH, FPAH and anorexigen-associated PAH patients (Morrell et al., 2018)</td>
</tr>
<tr>
<td>KCNK3</td>
<td>TASK-1; two-pore domain potassium channel</td>
<td>3.2% of HPAH cases, 1.3% of IPAH cases (Morrell et al., 2018)</td>
</tr>
<tr>
<td>CAV1</td>
<td>Caveolin-1; component of caveolae; involved in eNOS and BMPR2 signalling</td>
<td>Very rare</td>
</tr>
<tr>
<td>EIF2AK4</td>
<td>Serine/threonine kinase; phosphorylates α subunit of eukaryotic initiation factor 2</td>
<td>25% of patients with pulmonary veno-occlusive disease and pulmonary capillary haemangiomatosis (Liang et al., 2016)</td>
</tr>
<tr>
<td>TBX4</td>
<td>T-box 4; transcription factor involved in the regulation of developmental processes</td>
<td>7.5% of paediatric PAH cases; 1.3% of IPAH, FPAH and anorexigen-associated PAH patients (Morrell et al., 2018)</td>
</tr>
<tr>
<td>GDF2</td>
<td>BMP9; ligand for endothelial BMPR2</td>
<td>0.8% of IPAH, FPAH and anorexigen-associated PAH patients (Morrell et al., 2018)</td>
</tr>
<tr>
<td>SOX17</td>
<td>SRY-box 17; transcription factor involved in cellular differentiation</td>
<td>0.9% of IPAH, FPAH and anorexigen-associated PAH patients (Morrell et al., 2018)</td>
</tr>
<tr>
<td>AQP1</td>
<td>Aquaporin 1; widely expressed water channel</td>
<td>0.9% of IPAH, FPAH and anorexigen-associated PAH patients (Morrell et al., 2018)</td>
</tr>
<tr>
<td>ATP13A3</td>
<td>ATPase 13A3; unknown function</td>
<td>1.1% of IPAH, FPAH and anorexigen-associated PAH patients (Morrell et al., 2018)</td>
</tr>
</tbody>
</table>
1.7 Pharmacotherapy of PAH

Despite the advances in PAH therapies, there is currently no cure for PAH and lung transplantation offers the only hope for patients. Treatments, however, are clinically available, offering both symptomatic relief and prolonged survival. Three distinct pathways, involved in regulating pulmonary vascular homeostasis and dysregulated in PAH, are clinically targeted in the treatment of PAH. These are the prostacyclin (PGI$_2$), nitric oxide (NO) and endothelin-1 (ET-1) pathways (Figure 1.2). Consequently, drugs approved for the treatment of PAH fall into five classes: ET-1 receptor antagonists (ERAs; e.g. bosentan, macitentan and ambrisentan), phosphodiesterase type 5 (PDE5) inhibitors (e.g. sildenafil and tadalafil), soluble guanylate cyclase stimulators (e.g. riociguat), prostacyclin analogues (e.g. epoprostenol, treprostinil and iloprost) and most recently the prostacyclin (IP) receptor-selective agonist, selexipag (Lau et al., 2017). Calcium channel blockers are also used in PAH therapy, albeit rarely, as it is only indicated in patients who show a positive response to an acute vasodilatory challenge (Lau et al., 2017). The primary purpose of these therapies is to promote pulmonary vasodilation and bring about a reduction in PVR, PAP and right heart afterload. Although known widely for their vasodilatory effects, these agents are increasingly being recognised for their anti-proliferative and anti-remodelling effects (Clapp and Gurung, 2015).
Figure 1.3: The three signalling pathways targeted in the treatment of PAH (Lau et al., 2017). The endothelin 1, nitric oxide and prostacyclin pathways are dysregulated and targeted in the treatment of PAH. In the endothelin pathway (left), ET-1 is generated within the pulmonary endothelium from its precursor pro-endothelin 1. Endothelin 1 then binds to and activates the ET\(_A\) and ET\(_B\) receptors on the surface of smooth muscle cells. Activation of either receptor in SMCs leads to pulmonary arterial vasoconstriction and SMC proliferation. Selective ET\(_A\) receptor (e.g. ambrisentan) and dual ET\(_A\)/ET\(_B\) receptor antagonists (e.g. bosentan, macitentan) are clinically used in the treatment of PAH. In the nitric oxide pathway (middle), nitric oxide is liberated from L-arginine in the pulmonary endothelium and then diffuses to the underlying SMCs, where it activates soluble guanylate cyclase (sGC). sGC converts GTP to cGMP and the resultant increase in intracellular cGMP levels in SMCs causes pulmonary arterial vasodilation and inhibits SMC proliferation. NO signalling in SMCs is terminated by PDE5-mediated degradation of cGMP to GMP. Stimulators of sGC (e.g. riociguat) and inhibitors of PDE5 (e.g. sildenafil and tadalafil) are used clinically in the treatment of PAH patients to enhance NO signalling. Finally, in the prostacyclin pathway (right), prostacyclin is generated from arachidonic acid metabolites in the pulmonary endothelium and binds to IP receptors on SMCs. In SMCs, IP receptor activation results in an increase in intracellular cAMP to cause pulmonary arterial vasodilation and SMC proliferation. Prostacyclin analogues (e.g. epoprostenol, treprostinil, iloprost) and non-prostanoid IP receptor agonists (e.g. selexipag) are approved for the treatment of PAH.

1.7.1 Calcium channel blockers

Calcium channel blockers (CCBs) constitute a class of drugs that block L-type voltage-gated calcium channels (VGCCs; Medarov and Judson, 2015). In PASMCs, Ca\(^{2+}\) entry through VGCCs and the consequent rise in intracellular Ca\(^{2+}\) triggers vasoconstriction and CCBs block this Ca\(^{2+}\) influx to cause vasodilation. CCBs are restricted for use in PAH patients who exhibit an adequate response (≥10 mmHg decrease in mPAP with no
change or increase in CO and no change in systemic pressure) to an acute vasodilatory challenge (either inhaled NO or other short-acting vasodilators such as intravenous epoprostenol or adenosine), which is normally done at the time of the first diagnostic RHC. CCB therapy in PAH consists of high-dose oral diltiazem, nifidipine or amlodipine, and long-term responders to CCB therapy, which comprise <10% of IPAH patients, have a favourable prognosis and a mortality rate of 6% after 5 years (Sitbon et al., 2005).

1.7.2 Phosphodiesterase type 5 inhibitors
NO/cGMP signalling is terminated by phosphodiesterase type 5 (PDE5), which preferentially hydrolysates and inactivates cGMP. PDE5 is also upregulated in PAH patients and is thought to contribute to the depressed NO/cGMP signalling in the disease. Consequently, the NO/cGMP pathway in PAH can be augmented by inhibiting PDE5-mediated cGMP breakdown. Sildenafil was the first PDE5 inhibitor to be clinically validated for the treatment of PAH. Sildenafil causes rapid and potent pulmonary vasodilation to bring about a significant decrease in PVR and mPAP with very little effect on mean arterial pressure and improvement in cardiac output (Lepore et al., 2002; Michelakis et al., 2002). The magnitude of this vasodilatory effect of sildenafil was comparable to that of inhaled NO (Lepore et al., 2002; Michelakis et al., 2002). Sildenafil may also attenuate pulmonary vascular remodelling in patients with PAH. Indeed, sildenafil has been shown to inhibit PDGF-induced PASMC proliferation and to partially reverse experimental PH (monocrotaline- and chronic hypoxia-induced; Sebkho et al., 2003; Schermuly et al., 2004; Tantini et al., 2005). Side effects associated with the use of sildenafil include headache, flushing, dyspepsia and nasal decongestion (Barnett and Machado, 2006). Sildenafil suffers from having a relatively short half-life (4-6 hours), requiring three daily doses of 20 mg. With a longer half-life, tadalafil was later developed and approved for clinical use, allowing once-daily dosing and improving compliance amongst patients.

1.7.3 Soluble guanylate cyclase stimulators
Deficient NO/cGMP signalling in PAH can also be enhanced by directly stimulating sGC. Riociguat is a direct stimulator of sGC that is approved for use in the treatment of PAH and inoperable chronic thromboembolic pulmonary hypertension (CTEPH; Germani et al., 2017). In addition to stimulating sGC to increase cGMP production, riociguat increases the affinity of the sGC-NO interaction and stabilises the complex in its active confirmation (Germani et al., 2017). Riociguat significantly improves exercise performance and lowers PVR in patients with PAH (Ghofrani et al., 2013). Interestingly, riociguat is the only approved treatment option for CTEPH, consistently showing clinical
efficacy in placebo-controlled trials (Germani et al., 2017). Riociguat is generally well-tolerated by PAH patients (Germani et al., 2017). The most common side effects associated with the use of riociguat include headache, nasopharyngitis, gastrointestinal disturbance and peripheral oedema, occur early in the course of treatment and gradually dissipate (Germani et al., 2017). Hypotension has also been observed in patients receiving riociguat (Germani et al., 2017).

1.7.4 Prostacyclin and its stable analogues

Prostacyclin and its analogues are potent vasodilators, inhibitors of platelet activity and suppressors of smooth muscle cell proliferation. 

Prostacyclin analogues were originally thought to act exclusively via the IP receptor, which couples via Gs to activate adenylyl cyclase and subsequently elevate intracellular cAMP. Recent studies have shown that 

PGI$_2$ and its analogues activate not only IP receptors but also other prostanoid receptor subtypes and nuclear receptors, such as PPAR$_{\gamma}$. The predominant source of PGI$_2$ in the pulmonary vasculature is the endothelium. PGI$_2$ is generated from arachidonic acid in a multi-step process involving the enzymes cyclooxygenase and PGI$_2$ synthase (PGI$_2$S). Reduced PGI$_2$ synthesis has been reported in PAH patients, as indicated by reduced PGI$_2$S expression. Based on the rationale that exogenous PGI$_2$ may compensate for the loss of endogenous PGI$_2$ in PAH, epoprostenol, a synthetic PGI$_2$, was synthesised and approved for the treatment of patients with PAH (Sitbon and Vonk Noordegraaf, 2017).

Epoprostenol has a very short half-life of 3-5 minutes and must be administered by continuous intravenous infusion by means of an infusion pump together with a permanent tunnelled catheter (Sitbon and Vonk Noordegraaf, 2017). Epoprostenol has been shown to improve exercise capacity, haemodynamics and mortality in patients with severe PAH (Barst et al., 1996). The use of epoprostenol is limited by a multitude of adverse effects, including jaw pain, nausea and diarrhoea, and potentially serious, life-threatening complications such as infection at the site of infusion, sepsis and thromboembolism (Mitchell et al., 2014). Consequently, epoprostenol is often reserved to patients with severe PAH who do not improve on other available therapies (Sitbon and Vonk Noordegraaf, 2017).

As a result of its chemical instability and short half-life, epoprostenol was superseded by more chemically stable PGI$_2$ analogues with longer half-lives, namely beraprost, iloprost and treprostinil (Lau et al., 2017). Whereas beraprost can only be given orally, treprostinil and iloprost can be administered either subcutaneously, intravenously or by inhalation (Yerly et al., 2016). The efficacy of iloprost and treprostinil is superior to that of beraprost. Treprostinil was shown to significantly improve exercise capacity, pulmonary
haemodynamics and symptoms, particularly in patients who were more compromised at baseline (Skoro-Sajer et al., 2008). Due to their pharmacological profiles, these drugs, however, are associated with undesirable effects, reducing compliance amongst patients (Skoro-Sajer et al., 2008). Treprostinil was found not only to bind to and activate the IP receptor as once presumed but also to the EP₂ and DP₁ prostanoid receptors (Whittle et al., 2012). In PAH PASMCs, treprostinil has been shown to signal preferentially through the EP₂ receptor as a result of significantly diminished IP receptor levels coupled with increased expression of the EP₂ receptors (Falcetti et al., 2010; Patel et al., 2018). Treprostinil was also shown to inhibit PASMC proliferation via the nuclear receptor PPARγ. Indeed, due to their large ligand-binding pockets, PPARs can be activated by a variety of ligands, ranging from free fatty acids to their derivatives such as PGI₂ (Lim and Dey, 2002). Similar to treprostinil, ilorprost has been shown to bind to prostanoid receptors other than the vasodilatory IP receptor (Whittle et al., 2012). Iloprost was demonstrated to bind to and activate the EP₁ receptor to elevate intracellular calcium in expression systems (Whittle et al., 2012). Iloprost activating the EP₁ receptor could cause pulmonary vasoconstriction to offset its vasodilatory effect via the IP receptor (Whittle et al., 2012).

1.7.5 Non-prostanoid prostacyclin receptor agonists
A highly selective IP receptor agonist, termed seleixipag, was developed in the hope of reducing the adverse effects associated with PGI₂ analogues. Selexipag is hydrolysed in the liver into its active metabolite ACT-333679, which possesses a half-life of 8-14 hours and thus allows twice daily dosing (Lau et al., 2017). Selexipag was found to reduce morbidity from PAH and to improve exercise performance and pulmonary haemodynamics in the GRIPHON trial (Sitbon et al., 2015). Despite its high selectivity for the IP receptor, however, seleixipag produced dose-dependent adverse effects similar to those associated with PGI₂ therapy, including headache, jaw pain, diarrhoea, nausea and flushing (Noel et al., 2017). Moreover, the death rate among patients treated with seleixipag is similar to that seen in patients treated with other PAH therapeutics (Lau et al., 2017).

1.7.6 Endothelin receptor antagonists
Circulating levels of ET-1 are elevated in patients with PAH and correlate with disease severity. Moreover, ET-1 expression was shown to be strongly increased in the explanted lungs of PAH patients and animal models of PAH. ET-1 receptor antagonists (ETAs) were therefore developed to counteract the vasoconstrictive and mitogenic effects of ET-1 acting on ETₐ and ETₐ receptors in patients with PAH. Bosentan, the first ERA to
be approved for PAH, is a mixed ETₐ/ET₆ receptor antagonist (Gabbay et al., 2007). Bosentan has been shown to improve exercise capacity, pulmonary haemodynamics and time to clinical worsening in patients with IPAH but not those with CTD-associated PAH (Gabbay et al., 2007). The most common side effects observed in patients treated with oral bosentan were headache, flushing and syncope. Bosentan induces reversible hepatotoxicity, necessitating monthly assessment of liver function (Gabbay et al., 2007). Ambrisentan has a longer half-life than bosentan and is selective for the ETₐ receptor (Rivera-Lebron and Risbano, 2017). Ambrisentan demonstrated clinically efficacy in patients with IPAH and PAH associated with CTD and HIV, improving exercise performance, haemodynamics and time to clinical worsening (Rivera-Lebron and Risbano, 2017). Despite its ETₐ receptor selectivity, however, ambrisentan has a similar efficacy to bosentan and induces liver toxicity, also requiring monthly liver function assessments (Rivera-Lebron and Risbano, 2017). Most recently, macitentan, also a mixed ETₐ/ET₆ receptor antagonist, has been approved for PAH. With its high lipophilicity, increased receptor affinity and slow receptor dissociation rate, macitentan is better able at competing with ET-1 at its receptors than bosentan or ambrisentan (Lau et al., 2017). In spite of its higher affinity for the endothelin receptors, macitentan has a clinical efficacy in PAH patients that is comparable to both bosentan and ambrisentan (Bedan et al., 2018). The use of macitentan, however, is associated with fewer adverse effects than the use of either bosentan or ambrisentan (Bedan et al., 2018).

1.8 Two-pore domain potassium channels in PAH

Potassium (K⁺) channels are membrane-spanning proteins that constitute the largest family of ion channels in the human genome. They form pores within the membrane that allow the selective passage of K⁺ ions from one side of the membrane to the other. The selectivity of K⁺ channels for K⁺ ions is conferred by a conserved, 3-amino acid sequence (GYG) within the selectivity filter of their pore-forming α subunits. Many K channel α subunits can also associate with ancillary regulatory β subunits. Based on their membrane topology, K⁺ channels can be classified into three groups. These are voltage-gated K⁺ channels, which include Ca²⁺-activated K⁺ channels, inwardly rectifying K⁺ channels, which include ATP-sensitive K⁺ channels, and two-pore domain K⁺ (K₂P) channels (Kuang et al., 2015). Although members of all three K⁺ channel groups have been shown to be functionally expressed in the pulmonary vasculature, the focus of this thesis will be on the K₂P channel family, a member of which has been shown to be mutated in PAH patients.
Two-pore domain potassium channels, designated $K_{2P}$ channels, mediate “leak” or background potassium currents ($I_{K\text{N}}$) that underlie the high membrane permeability to $K^+$ at rest (Enyedi and Czirjak, 2010). They comprise a subfamily of potassium channels, characterised by the distinctive membrane topology of their constituent subunits (Enyedi and Czirjak, 2010). $K_{2P}$ channel subunits are each composed of four transmembrane domains (TM1 – TM4) and two pore-forming domains or re-entrant P-loops containing the $K^+$ selectivity filter (Enyedi and Czirjak, 2010). This contrasts with other potassium channel subfamilies, whose subunits each contain only one P-loop (Enyedi and Czirjak, 2010). Accordingly, two $K_{2P}$ assemble to form a functional, current-conducting channel. Members of the $K_{2P}$ channel subfamily are subdivided into six groups (TWIK, TREK, TASK, TALK, THIK and TRESK), based on sequence homology and functional resemblance.

1.8.1 TASK-1 channels
This thesis focuses on TASK-1, which belongs to the TWIK-related acid-sensitive $K^+$ channel (TASK) subgroup and is encoded by the gene KCNK3, which has been found to be mutated in patients with IPAH and HPAH (Ma et al., 2013). In the pulmonary vasculature, TASK-1 has been shown to be functionally expressed in PAECs and PASMCs. TASK-1 channels have also been shown to form functional channels in right ventricular cardiomyocytes. Several lines of evidence support a role for TASK-1 in regulating the resting membrane potential of PASMCs, which is a key determinant of vascular tone and diameter (Gurney et al., 2003). Arteries exist in a partially contracted, pressurised state and the relationship between membrane potential and vascular tone is so steep such that even small changes in membrane potential, like those caused by TASK-1 channel blockade, can alter the vessel diameter. Selective inhibitors of TASK-1, including anandamide, A293, and ML365, were shown to significantly inhibit $I_{K\text{N}}$ currents and cause depolarisation of the resting membrane potential in human PASMCs (Antigny et al., 2016; Gurney et al., 2003; Olschewski et al., 2006). Knockdown of TASK-1 in PASMCs was also reported to inhibit $I_{K\text{N}}$ and cause membrane depolarisation in a manner analogous to TASK-1 selective inhibitors (Olschewski et al., 2006). Whether this depolarisation is sufficient to activate sarcolemmal voltage-gated calcium channels and induce calcium influx remains largely unexplored. In pancreatic beta cells, TASK-1 blockade with A1899 was found to enhance glucose-induced calcium entry and consequent insulin secretion, indicating that TASK-1 currents serve to limit calcium influx in those cells (Dadi et al., 2014).
TASK-1 has also been shown to be functionally expressed in PAECs, as indicated by immunolabelling and patch-clamp experiments. Its physiological role in the pulmonary endothelium, however, remains unclear (Antigny et al., 2016). In the human heart, TASK-1 mRNA has been shown to be abundantly expressed in the atria and, to a lesser extent, in the ventricles (Limberg et al., 2011). TASK-1 blockade with A293 in human atrial cardiomyocytes has been shown to prolong the action potential duration, suggesting an important role for these channels in the repolarisation (phase 3) of atrial action potentials (Limberg et al., 2011). Moreover, K⁺ currents sensitive to the TASK-1 channel blocker A293 have been isolated in these cardiomyocytes, confirming the expression of functional TASK-1 channels in human atria (Limberg et al., 2011). Lambert et al. (2018) recently demonstrated functional TASK-1 expression in right ventricular cardiomyocytes and a role for TASK-1 in mediating the repolarisation of the right ventricular action potential similar to that identified by Limberg et al. (2011) in the atria.

**Biophysical properties and pH sensitivity of TASK-1 channels**

TASK-1 channels mediate a non-inactivating K⁺ current that is insensitive to changes in membrane potential (Olschewski et al., 2017). The shape of the current-voltage relationship for TASK-1 indicates that the channel rectifies outwardly (Olschewski et al., 2017). Elevating the extracellular concentration of K⁺ was found to increase the amplitude of not only the macroscopic inward TASK-1 current but also that of the outward current, suggesting that, in addition to being a charge carrier, extracellular K⁺ activated TASK-1 possibly by stabilising the open pore conformation of the channel. Recordings from single TASK-1 channels in cell-attached and inside-out membrane patches showed brief channel openings (1 ms), low opening probability or $P_o$ and unitary or single-channel conductance of 14-16 pS (Enyedi and Czirjak, 2010). As its name suggests, TASK-1 is highly sensitive to changes in extracellular pH (Duprat et al., 1997). In many cell types, including PASMCs, extracellular acidosis strongly and reversibly inhibits TASK-1 currents and causes membrane depolarisation, whereas extracellular alkalosis enhances the currents mediated by TASK-1 to hyperpolarise the membrane (Gurney et al., 2013). Site-directed mutagenesis studies revealed that the pH sensitivity of TASK-1 is largely conferred by protonation of histidine 98 (H98), which resides within the extracellular loop linking TM2 to the P-loop (Morton et al., 2003). Two other amino acid residues, H72 and lysine 2 (K2), were also demonstrated to contribute to the pH sensitivity of TASK-1, albeit to a lesser extent than H98 (Morton et al., 2003).

**Pharmacology of TASK-1 channels**

TASK-1 channels exhibit a distinctive pharmacological profile. They are relatively insensitive to the broad-spectrum K⁺ channel blockers tetraethylammonium (TEA), 4-
aminopyridine (4-AP), Cs⁺ and Ba²⁺, and to glibenclamide, a blocker of ATP-sensitive K⁺ (K<sub>ATP</sub>) channels (Enyedi and Czirják, 2010; Gurney et al., 2003). The endocannabinoid anandamide was found to inhibit TASK-1 currents independently of cannabinoid receptors in many cell types, including SMCs of the pulmonary artery (Enyedi and Czirják, 2010). Anandamide was originally reported to be highly selective for TASK-1 within the K<sub>2P</sub> channel subfamily and routinely used to isolate TASK-1 currents in a variety of cell types. However, it was later found that anandamide also inhibits currents mediated by TASK-3 and therefore cannot be used to discriminate between TASK-1 and TASK-3 homodimers. TASK-1 subunits can assemble either as homodimers or heterodimers with TASK-3 subunit to form functional channels. Interestingly, TASK-1 homodimers and TASK-1/TASK-3 heterodimers can be distinguished from TASK-3 homodimeric channels with the use of ruthenium red (Kang et al., 2004). In contrast to TASK-3 homodimeric channels, currents carried by TASK-1 homodimeric channels are insensitive to inhibition by ruthenium red. Ruthenium red had no effect on I<sub>NN</sub> in human PASMCs, providing further proof that TASK-3 is absent from these cells. TASK-1 channels are also among the numerous targets of general anaesthetics, particularly those which are administered through inhalation. Halothane, for instance, was shown to activate TASK-1 currents in COS-7 cells expressing TASK-1 (Tailey and Bayliss, 2002). Following membrane permeation, halothane is thought to interact with residues within the C-termini of TASK-1 subunits as deletion or substitution of the C-terminus was found to abolish the activation of TASK-1 by halothane (Patel et al., 1999; Tailey and Bayliss, 2002). Furthermore, halothane was demonstrated to enhance I<sub>NN</sub> (background K⁺ currents) mediated by TASK-1 channels in PASMCs (Gurney et al., 2003). Wildtype and mutant TASK-1 channels are also activated by the phospholipase A<sub>2</sub> inhibitor ONO-RS-082 (Ma et al., 2013). Interestingly, ONO-RS-082 was also reported to rescue the activity of mutant TASK-1 channels, with the exception of G203D (Ma et al., 2013).

**Regulation of TASK-1 channels in PASMCs and clinical relevance**

K<sub>2P</sub> channels are involved in many diverse physiological processes and are regulated by a wide array of stimuli. TASK-1 channels, for example, are regulated by hypoxia, pH, vasoactive mediators and metabolism. In PASMCs, macroscopic TASK-1 currents were found to be inhibited by acute hypoxia, resulting in sarcolemmal depolarisation (Gurney et al., 2003). The mechanisms underlying this inhibition remain unclear; although several indirect mechanisms, involving the mitochondria, have been proposed. Inhibition of TASK-1 as well as oxygen-sensitive, voltage-gated K⁺ channels (K<sub>1.2</sub>, K<sub>1.5</sub>, K<sub>2.1</sub> and K<sub>3.1</sub>) by acute hypoxia in PASMCs is thought to underlie hypoxic pulmonary vasoconstriction (HPV), a physiological phenomenon whereby small pulmonary arteries constrict in response to hypoxia (Enyedi and Czirják, 2010). HPV serves to divert blood
from hypoventilated regions of the lung to better ventilated regions. TASK-1 has also been suggested to play a role in chronic hypoxia-induced PH and vascular remodelling. Indeed, rats deficient in TASK-1 developed a more severe PH phenotype in response to chronic hypoxia than their wildtype counterparts (Antigny et al., 2016).

In relevance to PAH, background K^+ currents through TASK-1 channels were inhibited by the potent vasoconstrictor and mitogen ET-1 in human PASMCs (Seyler et al., 2012; Tang et al., 2009). ET-1 activates multiple downstream signalling pathways, including the PLC/IP_3/PKC pathway. At clinically relevant concentrations, ET-1 was therefore found to recruit the PLC/IP_3/PKC to phosphorylate TASK-1 and inhibit whole-cell background K^+ currents mediated by TASK-1 channels in PASMCs. However, another study showed that only the Rho kinase inhibitor Y-27632 could attenuate the inhibitory effect of ET-1 on TASK-1 currents, suggesting that ET-1 alternatively recruits the RhoA/Rho kinase pathway to inhibit TASK-1 currents in human PASMCs (Seyler et al., 2009). Moreover, two Rho kinase phosphorylation consensus sites were detected in the C-termini of TASK-1 subunits but only one was found to be required for the inhibitory effect of ET-1 on TASK-1 currents (Seyler et al., 2012). In Xenopus oocytes, inhibition of TASK-1 currents by ET-1 was mediated by the endothelin receptors ET_A and ET_B, both of which are expressed in human PASMCs and are antagonised in the treatment of PAH (Tang et al., 2009).

![Figure 1.4: Mechanisms of TASK-1 channel activation and inhibition in PASMCs.](image)

In PASMCs, TASK-1 (KCNK3) channel activation results in hyperpolarising outward potassium (K^+) currents, which reduce the membrane potential (Em) and switch off voltage-gated calcium channels to limit calcium entry. Direct activators of TASK-1 channels include extracellular alkalosis, volatile anaesthetics, such as halothane, and experimental drugs, such as ONO-RS-082. In PASMCs, TASK-1 channels can also be
indirectly activated by agonists that activate Gs-coupled receptors. For example, treprostinil and iloprost activate the IP prostanoid receptor to increase intracellular cAMP. cAMP activates the serine/threonine kinase PKA, which phosphorylates and activates TASK-1 channels. ET-1 signals via the endothelin receptors $\text{ET}_A$ and $\text{ET}_B$, which couple to $G_{q/11}$ to activate PLC$\beta$. PLC$\beta$ cleaves the membrane phospholipid PIP$_2$ to yield IP$_3$ and DAG. DAG activates PKC, which phosphorylates and inhibits TASK-1. $\text{ET}_A$ and $\text{ET}_B$ receptors can also couple to $G_{12/13}$ to activate RhoA. RhoA then activates the downstream effector RhoA-dependent kinase (ROCK). ROCK then phosphorylates and inhibits TASK-1 channels, leading to membrane depolarisation and opening of voltage-dependent calcium channels. Ca$^{2+}$ influx through voltage-dependent calcium channels elevates intracellular Ca$^{2+}$ and causes vasoconstriction and proliferation. TASK-1 channels are also inhibited by extracellular acidosis and hypoxia.

Ilorpost and treprostinil, both stable analogues of PGI$_2$, were demonstrated to enhance anandamide-sensitive background K$^+$ currents carried by TASK-1 channels in human PASMCs to cause membrane hyperpolarisation (Olschewski et al., 2006). Consensus PKA phosphorylation motifs were also identified within TASK-1 and treprostinil was shown to promote the phosphorylation of these sites (Olschewski et al., 2006). The effects of iloprost and treprostinil on TASK-1 currents were mimicked by 8-br-cAMP, a membrane-permeant form of the second messenger cAMP, and blocked by the PKA inhibitor KT5720, suggesting that, in human PASMCs, treprostinil signals primarily via the cAMP/PKA to increase TASK-1 currents (Olschewski et al., 2006). The receptors via which treprostinil signals to enhance TASK-1 currents in human PASMCs have not been determined and could include, prostanoid receptors of the IP or EP$_2$ subtypes or even nuclear receptors, such as PPAR$_\gamma$. Interestingly, cAMP-dependent PKA was reported to promote the forward trafficking of TASK-1 channel subunits from the ER, where they are synthesised, to the plasma membrane, where they assemble to form functional, current-conducting channels (Kilisch et al., 2016; Mant et al., 2011). PKA-mediated phosphorylation of serine 393 in the channel C-termini allows TASK-1 to interact with the scaffold protein 14-3-3 (Mant et al., 2011). This interaction is thought to mask the ER retention signal and promote the export of TASK-1 channel subunits to the Golgi, wherefrom they traffic to the plasma membrane (Mant et al., 2011; Kilisch et al., 2016). Treprostinil could therefore be increasing TASK-1 currents in human PASMCs by promoting the forward trafficking of TASK-1 channel subunits from the sarcoplasmic reticulum to the sarcolemma.

The effect of the pulmonary vasodilator NO on TASK-1 currents in human PASMCs has yet to be explored. NO is released from the endothelium and diffuses to the underlying
PASMCs, where it activates sGC to elevate cGMP. cGMP-elevating agents were shown to alter the pH sensitivity of TASK-1 channels in HEK293 cells loaded with PKG and in cholinergic neurons of the basal forebrain, increasing TASK-1 currents at physiological pH and attenuating TASK-1 inhibition by acidic pH (Toyoda et al., 2010). Whether cGMP or agents that elevate it (e.g. sildenafil and riociguat) have similar effects on TASK-1 currents in PASMCs remains, to the best of my knowledge, unexplored. It is worth noting that cGMP had no effect on TASK-1 currents in middle cerebral arterial smooth muscle cells (Lloyd et al., 2009). Moreover, riociguat was found to augment TASK-1 currents in tsA-201 cells expressing wildtype TASK-1 channels but not those carried by mutant TASK-1 channels (Cunningham et al., 2019). This suggests that riociguat might not be efficacious in PAH patients harbouring KCNK3 mutations.

1.8.2 TASK-1 channel dysfunction in PAH

Although most HPAH cases have been genetically accounted for, there remains a significant proportion (21%) with no identifiable genetic cause. In 2013, using whole-exome sequencing, Ma et al. identified novel heterozygous mutations in KCNK3, the gene encoding the two-pore domain potassium channel TASK-1, in patients with IPAH and HPAH, describing the first channelopathy in PAH (Ma et al., 2013). Homozygous KCNK3 mutations were later reported in patients with very aggressive and early-onset forms of PAH (Tejedor et al., 2017). All KCNK3 mutations severely reduced whole-cell TASK-1 currents recorded from COS-7 and PASMCs expressing both wildtype and mutant TASK-1 subunits to reproduce the heterozygosity observed in carriers of the mutations (Bohnen et al., 2017; Ma et al., 2013). Moreover, TASK-1 expression levels were found to be markedly reduced in the explanted lungs of patients with IPAH, but lacking disease-causing KCNK3 mutations (Antigny et al., 2016). TASK-1 currents were also shown to be attenuated in PAECs and PASMCs derived from the lungs of patients with HPAH due to BMPR2 mutations, suggesting that TASK-1 channel downregulation may represent a second hit required for PAH to manifest in carriers of BMPR2 mutations (Antigny et al., 2016).

Strikingly, neither right ventricular hypertrophy nor neomuscularisation of distal pulmonary arteries, both early signs of PAH, were observed in Kcnk3−/− mice (Manoury et al., 2011). Moreover, PASMCs acutely isolated from the lungs of these Kcnk3-deficient mice showed background K⁺ currents and resting membrane potentials indistinguishable from those measured in wildtype PASMCs (Manoury et al., 2011). Also, pulmonary arteries from Kcnk3−/− mice retained normal resting tone and vasoreactivity (Manoury et al., 2011). These findings suggest that, although expressed, TASK-1 failed to form
functional channels in mouse PASMCs (Manoury et al., 2011). This is in divergence with human and rat PASMCs and could be explained by the expression of other \( \text{K}_{\text{2p}} \) family members, particularly TWIK-2. Indeed, TWIK-2 channel knockout produced an early PAH-like phenotype in mice, as indicated by enhanced pulmonary vasoreactivity, elevated right ventricular systolic pressures and remodelling of the small pulmonary arteries (Pandit et al., 2014).

Recently, rats, chronically treated with A293, a selective blocker of TASK-1 channels, were shown to develop early manifestations of PAH, including muscularisation of distal pulmonary arteries (normally non-muscular) and elevated right ventricular systolic pressure (Antigny et al., 2016). Histological analysis of pulmonary arteries from these A293-treated rats revealed marked proliferation of PAECs, PASMCs and pulmonary adventitial fibroblasts (Antigny et al., 2016). Despite not influencing the resting tone of isolated rat pulmonary arteries, TASK-1 channel blockade with A293 was found to potentiate contractile responses to the thromboxane A\(_2\) mimetic U46619 (Antigny et al., 2016). To further confirm the involvement of TASK-1 in the pathogenesis of PAH, KCNK3-deficient rats were also generated using the CRISPR-Cas9 technique and, similarly to rats treated with A293, were found to spontaneously develop early signs of PAH, including neomuscularisation of distal pulmonary arteries and elevated RVSP (Lambert et al., 2019). Pulmonary arteries derived from the lungs of these KCNK3-deficient rats displayed exaggerated vasoreactivity and upregulated pro-proliferative MAPK signalling (Lambert et al., 2019). KCNK3-deficient rats also developed more severe PAH in response to chronic hypoxia (Lambert et al., 2019).

Right ventricular failure is the primary cause of death in PAH. Early in PAH, the RV undergoes hypertrophy to compensate for the increased afterload and maintain cardiac output (Lambert et al., 2018). Right ventricular hypertrophy, however, is rarely fully compensatory and persistent increase in afterload causes myocardial apoptosis, excessive fibrosis and increased expression of non-contractile proteins, all of which contribute to decreased RV systolic function (manifested as reduced right ventricular ejection fraction) and subsequently to dilatation and failure (Lambert et al., 2019). Lambert and colleagues recently demonstrated a role for TASK-1 channel dysfunction in PAH-associated right ventricular hypertrophy and dysfunction. TASK-1 channel expression was found to be severely reduced in right ventricular cardiomyocytes from PAH patients and monocrotaline or sugen/hypoxia-treated rats, even prior to the development of PH (Lambert et al., 2018). In right ventricular cardiomyocytes from monocrotaline-treated rats, reduced TASK-1 current density was accompanied by prolongation of the action potential duration (APD) and TASK-1 channel blockade with
A293 failed to further prolong the APD (Lambert et al., 2018). Chronic inhibition of TASK-1 with A293 in rats led to an increase in cell membrane capacitance, which is proportional to cell surface area, and RV cardiomyocyte diameter, both of which are indicative of RV hypertrophy (Lambert et al., 2018). In vivo TASK-1 channel blockade in rats also promoted RV fibrosis as demonstrated by Sirius red staining and increased mRNA expression of type 1 collagen (Lambert et al., 2018). In addition to these structural changes, TASK-1 channel inhibition reduced RV function as examined by echocardiography (Lambert et al., 2018).

In many tissues, TASK-1 is co-expressed and forms functional heterodimers with the closely related TASK-3 (encoded by the gene KCNK9), which are insensitive to inhibition by ruthenium red (Lopes et al., 2001; Czirjak and Enyedi, 2002). The lungs, however, are completely devoid of TASK-3, forcing TASK-1 to form functional homodimers and possibly explaining the pulmonary-specific manifestation of the TASK-1 mutations (Bohnen et al., 2017). It was, therefore, hypothesised that coassembly of TASK-1 with TASK-3 shields extrapulmonary tissues against the deleterious effects of TASK-1 mutations and that the absence of TASK-3 from the lungs underlies the pulmonary specificity of the phenotype observed in PAH patients with the heterozygous mutations (Bohnen et al., 2017). Interestingly, TASK-3 was found to assemble with mutant TASK-1 and produce functional heterodimeric channels, providing evidence in support of this hypothesis (Bohnen et al., 2017). TASK-3 is weakly expressed in the right ventricle and therefore cannot compensate for the loss of TASK-1 channel expression and activity in PAH patients lacking KCNK3 mutations (Lambert et al., 2018). Moreover, because of its low expression in the right ventricle, TASK-3 is also unlikely to rescue the function of mutant TASK-1 subunits in PAH patients harbouring KCNK3 mutations (Lambert et al., 2018).

### 1.9 Mitochondrial dysfunction in PAH

Mitochondria are cellular organelles, the major function of which is to generate ATP via the process of oxidative phosphorylation (Kuhlbrandt, 2015). They consist of two membranes and two aqueous compartments (Kulbrandt, 2015). The inner mitochondrial membrane (IMM) surrounds the mitochondrial matrix and forms invaginations called cristae, which contain the electron transport chain (ETC), consisting of the respiratory complexes I to IV and the F₁F₀-ATP synthase or complex V (Kulbrandt, 2015). The outer mitochondrial membrane (OMM) surrounds the IMM, forming a narrow intermembrane space (Kuhlbrandt, 2015). Most mitochondrial proteins are encoded by nuclear genes and are synthesised on ribosomes in the cytosol before being folded and integrated into
the mitochondrial reticulum (Hock and Kralli, 2009). The remainder is encoded by the mitochondrial DNA (mtDNA), which resides within the mitochondrial matrix (Hock and Kralli, 2009). Accumulating evidence suggests that mitochondrial dysfunction, particularly in pulmonary vascular cells, contributes to the pathogenesis of PAH. Changes in both the morphology and activity of the mitochondria have been reported in pulmonary vascular cells. These changes will be discussed in this section.

1.9.1 Mitochondrial dynamics in PAH

Although originally conceived as static, cable-like entities, it is now widely accepted that mitochondria exist in dynamic networks, composed of individual organelles that continuously join and divide by the processes of fusion and fission, respectively (Archer, 2013). Mitochondrial dynamics therefore refers to the ability of mitochondria to undergo fusion and fission and accounts for the wide morphological spectrum of mitochondrial structures (Archer, 2013; Dorn, 2019). Fusion serves to merge two mitochondria into one, whereas fission divides a single mitochondrion into two mitochondria. Although the primary function of mitochondria is to generate ATP, much of the impact of mitochondrial dynamics is on the non-bioenergetic role of mitochondria in regulating essential cellular processes, such as calcium and redox homeostasis, apoptosis and proliferation (Archer, 2013). For example, fission creates mitochondrial fragments, which, depending on the context, are more capable of producing ROS or promoting cell cycle progression into mitosis (Archer, 2013). Fusion results in a network of interconnected mitochondrial tubules with enhanced interactions with the ER and better ability at serving as calcium sinks (Archer, 2013). Mitochondrial fission and fusion are regulated by a small number of highly conserved GTP-hydrolysing proteins or GTPases, all of which are encoded by nuclear genes (Archer, 2013; Dorn, 2019).

Mediators of mitochondrial fusion

Mitochondrial fusion requires the fusion of the OMM followed by that of the IMM. Mitofusin-1 (MFN1) and mitofusin-2 (MFN2) mediate the fusion of the OMM, whereas optic atrophy 1 (OPA1) mediates the fusion of the IMM (Archer, 2013; Dorn, 2019). All three are transmembrane GTPases, with MFN1 and MFN2 spanning the OMM and OPA1 the IMM (Archer, 2013; Dorn, 2019). MFN2 also localises to the ER membrane (Archer, 2013; Dorn, 2019). MFN1 and MFN2 form homotypic or heterotypic interactions, tethering one mitochondrion to another or mitochondria to the ER (Archer, 2013). Mitochondria-ER bridges, mediated predominantly by MFN2, are thought to facilitate calcium uptake from the ER into the mitochondria and to create microdomains for mitochondrial fission, aided by the ER (Dorn, 2019). The dynamin-like GTPase OPA1 is
subject to proteolytic cleavage at two distinct sites S1 and S2, targeted by the metallopeptidases OMA1 and YMEL1, respectively (MacVicar and Langer, 2016). OPA1 cleavage generates a long OPA1 isoform, denoted L-OPA1, and a short OPA1 isoform, designated S-OPA1 (MacVicar and Langer, 2016). OMM fusion is mediated by L-OPA1, which remains anchored to the OMM and possesses fusogenic activity (MacVicar and Langer, 2016). Liberated S-OPA1, on the other hand, is thought to maintain the normal architecture of the mitochondrial cristae, invaginations of the IMM, where the ETC system resides (Lee et al., 2017).

Table 1.3: Mediators of mitochondrial fusion and their abnormality in PAH PASMCs.

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Function</th>
<th>Abnormality in PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitofusin-1</td>
<td>GTPase in OMM that tethers adjacent mitochondria and mitochondria to ER</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Mitofusin-2</td>
<td>GTPase in OMM that tethers adjacent mitochondria and mitochondria to ER</td>
<td>Downregulated (Marsboom et al., 2012)</td>
</tr>
<tr>
<td>Optic atrophy 1 (OPA1)</td>
<td>GTPase in IMM that mediates fusion of inner mitochondrial membrane</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Figure 1.5: The mechanism of fusion of two separate mitochondria into one mitochondrion (Dorn, 2019). (a) Two adjacent mitochondria with the fusion proteins MFN1 and MFN2 spanning their OMM and OPA1 spanning their IMM collide to allow mitochondrial tethering. (b) Mitochondrial tethering is mediated by the formation of intermembrane homo- or heterodimeric (e.g. MFN1-MFN1 or MFN2-MFN2) or heterodimeric (e.g. MFN1-MFN2) MFN complexes. (d) Energy released from GTP hydrolysis by MFNs
drives the fusion of the OMM. Fusion of the OMM is followed by that of the IMM. (c) OPA1 interact and hydrolyse GTP to GDP to fuel the fusion of the IMM. This completes the fusion of the two mitochondria into one and allows the mixing of their contents.

**Mediators of mitochondrial fission**

In most eukaryotes, the GTPase dynamin-related protein 1 (DRP1) and its adaptor proteins constitute the core machinery of mitochondrial fission (Breitzig et al., 2018). This is supported by the frequent finding that cells lacking DRP1 contain highly elongated mitochondrial networks, formed by ongoing fusion unopposed by fission activity (Breitzig et al., 2018). DRP1 is a soluble, cytosolic protein, composed of an N-terminal GTP-hydrolysing or GTPase domain, a middle domain and a C-terminal GTPase effector domain (GED) involved in self-assembly (Breitzig et al., 2018). Mitochondrial fission occurs upon DRP1 activation and recruitment to potential fission sites at the mitochondrial surface, where it self-assembles into multimers to form ring structures and hydrolyses GTP to mechanically constrict and ultimately sever the mitochondria (Breitzig et al., 2018). DRP1-mediated mitochondrial fission is thought to be facilitated by mitochondrial interaction with the ER. Images acquired using electron microscopy have revealed contact sites between the ER and the mitochondria, where the ER wraps around and constricts the mitochondria (Hakjoo and Yisang, 2014; Marchi et al., 2014). This step is thought to serve to reduce the average mitochondrial diameter from 300-500 nm to 150 nm to facilitate DRP1 ring formation and consequent fission (Hakjoo and Yisang, 2014).

DRP1 activity is regulated by post-translational phosphorylation and dephosphorylation at two key serine (S) residues. Each residue is targeted by different serine/threonine kinases and phosphatases. Reversible DRP1 phosphorylation by cAMP-dependent PKA at S637 (and its rat equivalent S656) within the C-terminal GED domain has been shown to inhibit DRP1 activity (Cribbs and Strack, 2007). This is supported by several lines of evidence. Firstly, forskolin, which stimulates adenylate cyclase and hence cAMP production, was found to increase DRP1 phosphorylation at S656 in rat PC12 cells (Cribbs and Strack, 2007). This was opposed by calcineurin, a Ca\(^{2+}\)-calmodulin-dependent protein phosphatase, which was activated by concomitant membrane depolarisation and calcium channel activation. Secondly, in cells expressing a pseudo-phosphorylated form of DRP1, in which S637 (or its rat equivalent S656) was mutated to phosphomimetic aspartate, mitochondrial fission and fragmentation were inhibited (Cribbs and Strack, 2007). In contrast, mitochondrial fission was increased in cells expressing a non-phosphorylatable form of DRP1, in which S637 was mutated to alanine (Cribbs and Strack, 2007).
Contrastingly, phosphorylation of S616, which also dwells within the GED domain of DRP1, has been shown to enhance the fission activity of DRP1. This is supported by the finding that mutation of S616 to non-phosphorylatable alanine inhibits mitochondrial fission, whereas mutating S616 to phosphomimetic glutamate exacerbated mitochondrial fission. In contrast to S637, which has thus far been shown to be phosphorylated exclusively by PKA, S616 is targeted by several kinases, linking mitochondrial fission to crucial cellular processes. For example, cyclin-dependent kinase 1 (CDK1), a regulator of cell cycle transition from the G2 to the M phase, has been demonstrated to phosphorylate DRP1 at S616 in various cell types, including PASMCs, synchronising mitochondrial fission with cell division (Marsboom et al., 2012; Taguchi et al., 2006). S616 is also phosphorylated by Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) and PKC\(\delta\), positioning mitochondrial fission downstream of increases in intracellular Ca\(^{2+}\) levels (Bo et al., 2018; Qi et al., 2010; Xu et al., 2016). Finally, the pro-survival serine/threonine kinase ERK2 has also been shown to phosphorylate DRP1 at S616 (Kashatus et al., 2015; Yu et al., 2011).

Figure 1.6: Modular structure and post-translational regulation of the mitochondrial fission protein DRP1. Dynamin-related protein 1 (DRP1) is a multidomain protein, composed of an N-terminal GTPase domain, a middle domain, a B-insert domain and a C-terminal GTPase effector domain (GED). DRP1 is regulated by post-translational phosphorylation at two key serine (S) residues within the GED domain. Phosphorylation at S616 activates DRP1, whereas phosphorylation at S637 inhibits DRP1. S637 is targeted exclusively by cAMP-dependent serine/threonine kinase protein kinase A (PKA), whereas S616 is subject to phosphorylation by a wide range of serine/threonine kinases, including cyclin-dependent kinase 1 (CDK1), extracellular signal regulated kinase 2 (ERK2), protein kinase C (PKC) and calmodulin-dependent kinase II (CaMKII). S637 is dephosphorylated by the calcium-calmodulin-dependent phosphatase calcineurin.
DRP1 lacks a pleckstrin homology (PH) domain and is therefore unable to bind directly to membrane phospholipids in the OMM. Instead, adaptor proteins, anchored to the OMM, serve as receptors for DRP1, enabling DRP1 recruitment to the mitochondrial surface. These include mitochondrial fission factor (MFF), mitochondrial fission protein 1 (FIS1) and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51). In addition to recruiting DRP1 to the OMM, MFF, MiD49 and MiD51 stimulate the GTPase activity of DRP1 (Chan, 2019). Conflicting evidence exists, however, regarding the role of these proteins in recruiting DRP1 to the mitochondria and mediating mitochondrial fission. FIS1, for example, has been demonstrated to be dispensable for DRP1 recruitment to the mitochondria. Recently, however, FIS1 has been shown to promote mitochondrial fission by inhibiting the fusion activity of mitochondrial fusion proteins (Yu et al., 2019). In addition to OMM localisation, MiD49 and MiD51 have been shown to be present in the cytosol, where they are thought to sequester DRP1 and prevent its mitochondrial recruitment. Indeed, in similarity to MiD49 and MiD51 double knockdown, overexpression of either MiD49 or MiD51 promoted mitochondrial fusion rather than fission (Palmer et al., 2013).

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Function</th>
<th>Abnormality in PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamin-related protein 1 (DRP1)</td>
<td>Cytosolic GTPase that is recruited to outer mitochondrial membrane when activated</td>
<td>Upregulated, increased phosphorylation at S616 (Marsboom et al., 2012)</td>
</tr>
<tr>
<td>Mitochondrial fission factor (MFF)</td>
<td>DRP1 receptor in outer mitochondrial membrane that recruits DRP1 from cytosol</td>
<td>Unknown</td>
</tr>
<tr>
<td>Mitochondrial fission 1 (FIS1)</td>
<td>DRP1 receptor in outer mitochondrial membrane that recruits DRP1 from cytosol</td>
<td>Upregulated (Marsboom et al., 2012)</td>
</tr>
<tr>
<td>Mitochondrial dynamics protein of 49 kDa (MiD49)</td>
<td>DRP1 receptor in outer mitochondrial membrane that recruits DRP1 from cytosol, also present in cytosol but role is unknown</td>
<td>Upregulated (Chen et al., 2018)</td>
</tr>
<tr>
<td>Mitochondrial dynamics protein of 51 kDa (MiD51)</td>
<td>DRP1 receptor in outer mitochondrial membrane that recruits DRP1 from cytosol, also present in cytosol but role is unknown</td>
<td>Upregulated (Chen et al., 2018)</td>
</tr>
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1.9.1.3 Impaired mitochondrial dynamics in PAH

In PAH, PASMC hyper-proliferation has been linked to increased mitochondrial fission due to heightened expression and activity of DRP1, as indicated by increased phosphorylation at S616 (Marsboom et al., 2012). Marsboom et al. (2012) attributed DRP1 hyper-phosphorylation at S616 to increased activity of the cell cycle regulator
CDK1 and its activator cyclin B1. Indeed, DRP1 inhibition with either the small molecule inhibitor Mdivi-1 or siRNA-mediated knockdown restored normal mitochondrial morphology, caused cell cycle arrest at the G2/M checkpoint and inhibited the proliferation of PAH PASMCs (Marsboom et al., 2012). Mdivi-1 also attenuated PAH in three different rodent models, including one where the transcription factor hypoxia-inducible factor-1α (HIF-1α) was chemically activated with cobalt chloride (Marsboom et al., 2012), implicating DRP1 in the pathogenesis of PAH. Prostacyclin analogues signal to activate PKA and may therefore induce the inhibitory phosphorylation of DRP1 at S637 to restore mitochondrial fusion in PAH PASMCs in a manner similar to Mdivi-1. Indeed, glucagon-like peptide 1 (GLP1), which, like prostacyclin analogues, signals to activate PKA, was reported to promote the inhibitory phosphorylation of DRP1 in aortic SMCs, inhibiting mitotic mitochondrial fission and cell proliferation induced by the growth factor PDGFB (Torres et al., 2016). Moreover, overexpression of DRP1 attenuated this anti-proliferative effect of GLP1, suggesting that GLP1 might be inhibiting vascular remodelling through a mechanism dependent on mitochondrial dynamics (Torres et al., 2016).

In addition to an increase in DRP1 expression and fission activity, increased recruitment of DRP1 to the mitochondria has been reported in PAH PASMCs. Marsboom et al. (2012) and Ryan et al. (2013) both found an increase in the mRNA expression of the DRP1 recruiter FIS1, one of the earliest DRP1 adaptor proteins identified. Moreover, the DRP1-binding partners MiD49 and MiD51 have both been recently reported to be upregulated in PASMCs from PAH patients, contributing to the excessive mitochondrial fragmentation observed in these cells (Chen et al., 2018). Overexpression of MiD49 or MiD51 in normal PASMCs caused fission and recapitulated the PAH phenotype, whereas silencing either MiD49 or MiD51 in PAH PASMCs attenuated excessive mitochondrial fission, inhibiting proliferation and promoting apoptosis (Chen et al., 2018). MicroRNA profiling in normal and PAH PASMCs revealed diminished levels of microRNA (miR)-34a-3p, which negatively regulates the expression of MiD49 and MiD51 (Chen et al., 2018). Accordingly, miR-34a-3p mimics were found to decrease MiD49 and MiD51 expression in PAH PASMCs, inhibiting mitochondrial fission and proliferation and promoting apoptosis (Chen et al., 2018). Contrastingly, silencing miR-34a-3p in normal PASMCs caused mitochondrial fission and promoted a PAH phenotype (Chen et al., 2018).

Opposing mediators of mitochondrial fission are those of mitochondrial fusion, including the OMM-spanning proteins MFN1 and MFN2 and the IMM-spanning protein OPA1. Transcript levels of MFN2, but not MFN1, were shown to be downregulated in PASMCs isolated from patients with PAH (Marsboom et al., 2012). This finding was later confirmed
by Ryan et al. (2013) in cultured PAH PASMCs and in the PASMC-rich medial layers of pulmonary arteries in the lungs of patients with PAH, suggesting that OMM fusion might be impaired in PAH PASMCs. Given the role of MFN2 in bridging between the mitochondria and the ER and facilitating communication between the two organelles, reduced MFN2 expression might also underlie the increased ER-mitochondria distance and reduced ER-to-mitochondria transfer of calcium and phosphatidylserine reported in PAH (Sutendra et al., 2013). Overexpressing MFN2 in PAH PASMCs reduced proliferation by hindering the mitochondrial fission that is required for and precedes cell cycle progression to mitosis (Ryan et al., 2013).

1.9.2 Mitochondrial respiration in PAH

The most important role of mitochondria in eukaryotic cells is the generation of ATP through oxidative phosphorylation (OxPhos; Kuhlbrandt, 2015). OxPhos refers to the use of oxygen to phosphorylate ADP into ATP and occurs in the infoldings of the IMM or cristae, which accommodate the five complexes (CI-CV) of the respiratory chain (Kuhlbrandt, 2015). Complexes I-IV are multi-subunit enzymes that function in concert to pump protons across the IMM and into the mitochondrial intermembrane space, setting up an electrochemical proton gradient that drives complex V or F₁F₀ ATP synthase to produce ATP (Kuhlbrandt, 2015). Complex I or NADH dehydrogenase is the largest of the complexes (Kuhlbrandt, 2015). It accepts electrons from NADH generated either in the cytosol or in the mitochondrial matrix by the tricarboxylic acid (TCA) cycle, oxidising NADH to NAD⁺ and pumping 4 protons across the IMM in the process (Kuhlbrandt, 2015). Unlike the rest of the complexes, complex II or succinate dehydrogenase is not a proton pump (Kuhlbrandt, 2015). It oxidises succinate and transfers the electrons released from this oxidation reaction to complex III or cytochrome c oxidoreductase (Kuhlbrandt, 2015). Complex III then passes electrons to the carrier cytochrome c and pumps 2 protons across the IMM. Cytochrome c transfers the electrons to complex IV or cytochrome c oxidase (Kuhlbrandt, 2015). Complex IV delivers the electrons to the final electron acceptor oxygen to convert it to water, pumping 4 protons in the process (Kuhlbrandt, 2015). Complex V uses the energy stored within the proton gradient established by complexes I-IV to phosphorylate ADP to ATP (Kuhlbrandt, 2015).

NADH and FADH₂, the primary donors of electrons to the ETC, are produced by the TCA cycle in the mitochondrial matrix. Briefly, the TCA cycle is a closed loop of reactions in which the last step generates the substrate that is used up in the first step. The substrate that drives the TCA cycle is the end-product of glycolysis pyruvate. Following its
production in the cytosol, pyruvate is transported into the mitochondria by means of mitochondrial pyruvate carriers (MPCs; McCommis and Finck, 2015). Once in the mitochondrial matrix, pyruvate is converted to acetyl coenzyme A (acetyl-CoA) by the enzyme complex pyruvate dehydrogenase (PDH). Acetyl-CoA is then incorporated into the TCA cycle. PDH is therefore known as the mitochondrial gatekeeper, coupling cytosolic glycolysis to mitochondrial OxPhos. PDH is a multi-subunit enzyme complex, composed of three enzymatic components: PDHE1, PDHE2 and PDHE3. PDH activity is inhibited by PDH kinase (PDK)-dependent phosphorylation of three serine residues within the alpha subunit of PDHE1 (PDHE1α; Park et al., 2018). Opposing this PDK-mediated inhibition is dephosphorylation by PDH phosphatases (PDPs; Park et al., 2018).

A normoxic shift in glucose metabolism from mitochondrial OxPhos to cytosolic glycolysis, reminiscent of the Warburg effect in cancer, has been reported in PAH pulmonary vascular cells, particularly PASMCs, and suggested to underlie the hyper-proliferative and apoptosis-resistant phenotype of these cells (Paulin and Michelakis, 2014). A number of mechanisms have been proposed to underlie the depressed mitochondrial OxPhos in PAH. Reduced PKM-mediated conversion of the glycolysis intermediate phosphoenolpyruvate to pyruvate has been demonstrated in ECs and fibroblasts derived from PAH patients. Diminished pyruvate transport into the mitochondria as a result of MPC downregulation has also been reported in fibroblasts isolated from PAH patients. Moreover, PDH, which converts pyruvate to the primary TCA substrate acetyl-CoA within the mitochondrial matrix, has been shown to be inhibited in PAH by several mechanisms. For example, increased expression of the PDK isoforms PDK1 and PDK2 concomitant with increased phosphorylation of the PDH subunit, PDHE1α, at the inhibitory residue S293 has been reported in the lungs of patients with PAH (Michelakis et al., 2017). PDH is a calcium-dependent enzyme and the reduced mitochondrial uptake of calcium as a result of downregulation of the mitochondrial calcium uniporter UCP2 is thought to deprive PDH of calcium and reduce its catalytic activity (Michelakis et al., 2017). ER stress in PAH has been proposed to contribute to the inhibition of PDH. ER stress results in impaired connectivity between the ER and the mitochondria, reducing the transfer of calcium from the ER to the mitochondria. Sirtuin-3 promotes the activity of PDH through deacetylation and its inhibition in PAH is thought to also contribute to the depressed function of PDH (Paulin et al., 2014; Michelakis et al., 2017).

Consistent with the central role of PDH inhibition, particularly by PDK-mediated phosphorylation, in driving Warburgian metabolism in PAH, the PDK inhibitor
dichloroacetate (DCA) was found to reverse the glycolytic shift in PAH PASMCs, rendering them susceptible to apoptosis and inhibiting their proliferation, and to attenuate monocrotaline-induced PH in rats (McMurtry et al., 2004). Interestingly, PDK inhibition with DCA was also found to rescue the expression of K\(_{v1.5}\), a channel which is severely downregulated in PAH, suggesting that K\(^+\) channel downregulation in PAH may be a consequence of depressed mitochondrial respiration (McMurtry et al., 2004). DCA was also trialled in a small group of patients, showing favourable effects on PVR, mPAP and exercise capacity. Lack of clinical efficacy of DCA was observed in a subset of these patients, in which loss-of-function SNPs in sirtuin 3 and UCP2, both regulators of PDH activity, were later identified (Michelakis et al., 2017).

1.9.3 Mitochondrial biogenesis in PAH

Mitochondrial biogenesis is a complex, tightly regulated process whereby cells add new mitochondrial mass to the established mitochondrial network (Jornayvaz and Shulman, 2014). It necessitates the synchronous transcription and translation of both nuclear and mitochondrial genes followed by the import and incorporation of proteins and lipids into the existing mitochondrial reticulum (Hock and Kralli, 2009). Mitochondrial biogenesis also requires the replication of mtDNA within the mitochondrial matrix. The clear majority of mitochondrial proteins are encoded by nuclear genes with only 13 mitochondrial proteins, all of which comprise subunits of the ETC complexes I, III, IV and V, encoded by the mitochondrial genome (Hock and Kralli, 2009). The master regulator of mitochondrial biogenesis is peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α), which lacks DNA-binding activity but interacts with and co-activates a number of transcription factors. Amongst the transcription factors co-activated by PGC1α are nuclear respiratory factors 1 (NRF1) and 2 (NRF2; Hock and Kralli, 2009). NRF1 was originally identified as a transcription factor, which binds to regulatory elements in the promoter region of cytochrome c, but has since then been shown to bind within promoter regions of many, other mitochondrial genes to drive their transcription (Hock and Kralli, 2009). Similar to NRF1, NRF2 induces the transcription of nuclear genes, encoding mitochondrial proteins, particularly constituent subunits of complex IV or cytochrome c oxidase of the respiratory chain (Hock and Kralli, 2009; Ongwijitwat et al., 2006). PGC1α together with NRF1 and NRF2 also regulate the expression of mitochondrial transcription factors A (TFAM), B1 (TFB1M) and B2 (TFB2M; Hock and Kralli, 2009). TFAM, TFB1M and TFB2M translocate to the mitochondria, where together they regulate replication and transcription of mtDNA (Hock and Kralli, 2009; Jornayvaz and Shulman, 2010).
Despite not being thoroughly investigated in PAH, there is evidence of decreased mitochondrial biogenesis in patients with PAH as well as rodent models of the disease. PGC1α expression, for example, was shown to be reduced in PASMCs from PAH patients compared to controls, suggesting a decrease in mitochondrial biogenesis (Ryan et al., 2013). This was accompanied by downregulation of the mitochondrial fusion protein MFN2 and mitochondrial fission (Ryan et al., 2013). Reduced PGC1α was also reported in the lungs of mice with chronic hypoxia-induced PH (Yeligar et al., 2018). Moreover, PPARγ knockdown and resultant PGC1α downregulation in PASMCs were found to reduce mitochondrial mass and bioenergetics. There is, however, conflicting evidence in the literature with regards to the expression of PPARγ in PAH PASMCs. Falcetti et al. (2010) demonstrated stronger PPARγ staining in the medial layers of pulmonary arteries in the lungs of patients with IPAH. Contrastingly, Ameshima et al. (2003) reported a significant downregulation of PPARγ expression in the lungs of patients with severe PAH.

1.9.4 Mitophagy in PAH

The elimination of mitochondria, particularly those that are damaged or dysfunctional, is mediated by a selective form of autophagy, aptly termed mitophagy (Pickles et al., 2018). Mitophagy therefore represents an important mitochondrial quality control mechanism (Pickles et al., 2018). In mitophagy, double-membraned autophagosomes engulf whole mitochondria or selectively target damaged regions (Pickles et al., 2018). Subsequently, autophagosomes fuse with lysosomes, in which mitochondrial degradation occurs (Pickles et al., 2018). In mammalian cells, mitophagy is preceded by DRP1-dependent mitochondrial fission, which divides mitochondria into fragments of sufficient size for encapsulation by autophagosomes (Graef, 2016). Mitophagy can, however, occur independently of DRP1-mediated mitochondrial fission (Graef, 2016). Interestingly, mitophagy is not the only lysosomal pathway implicated in the elimination of damaged mitochondria (Lemasters, 2014). For example, mitochondrial-derived vesicles, enriched in mitochondrial proteins, bud off and transit to multivesicular bodies (Lemasters, 2014). Thus far, two distinct pathways of mitophagy have been described: ubiquitin-dependent mitophagy and ubiquitin-independent (receptor-mediated) mitophagy (Fritsch et al., 2019).

In ubiquitin-dependent mitophagy, loss of mitochondrial membrane potential leads to the stabilisation and consequent accumulation of phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (PINK1) on the OMM (Pickles et al., 2018). PINK1 then recruits and phosphorylates parkin, an E3 ubiquitin ligase, at S65 (Pickles et al.,
PINK1 also phosphorylates ubiquitin and poly-ubiquitin chains conjugated to OMM proteins, which also recruit parkin for PINK1-dependent phosphorylation, leading to the amplification of the mitophagy signal (Palikaras et al., 2018). Once activated by PINK1, parkin, in concert with E2 ubiquitin-conjugating enzymes, decorates OMM proteins (e.g. MFN1, MFN2, VDAC1) with poly-ubiquitin chains (Pickles et al., 2018; Youle and Narendra, 2011). These ubiquitin chains are recognised by autophagy receptors (e.g. p62, NBR1, OPTN, NDP52; Pickles et al., 2018). Autophagy receptors contain ubiquitin-binding domains (UBDs) and LC3-interacting regions (LIR), allowing them to simultaneously bind to ubiquitin chains on mitochondrial OMM proteins and LC3 proteins on autophagosomes (Fritsch et al., 2019). In addition to Parkin, several other E3 ubiquitin ligases, such as Gp78, SMURF1, SIAH1, MUL1 and ARIH1, regulate ubiquitin-dependent mitophagy (Palikaras et al., 2018). Mitophagy can also occur independently of ubiquitin. In ubiquitin-independent or receptor-mediated mitophagy, mitochondrial OMM proteins serve as mitophagy receptors, which target dysfunctional mitochondria directly to autophagosomes (Palikaras et al., 2018). Containing LIR motifs, mitophagy receptors (e.g. BNIP3, FUNDC1, NIX) directly interact with LC3 and GABARAP proteins within the autophagosomal membrane, causing their autophagosomal sequestration and subsequent delivery to lysosomes for hydrolytic degradation (Palikaras et al., 2018).

The role of mitophagy in PAH pathogenesis remains largely unknown or very poorly understood. A study by Haslip et al. (2015) showed increased levels of PINK1 and parkin and conversion of the autophagosomal protein LC3B1 to LC3BII in the lungs of mice with intermittent hypoxia-induced PH, indicating increased PINK1/parkin-dependent mitophagy in this PH model (Haslip et al., 2015). This increase in PINK1/parkin mitophagy was augmented by endothelial-specific knockout of the mitochondrial calcium uniporter UCP2 (Haslip et al., 2019). Mitophagy has yet to be investigated in pulmonary vascular cells from PAH patients. Given that PINK1/parkin-dependent mitophagy is initiated by depolarisation of the mitochondrial membrane potential and that mitochondria have been shown to be hyperpolarised in PAH, namely in PASMCs, it is reasonable to postulate that mitophagic flux might be reduced in this disease, allowing the accumulation of dysfunctional mitochondria.
1.10 Glycolysis and pentose phosphate shunting in PAH

Glycolysis is a multistep, cytosolic pathway that converts glucose to pyruvate and precedes mitochondrial respiration. Glucose is less efficient in terms of ATP yield than mitochondrial respiration, generating two ATP molecules per molecule of glucose. Cellular influx of glucose occurs via diffusion facilitated by glucose transporters (GLUTs), of which four isoforms (GLUT1-4) are well established (Thorens and Mueckler, 2010). Glucose is then phosphorylated by hexokinases, particularly the most abundant isoforms hexokinase 1 and 2, resulting in its entrapment within the cell. Following glucose phosphorylation, G6P is converted to its isomer fructose-6-phosphate (F6P), which is then further phosphorylated in an irreversible reaction to fructose-1,6-bisphosphate (F1,6BP) by the enzyme phosphofructokinase 1 (PFK1). Conversion of F6P to F1,6P constitutes the rate-limiting step in glycolysis and represents the first point of commitment of glucose to the glycolytic pathway (Mor et al., 2011). The rate-limiting glycolytic enzyme PFK1 exists either as a homotetramer or a heterotetramer, owing to its three isoforms. These isoforms are the muscle isoform (PFKM), the liver isoform (PFKL) and the platelet isoform (PFKP; Mor et al., 2011). Given its rate-limiting function in glycolysis, PFK1 activity correlates positively with the rate of glycolysis and is tightly regulated by a myriad of metabolites (Mor et al., 2011). For example, PFK1 is inhibited by ATP and activated by AMP (Mor et al., 2011). This serves to prevent excess glucose degradation when ATP levels are ample (Mor et al., 2011). The most potent positive allosteric modulator of PFK1 is fructose-2,6-bisphosphate (F2,6BP; Mor et al., 2011). F2,6BP is thought to relieve the inhibitory effect of ATP on PFK1, uncoupling the link between cellular bioenergetics and glycolytic flux (Mor et al., 2011). Interestingly, F2,6BP is produced from the glycolysis intermediate F6P in a reversible reaction catalysed by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB; Lu et al., 2017). There are four PFKFB genes (PFKFB1-4), each encoding a different isoenzyme (Lu et al., 2017). Among these PFKFB isoenzymes, PFKFB3 functions only as a kinase due to its considerably higher affinity for F6P than F2,6BP (Lu et al., 2017).

To yield pyruvate, F1,6BP is first converted in a multi-step reaction to phosphoenolpyruvate, which is then converted to pyruvate by the enzyme pyruvate kinase (PK; Israelsen and Vander Heiden, 2015). To date, four PK isoforms have been identified, including two muscle isoforms (PKM1 and PKM2), a liver isoform (PFKL) and a red blood cell isoform (PKR; Vander Heiden, 2015). PKM1 and PKM2 are products of alternative splicing of the PKM gene (Israelsen and Vander Heiden, 2015). Whereas PKM1 constitutively exists as a high-activity tetramer, PKM2 can form either a high-
activity tetramer or a low-activity dimer (Vander Heiden, 2015). Therefore, the ratio of PKM2 to PKM1 determines the rate at which phosphoenolpyruvate is converted to pyruvate. An increase in the PKM2/PKM1 reduces overall PKM activity and inhibits the conversion of phosphoenolpyruvate to pyruvate (Israelsen and Vander Heiden, 2015). It also favours the accumulation of glycolysis intermediates, which then spill over into biosynthetic pathways (Israelsen and Vander Heiden, 2015). Under aerobic conditions, pyruvate is transported into the mitochondria by means of mitochondrial pyruvate carriers (MPCs; McCommis and Finck, 2015). In the mitochondrial matrix, pyruvate is converted to acetyl-CoA by the PDH enzyme complex. Acetyl-CoA then feeds into the TCA cycle to ultimately drive mitochondrial OxPhos. Under anaerobic conditions, pyruvate remains in the cytosol, where it is converted to lactate. Catalysing pyruvate’s conversion to lactate is the enzyme lactate dehydrogenase (LDH). LDH is a tetramer composed primarily of two subunits LDHA and LDHB (Valvona et al., 2015). LDHA has a higher affinity for pyruvate than lactate and essentially reduces pyruvate to lactate, oxidising NADH to NAD⁺. LDHB, in contrast, has a higher affinity for lactate and therefore preferentially converts lactate back to pyruvate, reducing NAD⁺ back to NADH (Valvona et al., 2015). The ratio of LDHA to LDHB therefore determines the direction of this reversible reaction. Once generated, lactate is then extruded from the cell by monocarboxylate transporters (MCTs) to acidify the extracellular milieu (Paven et al., 2019).

Analogous to the Warburgian shift observed in cancer cells, glucose flux through glycolysis has been shown to be elevated in pulmonary vascular cells from patients with PAH and proposed to underlie their hyper-proliferative phenotype (Caruso et al., 2017; Zhang et al., 2017). Increased glycolytic flux in PAH was thought to occur secondary to suppressed mitochondrial OxPhos to compensate for the reduced mitochondrial ATP production (Paulin and Michelakis, 2014). However, several benefits inherent to glycolysis cause highly proliferative cells, like pulmonary vascular cells from PAH patients, to favour glycolysis over mitochondrial OxPhos (Vander Heiden, 2011). Despite its lower efficiency at generating ATP than mitochondrial OxPhos, glycolysis can occur 10-100 times faster than OxPhos (Lunt and Vander Heiden, 2011). Interestingly, highly proliferative cells, such as cancer cells, seem to require ATP for cellular maintenance rather than proliferation. Moreover, proliferating cells require copious amounts of metabolic intermediates to support the biosynthesis of macromolecules, such as lipids, nucleic acids and proteins (Lunt and Vander Heiden, 2011). The accumulation of glycolysis intermediates, as a result of increased glucose flux through glycolysis coupled with suppressed mitochondrial OxPhos, is thought to fuel these biosynthetic pathways, many of which branch off glycolysis (Lunt and Vander Heiden, 2011).
Many mechanisms have been demonstrated to underlie this increased glycolytic flux. Using the radiotracer analogue of glucose $^{18}$F-D-glucose and positron emission tomography, glucose uptake has been shown to be increased in the lungs of patients with PAH as well as in the lungs of the monocrotaline-induced rat model of the disease (Hagan et al., 2011; Zhao et al., 2013). This increased glucose flux in PAH remains controversial, however, with one study reporting no difference in pulmonary glucose uptake between non-PAH patients and PAH patients. Supporting increased glucose uptake in PAH was increased expression of the glucose transporter GLUT1 (Zhao et al., 2013). In addition to increased glucose uptake, hexokinase 1 was found to be upregulated in PAH patients, particularly in PAFs, suggesting an increase in the conversion of glucose to G6P and its resultant cellular entrapment (Zhao et al., 2013). Recently, Calvier et al. (2017) reported increased expression of the PFK1 isoform PFKP in the pulmonary arteries of PAH patients. Moreover, TGFβ, a key driver of pulmonary vascular remodelling in PAH, was found to upregulate the expression of PFKP in control PASMCs (Calvier et al., 2017). Given its rate-limiting activity in glycolysis, an increase in the expression of PFKP was suggested to underlie the increased glycolytic flux in PAH (Calvier et al., 2017). PDH inhibition as a result of increased PDK-dependent phosphorylation of its subunit PDHE1α is also thought to hinder the mitochondrial uptake of pyruvate and to promote its cytosolic reduction to lactate. Indeed, studies have reported increased expression of the PDK isoforms PDK1 and PDK2 and increased inhibitory phosphorylation of PDHE1α at S293 in the lungs of patients with PAH (Michelakis et al., 2017; Zhao et al., 2013). A decrease in the expression of mitochondrial pyruvate transporter MPC1 has been reported in BOECs from patients with PAH, further contributing to reduced mitochondrial uptake of pyruvate and promoting a shift to glycolysis. Finally, the last step in glycolysis, which generates pyruvate and is catalysed by PKM, seems to be impaired in PAH. In the same year, both Caruso et al. (2017) and Zhang et al. (2017) reported an increase in the PKM2/PKM1 ratio in BOECs and PAFs derived from patients with PAH. As mentioned above, an increase in the ratio of PKM2 to PKM1 reduces the rate of conversion of phosphoenolpyruvate to pyruvate and creates a bottleneck effect that promotes the accumulation of glycolysis intermediates. These intermediates then channel into biosynthetic pathways to support the proliferative phenotype of PAH pulmonary vascular cells.

At the first step of glycolysis, G6P can be shunted into the oxidative arm of the pentose phosphate pathway (PPP), which generates the nucleotide precursor ribose-5-phosphate (R5P) and the reducing agent NADPH (Lunt and Vander Heiden, 2011). NADPH serves as a scavenger of ROS via reduced glutathione to maintain cellular redox homeostasis. NADPH is also used as a cofactor in the synthesis of fatty acids and
cholesterol (Lunt and Vander Heiden, 2011). The first step in the PPP is catalysed by the rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PD), which converts G6P to 6-phosphogluconolactone, reducing NADP* to NADPH in the process (Patra and Hay, 2014). 6-phosphogluconolactone is then further metabolised to yield R5P and a second molecule of NADPH. The production of the nucleotide precursor R5P and NADPH to support nucleic acid synthesis and lipid synthesis, respectively, render the PPP particularly critical for highly proliferative cells, such as cancer cells (Patra and Hay, 2014). Indeed, increased pentose phosphate shunting as indicated by upregulation of the rate-limiting enzyme G6PD, has been reported in many types of cancer, including breast, cervical, colon and lung cancers (Bokun et al., 1987; Hong et al., 2018; Jonas et al., 1992). In relevance to PAH, increased G6PD activity was reported in highly proliferative PASMCs from chronically hypoxic rats, suggesting that increased pentose phosphate shunting may be driving PASMC proliferation in PAH and that G6PD may constitute an attractive target for curbing PASMC proliferation in the disease (Chettimada et al., 2015).

1.10 Oxidative stress in PAH

The term reactive oxygen species (ROS) collectively refers to unstable free radicals, such as superoxide anion, hydroxyl radical and hypochlorite, as well as stable oxidants, such as hydrogen peroxide (DeMarco et al., 2010; Fulton et al., 2017). Free radicals are short-lived due to their high reactivity and are readily scavenged by a number of antioxidants and enzymes (DeMarco et al., 2010). Multiple sources are known to generate ROS within cells. These include the mitochondrial ETC complexes I and III, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), xanthine oxidases (XO), cyclooxygenases (COX), lipoxygenases (LO) and uncoupled NOS (DeMarco et al., 2010; Fulton et al., 2017). If not scavenged, free radicals can cause direct damage to cells by reacting with proteins, lipids and nucleic acids (DeMarco et al., 2010). Scavengers of ROS include the antioxidant reduced glutathione and the enzymes superoxide dismutases (SODs) and catalases (Mailloux et al., 2013). Reduced glutathione is generated in a two-step, cytosolic reaction, catalysed by glutamate cysteine ligase and glutathione synthase (Mailloux et al., 2013). Reduced glutathione then scavenges ROS with the aid of glutathione peroxidase (Mailloux et al., 2013). SODs convert superoxide anions to hydrogen peroxide, which is then broken down into water and oxygen by catalases (Mailloux et al., 2013). There are three isoforms of SOD (Fakai and Ushio-Fukai, 2011). SOD1 is located in the cytoplasm and the mitochondrial intermembrane space (Fakai and Ushio-Fukai, 2011). SOD2 resides within the mitochondrial matrix (Fakai and Ushio-Fukai, 2011). SOD3 is found extracellularly either
bound to cell surfaces or constituents of the extracellular matrix (Fakai and Ushio-Fukai, 2011).

In the healthy pulmonary vasculature, ROS plays a role in regulating physiological and cellular processes. One such physiological process is hypoxic pulmonary vasoconstriction (HPV; Moudgil et al., 2005). HPV occurs in response to acute alveolar hypoxia and serves to divert blood flow from poorly ventilated regions of the lung to better ventilated ones to optimise gas exchange (Moudgil et al., 2005). The role of ROS in mediating HPV remains controversial (Moudgil et al., 2005). Some studies have shown that ROS decreases during HPV and this favours the closure of K⁺ channels, such as the hypoxia-sensitive Kv1.5 and TASK-1 channels, and the consequent opening of voltage-gated calcium channels in PASMCs (Moudgil et al., 2005; Sham, 2002). Other studies have shown that ROS derived from the mitochondria or NOX increase during hypoxia and this increase in ROS triggers the cytosolic calcium increase in PASMCs that underlies HPV (Sommer et al., 2010; Ward, 2006; Waypa et al., 2006).

A state of oxidative stress has been demonstrated in the pulmonary vasculature of animal models of PH, predominantly the chronic hypoxia-induced PH model. Oxidative stress was also reported in patients with PAH and ROS has been proposed to promote pulmonary vasoconstriction and pulmonary vascular cell proliferation that underpins remodelling. Oxidative stress occurs when ROS production overwhelms antioxidant defences, creating an imbalance in the redox state of the cell and favouring oxidation. Oxidative stress is therefore caused by an increase in ROS generation with or without downregulation of ROS-scavenging systems. Several mechanisms have been proposed to contribute to oxidative stress in PAH. The NOX isoforms NOX1, NOX2 and NOX4 have been shown to be upregulated in rodent models of PH as well as in patients with PAH, suggesting increased generation of ROS by NOX (Mittal et al., 2009; Nisbet et al., 2009; Sanders and Hoidal, 2007; Veit et al., 2013). In addition to NOX, increased XO activity was reported in patients with IPAH (Spiekermann et al., 2009). Downregulation of mitochondrial SOD or SOD2 has also been reported in PAH, suggesting that reduced scavenging of mitochondrial ROS may also contribute to increased ROS levels and therefore oxidative stress in PAH (Archer et al., 2010). The levels of reduced glutathione, another scavenger of ROS, have been shown to remain unchanged in patients with PAH (Masri et al., 2008). However, the activity of glutathione peroxidase, the enzyme that utilises reduced glutathione to scavenge ROS, was found to be diminished (Masri et al., 2008). Downregulation of SOD2 and glutathione peroxidase suggests that, in addition to upregulation of ROS generating enzymes, suppression of antioxidant systems also contributes to oxidative stress in PAH.
1.11 Hypotheses, aims and objectives

PASMCs isolated from the remodelled pulmonary arteries of patients with PAH exhibit an abnormal hyper-proliferative and synthetic phenotype. The mechanisms underlying this pathological phenotype of PASMCs in PAH are not fully understood. The hypothesis is that reprogramming of mitochondrial function and dynamics, and loss of function of K⁺ channels, particularly TASK-1, drives this phenotypic switch of PASMCs in PAH to favour pulmonary vascular remodelling and prostacyclin mimetics, which constitute the gold standard treatment for patients with PAH, are able, to some extent, to reverse this mitochondrial reprogramming and K⁺ channel dysfunction to restore a normal phenotype.

The overall aim of this thesis was to assess mitochondrial dynamics, glycolysis, mitochondrial respiration and TASK-1 channel function in distal PASMCs derived from control and PAH patients and to unravel the mechanisms underlying any differences observed. The individual aims and objectives were as follows:

1. **Mitochondrial dynamics in PASMCs from PAH patients.** Mitochondrial morphology was assessed in control and PAH PASMCs. Protein expression of mediators of the opposing processes of mitochondrial fusion and fission was also explored in control and PAH PASMCs. The effects of prostacyclin mimetics on mitochondrial morphology and protein mediators of mitochondrial fusion and fission were determined.

2. **Glycolysis and pentose phosphate shunting in PASMCs from PAH patients.** Glucose flux through glycolysis in control and PAH PASMCs was assessed. Protein expression of key glycolytic enzymes in control and PAH PASMCs was also explored. Protein expression of the rate-limiting enzyme in the pentose phosphate pathway, which branches off glycolysis, in control and PAH PASMCs was also investigated to provide an indication of the level of glucose flux through the pentose phosphate pathway in PAH.

3. **Mitochondrial respiration, biogenesis and mitophagy in PASMCs from PAH patients.** Mitochondrial glucose oxidation was investigated in control and PAH PASMCs. Protein expression of mitochondrial mass markers, transcriptional regulators of mitochondrial biogenesis and key mitochondrial enzymes was investigated in control and PASMCs. Mitophagic flux as well as protein expression of markers of autophagy in control and PAH PASMCs was explored. ROS levels and protein expression of select ROS scavengers and generators in control and PAH.
PASMCs was measured. Finally, the effect of prostacyclin mimetics on mitochondrial respiration in PAH PASMCs was examined.

4. **TASK-1 channel dysfunction in PASMCs from PAH patients.** Expression of TASK-1 was assessed in control and PAH PASMCs. The effect of loss of TASK-1 channel function on PASMC proliferation and mitochondrial respiration was also investigated. The effect of loss of TASK-1 channel function on the anti-proliferative effects of prostacyclin mimetics on PAH PASMCs was explored. Finally, the effect of loss of TASK-1 channel function on PAEC apoptosis, an early event in PAH, was investigated.

This thesis therefore aimed to better understand the metabolic reprogramming of PASMCs in PAH and to unravel the mechanisms underlying it in the hope of identifying targets that could be used in the future treatment of this disease. It also aimed to further our understanding of the role of TASK-1 channels in the pathogenesis and treatment of PAH.
2. Materials and methods

2.1 Cell culture

2.1.1 Culturing, passaging and cryopreserving human PASMCs

Distal pulmonary arterial smooth muscle cells (PASMCs) were isolated from the lungs of either control subjects or patients with PAH undergoing transplant. The demographics and clinical characteristics of the patients are in Table 2.1. Lung tissue was taken after patient or relative consent and with Ethics Committee approval from Great Ormond Street Hospital (REC 05/Q0508/45), Papworth Hospital (REC H00/531/T) and the Assistance Publique – Hopitaux de Paris (IRB00006477, Agreement No. 11-045). Cryopreserved control or PAH PASMCs were revived and cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12; Gibco™) containing 10% Foetal Bovine Serum (FBS), the antibiotic penicillin (100 U/ml; Gibco™) and the antifungal agent streptomycin (100 µg/ml; Gibco™) in sterile, 75 cm² (T-75), cell culture flasks (Corning®) at 37°C in a humidified atmosphere with 5% CO₂. 100 U/ml penicillin and 100 µg/ml streptomycin are collectively denoted as 1% PenStrep in this thesis. Adherent PASMCs were liberated from the cell culture flasks for further passaging or cryopreservation by trypsinisation with 0.25% Trypsin-EDTA (TE; Gibco™). Briefly, 80-90% confluent PASMCs were washed twice with 1X Dulbecco’s Phosphate Buffered Saline (DPBS; Gibco™) and incubated with TE at 37°C for 2-3 minutes or until the cells have dislodged. Once the cells have detached, TE was neutralised with DMEM/F12 with 10% FBS and the cells were pelleted by centrifugation at 220 x g. The supernatant was discarded to remove TE and the cell pellet was re-suspended in 2 ml of DMEM/F12 with 10% FBS. For cell counting, 10 µl of the cell suspension was mixed with an equal volume of the vital stain Trypan Blue (0.4%; Sigma-Aldrich) and the mixture was pipetted into a C-chip disposable haemocytometer with the Neubauer improved grid (NanoEnTeK). Only cells which excluded the dye and remained unstained were counted. For further passaging, 250,000-500,000 PASMCs were cultured in DMEM/F12 with 10% FBS in a fresh T-75 flask. For cryopreservation, PASMCs were re-suspended in CryoSFM (PromoCell) at a density of 250,000-500,000 cells/ml and transferred to 1.2 ml cryogenic vials (cryovials; Nalgene®). Cryovials were placed in a Mr Frosty freezing container (Nalgene®) and stored at -80°C overnight. The cryovials containing the PASMCs were then transferred to liquid nitrogen for long-term storage.
#### Table 2.1: Demographics and clinical characteristics of PAH patients from whom distal PASMCs were derived.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (years)</th>
<th>Patient diagnosis</th>
<th>mPAP (mmHg)</th>
<th>PVRI (WU·m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
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<td>PAH associated with ASD</td>
<td>73</td>
<td>33</td>
</tr>
<tr>
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<td>5</td>
<td>PAH associated with ASD</td>
<td>89</td>
<td>39</td>
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<tr>
<td>Female</td>
<td>13</td>
<td>IPAH</td>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>IPAH</td>
<td>95</td>
<td>40</td>
</tr>
<tr>
<td>Female</td>
<td>35</td>
<td>IPAH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Male</td>
<td>43</td>
<td>IPAH</td>
<td>50</td>
<td>-</td>
</tr>
</tbody>
</table>

2.1.2 Culturing, passaging and cryopreserving human PAECs

Human pulmonary arterial endothelial cells were purchased from PromoCell. Prior to culturing the PAECs, T-75 flasks were coated with 1X Attachment Factor (AF; Gibco™) and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 30 minutes to provide a substrate upon which the PAECs can grow. AF was removed and cryopreserved PAECs were revived and cultured in Endothelial Cell Growth Medium (ECGM; PromoCell) supplemented with the Supplement Mix (PromoCell) and 1% PenStrep. Confluent PAECs were trypsinised, counted, passaged and cryopreserved as described above for PASMCs.

2.1.3 Induction of apoptosis in human PAECs

Human PAECs were cultured in ECGM (PromoCell) in AF-coated 6-well plates at 37°C in a humidified atmosphere with 5% CO₂ to full confluence. 16 hours prior to the application of the apoptotic stimulus, PAECs were bathed in Endothelial Cell Basal Medium (ECBM; PromoCell), containing 2% FBS and 1% PenStrep, but lacking any growth factors. During this incubation, cells were treated with or without 1 or 10 µM ML365 (Tocris Bioscience). After the 16-hour incubation, apoptosis was induced by incubating the cells with 10 ng/mL recombinant human TNFα (R&D Systems) and 20 µg/mL cycloheximide (Sigma-Aldrich) for 6 hours (Long et al., 2015).

2.2 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

2.2.1 RNA extraction and quantification

Total RNA was extracted from cells grown in monolayers using the RNeasy® Mini Kit (Qiagen) according to the manufacturer’s protocol. Briefly, cells were washed twice with 1X DPBS and lysed in RLT lysis buffer. An equal volume of 70% (v/v) ethanol was added to the lysates and mixtures were transferred to RNeasy spin columns placed in 2-ml collection tubes. The spin columns were centrifuged for 15 seconds at 8,000 x g to allow
the RNA to bind to the membrane. A series of 3 washes was then performed to wash the membrane-bound RNA prior to elution in RNase-free water. Buffer RW1 was first added and the columns were centrifuged for 15 seconds at 8,000 x g. Subsequently, Buffer RPE was added to the spin columns followed by centrifugation at 8,000 x g for 15 seconds. Once again, Buffer RPE was added but the spin columns were centrifuged for 2 minutes at 8,000 x g. To dry the membrane, the spin columns were placed in fresh collection tubes and centrifuged at 8,000 x g for 1 minute. Finally, elution was performed by placing the spin columns in RNase-free 1.5 ml collection tubes, adding 30 μl of RNase-free water and centrifuging the columns for 1 minute at 8,000 x g. The eluates were stored at -80°C until used. The concentration and purity of the RNA eluates were assessed using the NanoDrop™ 2000 Spectrophotometer (Thermo Scientific). RNA samples with an A\textsubscript{260}/A\textsubscript{280} ratio of ~1.8 were deemed of acceptable quality for downstream applications, such as RT-qPCR.

2.2.2 One-step RT-qPCR

The QuantiFast SYBR Green® RT-PCR Kit (Qiagen) together with QuantiTect Primer Assays (Qiagen) were used to rapidly and specifically analyse the mRNA expression of genes of interest. The QuantiFast SYBR Green® RT-PCR Kit allows reverse transcription of mRNA to cDNA and PCR amplification of the cDNA template to take place in a single tube. SYBR Green® is a green fluorescent dye that binds strongly to double-stranded DNA by intercalating between the DNA bases. It is used in qPCR because the fluorescence can be measured after each amplification cycle to determine how much DNA has been amplified. 12 μl reaction mixtures, each comprised of 2 μL of RNA eluate (50 – 100 ng), 1.2 μL of QuantiTect Primer Assays, 0.12 μL of QuantiFast RT Mix, 6 μL of QuantiFast SYBR Green RT-PCR Master Mix and 2.68 μL of RNase-free water, were prepared in 0.1 ml strip tubes (Qiagen). RT-qPCR protocol, consisting of reverse transcription at 50°C for 10 minutes, initial activation at 95°C for 5 minutes, and 40 cycles of denaturation at 95°C for 10 seconds and combined annealing/extension at 60°C for 30 seconds, was performed in a Corbett Rotor Gene 600 Cycler (Qiagen). Table 2.2 lists the QuantiTect Primer Assays (Qiagen) used in this study. Ct (threshold cycle) values were determined for the gene of interest and the housekeeping gene TBP. The delta Ct method (2\textsuperscript{-ΔCt}) method was used to determine the mRNA expression of genes of interest relative to the housekeeping gene TATA box-binding protein (TBP; Livak and Schmittgen, 2001; Radonica et al., 2004).
Table 2.2: QuantiTect Primer Assays used in this thesis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>QuantiTect Primer Assay</th>
<th>Cat No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNK3</td>
<td>Human</td>
<td>Hs_KCNK3_1_SG</td>
<td>QT00201264</td>
</tr>
<tr>
<td>TBP</td>
<td>Human</td>
<td>Hs_TBP_1_SG</td>
<td>QT00000721</td>
</tr>
</tbody>
</table>

2.3 Western blotting

2.3.1 Protein extraction and quantification

Cells grown in monolayers were washed thrice with ice-cold 1X PBS (Gibco™) and lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with cOmplete™ protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 2 (Sigma-Aldrich) and 3 (Sigma-Aldrich). Lysates were centrifuged at 600 x g for 10 minutes and supernatants were collected and stored at -20°C until used. Pierce™ BCA Assay (ThermoFisher Scientific) was used to determine the protein concentrations of the cell lysates. The Pierce™ BCA Assay utilises the well-known ability of proteins to chelate and reduce divalent copper (Cu²⁺) to monovalent copper (Cu¹⁺) in an alkaline milieu. This reaction, known as the biuret reaction, generates a light-blue complex. Cu¹⁺ then reacts with two molecules of bicinchoninic acid to yield a dark purple product, which exhibits absorbance at 562 nm. BCA working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). To generate the standard curve, pre-diluted Pierce™ Bovine Serum Albumin (BSA) standards (0-2000 µg/mL; ThermoFisher Scientific) together with the BCA working reagent were pipetted in triplicates into 96-well plates and incubated at 37°C for 30 minutes. Similarly, cell lysates of unknown protein concentrations were pipetted into 96-well plates and incubated with the BCA working reagent at 37°C for 30 minutes. Absorbance at 562 nm was measured using a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies). Absorbance values were averaged and then plotted against the concentrations of the BSA standards as shown in Figure 2.1. The equation of the regression line was generated and used to determine the protein concentrations of the cell lysates.
Figure 2.1: Standard curve for the Pierce™ BCA protein assay. Absorbance measured at 562 nm was plotted against the concentrations of the Pierce™ BSA standards. A regression line was fitted through the points to generate the regression equation that was used to determine the protein concentrations of the cell lysates.

2.3.2 SDS-PAGE and protein transfer to nitrocellulose membranes

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins within the cell lysates, based solely on molecular weight, allowing subsequent transfer and immunodetection of specific proteins. SDS (or LDS) denatures and confers upon the proteins a uniform negative charge, allowing them to migrate down the gel towards the positive electrode. Reducing agents (e.g. dithiothreitol) serve to disrupt intra- or inter-peptide disulphide linkages between cysteine residues and together with SDS help linearize proteins and impart an overall negative charge. Proteins were boiled in 1X NuPAGE™ LDS Sample Buffer (Invitrogen™) and 1X NuPAGE™ Sample Reducing Agent (Invitrogen™) at 70°C for 10 minutes. Equal amounts of protein (~25 μg) along with SeeBlue Plus2 Pre-stained Protein Standard (Invitrogen™) were loaded into the wells of NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen™) and run in NuPAGE™ MOPS SDS Running Buffer (1X; Invitrogen™) at 160 V for 50 minutes. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes (Amersham) in NuPAGE™ Transfer Buffer (1X; Invitrogen™) with 20% methanol at 30 V
for 2 hours. Successful transfer was confirmed using Ponceau S solution (Sigma-Aldrich), a reversible stain used to detect protein bands on nitrocellulose membranes. Ponceau S staining was removed by washing the membrane with 1X PBS with 0.1% (v/v) Tween 20 (Sigma-Aldrich) for 5 minutes with agitation.

2.3.3 Blocking and incubating the membrane with primary antibodies

Following successful protein transfer to nitrocellulose membranes, the membranes were blocked in various blocking buffers, depending on the primary antibody, for 1 hour at room temperature with gentle rocking. Blocking buffers included 1X Casein Blocking Buffer (Sigma-Aldrich) and 1X TBST (Cell Signalling) with 5% (w/v) skimmed milk (Marvel). After blocking, membranes were washed thrice with 1X TBST for 5 minutes each and incubated with the primary antibody in blocking buffer overnight at 4°C with rolling. Depending on the primary antibody used, membranes were incubated in either 1X Casein Blocking Buffer, 1X TBST with 5% (w/v) skimmed milk or 1X TBST with 5% (w/v) BSA (Sigma-Aldrich).
Table 2.3: Primary and secondary antibodies used for probing western blots in this thesis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Company</th>
<th>Cat No.</th>
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</thead>
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<td>GAPDH</td>
<td>Mouse</td>
<td>1:50,000</td>
<td>Abcam</td>
<td>ab8245</td>
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<tr>
<td>DRP1</td>
<td>Rabbit</td>
<td>1:1,000</td>
<td>Cell Signalling</td>
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</tr>
<tr>
<td>pDRP1 (S616)</td>
<td>Rabbit</td>
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<td>Cell Signalling</td>
<td>3455S</td>
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<td>pDRP1 (S637)</td>
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<td>Abcam</td>
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<td>Abcam</td>
<td>ab56889</td>
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<td>Beta tubulin</td>
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</table>
2.3.4 Washing and incubating the membrane with secondary antibodies

After overnight incubation with the primary antibody, membranes were washed thrice with 1X PBST for 5 minutes each to remove non-specific primary antibody binding. Membranes were then incubated with the appropriate HRP-conjugated secondary antibody in the same blocking buffer that was used for incubation with the primary antibody for 1 hour at room temperature with rolling. Membranes were washed thrice with 1X PBST for 5 minutes each to remove non-specific secondary antibody binding. The HRP-conjugated secondary antibody binds to the primary antibody that has bound to the protein of interest to allow for subsequent chemiluminescent detection.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Company</th>
<th>Cat No.</th>
</tr>
</thead>
<tbody>
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<td>Rabbit IgG, HRP-linked</td>
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<tr>
<td>Mouse IgG, HRP-linked</td>
<td>Horse</td>
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<td>7076S</td>
</tr>
</tbody>
</table>

2.3.5 Chemiluminescent detection and densitometry

Following incubation with HRP-conjugated secondary antibody, the proteins to which the primary antibody have bound were detected using enhanced chemiluminescence (ECL). In ECL, the HRP, which is conjugated to the secondary antibody, oxidises luminol to the chemiluminescent 3-aminophthalate in the presence of chemical enhancers, such as phenols. These chemical enhancers serve to increase the light output and prolong the duration of light emission. To detect proteins on nitrocellulose membranes in this thesis, equal volumes of Amersham ECL Western blotting Detection Reagents 1 and 2 (GE Healthcare) were mixed and applied to the membranes. Excess reagent was drained off and the membranes were wrapped up in SaranWrap and placed in X-ray film cassettes. The membranes were exposed to sheets of Amersham Hyperfilm ECL (GE Healthcare), which were then developed and fixed in the dark. Densitometric analysis of the protein bands was performed using ImageJ.

2.4 Immunohistochemistry

Immunohistochemistry (IHC) is a qualitative method used for demonstrating the presence and location of proteins in tissue sections. Despite being less quantitative than other immunoassays, such as western blotting, IHC generates a visual output that enables the observation of protein expression and localisation in the context of intact tissue. IHC utilises antibodies that recognise specific epitopes within proteins of interest. Given the high specificity of antibodies, their binding is restricted to proteins of interest in the tissue sections. The antibody-antigen is subsequently visualised using...
2.4.1 Deparaffinisation and rehydration of tissue sections

Formalin-fixed, paraffin-embedded lung sections (approximately 5 μm in thickness) derived from control subjects or patients with PAH were used. Paraffin wax was removed by immersing the slides in xylene for 10 minutes. The slides were then passed through xylene twice followed by decreasing concentrations of ethanol (100%-75% (v/v)) and ultimately water to gradually rehydrate the tissue sections.

2.4.2 Heat-induced antigen retrieval

Formaldehyde fixation crosslinks proteins within tissues by promoting the formation of methylene bridges. Consequently, the epitopes, to which antibodies bind, are masked or buried, rendering them inaccessible and restricting the antigen-antibody interactions that are required for successful immunostaining. Antigen retrieval serves to sever these methylene bridges, exposing antigenic sites and permitting efficient antibody binding. There are two primary methods of antigen retrieval: heat-induced antigen retrieval and protease-induced antigen retrieval. In this study, heat-induced antigen retrieval was employed to gently expose the epitopes and allow primary antibody binding. Depending on the primary antibody used, slides were heated in either citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) or Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9.0) for 10-20 minutes in a microwave set to full power. Slides were then cooled in running tap water for 10 minutes.

2.4.3 Novolink™ Polymer Detection System

Following antigen retrieval, the Novolink™ Polymer Detection System (Leica Biosystems) was used to stain the tissues for proteins of interest with mouse or rabbit IgG primary antibodies (listed in Table 2.5). Endogenous peroxidases within the tissue sections were neutralised using the Peroxidase Block for 5 minutes to minimise background staining caused by the HRP-conjugated secondary antibodies. The sections were washed twice with 1X TBS for 5 minutes each followed by incubation with the Novocastra™ Protein Block for 5 minutes to reduce non-specific binding of the primary antibody. Sections were washed twice with 1X TBS and incubated with the primary antibody, optimally diluted in Antibody Diluent (Dako), for 1 hour at room temperature. Dilution factor for each primary antibody was optimised for maximal signal and minimal chromogenic detection. Chromogenic detection requires the use of a secondary antibody conjugated to an enzyme that catalyses the cleavage of a substrate to yield a coloured precipitate at the site of the protein.
background. Sections were washed twice with 1X TBS prior to incubation with the Post Primary (rabbit anti-mouse IgG) for 30 minutes. After two 5-minutes washes with 1X TBS, sections were incubated with the Novolink™ Polymer for 30 minutes. Sections were washed twice with 1X TBS for 5 minutes each with gentle rocking and developed with DAB working solution (50 μl of DAB Chromogen to 1 ml of Novolink™ DAB Substrate Buffer) for 5 minutes.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Company</th>
<th>Cat No.</th>
</tr>
</thead>
<tbody>
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<td>SDHA</td>
<td>Mouse</td>
<td>1:1,000</td>
<td>Abcam</td>
<td>ab14715</td>
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<td>PGC1α</td>
<td>Rabbit</td>
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<td>Novus Biologicals</td>
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<td>ProteinTech</td>
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<td>Normal rabbit IgG</td>
<td>Rabbit</td>
<td>NA</td>
<td>Cell Signalling</td>
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</tr>
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</table>

### 2.4.4 Counterstaining with haematoxylin, dehydration and mounting of tissue sections

Sections were rinsed with water for 5 minutes and counterstained with Meyer’s haematoxylin, a nuclear stain, for 2 minutes. Sections were rinsed again with water and dipped briefly in 0.3% (v/v) acid alcohol. Sections were rinsed with water and dehydrated by passing them through increasing concentrations of ethanol (75-100% (v/v)) and xylene. Sections were mounted with DPX Mounting Medium (ThermoFisher Scientific) and imaged using the Nanozoomer Digital Scanner.

### 2.5 Immunofluorescence

PASMCs were seeded at a density of $2 \times 10^4$ cells/well in four-well chamber slides (Nunc) and maintained in 10% FBS for 48 hours to allow the cells to adhere and acquire their morphology. Cells were then starved in 0.1% FBS for 48 hours to induce quiescence followed by fixation in 4% (w/v) paraformaldehyde (PFA) in 1X PBS for 15 minutes and three 5-minute washes with 1X PBS. Cells were permeabilised with 0.3% (v/v) Triton X-100 in 1X PBS to allow primary antibody permeation and blocked in 5% (w/v) normal serum (derived from the same species in which the fluorophore-conjugated secondary antibody was raised) in 1X PBS for 1 hour at room temperature to block non-specific binding. Subsequently, cells were incubated with the primary antibody, optimally diluted in 1X PBS with 1% (w/v) BSA and 0.3% (v/v) Triton X-100, at 4°C overnight. After
incubation with the primary antibody, cells were washed thrice with 1X PBS and incubated with the fluorophore-conjugated secondary antibody and the F-actin probe Texas Red™-X Phalloidin (1:200; Invitrogen) diluted in 1X PBS with 1% BSA and 0.3% Triton X-100 in the dark for 1 hour at room temperature. Once again, cells were washed thrice with 1X PBS and mounted with ProLong™ Gold Antifade Mountant (Invitrogen™) containing the nuclear stain 4,6-diamidino-2-phenylindole (DAPI). Finally, cells were imaged with a Nikon Eclipse Ti-E inverted confocal laser scanning microscope.

Table 2.6: Primary and secondary antibodies used for immunofluorescence in this thesis.

<table>
<thead>
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<th>Antibody</th>
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2.6 Live-cell imaging

2.6.1 Live-cell assessment of mitochondrial morphology

Mitochondrial networks are not static as once believed but rather undergo fission and fusion. Fission results in the fragmentation of the mitochondrial network, whereas fusion serves to elongate it. To examine the morphology of the mitochondrial networks in PASMCs, tetramethylrhodamine methyl ester (TMRM) was utilised in conjunction with fluorescence microscopy. TMRM is a membrane-permeant, cationic, red-orange fluorescent dye that accumulates in active mitochondria. Its sequestration by the mitochondria is dependent upon the mitochondrial membrane potential. Collapse of the mitochondrial membrane potential causes the dye to dissipate to the cytosol and the fluorescence to drop. PASMCs were seeded at low density in glass-bottom μ-dishes (Ibidi) and cultured for 48 hours in 10% FBS. After starving them in 0.1% FBS for 48 hours, PASMCs were rinsed twice with HBSS and incubated with 25 nM TMRM and 10 µg/ml Hoechst 33342, a nuclear stain, in HBSS for 30 minutes. PASMCs were then imaged at x60 magnification with a Nikon Eclipse Ti-E inverted confocal laser-scanning microscope.

2.6.2 Live-cell assessment of mitophagy

Mitophagy has emerged as an important quality control mechanism whereby cells eliminate damaged or dysfunctional mitochondria. The Mitophagy Detection Kit (Dojindo) was used to examine basal and induced mitophagy in quiesced, live PASMCs. The kit consists of two fluorescent dyes: the Mtphagy Dye, which accumulates in intact mitochondria and tethers covalently, and the Lyso Dye, which stains lysosomes. The
Mtphagy Dye emits weak red fluorescence in the mitochondria but fluoresces more strongly when the mitochondria are autophaged and end up in the acidic environment of lysosomes. The Lyso Dye accumulates in lysosomes where it fluoresces green. PASMCs were seeded at low density in four-well μ-Slides with a coverslip bottom (Ibidi) and maintained in 10% FBS for 48 hours. PASMCs were then starved in 0.1% FBS for 24 hours followed by incubation with 100 nM Mtphagy Dye in HBSS for 30 minutes. PASMCs were washed twice with HBSS and incubated in 0.1% FBS for 24 hours to allow mitophagy to occur. After 24 hours, PASMCs were washed twice with HBSS and incubated with 1 μM Lyso Dye for 30 minutes in HBSS to stain the lysosomes. PASMCs were washed twice with HBSS and imaged with a Nikon Eclipse Ti-E inverted confocal laser-scanning microscope, equipped with an x60 (oil immersion) objective.

**Figure 2.2: Principle of the Mitophagy Detection Kit.** The Mitophagy Detection Kit (Dojindo) allows the visualisation of mitophagic flux in live cells. It utilises two dyes: Lyso Dye and Mtphagy Dye. The Lyso Dye accumulates in lysosomes and fluoresces green. The Mtphagy Dye accumulates in mitochondria, where it tethers covalently and weakly fluoresces red. The Mtphagy Dye fluoresces more strongly when the mitochondria are sequestered in the acidic interiors of lysosomes following engulfment and delivery by autophagosomes.
2.6.3 Live-cell assessment of ROS production

To assay ROS production in live PASMCs, the superoxide indicator dihydroethidium (DHE) was used (Knock et al., 2018). DHE exhibits blue fluorescence in the cytosol prior to oxidation to ethidium. Once oxidised to ethidium, it intercalates within nuclear DNA and consequently stains the nucleus bright fluorescent red. PASMCs were seeded at low density in glass-bottom μ-dishes (Ibidi) and cultured for 48 hours in 10% FBS before being quiesed in 0.1% FBS for an additional 48-hour period. PASMCs were then rinsed twice with HBSS and loaded with 10 μM dihydroethidium (DHE) in HBSS for 10 minutes. After 10 minutes of incubation, PASMCs were imaged with a Nikon Eclipse Ti-E inverted confocal laser-scanning microscope, equipped with an x60 (oil immersion) objective. The number of nuclei that stained positively for OxDHE were counted and expressed as a percentage of the total number of nuclei.

2.7 Cell proliferation assay

To assess PASMC proliferation, the conventional haemocytometer counting method was used (Falcetti et al., 2010). PASMCs were seeded at a density of 2 × 10^4 cells/ml in 6-well plates in 10% FBS and left to adhere overnight. PASMCs were then starved in 0.1% FBS for 48 hours. This serum starvation step serves to arrest the PASMCs at the G1 phase of the cell cycle. After 48-hour serum starvation, PASMCs were treated as indicated in the results in 10% FBS for 96 hours. PASMCs were trypsinised with 0.25% TE for 2-3 minutes or until the cells have detached. Trypsin was neutralised with 10% FBS and PASMCs were blindly counted using the vital stain Trypan Blue and C-chip disposable haemocytometers. Unstained cells were counted in the four outer squares of the grid and the mean cell count was multiplied by the dilution factor (to correct for the dilution in trypan blue) and 10^4 to yield the cell count per ml.

2.8 Seahorse XFp Extracellular Flux Analyser

The Seahorse XFp Extracellular Flux Analyser (Agilent) is used to measure oxygen consumption rate (OCR), which is an indicator of mitochondrial respiration, and extracellular acidification rate (ECAR), which is largely a consequence of glycolysis, of live cells seeded in an 8-well miniplate. It employs solid state sensor probes, which reside in the medium 200 μm above the cell monolayers within the wells of the miniplate. This isolates a miniscule volume (about 2 μl) of medium superior to the cell monolayer, within which real-time measurements of changes to the concentrations of both dissolved oxygen and free protons can be made.
2.8.1 Seahorse XFp Cell Mito Stress Test

The Seahorse XF Cell Mito Stress Test (Agilent) allows the assessment of key parameters of mitochondrial respiration in live cells, including basal respiration, ATP-linked respiration, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration. To reveal these parameters, the Seahorse XF Cell Mito Stress Test uses inhibitors that selectively target components of the ETC. The compounds oligomycin A, FCCP and a mix of rotenone and antimycin A are serially injected and OCR measurements are made after each injection. PASMCs were seeded at an optimal density of 7,500 cells per well in 6 wells of an 8-well Seahorse XFp Cell Culture Miniplate (Agilent). Two wells were kept blank (i.e. filled with cell-free media) to allow for accurate background detection and the 8 moats surrounding the wells were filled with 1X DPBS. PASMCs were allowed to adhere overnight at 37°C in a humidified atmosphere with 5% CO₂. A Seahorse XFp Cartridge (Agilent) was hydrated by adding Seahorse XF Calibrant Solution to the 8 wells and moats of the utility plate, upon which the cartridge lid rests, and incubating the XFp Cartridge overnight at 37°C in a humidified, non-CO₂ incubator. The following day, the PASMC monolayers were transferred into Seahorse XF Base Medium (Agilent), containing 1 mM sodium pyruvate (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 10 mM D-(+)-glucose (Sigma-Aldrich), and incubated at 37°C in a humidified, non-CO₂ incubator for 1 hour. This 1-hour non-CO₂ incubation step serves to degas the plate, permitting CO₂ diffusion from the cells, medium and plate. During this 1-hour incubation, the hydrated Seahorse XFp Sensor Cartridge was loaded with oligomycin A in port A, carbonyl-cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) in port B and rotenone and antimycin A in port C, and loaded into the Seahorse XFp Analyser for calibration. Following cartridge calibration, the cell culture miniplate was loaded into the Seahorse XFp Analyser and the Seahorse XF Cell Mito Stress Test was run to measure OCRs prior to and after the serial injections of the inhibitors. The final concentrations of oligomycin A, FCCP and the combination of rotenone and antimycin A in the wells were 1.5 µM, 1.5 µM and 0.5 µM, respectively. OCRs measured in PASMCs were normalised to their protein content, which was measured using the Pierce™ BCA Protein Assay (see Section 2.3.1). The Seahorse XF Cell Mito Stress Report Generator was used to automatically calculate the Seahorse XF Cell Mito Stress Test parameters, which are summarised in Figure 2.3, from the normalised OCRs.
Figure 2.3: Targets of compounds used in the Seahorse XF Cell Mito Stress Test (Agilent Technologies, 2019). The Seahorse XF Cell Mito Stress Test involves 3 consecutive injections of oligomycin A, FCCP and a combination of both rotenone and antimycin A. Oligomycin A inhibits complex V, the ATP synthase, of the mitochondrial electron transport chain and is injected first. Oligomycin A decreases OCR, revealing ATP-linked respiration. FCCP is a protonophore and therefore forms pores within the inner mitochondrial membrane that selectively allow proton permeation from either side of the membrane. FCCP increases OCR maximally after the dip caused by oligomycin A to reveal the maximum extent to which the cells can respire. Rotenone and antimycin A inhibit complexes I and III of the ETC, respectively, shutting down the entire chain and completely inhibiting mitochondrial OCR. Rotenone and antimycin A therefore reveal non-mitochondrial OCR.
Figure 2.4: Profile of key parameters of mitochondrial function as assessed by Seahorse XF Cell Mito Stress Test (Agilent Technologies, 2019). Consecutive injections of the compounds indicated above reveal basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial oxygen consumption. Basal respiration shows the energetic demand of cells under baseline conditions. It represents the difference between OCR measured prior to the injections of the compounds and OCR measured after the injection of rotenone and antimycin A. ATP production represents the portion of basal respiration used to drive ATP synthesis. It is the difference between OCR measured prior to the injection of the compounds and OCR measured after the injection of oligomycin A, which inhibits the ATP synthase of the respiratory chain. Proton leak is the portion of basal respiration that is not coupled to ATP production and is a sign of mitochondrial damage. It is the difference between OCR measured after the addition of oligomycin A and OCR measured following the injection of rotenone and antimycin A. Maximal respiration is the maximal rate of respiration that cells can achieve. It is calculated as the difference between OCR measured after the addition of the uncoupler FCCP and OCR measured following the injection of rotenone and antimycin A. The capability of cells to respond to an energetic demand is indicated by their spare respiratory capacity. Spare respiratory capacity is the difference between OCR measured prior to the addition of the compounds and OCR measured after FCCP injection. Lastly, non-mitochondrial respiration represents oxygen consumed by processes other than mitochondrial oxidative phosphorylation, such as cytosolic oxidases. It is the OCR that remains after shutting
down the respiratory chain with rotenone and antimycin A, inhibitors of complexes I and III, respectively.

Although 1.5 µM oligomycin A and 0.5 µM rotenone/antimycin A are recommended for use in most cell types, a titration experiment was performed to determine the concentration of FCCP that optimally induces maximal respiration in PASMCs. This is because too much FCCP can inhibit mitochondrial function and diminish OCR. PASMCs were seeded in a Seahorse XFp Cell Culture Miniplate as described above and incubated overnight at 37°C in a humidified incubator with 5% CO₂. A Seahorse XFp Sensor Catridge was also hydrated as described above and incubated overnight at 37°C in a humidified, non-CO₂ incubator. The following day, the media in the wells were changed to Seahorse XF Base Medium, containing 1 mM sodium pyruvate, 2 mM L-glutamine and 10 mM D-(+)-glucose, and incubated at 37°C in a humidified, non-CO₂ incubator for 1 hour. The hydrated cartridge was loaded with oligomycin A in port A and increasing concentrations of FCCP in port B and loaded into the Seahorse XFp Analyser for calibration. After cartridge calibration, the miniplate was loaded and OCRs were measured before and after the serial injections of oligomycin A and FCCP. As shown in Figure 2.4, OCRs obtained in response to FCCP were plotted against the concentration of FCCP. 1.5 µM FCCP was found to optimally induce maximal OCR in PASMCs and was used in the Seahorse XFp Cell Mito Stress Test.
Figure 2.5: Empirical determination of the optimal FCCP concentration for use in the Seahorse XF Cell Mito Stress Test. Using the Seahorse XFp Extracellular Flux Analyser, OCRs were measured in PASMCs after the sequential additions of oligomycin A and increasing concentrations of FCCP. OCRs were then plotted against the concentrations of FCCP injected as shown in the figure above.

2.8.2 Seahorse XFp Glycolysis Stress Test

The Seahorse XF Glycolysis Stress Test was used to measure the glycolytic function of live PASMCs derived from either control subjects or PAH patients. PASMCs were seeded at an optimal density of 7,500 cells per well in 6 wells of an 8-well Seahorse XFp Cell Culture Miniplate (Agilent). Two wells were kept blank (i.e. filled with cell-free media) to allow for accurate background detection and the 8 moats surrounding the wells were filled with 1X DPBS. PASMCs were allowed to adhere overnight at 37°C in a humidified atmosphere with 5% CO₂. The Seahorse XFp Cartridge (Agilent) was hydrated by adding Seahorse XF Calibrant Solution (Agilent) to the 8 wells and moats of the utility plate, upon which the cartridge lid rests, and incubating the XFp Cartridge overnight at 37°C in a humidified, non-CO₂ incubator. The following day, the PASMC monolayers were transferred into Seahorse XF DMEM medium pH 7.4 (buffered with 5 mM HEPES, Agilent), containing 2 mM L-glutamine (Sigma-Aldrich), and incubated at 37°C in a humidified, non-CO₂ incubator for 1 hour. During this 1-hour incubation, the hydrated Seahorse XFp Sensor Cartridge was loaded with D-(+)-glucose in port A, oligomycin A in port B and 2-DG in port C, and loaded into the Seahorse XFp Analyser for calibration. After cartridge calibration, the cell culture miniplate was loaded into the Seahorse XFp
Flux Analyser and the Seahorse XFp Glycolysis Stress Test was run to measure ECARs before and after the sequential injections of the glycolysis modulators glucose, oligomycin A and 2-deoxy-D-glucose (2-DG). The final concentrations of glucose, oligomycin A and 2-DG in the wells were 10 mM, 1 µM and 50 mM, respectively. ECARs measured in PASMCs were normalised to their protein content, which was determined using the Pierce™ BCA Protein Assay (see Section 2.3.1). The Seahorse XF Glycolysis Stress Test Report Generator was used to calculate the assay parameters of the XFp Glycolysis Stress Test, which are summarised in Figure 2.6, from the normalised ECAR data.

![Diagram of glycolysis](image)

Figure 2.6: Modulators of glycolysis used in the Seahorse XF Glycolysis Stress Test (Agilent Technologies, 2019). The Seahorse XF Glycolysis Stress Test involves 3 consecutive injections of glucose, oligomycin A and 2-DG. Glucose is injected first and stimulates glycolysis, resulting in an increase ECAR. Subsequent to glucose, injection of oligomycin A inhibits the mitochondrial ATP synthase resulting in an increased dependence on glycolysis for ATP production and a further increase in ECAR. 2-DG is a competitive inhibitor of the first glycolytic enzyme hexokinase, which converts glucose to glucose-6-phosphate. 2-DG shuts down glycolysis and is injected last to reduce ECAR.
Figure 2.7: Profile of key parameters of glycolytic function as assessed by Seahorse XF Glycolysis Stress Test (Agilent Technologies, 2019). Sequential additions of the compounds shown in the figure above allow the assessment of glycolysis, glycolytic capacity, glycolytic reserve and non-glycolytic acidification. Glycolysis is the process by which cells convert glucose to pyruvate. Glycolysis represents the difference between ECAR measured prior to the injection of the compounds and after the injection of saturation amounts of glucose, which is the major substrate for glycolysis. Glycolytic capacity is the maximum ECAR reached by cells following the addition of oligomycin A, which shuts down mitochondrial oxidative phosphorylation and drives cells to use glycolysis to its maximal capacity. Glycolytic capacity is therefore measured as the difference between ECAR measured prior to the addition of the compounds and ECAR measured after injection of oligomycin A. Glycolytic reserve indicates the extent to which cells can elevate glycolysis to respond to an energetic demand. It is the difference between ECAR measured following the addition of glucose and ECAR measured after the injection of oligomycin A. Finally, non-glycolytic acidification reflects sources of extracellular acidification other than glycolysis.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. In the results, values are expressed as means ± standard error of the mean (SEM) and \( n \) denotes the number of different control or PAH patients. Unpaired student’s t test was used to compare the means of two unrelated groups. One-way analysis of variance (ANOVA) with Bonferroni
post hoc test for multiple comparisons was used to compare the means of three or more independent groups. Two-way ANOVA with Sidak post hoc test for multiple comparisons was used to compare the mean differences between groups that have been split on two independent variables (e.g. treatment and time). Paired student’s t test or repeated measures one-way ANOVA with Tukey’s post hoc test were used where groups were normalised to a control group. 95% confidence intervals (CI) were used to compare between two mean differences. Only significant p values (i.e. $p < 0.05$) were indicated on the figures.
3. Mitochondrial dynamics in PASMCs from patients with PAH

3.1 Introduction

Vascular remodelling in PAH is characterised by proliferation of PASMCs within the medial layers of the small pulmonary arteries (Ryan et al., 2016). PASMC hyper-proliferation in PAH has been linked to changes in mitochondrial morphology (Ryan et al., 2016). Marsboom et al. (2012) were the first to demonstrate that the mitochondrial network is excessively fragmented in PASMCs, derived from patients with PAH, and to unravel the mechanisms underlying this increased mitochondrial fission. They showed that DRP1, the primary mitochondrial fission protein, is upregulated both at the mRNA and protein levels in PAH PASMCs (Marsboom et al., 2012). They also showed that DRP1 is hyper-phosphorylated at S616 as a consequence of increased activity of the cell cycle regulator CDK1 together with its partner cyclin B1 (Marsboom et al., 2012). As discussed in the introduction, phosphorylation at S616 serves to activate DRP1 and cause downstream mitochondrial fission (Taguchi et al., 2007). Moreover, DRP1 phosphorylation at S616 is mediated by a number of pro-proliferative serine/threonine kinases, including CDK1 and ERK2 (Kashatus et al., 2015; Taguchi et al., 2007). Inhibiting DRP1 either via siRNA-mediated knockdown or the pharmacological inhibitor Mdivi-1 induced cell cycle arrest at the G2/M checkpoint in PAH PASMCs and inhibited their proliferation (Marsboom et al., 2012).

In order to mediate fission, DRP1, a soluble, cytosolic protein, has to be recruited to fission sites at the OMM, where it self-assembles into ring structures that constrict and ultimately sever the mitochondria (Atkins et al., 2016; Loson et al., 2013). Several DRP1-interacting proteins have been identified within the OMM that serve to recruit DRP1 from the cytosol. These include the classical DRP1 recruiters FIS1 and MFF and the more recently identified MiD49 and MiD51 (Atkins et al., 2016; Loson et al., 2013). Marsboom et al. (2012) showed an increase in the transcript level of FIS1 and suggested increased mitochondrial recruitment of DRP1. A more recent study demonstrated that MiD49 and MiD51 are both upregulated in PAH due to epigenetic alterations (Chen et al., 2018). The relative protein expressions of MiD49 and MiD51 were found to be increased in PASMCs derived from PAH patients, strongly suggesting once again increased recruitment of DRP1 to fission sites at the OMM (Chen et al., 2018). Similar to DRP1 silencing, siRNA-mediated knockdowns of either MiD49 or MiD51 promoted mitochondrial elongation, induced cell cycle arrest at the G2/M checkpoint, inhibited
proliferation and induced apoptosis of PAH PASMCs (Chen et al., 2018). MiD49 or MiD51 knockdown also resulted in DRP1 dephosphorylation at the activating residue serine 616 (Chen et al., 2018). This has been suggested to be due to decreased synthesis of the PASMC mitogen PDGF, which signals to activate ERK2 to phosphorylate DRP1 at S616 (Chen et al., 2018). Indeed, a separate study has demonstrated DRP1 stimulatory phosphorylation and consequent mitochondrial fission downstream of PDGF in VSMCs (Wang et al., 2015).

The transmembrane GTPases MFN1 and MFN2 mediate fusion of the OMM by tethering adjacent mitochondria and utilising the energy liberated from GTP hydrolysis to induce OMM fusion (Archer, 2013). Mitochondrial tethering results from the formation of homotypic (MFN1-MFN1 or MFN2-MFN2) or heterotypic (MFN1-MFN2) MFN interactions (Archer, 2013). MFN2 also localises to the ER, where it forms bridges between the ER and the mitochondria, facilitating Ca\(^{2+}\) uptake into the mitochondria (de Brito and Scorrano, 2008). In contrast to DRP1, Marsboom et al. (2012) found a decrease in the mRNA expression of MFN2 in PAH PASMCs. The mRNA expression of MFN1, however, was not different in PAH PASMCs, when compared to control PASMCs (Marsboom et al., 2012). To complete mitochondrial fusion, the dynamin-like GTPase OPA1 mediates IMM fusion (Archer, 2013). It also serves a critical role in preserving normal architecture of mitochondrial cristae (invaginations of the IMM) and therefore proper functioning of the electron transport system, which resides within these IMM folds (Lee et al., 2017). OPA1 undergoes proteolytic processing in healthy cells to generate a long isoform, denoted L-OPA1, and a short isoform, designated S-OPA1 (MacVicar and Langer, 2016). L-OPA1 remains tethered to the IMM and possesses fusogenic activity, whereas S-OPA1 is liberated to regulate the structure of mitochondrial cristae (MacVicar and Langer, 2016). However, the expression and proteolytic processing of OPA1 in PAH PASMCs have yet to be examined.

Clinically used prostacyclin mimetics, including the stable prostacyclin analogue treprostinil and the non-prostanoid IP receptor agonist selexipag, have been shown to possess varying degrees of anti-proliferative effects on PASMC proliferation (Falcetti et al., 2010; Patel et al., 2018). Given their anti-proliferative properties, it was postulated that prostacyclin mimetics might promote mitochondrial elongation to reverse the mitotic fragmentation observed in PAH PASMCs. Prostacyclin mimetics signal via membrane GPCRs that couple to Gs, resulting in intracellular cAMP generation and cAMP-dependent PKA activation. PKA has been shown to phosphorylate DRP1 at S637 within its GED domain to inhibit its fission activity (Cereghetti et al., 2008; Cribbs and Strack, 2007). Accordingly, it seems plausible that prostacyclin mimetics would induce inhibitory
DRP1 phosphorylation at S637 to promote mitochondrial fusion and consequent elongation. Moreover, other cAMP-elevating agents, such as glucagon-like peptide 1, have been shown to induce inhibitory DRP1 phosphorylation in VSMCs and to attenuate experimental PH in mice (Honda et al., 2018; Torres et al., 2016). This chapter sought to confirm the increased mitochondrial fission that was previously reported in PAH PASMCs. It also compares the protein expression of various mediators of mitochondrial fission and fusion in PAH PASMCs to that in control PASMCs and explores the influence of prostacyclin mimetics on mitochondrial dynamics and the mitochondrial fission and fusion machineries.
3.2 Results

3.2.1 Mitochondrial dynamics in control and PAH PASMCs

Mitochondria were previously shown to be more fragmented in PASMCs isolated from PAH patients than in their control counterparts, as indicated by a considerable increase in the number of individual mitochondria normalised to the entire mitochondrial network in PAH PASMCs (Marsboom et al., 2012). To confirm this finding, the mitochondrial dye TMRM was employed together with confocal microscopy to image the mitochondrial network in both control and PAH PASMCs. Mitochondrial morphometry was then performed using a freely available ImageJ macro tool, developed and described by Valente et al. (2017), and aptly named Mitochondrial Network (MiNA) toolset. Measurements, generated by MiNA include, mitochondrial footprint, number of mitochondrial networks (mitochondrial structures with at least one node and three branches), number of mitochondrial individuals (punctate, rod, and large, round structures), branch length and number of branches per mitochondrial network. The numbers of mitochondrial networks and individual mitochondria were normalised to 100 \( \mu m^2 \) mitochondrial footprint. As shown in Figure 3.1, in comparison to control PASMCs, PAH PASMCs contained significantly higher numbers of both mitochondrial networks (4 ± 1 counts/100 \( \mu m^2 \) in control PASMCs; 6 ± 1 counts/100 \( \mu m^2 \) in PAH PASMCs; \( p = 0.0430; n = 3 \); Figure 3.1D) and individuals (31 ± 6 counts/100 \( \mu m^2 \) in control PASMCs; 54 ± 4 counts/100 \( \mu m^2 \) in PAH PASMCs; \( p = 0.0088; n = 3 \); Figure 3.1E), indicative of increased fission and fragmentation of the mitochondria. Moreover, the mitochondrial branches appeared to be longer (2.00 ± 0.11 \( \mu m \) in control PASMCs; 1.76 ± 0.01 \( \mu m \) in PAH PASMCs; \( p = 0.0418; n = 3 \); Figure 3.1B) and more numerous (18 ± 1 counts in control PASMCs; 5 ± 1 counts in PAH PASMCs; \( p = 0.0009; n = 3 \); Figure 3.1C) in control PASMCs than in their PAH counterparts.
Figure 3.1: Mitochondrial network is more fragmented in PAH PASMCs than in their control counterparts. Control (n = 3) and PAH (n = 3) PASMCs were seeded at low density in glass-bottom μ-dishes and maintained in 10% FBS for 48 hours. PASMCs were then quiesced in 0.1% FBS for 48 hours followed by incubation with the nuclear and mitochondrial dyes, Hoechst 33342 and TMRM, respectively, in HBSS for 30 minutes. (A) Stained, live PASMCs were then imaged using confocal laser scanning microscopy at ×60 magnification. Mitochondrial morphometry was performed using ImageJ together with the MiNA toolset. (B) Mitochondrial branch length, (C) number of branches per mitochondrial network, (D) number of mitochondrial networks and (E) number of individual mitochondria were determined in control and PAH PASMCs. Numbers of mitochondrial networks and individuals were both normalised to 100 μm² mitochondrial footprint. Values are means ± SEM. Unpaired student’s t-test was used in B, C, D and E. p < 0.5 was considered statistically significant.
3.2.2 Protein expression and activating phosphorylation of DRP1 in control and PAH PASMCs

DRP1 is the primary mediator of mitochondrial fission in mammalian cells. It has previously been demonstrated that the transcript and protein levels of DRP1 are upregulated in PASMCs derived from patients with PAH and this upregulation is thought to partly underlie the hyperproliferative phenotype of these cells (Marsboom et al., 2012; Ryan et al., 2013). To confirm the DRP1 upregulation that was previously reported in PAH, control and PAH PASMCs were starved in 0.1% FBS for 48 hours and proteins were isolated and immunoblotted for DRP1. As shown in Figure 3.2B, the relative protein level of DRP1 in PAH PASMCs was considerably higher in PAH PASMCs, compared to their control counterparts (p = 0.0386; n = 3 for control PASMCs; n = 6 for PAH PASMCs). DRP1 activity is predominantly regulated by post-translational phosphorylation of two serine residues, both of which reside within the C-terminal GED of the modular protein. Phosphorylation of serine 616 has been shown to activate DRP1 and, in PAH PASMCs, DRP1 has been reported to be hyper-phosphorylated at this stimulatory serine residue, suggesting increased activity in addition to increased protein expression (Marsboom et al., 2012). However, the increased level of pDRP1\textsuperscript{S616} in PAH PASMCs could be a consequence of increased protein expression of DRP1. Therefore, relative pDRP1\textsuperscript{S616} levels were quantified in control and PAH PASMCs using immunoblotting and densitometry and the ratios of pDRP1\textsuperscript{S616} to DRP1 were determined. Control and PAH PASMCs were also stained for pDRP1\textsuperscript{S616} using immunofluorescence. As shown in Figure 3.3, PAH PASMCs stained more strongly for pDRP1\textsuperscript{S616} than control PASMCs. Moreover, the ratio of pDRP1\textsuperscript{S616} to DRP1 in PAH PASMCs was twofold higher than that in control PASMCs (p = 0.0337; n = 3 for control PASMCs; n = 6 for PAH PASMCs; Figure 3.2D). This suggests that increased protein expression of DRP1 is accompanied by hyper-phosphorylation at S616 as observed in the fluorescently stained PAH PASMCs.
Figure 3.2: DRP1 protein expression and phosphorylation at the activating residue S616 in control and PAH PASMCs. Control (n = 3) and PAH (n = 6) PASMCs were cultured in 10% FBS to full confluence, serum-starved 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for pDRP1\textsuperscript{S616} and total DRP1. Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to quantify the protein levels of pDRP1\textsuperscript{S616} (C) and total DRP1 (B) relative to the loading control GAPDH in control and PAH PASMCs. (D) The ratios of pDRP1\textsuperscript{S616} to total DRP1 were also determined. Unpaired student’s t-test was used in B, C and D. p < 0.05 was considered statistically significant.
Figure 3.3: Immunofluorescent staining of pDRP$^{S616}$ in cultured control and PAH PASMCs. Control ($n = 3$) and PAH ($n = 3$) were seeded at low-density in 8-chamber slides and maintained in 10% FBS for 48 hours. PASMCs were then quiesced in 0.1% FBS for 48 hours. PASMCs were fixed and stained for pDRP$^{S616}$ (green) and F-actin (red) with phalloidin. Nuclei were stained with DAPI (blue). PASMCs were imaged at x60 magnification using laser scanning confocal microscopy.
After confirming DRP1 upregulation in cultured PAH PASMCs, immunohistochemical staining of lung sections from control subjects and PAH patients was performed to assess DRP1 protein expression within the medial layers of small pulmonary arteries (<500 μm in diameter), where the PASMCs reside, as well as elsewhere in the vessel wall. Stained sections were imaged using the Nanozoomer Digital Scanner. Pulmonary arteries were stratified into three groups according to vessel diameter. As shown in Figure 3.4A, small pulmonary arteries of various diameters stained, albeit weakly, for DRP1, when compared to those stained with normal IgG (Figure 3.4C). Moreover, DRP1 staining was evident in all three concentric layers of the artery; however, DRP1 staining appeared to be strongest in the medial layers of the arteries. In contrast to the control lung sections, the remodelled pulmonary arteries in the PAH lung sections stained more strongly for DRP1 (Figure 3.4B). The difference in DRP1 staining was most evident in pulmonary arteries with diameters ranging from 0 to 50 μm, the predominant sites of disease pathology. These results are consistent with those obtained by immunoblotting proteins isolated from control and PAH PASMCs for DRP1.
Figure 3.4: Immunohistochemical staining of DRP1 in lung sections from control and PAH patients. Formalin-fixed, paraffin-embedded lung sections from control (A; n = 3) and PAH (B; n = 3) patients were stained for DRP1 using immunohistochemistry. (C) Lung sections were also stained with normal rabbit IgG to confirm specificity of the DRP1 staining. Sections were counterstained with the nuclear stain Meyer’s haematoxylin. High resolution images were acquired using the Nanozoomer Digital Scanner. Pulmonary arteries were stratified into 3 groups according to vessel diameter.
3.2.3 Protein expression of DRP1 mitochondrial recruiters in control and PAH PASMCs

DRP1 recruitment to the OMM and subsequent homo-oligomeric assembly into ring structures around mitochondrial puncta are required for mitochondrial fission to occur (Loson et al., 2013). DRP1-interacting proteins, which reside within the OMM, accumulate at fission sites and mediate DRP1 recruitment (Loson et al., 2013). These DRP1 binding partners are essential components of the mitochondrial fission machinery and include FIS1, MFF, MiD49 and MiD51 (Loson et al., 2013). FIS1, MiD49 and MiD51 were previously found to be upregulated in PAH PASMCs, suggesting increased DRP1 recruitment to the mitochondria (Chen et al., 2018; Marsboom et al., 2012). However, the expression of MFF in PAH PASMCs has yet to be investigated. Proteins were extracted from control and PAH PASMCs, quiesced in 0.1% FBS for 48 hours, and immunoblotted for FIS1, MFF, MiD49 and MiD51 (Figure 3.5A). The relative protein expression of FIS1 was twofold higher in PAH PASMCs than in their control counterparts ($p = 0.0168$; $n = 3$ for control PASMCs; $n = 4$ for PAH PASMCs; Figure 3.5B). The protein expression of MFF, MiD49 and MiD51, however, in control PASMCs was comparable to that in PAH PASMCs.
Figure 3.5: Protein expression of the DRP1 binding partners in control and PAH PASMCs. Control (n = 3) and PAH (n = 4) PASMCs were grown in 10% FBS to full confluence, serum-starved in 0.1% for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for the DRP1 binding partners FIS1, MFF, MiD49 and MiD51. Densitometry was used to quantify the protein levels of FIS1 (B), MFF (C), MiD49 (D) and MiD51 (E) relative to the loading control GAPDH. Unpaired, student’s t-test was used in B, C, D and E. p < 0.05 was considered statistically significant.
3.2.4 Protein expression of the OMM fusion proteins MFN1 and MFN2 in control and PAH PASMCs

Tethering of adjacent mitochondria and subsequent fusion of the OMM is mediated by two transmembrane GTPases MFN1 and MFN2 (Archer, 2013). MFN1 and MFN2 also bridge between the mitochondria and the endoplasmic reticulum, facilitating Ca$^{2+}$ uptake into the mitochondria. In PAH PASMCs, the transcript level of MFN2 was reported to be downregulated whereas that of MFN1 remained unchanged (Marsboom et al., 2012). The protein levels of these OMM fusion proteins, however, have not been assessed. Therefore, as shown in Figure 3.6A, proteins, isolated from quiescent control and PAH PASMCs, were blotted for MFN1 and MFN2. Blots were reprobed for GAPDH to ensure equal loading. The relative levels of MFN1 and MFN2 in PAH PASMCs, as determined by densitometry, were comparable to those in control PASMCs, suggesting that the OMM fusion machinery remains unaltered in PAH. This is in divergence with the finding that MFN2 expression is reduced in PAH PASMCs but confirms the unaltered expression of MFN1 (Marsboom et al., 2012).
Figure 3.6: Protein expression of the OMM fusion proteins MFN1 and MFN2 in control and PAH PASMCs. Control (n = 3) and PAH (n = 6) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for the OMM fusion proteins MFN1 and MFN2. Blots were reprobed with GAPDH to ensure equal loading. Densitometry was used to quantify the protein levels of MFN1 (B) and MFN2 (C) relative to the loading control GAPDH. Unpaired student’s t-test was used in B and C. p < 0.05 was considered statistically significant.
3.2.5 Protein expression and proteolytic processing of the IMM fusion protein OPA1 in control and PAH PASMCs

At the IMM, fusion is mediated by the transmembrane, dynamin-like GTPase OPA1. In healthy cells (MacVicar and Langer, 2016), OPA1 undergoes proteolytic cleavage by the zinc metallopeptidase OMA1 to yield a long isoform (L-OPA1), which remains bound to the IMM, and a soluble, short isoform (S-OPA1; MacVicar and Langer, 2016)). L-OPA1 possesses fusion activity, whereas S-OPA1 has minimal fusogenic activity and is thought to maintain normal architecture of the IMM folds or cristae, within which the ETC resides (MacVicar and Langer, 2016). Mitochondrial dysfunction enhances the activity of OMA1, increasing the proteolytic cleavage of OPA1. In PAH PASMCs, increased proteolytic processing of OPA1 may underlie excessive mitochondrial fragmentation. To investigate this notion, proteins, isolated from quiescent control and PAH PASMCs, were immunoblotted for OPA1 and GAPDH. As shown in Figure 3.7A, upon immunoblotting, OPA1 appeared as two distinct bands, representing the long and short isoforms. The relative protein level of total OPA1 in PAH PASMCs was similar to that in control PASMCs. The protein level of S-OPA1 relative to GAPDH was 2-fold higher (p = 0.0234; n = 3 for control PASMCs; n = 6 for PAH PASMCs; Figure 3.7D) in PAH PASMCs than in their control counterparts whereas the level of L-OPA1 remained unchanged. The ratio of S-OPA1 to total OPA1 was also markedly elevated in PAH PASMCs (p = 0.0050; n = 3 for control PASMCs; n = 6 for PAH PASMCs; Figure 3.7F). Despite being statistically non-significant, the ratio of L-OPA1 to total OPA1 was slightly lower in PAH PASMCs. This suggests increased proteolytic cleavage of the fusogenic isoform of OPA1 and reduced IMM fusion in PAH PASMCs.
Figure 3.7: Protein expression and proteolytic processing of the IMM fusion protein OPA1 in control and PAH PASMCs. Control (n = 3) and PAH (n = 6) PASMCs were cultured in 10% FBS to full confluence, starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for OPA1. Immunoblotting revealed the long and short isoforms of OPA1, denoted L-OPA1 and S-OPA1, respectively. Densitometry was used to quantify the protein levels of total OPA1 (B), L-OPA1 (C) and S-OPA1 (D) relative to the loading control GAPDH in control and PAH PASMCs. Ratios of L-OPA1 (E) and S-OPA1 (F) to total OPA1 were also determined. Values are means ± SEM. Unpaired student’s t-test was used in B, C, D, E and F. p < 0.05 was considered statistically significant.
3.2.6 Treprostinil, MRE-269 and butaprost induce mitochondrial fusion and consequent elongation in PAH PASMCs

The prostacyclin mimetics treprostinil and MRE-269 and the EP2 receptor agonist butaprost have been demonstrated to inhibit PASMC proliferation and may therefore attenuate the mitotic mitochondrial fragmentation that was observed in PAH PASMCs (Falcetti et al., 2010; Patel et al., 2018). Treprostinil and butaprost have been shown to inhibit proliferation of PAH PASMCs more strongly than MRE-269 (Patel et al., 2018). Consequently, treprostinil and butaprost were postulated to attenuate mitochondrial fragmentation to a greater extent than MRE-296. PAH PASMCs were seeded at low density in glass-bottom μ-dishes, serum-starved in 0.1% FBS for 48 hours and treated with DMSO, 100 nM treprostinil, 100 nM butaprost or 100 nM MRE-269 in 0.1% FBS for 3 hours. Treated PAH PASMCs were then stained with the mitochondrial dye TMRM and the nuclear dye Hoechst 33342 in HBSS for 30 minutes and imaged using a confocal laser scanning microscope. Mitochondrial morphometry was performed with the ImageJ macro MiNA. Treatment of PAH PASMCs with treprostinil increased the mean length of mitochondrial branches by 0.28 ± 0.07 μm (p = 0.0091; n = 4; Figure 3.8B) and the mean number of branches of each mitochondrial network by 7 ± 1 counts (p = 0.0031; n = 4; Figure 3.8C). Treprostinil also reduced the mean number of mitochondrial networks and individuals for every 100 μm² mitochondrial footprint by 2 ± 1 counts/100 μm² (p = 0.0240; n = 4; Figure 3.8D) and 26 ± 7 counts/100 μm² (p = 0.0166, n = 4; Figure 3.8E), respectively. Taken together, these results show that treprostinil promotes the formation of fewer, highly branched mitochondrial networks in PAH PASMCs, suggesting increased mitochondrial fusion and elongation and attenuation of mitotic mitochondrial fragmentation.

In a similar fashion to treprostinil, treatment of PAH PASMCs with the non-prostanoid IP receptor agonist MRE-269 led to an increase of 8 ± 2 counts (p = 0.0050; n = 4) in the mean number of branches of each mitochondrial network (Figure 3.9C). The EP2 receptor agonist butaprost also increased the mean number of branches by 6 counts ± 2 (p = 0.0166; n = 4). However, unlike treprostinil, both MRE-269 and butaprost failed to increase the mean mitochondrial branch length (Figure 3.9B). MRE-269 reduced the number of mitochondrial networks and individuals for every 100 μm² mitochondrial footprint by 2 ± 1 (p = 0.0197; n = 4; Figure 3.9D) and 26 ± 7 (p = 0.0071; n = 4; Figure 3.9E), respectively. Moreover, butaprost reduced the number of mitochondrial networks and individuals for every 100 μm² mitochondrial footprint by 2 ± 1 (p = 0.0313; n = 4) and 26 ± 7 (p = 0.0078; n = 4), respectively.
Figure 3.8: Treprostinil attenuates mitochondrial fission and fragmentation in PAH PASMCs. PAH (n = 4) PASMCs were seeded at low density in glass-bottom μ-dishes and maintained in 10% FBS for 48 hours. PASMCs were then quiesced in 0.1% FBS for 48 hours and treated with either DMSO or 100 nM treprostinil in 0.1% FBS for 3 hours. (A) Treated PAH PASMCs were incubated with the nuclear and mitochondrial dyes, Hoechst 33342 (blue) and TMRM (red), respectively, in HBSS for 30 minutes and imaged using confocal laser scanning microscopy. Mitochondrial morphometry was performed using ImageJ together with the MiNA toolset. (B) Mitochondrial branch length, (C) number of branches per mitochondrial network, (D) number of mitochondrial networks and (E) number of individual mitochondria were determined. Numbers of mitochondrial networks and individuals were both normalised to 100 μm² mitochondrial footprint. Values are means ± SEM. Unpaired student’s t-test was used in B, C, D and E. p < 0.5 was considered statistically significant.
Figure 3.9: MRE-269 and butaprost attenuate mitochondrial fission and fragmentation in PAH PASMCs. PAH (n = 4) PASMCs were seeded at low density in glass-bottom μ-dishes and maintained in 10% FBS for 48 hours. PASMCs were then quiesced in 0.1% FBS for 48 hours and then treated with either DMSO, 100 nM MRE-269 or 100 nM butaprost in 0.1% FBS for 3 hours. (A) Treated PAH PASMCs were incubated with the nuclear and mitochondrial dyes, Hoechst 33342 and TMRM, respectively, in HBSS for 30 minutes and imaged using confocal laser scanning microscopy. Mitochondrial morphometry was performed using ImageJ together with the MiNA toolset. (B) Mitochondrial branch length, (C) number of branches per mitochondrial network, (D) number of mitochondrial networks and (E) number of individual mitochondria were determined. Numbers of mitochondrial networks and individuals were both normalised to 100 μm² mitochondrial footprint. One-way ANOVA with Bonferroni’s test for multiple comparisons was used in B, C, D and E. p < 0.5 was considered statistically significant.
3.2.7 Treprostinil does not influence DRP1 protein expression, but induces phosphorylation at S637 and inhibits phosphorylation at S616 in PAH PASMCs

Given that treprostinil promotes mitochondrial fusion and consequent elongation in PAH PASMCs, it was worth investigating whether treprostinil reduces the expression of DRP1, the primary effector of mitochondrial fission, and/or inhibits its fission activity by influencing its phosphorylation at two regulatory serine residues in PAH PASMCs. Quiescent PAH PASMCs were treated with either DMSO or 100 nM treprostinil in 0.1% FBS for 0, 0.5, 1, 3, 6 and 24 hours, as indicated in the figures below. Proteins were extracted and immunoblotted for pDRP1<sup>S637</sup>, pDRP1<sup>S616</sup> and total DRP1. Treprostinil had no significant effect on the protein expression of DRP1 at the different time points (Figure 3.10). Treprostinil did, however, induce inhibitory DRP1 phosphorylation at S673 in a time-dependent manner. As shown in Figure 3.11, phosphorylation of DRP1 at S637 increased 30 minutes after treatment with 100 nM treprostinil, with maximal phosphorylation occurring at 3 hours after treatment. Moreover, treprostinil-induced DRP1 phosphorylation at S637 lasted for at least 6 hours but subsided 24 hours after treatment. Treprostinil also inhibited stimulatory DRP1 phosphorylation at S616. As illustrated in Figure 3.12, 100 nM treprostinil considerably reduced the level of pDRP1<sup>S616</sup> 1 hour after treatment and this inhibition persisted for 24 hours after treatment. These results suggest that treprostinil attenuates mitochondrial fission in PAH PASMCs by influencing the post-translational phosphorylation of DRP1 rather than its protein expression.
Figure 3.10: Treprostinil has no effect on the protein expression of DRP1 in PAH PASMCs. PAH PASMCs (n = 3) were grown in 10% FBS to full confluence in 6-well plates and starved for 48 hours in 0.1% FBS. Quiesced PAH PASMCs were then treated with either DMSO or 100 nM treprostinil for 0, 0.5, 1, 3, 6 and 24 hours. (A) Proteins were isolated and immunoblotted for DRP1. Blots were reprobed for GAPDH to ensure equal loading. (B) Densitometry was used to determine the levels of DRP1 relative to GAPDH. One-way ANOVA with Bonferroni’s test for multiple comparisons was used in B. Values are means ± SEM. p < 0.05 was considered statistically significant.
Figure 3.11: Treprostinil stimulates DRP1 phosphorylation at the inhibitory residue S637 in a time-dependent manner in PAH PASMCs. PAH PASMCs \((n = 3)\) were grown in 10% FBS to full confluence in 6-well plates and starved for 48 hours in 0.1% FBS. Quiescent PAH PASMCs were then treated with either DMSO or 100 nM treprostinil for 0, 0.5, 1, 3, 6 and 24 hours as indicated. (A) Proteins were isolated and immunoblotted for pDRP1\(^{S637}\) and total DRP1. (B) Densitometry was used to determine the levels of pDRP1\(^{S637}\) relative to total DRP1 in control and PAH PASMCs. Values are means ± SEM. Two-way ANOVA with Sidak’s test for multiple comparisons was used in B. \(p < 0.05\) was considered statistically significant.
Figure 3.12: Treprostinil inhibits DRP1 phosphorylation at the activating residue S616 in a time-dependent manner in PAH PASMCs. PAH PASMCs ($n = 3$) were grown in 10% FBS to full confluence in 6-well plates and starved for 48 hours in 0.1% FBS. Quiescent PAH PASMCs were then treated with either DMSO or 100 nM treprostinil for 0, 0.5, 1, 3, 6 and 24 hours. (A) Proteins were isolated and immunoblotted for $\text{pDRP1}^{\text{S616}}$ and total DRP1. (B) Densitometry was used to determine the levels of $\text{pDRP1}^{\text{S616}}$ relative to total DRP1 in control and PAH PASMCs. Values are means ± SEM. Two-way ANOVA with Sidak's test for multiple comparisons was used in B. $p < 0.05$ was considered statistically significant.
3.2.8 Treprostinil reduces the protein levels of the DRP1 binding proteins FIS1 and MiD49, but not those of MFF and MiD51, in PAH PASMCs

Treprostinil might be promoting mitochondrial elongation in PAH PASMCs by inhibiting the increased recruitment of DRP1 to the mitochondria. The effect of treprostinil on the protein expression of the classical DRP1 mitochondrial recruiters FIS1 and MFF and the more recently identified DRP1 adapter proteins MiD49 and MiD51 was therefore assessed. PAH PASMCs, quiesced in 0.1% FBS for 48 hours, were treated with 100 nM treprostinil for 0, 0.5, 1, 3, 6 and 24 hours and proteins were isolated and immunoblotted for the aforementioned DRP1 recruiters. The protein levels of MiD51 and MFF relative to GAPDH in PAH PASMCs did not change over the course of treatment with 100 nM treprostinil. However, the relative protein levels of MiD49 and FIS1 in PAH PASMCs did fall 6 (p = 0.0383; n = 3; Figure 3.13B) and 24 hours (p = 0.0062; n = 4; Figure 3.14B), respectively, after treatment with 100 nM treprostinil.
Figure 3.13: Treprostinil reduces the protein expression of the novel DRP1 adapter protein MiD49, but not its close relative MiD51, in PAH PASMCs. PAH PASMCs (n = 3-4) were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. Quiescent PAH PASMCs were treated with either DMSO or 100 nM treprostinil in 0.1% FBS for 0, 0.5, 1, 3, 6 and 24 hours. (A) Proteins were isolated and immunoblotted for MiD49, MiD51 and GAPDH. (B) Densitometry was used to determine the levels of MiD49 and MiD51 relative to GAPDH. (C) Proteins were also immunoblotted for MFF and β-tubulin and (D) levels of MFF relative to β-tubulin were determined by densitometry. Values are means ± SEM. Two-way ANOVA with Sidak’s test for multiple comparisons were used in B and D. p < 0.05 was considered statistically significant.
Figure 3.14: Treprostinil inhibits the protein expression of the classical DRP1 mitochondrial recruiter FIS1, but not MFF, in PAH PASMCs. PAH PASMCs (n = 3-4) were cultured in 10% FBS to full confluence and starved in 0.1% FBS for 48 hours. Quiescent PAH PASMCs were treated with either DMSO or 100 nM treprostinil for 0, 0.5, 1, 3, 6 and 24 hours. (A) Proteins were isolated and immunoblotted for FIS1 and GAPDH. (B) Densitometry was used to determine the levels of FIS1 relative to GAPDH. (C) Proteins were also immunoblotted for MFF and β-tubulin and (D) levels of MFF relative to β-tubulin were determined by densitometry. Values are means ± SEM. Two-way ANOVA with Sidak’s test for multiple comparisons were used in B and D. $p < 0.05$ was considered statistically significant.
3.2.9 Treprostinil signals via either the IP or EP₂ receptor to activate PKA and induce DRP1 phosphorylation at S637 in PAH PASMCs

Treprostinil has been shown to bind with high affinity and potently activate the IP, EP₂ and DP₁ prostanoid receptors heterologously expressed in HEK293 cells (Whittle et al., 2012). In PAH PASMCs, however, using siRNA and selective receptor antagonists, treprostinil has been demonstrated to inhibit proliferation predominantly via the EP₂ receptor and weakly via the IP receptor, with no involvement of the DP₁ receptor (Falcetti et al., 2010; Patel et al., 2018). Moreover, EP₂ receptors were proposed to constitute the dominant pharmacological target of treprostinil in PAH PASMCs because of reduced IP receptor expression and enhanced EP₂ receptor expression (Falcetti et al., 2010; Patel et al., 2018). Given these findings, it was postulated that treprostinil stimulates DRP1 phosphorylation at S637 largely via the EP₂ receptor and to a lesser extent via the IP receptor. To investigate this hypothesis, PAH PASMCs, starved in 0.1% FBS for 48 hours, were first pre-treated with either DMSO, 1 μM RO1138452 (IP receptor-selective antagonist), 1 μM PF04418948 (EP₂ receptor-selective antagonist) or a combination of both for 30 minutes to allow the antagonists to equilibrate with their respective receptors. Subsequently, pre-treated PAH PASMCs were incubated with either DMSO or 100 nM treprostinil in the absence or presence of the antagonists for 3 hours. As shown in Figure 3.15, stimulation of PAH PASMCs with 100 nM treprostinil resulted in a significant increase in the levels of pDRP1\textsuperscript{S637}. Surprisingly, when given individually, 1 μM RO1138452 and 1 μM PF04418948 had no significant effects on treprosinil-induced DRP1 phosphorylation at S637, whereas, in combination, the antagonists completely blocked DRP1 phosphorylation. This finding suggests that, in PAH PASMCs, treprostinil recruits either the IP or EP₂ receptor to induce inhibitory DRP1 phosphorylation at S637.

The IP and EP₂ receptors both couple via Gs to activate membrane-bound adenylate cyclase, elevating intracellular cAMP levels. Moreover, DRP1 phosphorylation at S637 is mediated by PKA, which is cAMP-dependent (Cribbs and Strack, 2007). Treprostinil has been shown to elevate intracellular cAMP in PAH PASMCs via the IP and EP₂ receptors, consequently activating cAMP-dependent PKA (Patel et al., 2018). To determine whether treprostinil-induced DRP1 phosphorylation at S637 is PKA-dependent, quiescent PAH PASMCs were pre-treated with either DMSO or 10 μM H89, a PKA inhibitor, for 30 minutes followed by incubation with either DMSO or 100 nM treprostinil without or with 10 μM H89 for 3 hours. H89 blocked treprostinil-induced DRP1 phosphorylation at S637, suggesting complete dependence on PKA.
Figure 3.15: Treprostinil recruits either the IP or EP\textsubscript{2} receptor to stimulate PKA-dependent DRP1 phosphorylation at the inhibitory residue S637 in PAH PASMCs. PAH PASMCs \((n = 4-6)\) were cultured in 10% FBS to full confluence and starved in 0.1% FBS for 48 hours. Quiescent PAH PASMCs were then pre-treated with either DMSO, 1 μM RO11138452, 1 μM PF04418948, a combination of both antagonists or 10 μM H89 for 30 minutes. After pre-treatment, PAH PASMCs were stimulated with 100 nM treprostinil in the presence of DMSO, 1 μM RO11138452, 1 μM PF04418948, a combination of both antagonists or 10 μM H89 for 3 hours. (A) Proteins were isolated and immunoblotted for pDRP1\textsuperscript{S637} and total DRP1. (B) Densitometry was used to determine the levels of pDRP1\textsuperscript{S637} relative to total DRP1. (C) Values are means ± SEM. One-way ANOVA with Bonferroni’s test for multiple comparisons was used in B. \(p < 0.05\) was considered statistically significant.
3.2.10 Treprostinil signals via either the IP or EP2 receptor to activate PKA and inhibit DRP1 phosphorylation at S616 in PAH PASMCs

After identifying the prostanoid receptors and the serine-threonine kinase that mediate treprostinil-induced DRP1 phosphorylation at S637, the receptors and downstream effectors, involved in the treprostinil-induced inhibition of DRP1 phosphorylation at S616, were dissected. PAH PASMCs, starved in 0.1% FBS for 48 hours, were first pre-treated with either DMSO, 1 μM RO1138452 (IP receptor-selective antagonist), 1 μM PF04418948 (EP2 receptor-selective antagonist), a combination of both or 10 μM H89 (PKA inhibitor) for 30 minutes to allow the antagonists to equilibrate with their cognate receptors. Subsequently, pre-treated PAH PASMCs were incubated with either DMSO or 100 nM treprostinil in the absence or presence of the antagonists for 3 hours. As shown in Figure 3.16, stimulation of PAH PASMCs with 100 nM treprostinil resulted in a significant decrease in the levels of pDRP1\textsuperscript{S616} relative to total DRP1. When given individually, 1 μM RO1138452 and 1 μM PF04418948 had no significant effect on treprostinil-induced inhibition of DRP1 phosphorylation at S616, whereas, in combination, the antagonists completely blocked this inhibition. Moreover, pre-treatment with the PKA inhibitor H89 blocked the inhibitory effect of treprostinil on DRP1 phosphorylation at S616. Collectively, these findings suggest that, in PAH PASMCs, treprostinil recruits either the IP or EP\textsubscript{2} receptor to activate PKA and inhibit stimulatory DRP1 phosphorylation at S616.
Figure 3.16: Treprostinil recruits either the IP or EP\textsubscript{2} receptor to inhibit DRP1 phosphorylation at the activating residue S616 in PAH PASMCs. PAH PASMCs ($n$ = 4-6) were grown in 10% FBS to full confluence and serum-starved for 48 hours in 0.1% FBS. PAH PASMCs were then pre-treated with either DMSO, 1 μM RO1138452, 1 μM PF04418948, both or 10 μM H89 for 30 minutes. After pre-treatment, PAH PASMCs were stimulated with 100 nM treprostinil in the presence of DMSO, 1 μM RO1138452, 1 μM PF04418948, both or 10 μM H89 for 3 hours. (A) Proteins were isolated and immunoblotted for pDRP1\textsuperscript{S616} and total DRP1. (B) Densitometry was used to determine the levels of pDRP1\textsuperscript{S616} relative to total DRP1. Values are means ± SEM. One-way ANOVA with Bonferroni’s test for multiple comparisons was used in B. p < 0.05 was considered statistically significant.
3.2.11 MRE-269 and butaprost induce DRP1 phosphorylation at S637 via the IP and EP$_2$ receptors, respectively

MRE-269, the active metabolite of the prostacyclin mimetic and PAH therapeutic agent selexipag, has been shown to elevate intracellular cAMP levels and inhibit PASMC proliferation exclusively via the IP receptor (Patel et al., 2018). Consequently, MRE-269 was postulated to recruit the IP receptor to activate PKA and induce downstream DRP1 phosphorylation at the inhibitory residue S637 in PAH PASMCs. To address this hypothesis, PAH PASMCs were quiesced in 0.1% FBS for 48 hours and pretreated with either DMSO or 1 μM RO1138452, an IP receptor-selective antagonist, for 30 minutes to allow the antagonist to equilibrate with its cognate receptors. Pre-treated PASMCs were then incubated with DMSO or 100 nM MRE-269 in the absence or presence of 1 μM RO1138452. Proteins were isolated and immunoblotted for pDRP$_{1}^{S637}$ and total DRP1. Low levels of pDRP$_{1}^{S637}$ were observed in DMSO-treated PASMCs, whereas 100 nM MRE-269 significantly increased the levels of pDRP$_{1}^{S637}$. As illustrated in Figure 3.17, pre-treatment with 1 μM RO1138452 completely blocked the phosphorylation induced by 100 nM MRE-269, indicating that MRE-269-induced DRP1 phosphorylation at S637 is mediated solely by the IP receptor in PAH PASMCs.
**Figure 3.17: MRE-269 stimulates DRP1 phosphorylation at the inhibitory residue S637 via the IP receptor in PAH PASMCs.** PAH PASMCs (n = 4) were cultured to full confluence in 10% FBS and then serum-starved in 0.1% FBS for 48 hours. Quiescent PAH PASMCs were pre-treated with either DMSO or 1 μM RO1138452, an IP receptor-selective antagonist, for 30 minutes followed by incubation with either DMSO or 100 nM MRE-269 in the absence or presence of 1 μM RO1138452 for 3 hours. (A) Proteins were extracted and immunoblotted for pDRP1\(^{S637}\) and total DRP1. (B) Densitometry was used to quantify the levels of pDRP1\(^{S637}\) relative to total DRP1. Values are means ± SEM. One-way ANOVA with Bonferroni’s test for multiple comparisons was used in B. p < 0.05 was considered statistically significant.

In PAH PASMCs, EP\(_2\) receptor agonism with either treprostinil or the EP\(_2\) receptor agonist butaprost also leads to intracellular cAMP generation and consequent PKA activation (Patel et al., 2018). Therefore, butaprost was also investigated for its ability to induce inhibitory DRP1 phosphorylation at S637 via the EP\(_2\) receptor. Quiescent PAH PASMCs were pre-treated with either DMSO or 1 μM PF04418948 for 30 minutes to allow antagonist equilibration with the target receptors. Following pre-treatment, PAH PASMCs were stimulated with 100 nM butaprost without or with 1 μM PF04418948 for 3 hours. As shown in Figure 3.18, 100 nM butaprost significantly increased the level of pDRP\(^{S637}\), compared to the vehicle DMSO. Pre-treatment with 1 μM PF04418948 completely blocked the butaprost-induced DRP1 phosphorylation. These results suggest that butaprost induces inhibitory DRP1 phosphorylation at S637 exclusively via the EP\(_2\) receptor.
Figure 3.18: Butaprost induces DRP1 phosphorylation at the inhibitory residue S637 via the EP$_2$ receptor in PAH PASMCs. PAH PASMCs ($n = 3$) were cultured to full confluence in 10% FBS and then quiesced in 0.1% FBS for 48 hours. Quiescent PAH PASMCs were pre-treated with either DMSO or 1 μM PF04418948, an EP$_2$ receptor-selective antagonist, in 0.1% FBS for 30 minutes followed by incubation with either DMSO or butaprost in the absence or presence of 1 μM PF04418948 for 3 hours. (A) Proteins were extracted and immunoblotted for pDRP1$^{S637}$ and total DRP1. (B) Densitometry was used to quantify the levels of pDRP1$^{S637}$ relative to total DRP1. Values are means ± SEM. One-way ANOVA with Bonferroni’s test for multiple comparisons was used in B. $p < 0.05$ was considered statistically significant.
3.2.12 Treprostinil upregulates the OMM fusion protein MFN1 but not MFN2 in PAH PASMCs

Treprostinil was proposed to promote mitochondrial fusion and elongation in PAH PASMCs by upregulating the protein expression of the OMM fusion proteins MFN1 and/or MFN2. Quiescent PAH PASMCs were treated with 100 nM treprostinil for 0, 0.5, 1, 3, 6 and 24 hours and lysed for protein. Proteins were then immunoblotted for the OMM fusion proteins MFN1 and MFN2. Densitometry was performed to determine the levels of MFN1 and MFN2 relative to GAPDH. As demonstrated in Figure 3.19, the relative protein levels of MFN1 were increased 30 minutes after treatment with 100 nM treprostinil. This increase in MFN1 protein expression persisted for 24 hours following treatment with treprostinil. However, treprostinil failed to upregulate the protein expression of MFN2.
Figure 3.19: Treprostinil upregulates the OMM fusion protein MFN1, but not MFN2, in PAH PASMCs. PAH PASMCs (n = 3) were cultured in 10% FBS to full confluence and quiesced in 0.1% FBS for 48 hours. Quiescent PAH PASMCs were then treated with either DMSO or treprostinil in 0.1% FBS for 0, 0.5, 1, 3, 6 and 24 hours and lysed for protein. Proteins were immunoblotted for MFN1 (A) and MFN2 (C). Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to quantify the levels of MFN1 (B) and MFN2 (D) relative to the loading control GAPDH. Values are means ± SEM. Two-way ANOVA with Sidak’s test for multiple comparisons were used in B and D. p < 0.05 was considered statistically significant.
3.2.13 Treprostinil has no effect on the protein expression or proteolytic processing of the IMM fusion protein OPA1 in PAH PASMCs

The inhibitory effect of treprostinil on mitochondrial fission together with the increased proteolytic cleavage of OPA1 and the consequent increase in the expression of S-OPA1 in PAH PASMCs suggest that treprostinil could be promoting mitochondrial elongation by reducing OPA1 proteolytic processing. Proteins from quiescent PAH PASMCs treated with either DMSO or 100 nM treprostinil for 0, 0.5, 1, 3, 6 and 24 hours were immunoblotted for OPA1. Protein levels of total OPA1, L-OPA1 and S-OPA1 relative to GAPDH were determined using densitometry. As shown in Figure 3.20, despite not reaching statistical significance, treprostinil reduced the relative protein expression of S-OPA1 24 hours after treatment without affecting the expression of the long isoform. This suggests that treprostinil may be inhibiting the proteolytic cleavage of OPA1 in PAH PASMCs to promote mitochondrial fusion and elongation.
Figure 3.20: Treprostinil has no effect on the protein expression and proteolytic processing of the IMM fusion protein OPA1 in PAH PASMCs. PAH PASMCs ($n = 3$) were cultured in 10% FBS to full confluence and quiesced in 0.1% FBS for 48 hours. Quiescent PAH PASMCs were then treated with either DMSO or 100 nM treprostinil in 0.1% FBS for 0, 0.5, 1, 3, 6 and 24 hours and lysed for protein. (A) Proteins were immunoblotted for OPA1 and OPA1 appeared as two distinct bands, denoted L-OPA1 and S-OPA1. Densitometry was used to quantify the relative levels of OPA1 (B), L-OPA1 (C) and S-OPA1 (D) relative to the loading control GAPDH. Values are means ± SEM. Two-way ANOVA with Sidak’s test for multiple comparisons were used in B and D. $p < 0.05$ was considered statistically significant.
3.3 Discussion

Mitochondria are remarkably dynamic organelles and the term mitochondrial dynamics refers to their ability to undergo tightly regulated cycles of the opposing processes of fission and fusion (Archer, 2013). Consequently, mitochondria can have a wide range of morphologies, appearing as small spheres, short or long rods, or interconnected tubules. The rapid and transient morphological changes in mitochondrial networks are crucial for many cellular processes, including proliferation, apoptosis and mitophagy (Chan, 2019). For example, mitochondrial fission has been shown to precede the M phase of the cell cycle and its abrogation arrests the cell cycle at the G2/M checkpoint (Mishra and Chan, 2014). Mitochondrial fission serves to fragment the mitochondria into structures small enough to be propagated to and inherited by daughter cells (Mishra and Chan, 2014). Marsboom et al. (2012) was the first to demonstrate that underlying the hyper-proliferative phenotype of PAH PASMCs are highly fragmented mitochondria, generated as a result of a tip in the balance between the processes of mitochondrial fission and fusion in favour of fission. Consistently, in this chapter, staining of live control and PAH PASMCs with the mitochondrial dye TMRM revealed morphological changes in the mitochondria akin to those observed by Marsboom et al. (2012). PAH PASMCs were shown to contain higher numbers of individual mitochondria, which encompass punctate, rod, and large, round mitochondrial structures, and mitochondrial networks or mitochondrial structures with at least one central node and three branches than control PASMCs. Moreover, mitochondrial branches appeared to be longer and more numerous in control PASMCs than in their PAH equivalents. Collectively, these results suggest that mitochondrial fission and fragmentation are increased in PAH PASMCs. This increased mitochondrial fission in PAH PASMCs is consistent with that previously reported by Marsboom et al., (2012).

Mitochondrial fission is the process by which a mitochondrion divides into two smaller mitochondria. The primary mediator of mitochondrial fission is DRP1, a large monomeric GTPase (Chan, 2019). DRP1 monomers are recruited from the cytosol to potential fission sites on the mitochondrial surface, where they assemble into ring or spiral structures that wrap around and constrict mitochondrial tubules (Archer, 2013). Because of the increased mitochondrial fission observed in PAH PASMCs and the central role of DRP1 in mediating mitochondrial fission, it was posited that DRP1 expression and activity might also be elevated in PAH PASMCs. Indeed, protein expression of DRP1 was found to be considerably higher in cultured PAH PASMCs than in their control counterparts. Moreover, stronger DRP1 expression was observed in the medial layers of distal pulmonary arteries in PAH lung tissue sections than in those derived from control...
subjects. This is in agreement with the increased DRP1 expression in PAH PASMCs reported by Marsboom et al. (2012). DRP1 activity is increased by phosphorylation at S616, which is mediated by a variety of serine/threonine kinases. For example, during the cell cycle, DRP1 is phosphorylated at S616 by CDK1 complexed with its activator cyclin B to induce mitochondrial fission and permit G2-to-M transition (Taguchi et al., 2017). In addition to increased DRP1 protein expression in PAH PASMCs, DRP1 was found to be hyper-phosphorylated at serine 616, suggesting that increased DRP1 levels is accompanied by increased activity (Marsboom et al., 2012). Marsboom et al. (2012) reported a similar finding and implicated the cell cycle regulator CDK1 and its activator cyclin B as the key drivers of DRP1 hyper-phosphorylation in PAH PASMCs. Marsboom et al. (2012) showed that inhibiting CDK1 with RO-3306 markedly reduced DRP1 phosphorylation at serine 616. However, inhibiting other serine/threonine kinases known to phosphorylate DRP1 at S616, such as CaMKII, had no effect on DRP1 phosphorylation in PAH PASMCs (Marsboom et al., 2012). Recently, the pro-proliferative kinase ERK2 has also been shown to phosphorylate DRP1 at S616 and may therefore contribute to the hyper-phosphorylation observed in PAH PASMCs (Kashatus et al., 2015).

DRP1 lacks a pleckstrin homology domain and is therefore unable to interact with phospholipids, namely phosphatidylinositols, within the OMM. Instead, DRP1 adaptor proteins or receptors, anchored within the OMM, recruit DRP1 from the cytosolic pool of proteins to potential fission sites on the mitochondrial surface (Kashatus, 2016). Among these proteins are the classical DRP1-binding proteins FIS and MFF and the more recently identified DRP1-binding partners MiD49 and MiD51 (Loson et al., 2013). Marsboom et al. (2012) demonstrated increased expression of FIS1 in PAH PASMCs, suggesting that increased DRP1 recruitment to the mitochondria may also account for the increased mitochondrial fission. FIS1 was also found to be upregulated in our PAH PASMC isolates. However, given the minor role of FIS1 in mitochondrial fission, as indicated by slightly altered or unaltered mitochondrial fission in cells lacking FIS1, it is unlikely that increased FIS1-mediated DRP1 recruitment accounts for the excessive mitochondrial fragmentation observed in PAH PASMCs in this chapter (Otera et al., 2010). MFF, MiD49 and MiD51 have more substantial roles in mediating DRP1 recruitment to the mitochondria. Although MiD49 and MiD51 have both been previously shown to be upregulated in PAH PASMCs and to account for the increased mitochondrial fission, the expression of MFF in PAH PASMCs has not been explored. In this chapter, MFF proteins levels in PAH PASMCs were found to be similar to those in control PASMCs, suggesting that MFF is unlikely to contribute to the increased mitochondrial fission. The DRP1 recruitment activity of MFF is enhanced by post-translational
phosphorylation at serine 146. Increased phosphorylation of MFF and subsequent DRP1 recruitment may therefore alternatively underlie the increased mitochondrial fission in PAH PASMCs. This chapter also examined the protein levels of the novel DRP1 recruiters MiD49 and MiD51 in PAH PASMCs in an attempt to replicate the findings of Chen et al. (2018). However, no difference in the expression of either MiD49 or MiD51 between control and PAH PASMCs was observed in this chapter. Conflicting evidence exists in the literature concerning the roles of MiD49 and MiD51 in recruiting DRP1 to the mitochondria and mediating fission. Double knockdown of MiD49 and MiD51 inhibited mitochondrial DRP1 recruitment and resulted in mitochondrial elongation, supporting a role for these two proteins in mitochondrial fission. Paradoxically, overexpressing either MiD49 or MiD51 also surprisingly resulted in mitochondrial elongation (Palmer et al., 2013). Indeed, MiD49 and MiD51 have been shown to localise to the cytosol, where they are thought to sequester DRP1 and maintain it in an inactive conformation, preventing it from being recruited to the mitochondria. However, overexpressing MiD49 or MiD51 in normal PASMCs induced mitochondrial fission consistent with the roles of MiD49 and MiD51 in recruiting DRP1 to the mitochondria (Chen et al., 2018).

Conversely to mitochondrial fission, mitochondrial fusion involves the merging of two mitochondria into one mitochondrion (Scott and Youle, 2010). It requires the end-to-end collision of mitochondria and the fusion of the OMM followed by that of the IMM culminating in content mixing to distribute matrix components throughout the newly formed mitochondrion (Scott and Youle, 2010). Fusion of the OMM is mediated by the two GTPases MFN1 and MFN2, whereas that of the IMM is mediated by the GTPase OPA1 (Archer, 2013; Scott and Youle, 2010). MFN1 and MFN2 form homotypic or heterotypic interactions to tether adjacent mitochondria and promote the fusion of their OMM (Scott and Youle, 2010). Given the increased mitochondrial fission in PAH PASMCs, it was postulated that the fusion machinery, comprised of these three GTPases, might be downregulated. Marsboom et al. (2012) demonstrated a reduction in the mRNA levels of MFN2 but not MFN1 in PAH PASMCs compared to control PASMCs. Contrastingly, this chapter demonstrated that the protein levels of MFN1 and MFN2, albeit slightly increased in PAH PASMCs, are not significantly different from those in control PASMCs, suggesting that the OMM fusion machinery remains intact in PAH PASMCs. Following IMM fusion, OPA1 mediates the fusion of the IMM, allowing continuity between the matrices of the two merging mitochondria. OPA1 undergoes proteolytic cleavage to generate long and short isoforms, denoted S-OPA1 and L-OPA1, respectively (Scott and Youle, 2010; MacVicar and Langer, 2016). L-OPA1 remains anchored to the IMM and possesses fusogenic activity, whereas soluble S-OPA1 shows
little or no fusion activity and is instead thought to maintain the structure of the IMM invaginations or cristae, within which the ETC resides (MacVicar and Langer, 2016). OPA1 protein expression and proteolytic processing have not been previously assessed in PAH PASMCs. This chapter demonstrated that total OPA1 protein levels in PAH PASMCs were comparable to those in control PASMCs. However, the levels of the short isoform were higher in PAH PASMCs than in control PASMCs, suggesting increased cleavage of OPA1. OPA1 contains two cleavages sites S1 and S2 that are targeted by the proteases OMA1 and YMEL1, respectively (MacVicar and Langer, 2016). Increased expression and/or activity of either or both of these two proteases in PAH PASMCs may account for the increased levels of S-OPA1. However, this has yet to be determined and requires further investigation. Given the role of S-OPA1 in stabilising the structure of mitochondrial cristae, within which the complexes of the ETC are embedded (Mishra et al., 2014), increased S-OPA1 levels may contribute to the increased mitochondrial oxygen consumption observed in Chapter 5.

Several studies suggest that mitochondrial fission is required for cell cycle progression to mitosis and is increased in many different highly proliferative cells, such as PAH PASMCs (Marsboom et al., 2012; Tian et al., 2018). Indeed, inhibiting DRP1 with either siRNA-mediated knockdown or the small molecule inhibitor Mdivi-1 was found to promote mitochondrial elongation, induce G2/M cell cycle arrest and inhibit proliferation in PAH PASMCs (Marsboom et al., 2012). Prostacyclin analogues are clinically used in the treatment of PAH and, in addition to their well-established vasodilatory effects on pulmonary arteries, have been shown to inhibit the proliferation of PASMCs by inducing cell cycle arrest at the G1/S checkpoint (Wharton et al., 2000). This chapter investigated whether the stable prostacyclin analogue treprostinil attenuates excessive mitochondrial fragmentation in PAH PASMCs. Acute treatment of PAH PASMCs with treprostinil was found to rapidly decrease the numbers of mitochondrial individuals and networks in PAH PASMCs. Treprostinil also increased the lengths of mitochondrial branches and the number of mitochondrial branches per mitochondrial network. Collectively, these results suggest that, in PAH PASMCs, treprostinil promotes the formation of fewer, larger, highly branched mitochondrial networks. DRP1 activity is decreased by phosphorylation at S637 (Chang and Blackstone, 2007). This inhibitory DRP1 phosphorylation is mediated by the cAMP-dependent serine/threonine kinase PKA (Chang and Blackstone, 2007). Given the pro-fusion effect of treprostinil on mitochondrial dynamics and its ability to evoke an increase in intracellular cAMP levels in PAH PASMCs, it was suggested that treprostinil might be inhibiting DRP1 activity by stimulating its phosphorylation at S637. Prior to the addition of treprostinil, very little if any DRP1 phosphorylation at S637 was observed in PAH PASMCs, suggesting that DRP1 could be maintained in a
dephosphorylated state by calcineurin or the breakdown of constitutively generated cAMP by PDEs. Indeed, calcineurin and the cAMP-degrading PDEs PDE1 and PDE3 have been reported to be upregulated in PASMCs from patients with PAH. This could therefore account for the lack of DRP1 phosphorylation at S637 in the untreated PAH PASMCs. Treprostinil rapidly induced DRP1 phosphorylation at S637 in PAH PASMCs without affecting total DRP1 protein levels. However, this DRP1 phosphorylation was transient and only lasted for 6 hours, disappearing completely after 24 hours. Treprostinil binds to cell-surface prostanoid receptors, which couple to and activate membrane-bound adenyl cyclase (Falcetti et al., 2010). In order to phosphorylate DRP1 at S637, cAMP generated near the plasma membrane would have to diffuse to the mitochondria, encountering on its way cAMP-hydrolysing PDEs. Degradation of treprostinil-induced cAMP could therefore underlie the transiency of its stimulation of DRP1 inhibitory phosphorylation at S637. Treprostinil also inhibited DRP1 phosphorylation at S616. In contrast to the short-lived treprostinil-induced DRP1 phosphorylation at S637, the inhibition of DRP1 phosphorylation at S616 by treprostinil lasted for 24 hours and was therefore more sustained. Taken together, these results suggest that treprostinil inhibits DRP1 activity in PAH PASMCs by stimulating its inhibitory phosphorylation at S637 and inhibiting its stimulatory phosphorylation at S616. These results also indicate that the sustained inhibition of DRP1 phosphorylation by treprostinil is more likely to account for its anti-proliferative effect on PAH PASMCs than the transitory stimulation of DRP1 phosphorylation at S637.

Treprostinil has been shown to bind with high affinity to the EP2, DP1 and IP prostanoid receptors but to inhibit the proliferation of PAH PASMCs predominantly via the EP2 and to a much lesser degree via the IP receptor (Patel et al., 2018; Whittle et al., 2012). Selective receptor antagonists were used to identify the receptors through which treprostinil signals to induce DRP1 phosphorylation at S637 and to inhibit that at S616. Individually, the IP receptor-selective antagonist RO1138452 and the EP2 receptor-selective antagonist PF04418948 had no effect on treprostinil-induced phosphorylation of DRP1 at S637. When given together, however, the antagonists completely blocked treprostinil-induced DRP1 phosphorylation at S637, suggesting that treprostinil recruits either the IP or EP2 receptor to stimulate inhibitory DRP1 phosphorylation at S637. Similarly, antagonising the IP and EP2 receptors individually failed to reverse the treprostinil-mediated inhibition of DRP1 phosphorylation at S616. In combination, the IP and EP2 receptor antagonists fully reversed the inhibition of DRP1 phosphorylation at S616 that was caused by treprostinil, indicating that treprostinil can activate either receptor to inhibit this stimulatory phosphorylation event. The IP and EP2 receptors couple via Gs to activate membrane-bound AC and elevate intracellular cAMP, which in
turn activates PKA (Clapp and Gurung, 2015). To determine whether PKA mediates the effect of treprostinil on DRP1 phosphorylation at both serine residues downstream of the IP and EP$_2$ receptors, the relatively PKA-selective inhibitor H89 was used. Inhibition of PKA blocked treprostinil-induced DRP1 phosphorylation at S637. PKA inhibition also completely reversed the treprostinil-mediated inhibition of DRP1 phosphorylation at S616. Taken together, these results support a new pathway for the inhibition of DRP1 activity in PAH PASMCs, in which IP or EP$_2$ receptor agonism signals via PKA to induce DRP1 phosphorylation at the inhibitory residue S637 and inhibit that at the stimulatory residue S616.

To confirm the roles of the IP and EP$_2$ receptors in inhibiting DRP1 activity in PAH PASMCs, agonists selective for the IP and EP$_2$ receptor were used. MRE-269 is the active metabolite of the non-prostanoid IP receptor agonist and PAH drug selexipag. Similar to treprostinil, MRE-269 induced inhibitory phosphorylation of DRP1 at S637 in PAH PASMCs. This was blocked by IP receptor antagonism with RO1138452, confirming the selectivity of MRE-269 for the IP receptor and the ability of the IP receptor to signal to inhibit DRP1 via S637 phosphorylation in PAH PASMCs. This also suggests that, despite the downregulation of the IP receptor in PAH PASMCs (Falcetti et al., 2010), the number of IP receptors available are sufficient to deliver a signal that promotes DRP1 phosphorylation at S637. The EP$_2$ receptor agonist butaprost also induced DRP1 phosphorylation at S637. This was blocked by the EP$_2$ receptor antagonist PF04418948, further supporting the role of EP$_2$ receptor in inhibiting DRP1 activity through stimulation of inhibitory S637 phosphorylation. Butaprost and MRE-269 also attenuated mitochondrial fission in PAH PASMCs. Butaprost and MRE-269 both similarly decreased the numbers of mitochondrial networks and mitochondrial individuals in PAH PASMCs. They also increased mitochondrial branching but, in contrast to treprostinil, failed to significantly increase the lengths of the mitochondrial branches.

Treprostinil could be inhibiting mitochondrial fission in PAH PASMCs by reducing the mitochondrial recruitment of DRP1. The effect of treprostinil on the expression of mitochondrial DRP1 receptors was therefore assessed. Treprostinil was found to significantly reduce the protein levels of FIS1 and MiD49 but not those of MFF and MiD51. This treprostinil-induced downregulation of FIS1 and MiD49 occurred 24 and 6 hours after treatment, respectively, suggesting that treprostinil could be influencing DRP1 recruitment to mitochondrial fission sites as well as its activity. However, this effect is unlikely to underlie the pro-fusion effect of treprostinil on the mitochondria observed in this chapter, as the PAH PASMCs were only treated with treprostinil for 3 hours prior to staining and imaging of their mitochondrial networks. FIS1 expression has been shown
to be sensitive to intracellular calcium levels (Wu et al., 2017). For instance, a reduction in calcium release from the ER in mouse neonatal cardiomyocytes has been shown to suppress the expression of FIS1 (Wu et al., 2017). Treprostinil could therefore be reducing FIS1 protein levels by inhibiting calcium release from the ER, which has also been shown to be increased in PAH PASMCs. Despite not influencing the protein expression of MFF, treprostinil could still be inhibiting the DRP1 recruitment activity of MFF by reducing its AMPK-mediated activating phosphorylation at S146 (Ducommun et al., 2015). Indeed, PKA, one of the downstream effectors of treprostinil signalling, has been shown to phosphorylate and inhibit the catalytic α subunit of AMPK (Djouder et al., 2010).

The effect of treprostinil on the fusion machinery was also examined. Treprostinil rapidly increased the proteins levels of MFN1 in PAH PASMCs. The rapidity with which treprostinil upregulated MFN1 protein levels indicated that treprostinil might have enhanced the stability of the protein rather than its synthesis. However, the treprostinil-evoked increase in MFN1 protein levels lasted for 24 hours, suggesting a possible additional induction of MFN1 expression. MFN1 is subject to ubiquitination by a number of E3 ubiquitin ligases, including MARCH5. Prior to the G2/M transition of the cell cycle, MARCH5, facilitated by the cyclin B1/CDK1 complex, has been shown to ubiquitinate and degrade MFN1 (Park and Cho, 2012). Prostacyclin analogues inhibit vascular SMC proliferation by inducing cell cycle arrest at the G1/S checkpoint (Wharton et al., 2000). Treprostinil could therefore be increasing the protein levels of MFN1 in PAH PASMCs by inducing cell cycle arrest at the G1/S checkpoint and preventing cell cycle progression to the G2 phase, where MFN1 is ubiquitinated and degraded by MARCH5 in preparation for mitosis. The AC activator and cAMP-elevating agent forskolin has been shown to increase the mRNA expression of MFN1 in immature Schwann cells (Cowell et al., 2008). This suggests that treprostinil, also a cAMP-elevating agent, could be increasing the protein levels of MFN1 in PAH PASMCs by enhancing the transcription of its nuclear gene. The MFN1 gene is targeted by the transcriptional coactivator PGC1α, the expression of which has been shown to be induced by cAMP-elevating agents and activators of the nuclear receptor PPARγ (Cowell et al., 2008). In addition to its ability to elevate intracellular cAMP, treprostinil has been suggested to activate PPARγ either through the cell-surface IP receptor or direct binding to PPARγ (Clapp and Gurung, 2015; Falcetti et al., 2007; Falcetti et al., 2010). Activation of PPARγ and consequent induction of PGC1α expression could constitute another potential mechanism for the treprostinil-induced increase in MFN1 protein levels in PAH PASMCs. In contrast to MFN1, treprostinil had no effect on the protein levels of MFN2. Indeed, MFN2 has also been shown to be unresponsive to the cAMP-elevating agent forskolin in Schwann cells (Park
and Cho, 2012). Altogether, these results suggest that treprostinil could be attenuating mitochondrial fission in PAH PASMCs by enhancing MFN1-mediated tethering of adjacent mitochondria and the fusion of their OMMs. Albeit not reaching statistical significance, treprostinil was also found to reduce the levels of the short isoform of OPA1 in PAH PASMCs without affecting those of the long isoform, suggesting that treprostinil might be reducing OPA1 cleavage to promote mitochondrial elongation.

In conclusion, increased mitochondrial fission has once more been demonstrated in PASMCs isolated from patients with PAH. Underlying this increased mitochondrial fission is increased protein expression of DRP1 accompanied by hyper-phosphorylation at the activating residue S616. Among the mitochondrial recruiters of DRP1, only FIS1 was found to be upregulated in PAH PASMCs. Proteins mediating fusion of the OMM (e.g. MFN1 and MFN2) were unaltered in PAH PASMCs. However, increased proteolytic cleavage of OPA1, which mediates IMM fusion, was observed in PAH PASMCs. The stable prostacyclin analogue treprostinil was able to attenuate mitochondrial fission in PAH PASMCs by inhibiting the stimulating phosphorylation of DRP1 at S616 while inducing that at the inhibitory residue S637. These effects were mediated by the IP or EP₂ receptors and were dependent on the cAMP-dependent serine/threonine kinase PKA. Individual IP or EP₂ receptor agonists were also found to attenuate mitochondrial fission in PAH PASMCs by stimulating the phosphorylation of DRP1 at the inhibitory residue S637. Treprostinil also inhibited the expression of the mitochondrial DRP1 recruiters MiD49 and FIS1, suggesting that reduced DRP1 recruitment to mitochondrial fission sites may additionally underlie the inhibitory effect of treprostinil on mitochondrial fission. Treprostinil upregulated the protein levels of the OMM fusion protein MFN1 but not MFN2 in PAH PASMCs. These findings suggest that prostacyclin analogues may be targeting the mitotic mitochondrial fission in PAH by switching off DRP1, inhibiting its recruitment to the mitochondria and upregulating MFN1 to promote OMM fusion.
4. Glycolysis and pentose phosphate shunting in PASMCs from patients with PAH

4.1 Introduction

Glycolysis refers to the cytosolic pathway that metabolises glucose to pyruvate, independently of oxygen (Li et al., 2015; Lunt et al., 2011). Following influx through GLUTs, glucose is converted to G6P by hexokinases, trapping it inside the cell. Hexokinases comprise a family of four isozymes, with hexokinases 1 and 2 being the most abundant (Li et al., 2015; Navale and Paranjape, 2016). After hexokinase-mediated glucose phosphorylation, G6P can be either converted to its isomer F6P, directing it further down the glycolytic pathway, or shunted into the PPP to generate R5P and the reducing agent NADPH (Li et al., 2015; Lunt et al., 2011). The step that commits glucose to the glycolytic pathway is catalysed by PFK1, which further phosphorylates F6P to F1,6BP (Mor et al., 2011). PFK1 functions as a tetramer and its constituent subunits are encoded by three genes. PFK1 subunits include the muscle isoform (PFKM), the liver isoform (PFKL) and the platelet isoform (PFKP), which can assemble to form either homotetrameric or heterotetrameric complexes (Mor et al., 2011). F6P can also be converted to the metabolite F2,6BP by the bifunctional enzyme PFKFB, of which there are four isoforms (PFKFB1-4; Lu et al., 2017; Mor et al., 2011). F2,6BP acts as a positive allosteric modulator of PFK1, enhancing its catalytic activity and increasing the rate at which glycolysis occurs (Lu et al., 2017; Mor et al., 2011). Further down the glycolytic pathway F1,6BP is converted to phosphoenolpyruvate in a multi-step reaction (Israelsen and Vander Heiden, 2015). Phosphoenolpyruvate is then converted to the end-product of glycolysis pyruvate by PK, of which four isoforms have thus far been identified: the two muscle isoforms (PKM1 and PKM2), the liver isoform (PKF) and the red blood cell isoform (PKR; Israelsen and Vander Heiden, 2015). At the end of glycolysis, pyruvate can be either transported into the mitochondria by MPCs, where it partakes in the TCA cycle, or converted to lactate by LDH in the cytosol (Li et al., 2015; McCommis and Finck et al., 2015). Lactate is extruded by means of MCTs and, through the liberation of protons, acidifies the extracellular milieu (Payen et al., 2019).

Glycolytic flux has repeatedly been shown to be upregulated in pulmonary vascular cells, particularly PASMCs, derived from patients with PAH (Archer et al., 2017; Kovacs et al., 2019). It is widely thought that increased glycolysis occurs secondary to depressed
mitochondrial respiration. Reduced pyruvate transport into the mitochondria coupled with inhibition of pyruvate dehydrogenase, which feeds pyruvate into the TCA cycle, by increased PDK-dependent phosphorylation has been demonstrated in PAH and suggested to divert pyruvate from mitochondrial oxidation to cytosolic reduction to lactate (Paulin and Michelakis, 2014; Michelakis et al., 2017). However, recent evidence suggests that increased glycolysis in PAH PASMCs serves to support biosynthetic pathways that produce the building blocks required for the generation of daughter cells. The mechanisms of increased glucose flux through glycolysis in PAH are beginning to be unravelled. Increased glucose uptake as a result of increased expression of GLUT1 was reported in the lungs of monocrotaline-treated rats and PAH patients and suggested to drive the increased glycolytic flux in PAH (Marsboom et al., 2012; Zhao et al., 2013). However, one study could not replicate this increased pulmonary glucose uptake in patients with PAH and other mechanisms downstream of glucose influx through GLUTs may underlie the increased glycolytic flux in PAH (Ruiter et al., 2013). For example, the cellular entrapment of glucose by its conversion to G6P may be increased in PAH as a consequence of upregulation of hexokinases, particularly the predominant isoforms hexokinase 1 and 2. Indeed, increased expression of HK1 was demonstrated in monocrotaline-induced PH in rats (Zhang et al., 2014).

Given the rate-limiting nature of the PFK1-catalysed step in glycolysis, studies have focused on elucidating whether an increase in the expression of PFK1 subunits concomitant with increased activity underlie the elevated glycolytic flux in PASMCs from patients with PAH. The PFK1 subunit PFKP, which is abundantly expressed in tumour cells, has been shown to be upregulated by the growth factor TGFβ, a key player in PAH pathogenesis, in normal PASMCs (Calvier et al., 2017). This upregulation was coupled with an increase in mitochondrial oxidative phosphorylation and proliferation (Calvier et al., 2017). An increase in PFKP protein expression was also demonstrated in pulmonary arteries from patients with PAH (Calvier et al., 2017). However, the effects of TGFβ on the expression of the other two PFK1 subunits, PFKM and PFKL, and glycolysis were not assessed. Intriguingly, it remains unknown whether, in addition to the PFKP isoform, PASMCs express the PFKM and PKFL isoforms. An increase in the expression of either PFKM, PFKL or both could therefore also contribute to the increased glycolytic rate observed in PAH PASMCs.

The rate-limiting, glycolytic enzyme PFK1 is activated by its positive allosteric modulator F2,6BP. F2,6BP relieves the ATP-mediated inhibition of PFK1 that serves to limit glycolytic flux under aerobic conditions (Ros and Schulze, 2013). In vascular cells, F2,6BP is generated from the glycolysis intermediate F6P by the action of the enzyme
PKFB3 (Kovacs et al., 2019). Increased PKFB3 expression concomitant with an increase in the levels of F2,6BP leads to an elevation of PFK1 activity and therefore glycolysis (Ros et al., 2013). Induction of PKFB3 expression has been reported to underpin the metabolic shift to glycolysis in many human cancers (Atsumi et al., 2002). It was postulated that increased PKFB3 expression might also contribute to the increased glycolytic flux previously reported in pulmonary vascular cells, including PASMCs, from patients with PAH. Indeed, increased PKFB3 expression was demonstrated in PAECs isolated from patients with PAH as well as PASMCs (Kovacs et al., 2019). In addition to its canonical role in regulating PFK1, PKFB3 has been shown to localise to the nucleus, where it generates F2,6BP to activate the cell cycle regulator CDK1 and consequently promote G2-to-M transition of the cell cycle (Yalcin et al., 2014). F2,6,BP through CDK1 also promotes an anti-apoptotic state by phosphorylating p27 and targeting it for proteasomal degradation (Yalcin et al., 2014). Furthermore, PKFB3 has been reported to interact with CDK4, which promotes cell cycle progression from the G1 phase to the S phase (Jia et al., 2018).

Another rate-limiting step in glycolysis downstream of PFK1 and catalysed by PKM has been recently investigated in PAFs and blood outgrowth endothelial cells (BOECs) derived from patients with PAH (Caruso et al., 2017; Zhang et al., 2017). Like PAH PASMCs, PAFs and BOECs from PAH patients were shown to exhibit a Warburgian shift from mitochondrial oxidative phosphorylation to cytosolic glycolysis (Caruso et al., 2017; Zhang et al., 2017). This metabolic reprogramming has been suggested to contribute to their highly proliferative phenotype (Caruso et al., 2017; Zhang et al., 2017). PKM catalyses the final step in glycolysis, which is the conversion of phosphoenolpyruvate to pyruvate (Israelsen and Vander-Heiden, 2015). PKM exists as two isoforms PKM1 and PKM2, both products of the alternatively spliced PKM gene (Israelsen and Vander Heiden, 2015). PKM1 constitutively forms a high-activity tetramer, whereas PKM2 can form either a low-activity dimer or a high-activity tetramer (Israelsen and Vander Heiden, 2015). An increase in the ratio of PKM2 to PKM1 with a resultant reduction in PKM activity has been demonstrated in BOECs and PAFs from patients with PAH (Caruso et al., 2017; Zhang et al., 2017). Normalisation of the PKM2:PKM1 ratio via PKM2 silencing inhibited lactate production and restored a normal metabolic phenotype in PAH PAFs and BOECs, suggesting a critical role for PKM2 in mediating the Warburgian switch towards glycolysis in PAH (Caruso et al., 2017; Zhang et al., 2017). Whether a similar increase in the PKM2:PKM1 ratio occurs in the highly glycolytic PASMCs from PAH patients remains unexplored. Interestingly, a decrease in PKM activity is also thought to allow for the accumulation of glycolysis intermediates and their subsequent channelling into the pentose phosphate shunt to support nucleotide synthesis required by rapidly
dividing cells, such as pulmonary vascular cells from PAH patients (Israelson and Vander-Heiden, 2015).

Once generated by glycolysis, pyruvate is either transported into the mitochondria by MPCs or converted to lactate in the cytosol (Valvona et al., 2016). Under aerobic conditions, pyruvate enters the mitochondria, where it is converted to acetyl-CoA and fed into the TCA cycle. When oxygen becomes scarce, pyruvate tends to accumulate in the cytosol, where it is converted to lactate. Lactate dehydrogenase (LDH) is the enzyme responsible for the interconversion of pyruvate to lactic acid in the cytoplasm (Valvona et al., 2016). It exists as a tetramer, composed predominantly of two different subunits LDHA and LDHB. LDHA primarily reduces pyruvate to lactate, oxidising NADH to NAD⁺ in the process, whereas LDHB converts lactate back to pyruvate, converting NAD⁺ back to NADH (Feng et al., 2018). High levels of LDH were reported in the sera of patients with IPAH and associated with increased severity and mortality (Hu et al., 2015). However, it remains to be determined whether the expression of LDHA and LDHB is altered in vascular cells, particularly PASMCs, in patients with PAH. It was postulated that an increase in the ratio of LDHA to LDHB might underlie the high glycolytic flux to lactate in PAH, which occurs even under normoxic conditions. Indeed, an abnormally high LDHA:LDHB ratio has been demonstrated in cancer cells and proposed to contribute to the metabolic switching of these cells to aerobic glycolysis. Moreover, transcript levels of LDHA were found to be upregulated in BOECs isolated from patients with HPAH compared to those isolated from control subjects (Caruso et al., 2017). LDHA activity can also be increased by phosphorylation at tyrosine (Y) 10 downstream of growth factor receptors (e.g. FGFR1; Kachel et al., 2015) and soluble tyrosine kinases (e.g. Src; Jin et al., 2017). Phosphorylation-driven activation of LDHA has been shown to strongly correlate with tumour invasiveness and metastasis and could therefore also be driving the proliferation of pulmonary vascular cells in PAH (Jin et al., 2017).

Glycolysis intermediates can also spill over into biosynthetic pathways branching off glycolysis. One such pathway is the pentose phosphate shunt, particularly its oxidative arm, in which the glycolysis intermediate G6P is metabolised to yield the reducing agent NADPH and the nucleotide precursor ribulose-5-phosphate. NADPH is not only heavily involved in fatty acid and cholesterol synthesis but is also required for the production of reduced glutathione (GSH), an important scavenger of ROS (Kuwalik et al., 2017). The production of nucleotide precursors to support elevated nucleic acid synthesis and NADPH to drive lipid and cholesterol synthesis make the PPS critical for highly proliferative cells (Lunt and Vander Heiden, 2011). Indeed, heightened pentose phosphate shunting has been reported in a wide variety of cancer cells and associated
with invasion, metastasis and angiogenesis (Kowalik et al., 2017). Conversion of G6P to 6-phosphogluconate by G6PD comprises the first, rate-limiting step in the PPS (Kowalik et al., 2017). Increased expression and/or activity of G6PD therefore leads to an increase in the rate at which PPS occurs. Upregulation of G6PD expression has been reported in different types of cancer, including those of the colon, lung and breast. In addition to increased G6PD expression, increased O-GlcNAcylation of G6PD, which leads to G6PD activation and increased glucose flux through the PPS, has also been demonstrated in human non-small cell lung cancer (Rao et al., 2015). Similarly, pulmonary vascular cells from PAH patients require copious amounts of nucleotides, lipids and cholesterol to support their highly synthetic and proliferative phenotype and may also therefore upregulate PPS and other biosynthetic pathways to meet this increased demand for macromolecules.

This results chapter aimed to reassess glucose flux through glycolysis in control and PAH PASMCs to confirm the increased glycolytic flux previously observed. This chapter also sought to elucidate the mechanisms underlying the increase in the rate of glycolysis in PAH. For example, the protein expression of GLUT1, which mediates cellular entry of the glycolysis substrate glucose in PASMCs, was first determined. The protein expression of hexokinases 1 and 2, which phosphorylate glucose to G6P and consequently trap it inside the cell, was also investigated. The platelet isoform of PFK1, the rate-limiting enzyme in glycolysis, has been shown to be expressed in PASMCs and upregulated in PAH. It is unknown, however, whether the other two PFK1 isoforms PFKM and PFKL are expressed in PASMCs. This chapter therefore also sought to examine the expression of these two PFK1 isoforms as well as PFKP in control and PAH PASMCs. Moreover, PFKL and PFKM expression was also examined in lung sections from control subjects and patients with PAH. PFK1 activity is positively regulated by the metabolite F2,6BP, which is predominantly generated by the enzyme PFKFB3. The protein expression of PFKFB3 was explored in control and PAH PASMCs as well as in lung sections from control subjects and PAH patients. PKM catalyses the final rate-limiting step in glycolysis, which converts phosphoenolpyruvate to pyruvate. Increased PKM2-to-PKM1 ratio, which is indicative of decreased PKM activity, was demonstrated in fibroblasts and BOECs from PAH patients. This chapter sought to determine whether there is a similar increase in the ratio of PKM2 to PKM1 in PAH PASMCs. In the cytosol, pyruvate is converted to lactate by the enzyme LDHA. Lactate is also converted back to pyruvate by LDHB. The expression of these two LDH isoenzymes was determined in control and PAH PASMCs. Finally, in addition to generating pyruvate, glycolysis intermediates can be shunted into biosynthetic pathways, such as the PPS. G6PD is the rate-limiting enzyme in the PPS and its expression in PAH PASMCs has yet to be
investigated. This chapter therefore compared the protein expression of G6PD in control PASMCs with that in PAH PASMCs. The expression of G6PD in lung sections from control subjects and PAH patients was also examined.
4.2 Results

4.2.1 Glycolytic flux in control and PAH PASMCs

PASMCs from PAH patients have previously been shown to metabolise glucose predominantly via glycolysis, thus producing higher levels of lactic acid than their normal counterparts even under oxygen-rich or aerobic conditions (Kovacs et al., 2019). This metabolic shift from mitochondrial respiration to glycolysis for ATP production is analogous to the phenomenon observed by Otto Warburg in cancer cells (Paulin and Michelakis, 2014). To confirm this increased glycolytic flux in PAH PASMCs, the Seahorse XFp Glycolysis Stress Test was performed to measure ECARs in quiescent control and PAH PASMCs and assess key parameters of glycolytic flux, including non-glycolytic acidification, glycolysis, glycolytic capacity and glycolytic reserve. As shown in Figure 4.1A, PAH PASMCs exhibited considerably higher ECARs than their control counterparts. Basally, ECARs were higher in PAH PASMCs than in control PASMCs, as indicated by the two-fold increase in non-glycolytic acidification (p = 0.0388; n = 3 for control PASMCs; n = 3 for PAH PASMCs; Figure 4.1B). Injection of glucose, which induces cytosolic glycolysis, resulted in a much higher rise in ECAR in PAH PASMCs than in control PASMCs, indicating increased glycolysis in PAH PASMCs (p = 0.0491; n = 3 for control PASMCs; n = 3 for PAH PASMCs; Figure 4.1C). Oligomycin, which inhibits mitochondrial pyruvate metabolism and diverts pyruvate to glycolysis, resulted in a further increase ECAR in PAH PASMCs but not in control PASMCs. Glycolytic capacity was therefore negligible in control PASMCs and markedly elevated in PAH PASMCs (p = 0.0299; n = 3 for control PASMCs; n = 3 for PAH PASMCs; Figure 4.1D). Moreover, no glycolytic reserve was observed in control PASMCs, whereas PAH PASMCs showed a considerably higher glycolytic reserve (p = 0.0269; n = 3 for control PASMCs; n = 3 for PAH PASMCs; Figure 4.1E).
Figure 4.1: Glycolytic flux in control and PAH PASMCs. Control (n = 3) and PAH (n = 5) PASMCs were seeded in Seahorse XFp Cell Culture Miniplates and maintained in 10% FBS overnight. PASMCs were then serum-starved in 0.1% FBS for 48 hours to induce quiescence and transferred to glycolysis stress test medium. (A) ECARs were measured in quiescent control and PAH PASMCs using the Seahorse XFp Analyser. Glucose, oligomycin and 2-DG were serially injected as part of the Seahorse XFp Glycolysis Stress Test to assess key parameters of glycolytic flux, including non-glycolytic acidification (B), glycolysis (C), glycolytic capacity (D) and glycolytic reserve (E). Values are means ± SEM. Unpaired student’s t-test was used in B, C, D and E. p < 0.05 was considered statistically significant.
4.2.2 Protein expression of GLUT1 in control and PAH PASMCs

Several lines of evidence suggest increased cellular uptake of glucose in rodent models of PH and PAH patients via upregulation of the glucose transporter GLUT1, particularly in PASMCs (Marsboom et al., 2012; Zhao et al., 2013). One study, however, showed that glucose uptake in the lungs of patients with IPAH was indistinguishable from glucose uptake in the lungs of patients with myocardial infarction, reflecting the metabolic heterogeneity amongst PAH patients (Ruiter et al., 2013). The increased glucose influx previously reported is thought to account for the increased glucose flux through glycolysis in PAH PASMCs. In an attempt to replicate this finding, proteins from quiescent control and PAH PASMCs were immunoblotted for GLUT1 as shown in Figure 4.2. Contrary to previous evidence, the protein expression of GLUT1 relative to GAPDH in PAH PAMSCs was similar to that in control PASMCs.

**Figure 4.2:** Protein expression of GLUT1 in control and PAH PASMCs. Control ($n = 3$) and PAH ($n = 6$) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for GLUT1. Blots were reprobed with GAPDH to ensure equal loading. (B) Densitometry was used to quantify the protein levels of GLUT1 relative to the loading control GAPDH. Unpaired student’s t-test was used in B. Values are means ± SEM. $p < 0.05$ was considered to be statistically significant.
4.2.3 Protein expression of hexokinases 1 and 2 in control and PAH PASMCs

Hexokinases 1 and 2 are cytosolic enzymes that catalyse the first step in the glycolytic pathway. Using ATP as a phosphate donor, they phosphorylate glucose to G6P, resulting in its entrapment within the cell. Given the increased glycolytic flux in PAH PASMCs, it was hypothesised that the expression of these two enzymes might be upregulated in PASMCs isolated from PAH patients. To test this hypothesis, control and PAH PASMCs were cultured in 10% FBS to full confluence, quiesced in 0.1% FBS for 48 hours and lysed for protein. Proteins from quiescent control and PAH PASMCs were then immunoblotted for hexokinases 1 and 2, as shown in Figure 4.3A. The levels of hexokinases 1 and 2 relative to GAPDH in PAH PASMCs were akin to those in control PASMCs (Figure 4.3B and Figure 4.3C). This indicates that the first step in glycolysis remains unaffected and does not underlie the increased glycolytic flux in PAH PASMCs.
Figure 4.3: Protein expression of hexokinases 1 and 2 in control and PAH PASMCs. Control ($n = 3$) and PAH ($n = 6$) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for hexokinases 1 and 2. Blots were reprobed with GAPDH to ensure equal loading. Densitometry was used to quantify the protein levels of hexokinases 1 (B) and 2 (C) relative to the loading control GAPDH. Unpaired student's t-test was used in B and C. Values are means ± SEM. $p < 0.05$ was considered statistically significant.
4.2.4 Protein expression of the PFK1 subunits PFKL, PFKM and PFKP in control and PAH PASMCs

The cytosolic enzyme PFK1 catalyses the rate-limiting step in glycolysis. Consequently, PFK1 levels and activity positively correlate with glycolytic flux. PFK1 converts F6P to F1,6BP, committing glucose to the glycolytic pathway, and is encoded by three different genes, each of which give rise to one of three PFK1 isoforms: PFKM, PFKL and PFKP (Mor et al., 2011). PFK1 functions as a tetramer and the three different isoforms can assemble to form either homotetramers or heterotetramers depending on the cell type (Mor et al., 2011). PFKP has previously been shown to be expressed in PASMCs; the expression of the other two PFK1 isoforms in PASMCs has yet to be assessed (Calvier et al., 2017). To determine whether the constituent subunits of PFK1 are upregulated in PAH PASMCs, proteins from quiescent control and PAH PASMCs were immunoblotted for PFKM, PFKL and PFKP. The levels of each isoenzyme relative to GAPDH were determined by densitometry. Despite not reaching statistical significance, the protein levels of PFKM relative to GAPDH in PAH PASMCs were twice as much as those in control PASMCs (Figure 4.4B). Moreover, the medial layers of pulmonary arteries in PAH lung sections stained more strongly for PFKM than those of pulmonary arteries in control lung sections (Figure 4.5). As shown in Figure 4.4C, relative PFKL protein expression was found to be four-fold higher in PAH PASMCs than in their control counterparts ($p = 0.0016; n = 3$ for control PASMCs; $n = 6$ for PAH PASMCs). Consistent with the increase PFKL protein expression in cultured PAH PASMCs, stronger staining for PFKL was also observed in the medial layers of distal pulmonary arteries in PAH lung sections compared to those in control lung sections (Figure 4.6). In contrast to both PFKM and PFKL, the relative expression of the PFK1 isoenzyme PFKP in PAH PASMCs was similar to that in their control counterparts.
Figure 4.4: Protein expression of the PFK1 isoforms PFKM, PFKL and PFKP in control and PAH PASMCs. Control \((n = 3 - 4)\) and PAH \((n = 5)\) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for PFKM, PFKL and PFKP. Blots were reprobed with GAPDH to ensure equal loading. Densitometry was used to quantify the protein levels of PFKM (B), PFKL (C) and PFKP (D) relative to the loading control GAPDH in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in B, C and D. \(p < 0.05\) was considered statistically significant.
Figure 4.5: Immunohistochemical staining for PFKM in lung sections from control and PAH patients. Formalin-fixed, paraffin-embedded lung sections from control (A; \(n = 3\)) and PAH (B; \(n = 3\)) patients were stained for the PFKM using immunohistochemistry. (C) Lung sections were also stained with normal rabbit IgG to confirm specificity of the PFKM staining. Sections were counterstained with the nuclear stain Meyer's haematoxylin. High resolution images were acquired using the Nanozoomer Digital Scanner. Pulmonary arteries were stratified into 3 groups according to vessel diameter.
Figure 4.6: Immunohistochemical staining for PFKL in lung sections from control and PAH patients. Formalin-fixed, paraffin-embedded lung sections from control (A; \( n \) = 3) and PAH (B; \( n \) = 3) patients were stained for the PFKL using immunohistochemistry. (C) Lung sections were also stained with normal rabbit IgG to confirm specificity of the PFK1 isoenzyme PFKL staining. Sections were counterstained with the nuclear stain Meyer's haematoxylin. High resolution images were acquired using the Nanozoomer Digital Scanner. As indicated, pulmonary arteries were stratified into 3 groups according to vessel diameter.
4.2.5 Protein expression of PFKFB3 in control and PAH PASMCs

PFKFB3 is a bifunctional enzyme that possesses both kinase and phosphatase activity (Ros and Schulze, 2013). Despite its bifunctional nature, PFKFB3 primarily converts F6P to F2,6BP, which functions as a positive allosteric modulator of PFK1 (Ros and Schulze, 2013). Since PFK1 catalyses the rate-limiting step in glycolysis, it was postulated that increased levels and activity of PFKFB3 concomitant with increased levels of F2,6BP could underlie the increased glycolytic flux that was observed in PAH PASMCs (Ros and Schulze, 2013). Indeed, as shown in Figure 4.7, immunoblotting of proteins, isolated from quiescent control and PAH PASMCs, revealed a 3-fold increase in the level of PFKFB3 relative to GAPDH in PAH PASMCs compared to their control counterparts (p = 0.0037; n = 3 for control PASMCs; n = 6 for PAH PASMCs). Lung sections from three control subjects and three PAH patients were also stained for PFKFB3 to assess expression within the medial layers of small pulmonary arteries (< 500 μm in diameter), sites of extensive remodelling and luminal narrowing in PAH. When compared to lung sections stained with normal IgG, PFKFB3 was found to be expressed in all three layers of the pulmonary arteries, irrespective of vessel size. Moreover, as shown in Figure 4.8, markedly stronger staining for PFKFB3 was observed in the medial layers of the pulmonary arteries in the PAH lung sections than in those of the pulmonary arteries in the control lung sections, consistent with the increased protein expression observed in cultured PAH PASMCs. The difference in staining was most evident in pulmonary arteries with diameters ranging from 0 to 50 μm. Interestingly, PAECs, which comprise the innermost layer of the pulmonary artery, were also found to stain more strongly for PFKFB3 in PAH lung sections than in control lung sections. In the smallest pulmonary arteries shown, PFKFB3 strikingly appears to localise to the nucleus, as indicated by colocalisation with the haematoxylin stain.
Figure 4.7: Protein expression of PFKFB3 in control and PAH PASMCs. Control \((n = 3)\) and PAH \((n = 6)\) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for PFKFB3. Blots were reprobed with GAPDH to ensure equal loading. (B) Densitometry was used to quantify the protein levels of PFKFB3 relative to the loading control GAPDH in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in B. \(p < 0.05\) was considered statistically significant.
Figure 4.8: Immunohistochemical staining for PFKFB3 in lung sections from control subjects and PAH patients. Formalin-fixed, paraffin-embedded lung sections from control (A; n = 3) and PAH (B; n = 3) patients were stained for PFKFB3 using immunohistochemistry. (C) Lung sections were also stained with normal rabbit IgG to confirm specificity of the PFKL staining. Sections were counterstained with the nuclear stain Meyer's haematoxylin. High resolution images were acquired using the Nanozoomer Digital Scanner. As indicated, pulmonary arteries were stratified into 3 groups according to vessel diameter.
4.2.6 Protein expression of PKM1 and PKM2 in control and PAH PASMCs

PKM catalyses the final step in glycolysis, in which phosphoenolpyruvate is converted to pyruvate (Israelson and Vander-Heiden, 2015). Pyruvate is then either converted to lactic acid or fed into the mitochondria to partake in the TCA cycle (Israelson and Vander-Heiden, 2015). Therefore, pyruvate occupies the junction between cytosolic glycolysis and mitochondrial respiration (Israelson and Vander-Heiden, 2015). PKM exists as two isoforms PKM1 and PKM2, generated as a consequence of alternative splicing of the PKM gene. PKM1 constitutively occurs in a highly active tetrameric form, whereas PKM2 exists either as a low-activity dimer or a high-activity tetramer (Israelson and Vander-Heiden, 2015). Several lines of evidence suggest that an increased PKM2 to PKM1 ratio underlies increased glycolysis and depressed mitochondrial respiration in proliferating cells. It was therefore postulated that, in PAH PASMCs, the ratio of PKM2 to PKM1 might be increased, accounting for the increased glycolytic flux. Immunoblotting of proteins from quiescent control and PAH PASMCs revealed a significant reduction in the protein expression of PKM1 relative to GAPDH in PAH PASMCs (p = 0.0027; n = 3 for control PASMCs; n = 6 for PAH PASMCs; Figure 4.9B). However, as shown in Figure 4.9C, the protein levels of PKM2 relative to GAPDH in PAH PASMCs were comparable to those in control PASMCs. Furthermore, the PKM2 to PKM1 ratio was two-fold higher in PAH PASMCs than in control PASMCs (p = 0.0073; n = 3 for control PASMCs; n = 6 for PAH PASMCs; Figure 4.9D).
Figure 4.9: Protein expression of the PKM isoforms PKM1 and PKM2 in control and PAH PASMCs. Control (n = 3) and PAH (n = 5) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for PKM1 and PKM2. Blots were reprobed with GAPDH to ensure equal loading. Densitometry was used to quantify the protein levels of PKM1 (B) and PKM2 (C) relative to the loading control GAPDH in control and PAH PASMCs. (D) The ratios of PKM2 to PKM1 in control and PAH PASMCs were also determined. Unpaired student’s t-test was used in B, C and D. Values are means ± SEM. p < 0.05 was considered statistically significant.
4.2.7 Protein expression and stimulatory phosphorylation of LDHA in control and PAH PASMCs

Once generated, pyruvate can be either converted to acetyl coenzyme A by the enzyme PDH or lactate by the enzyme LDH. LDH is a cytosolic tetramer, the constituent subunits of which are encoded by two genes LDHA and LDHB. LDH catalyses the reversible conversion of pyruvate to lactate, oxidising NADH to NAD$^+$ in the process. Lactate is then exported outside the cell to acidify the extracellular space through the release of H$^+$. Interestingly, LDHA and LDHB display different substrate affinities. LDHA has a higher affinity for pyruvate and therefore tends to convert pyruvate to lactate. LDHB, in contrast, has a higher affinity for lactate and preferentially converts lactate back to pyruvate. LDHA activity is regulated by post-translational modification, namely stimulatory phosphorylation at tyrosine 10. Increased expression and/or stimulatory phosphorylation of LDHA might therefore contribute to the increased ECARs observed in PAH PASMCs.

To investigate this hypothesis, proteins from quiescent control and PAH PASMCs were immunoblotted for LDHA and LDHA phosphorylated at Y10 (Figure 4.10). The protein expression of LDHA relative to GAPDH in PAH PASMCs was similar to that in control PASMCs. Moreover, stimulatory LDHA phosphorylation at Y10 in PAH PASMCs was indistinguishable from that in control PASMCs, as indicated by the lack of significant changes in the levels of pLDHA$^{Y10}$ relative to GAPDH and ratios of pLDHA$^{Y10}$ to LDHA in PAH PASMCs.
Figure 4.10: Protein expression and phosphorylation of LDHA in control and PAH PASMCs. Control \((n = 3)\) and PAH \((n = 4)\) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for LDHA and pLDHA\(^{Y10}\). Blots were reprobed with GAPDH to ensure equal loading. Densitometry was used to quantify the protein levels of LDHA (B) and pLDHA\(^{Y10}\) (C) relative to the loading control GAPDH in control and PAH PASMCs. (D) The ratios of pLDHA\(^{Y10}\) to LDHA in control and PAH PASMCs were also determined. Values are means ± SEM. Unpaired student’s t-test was used in B, C and D. \(p < 0.05\) was considered statistically significant.
4.2.8 Protein expression of LDHB in control and PAH PASMCs

LDHB preferentially converts lactate back to pyruvate, reducing \( \text{NAD}^+ \) back to \( \text{NADH} \) in the process. Given the reversibility of pyruvate conversion to lactate, it was hypothesised that reduced LDHB expression might underlie the increased glycolytic flux observed in PAH PASMCs. Therefore, proteins from control and PAH PASMCs were immunoblotted for LDHB. As shown in Figure 4.11, the protein levels of LDHB relative to GAPDH in PAH PASMCs were similar to those in control PASMCs, suggesting that the conversion of lactate back to pyruvate remains intact in PAH PASMCs.

**Figure 4.11: Protein expression of LDHB in control and PAH PASMCs.** Control \( (n = 3) \) and PAH \( (n = 4) \) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for LDHB. Blots were reprobed with GAPDH to ensure equal loading. (B) Densitometry was used to quantify the protein levels of LDHA relative to the loading control GAPDH in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in B, \( p < 0.05 \) was considered statistically significant.
4.2.9 Protein expression of G6PD in control and PAH PASMCs

After phosphorylation by hexokinases, glucose can be either metabolised via the glycolytic pathway to generate pyruvate or shunted into the pentose phosphate pathway, the main products of which are ribose-5-phosphate, a precursor for nucleotides, and the reducing agent NADPH (Lunt and Vander Heiden, 2011). PAH PASMCs have been shown to proliferate at much higher rates and to produce more ROS than their control counterparts. Therefore, the pentose phosphate pathway could be upregulated in PAH PASMCs to generate enough nucleotides to support the increased proliferation and NADPH to neutralise the increased ROS production. G6PD catalyses the rate-limiting step in the PPP. To assess whether the PPP is upregulated in PAH PASMCs, proteins from control and PAH PASMCs were immunoblotted for the rate-limiting enzyme G6PD.

As shown in Figure 4.12, the protein level of G6PD relative to GAPDH was three-fold higher in PAH PASMCs than in control PASMCs (p = 0.0443; n = 3 for control PASMCs; n = 5 for PAH PASMCs). Consistently, immunohistochemical staining for G6PD in lung sections from 3 control subjects and 3 PAH patients revealed increased protein expression of G6PD in PASMCs within the medial layers of the distal pulmonary arteries in the PAH lung sections. Moreover, similar to PFKFB3, the PAECs were found to stain more strongly for G6PD in PAH lung sections than in control lung sections.
Figure 4.12: Protein expression of G6PD in control and PAH PASMCs. Control ($n = 3$) and PAH ($n = 5$) PASMCs were cultured in 10% FBS to full confluence, serum-starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for G6PD. Blots were reprobed with GAPDH to ensure equal loading. (B) Densitometry was used to quantify the protein levels of G6PD relative to the loading control GAPDH in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in B. $p < 0.05$ was considered statistically significant.
**Figure 4.13: Immunohistochemical staining for G6PD in lung sections from control subjects and PAH patients.** Formalin-fixed, paraffin-embedded lung sections from control (A; n = 3) and PAH (B; n = 3) patients were stained for PFKFB3 using immunohistochemistry. (C) Lung sections were also stained with normal rabbit IgG to confirm specificity of the PFKL staining. Sections were counterstained with the nuclear stain Meyer’s haematoxylin. High resolution images were acquired using the Nanozoomer Digital Scanner. As indicated, pulmonary arteries were stratified into 3 groups according to vessel diameter.
4.3 Discussion

Aerobic glycolysis has been shown to be upregulated in many hyper-proliferative cells, ranging from cancer cells to pulmonary vascular cells derived from patients with PAH (Caruso et al., 2017; Jiang, 2017; Kovacs et al., 2019; Zhang et al., 2017). Glycolysis is a multi-step, cytosolic pathway that converts glucose to pyruvate (Li et al., 2015; Lunt et al., 2011). Pyruvate can then be transported into the mitochondria, where it is oxidatively metabolised to carbon dioxide in the TCA cycle to generate ATP through the process of oxidative phosphorylation (Jiang, 2017; Li et al., 2015; Lunt et al., 2011). Alternatively, pyruvate can remain in the cytosol, where it is reduced to lactic acid, which is then extruded via MCTs to acidify the extracellular milieu through the liberation of protons (Jiang, 2017). This chapter utilised the Seahorse XFp Extracellular Flux Analyser to confirm the previously reported increase in glycolytic flux in PAH PASMCs. Basally and prior to the sequential injection of the glycolysis substrate glucose and the inhibitors oligomycin A and 2-DG, ECARs measured were found to be much higher in PAH PASMCs than in their control counterparts. Basal ECARs represent extracellular acidification caused by carbon dioxide derived from mitochondrial respiration. In chapter 4, basal respiration has been reported to be elevated in PAH PASMCs as a result of increased mitochondrial mass. This is likely to contribute to the increased basal ECARs through the increased generation of carbon dioxide and the subsequent formation of carbonic acid catalysed by carbonic anhydrase. Indeed, inhibition of carbonic anhydrase with acetazolamide has been reported to ameliorate sugen 5416/hypoxia-induced PH in rats and to inhibit dedifferentiation of PASMCs from patients with PAH, suggesting a role for carbonic anhydrase and possibly even non-glycolytic acidification in the pathogenesis of PAH (Hudallah et al., 2019). It remains to be determined, however, whether carbonic anhydrase expression is increased in PASMCs from PAH patients. Injection of exogenous glucose to stimulate glycolysis resulted in a considerably larger increase in ECARs in PAH PASMCs than in controls, confirming that glycolysis occurs at a much higher rate in PAH PASMCs. Inhibiting mitochondrial oxidative phosphorylation through the injection of the mitochondrial ATP synthase or complex V inhibitor oligomycin A further increased ECARs in PAH PASMCs but not in control PASMCs. This revealed that PAH PASMCs display a much greater glycolytic capacity than control PASMCs. PAH PASMCs were also found to be more capable of upregulating glycolysis to respond to energy demand than their control equivalents, as indicated by a greater glycolytic reserve. Finally, non-glycolytic acidification, unmasked by shutting down the glycolytic pathway with the hexokinase inhibitor 2-DG, was also several-fold higher in PAH PASMCs than in control PASMCs. Like basal acidification, non-glycolytic acidification is thought to be a consequence of carbon dioxide generation in the TCA cycle followed by
conversion to carbonic acid by carbonic anhydrase. Collectively, these findings confirm that PAH PASMCs upregulate glycolysis to potentially support their highly synthetic and proliferative phenotype.

Compared to mitochondrial oxidative phosphorylation, which generates up to 36 molecules of ATP per molecule of glucose, glycolysis is highly inefficient in terms of ATP yield, producing only 2 molecules of ATP per molecule of glucose (Lunt and Vander Heiden, 2011). In spite of its low efficiency at generating ATP, glycolysis occurs at a much higher rate and therefore produces more ATP than oxidative phosphorylation (Lunt and Vander Heiden, 2011). An inefficient but faster pathway for ATP production may be desired by highly proliferating cells, such as PAH PASMCs, to meet their high energy demands. However, the role of increased glycolysis in proliferating cells extends beyond ATP production and pyruvate generation (Lunt and Vander Heiden, 2011). Several intermediates along the glycolytic pathway feed into branching biosynthetic pathways to generate the building blocks of many cellular components (e.g. nucleotides, amino acids and lipids) and the function of upregulated glycolysis in proliferating cells may consequently be to maintain the levels of these intermediates and support biosynthesis (Lunt and Vander Heiden, 2011). At the tip of glycolysis, G6P can be shunted into the pentose phosphate pathway, the major function of which is to produce the nucleotide precursor ribose-5-phosphate and the reducing agent NADPH (Lunt and Vander Heiden, 2011). The status and potential role of the pentose phosphate pathway in PAH PASMCs is discussed below. Further down the glycolytic pathway, the intermediate glyceraldehyde-3-phosphate is converted to dihydroxyacetone phosphate, which is the precursor to glycerol-3-phosphate (Lunt and Vander Heiden, 2011). Glycerol-3-phosphate is important for the synthesis of phospholipids and triacylglycerols that constitute major structural lipids in cell membranes (Lunt and Vander Heiden, 2011). Finally, downstream of glyceraldehyde-3-phosphate, the intermediate 3-phosphoglycerate provides the carbons for the synthesis of the amino acids cysteine, glycine and serine (Lunt and Vander Heiden, 2011). Given that proteins comprise around 50% of dry mass in mammalian cells, a large amino acid requirement is imposed on highly proliferative cells to support the generation of daughter cells (Hosios et al., 2016). Therefore, the increased glycolytic flux in PAH PASMCs may also feed into amino acid synthesis pathways to generate sufficient amino acids to support proliferation.

Uptake of the glycolysis substrate glucose is mediated primarily by a family of four membrane proteins, termed glucose transporters (GLUTs). These transporters facilitate the diffusion of glucose across the plasma membrane. Glucose uptake was found to be significantly elevated in PAH lungs primarily due to upregulation of the ubiquitously
expressed glucose transporter GLUT1 (Marsboom et al., 2012; Zhao et al., 2013). Inconsistently with previous findings, the protein levels of GLUT1 in PAH PASMCs were similar to those in control PASMCs. This finding, however, does not necessarily suggest that glucose uptake is reduced in our PAH PASMC isolates. Indeed, the increased glycolytic flux observed suggests otherwise. Increased glucose influx in PAH PASMCs may alternatively be explained by increased trafficking of GLUT1 from intracellular membrane-bound compartments to the plasma membrane. A number of pro-inflammatory cytokines have been shown to increase the activity of GLUT1 and its trafficking to the membrane (Wiemen et al., 2007; Wofford et al., 2008). The elevation of these cytokines in PAH may have a similar effect on GLUT1 in PASMCs. Following cellular uptake, glucose must be phosphorylated to G6P by hexokinases, of which there are four isoforms, to prevent its transport out of the cell and to prime it for entry not only into the glycolytic pathway but also anabolic pathways, such as the pentose phosphate pathway. This chapter explored whether upregulation of hexokinases 1 and 2, the two predominant isozymes, contributes to the increased glycolytic flux observed in PAH PASMCs. Contrary to what was hypothesised, the protein levels of hexokinases 1 and 2 in PAH PASMCs were comparable to those in their control counterparts, suggesting that the first glycolytic step remains unaltered in PAH PASMCs. Interestingly, hexokinases 1 and 2 have been shown to interact with the OMM protein VDAC1, which has been reported to be upregulated in PAH PASMCs in Chapter 4. This interaction with VDAC1 is thought to position these hexokinases near sites of ATP extrusion from the mitochondria, allowing them preferential access to ATP for the phosphorylation of glucose to G6P (Pastorino and Hoek, 2008; Shoshan et al., 2017). The increased coupling between VDAC1 and hexokinases 1 and 2 in PAH PASMCs due to upregulation of the former may promote the phosphorylation of glucose and contribute to the increased glycolytic flux that has been observed.

For glucose to be directed down the glycolytic pathway, G6P must first be converted in a reversible reaction to its isomer F6P, which is then further phosphorylated to F1,6BP by the enzyme PFK1 (Mor et al., 2011). Due to its irreversibility, this step represents the first point of commitment of glucose to the glycolytic pathway (Mor et al., 2011). Moreover, the rate-limiting nature of this PFK1-mediated catalysis renders it an essential metabolic control node for glucose metabolism (Mor et al., 2011). PFK1 is encoded by three distinct genes, each of which give rise to a different isoform. The isoforms include PFKM (muscle), PFKL (liver) and PFKP (platelet), which can assemble into either homotetramers or heterotetramers to form the functional enzyme (Mor et al., 2011). Of the three PFK1 isoforms, only PFKP has been reported to be expressed in PASMCs and to be upregulated by the growth factor TGFβ (Calvier et al., 2017). This chapter sought
to investigate whether the other two PFK1 isoforms PFKL and PFKM are expressed in PASMCs as well as in the layers of distal pulmonary arteries. Immunoblotting revealed considerable PFKL and PFKM protein expression in cultured PASMCs. Moreover, immunohistochemical staining for PFKL and PFKM in lung sections showed expression of both isoforms in all three concentric layers of distal pulmonary arteries as well as in the surrounding pulmonary interstitium. Expression of all three PFK1 isoforms in cultured PASMCs as well as in the medial layers of pulmonary arteries suggests that the enzyme exists as a multitude of diverse homotetrameric and heterotetrameric complexes in PASMCs. Given the increased glycolytic flux observed in PAH PASMCs and the rate-limiting activity of PFK1, it was postulated that the expression of all three PFK1 isoforms might be upregulated in PAH PASMCs. Immunoblotting revealed increased protein levels of PFKL and PFKM but not PFKP. Upregulation of the PFK1 isoforms PFKL and PFKM is likely to increase the rate-determining activity of the PFK1 complex and thus underlie the increased glycolytic influx in PASMCs. Whether the subunit composition of PFK1 has any influence on its activity remains to the best of my knowledge unexplored. Although, a study in dogs has shown that PFK1 activity is greater in the brain, where all three PFK1 isoforms are expressed, than in the liver, which exclusively expresses the PFKL isoform (Kanai et al., 2019).

Interestingly and uniquely among the PFK1 isoforms, PFKL has been shown to assemble into homomeric filaments in close proximity to the plasma membrane, allowing for localised ATP production to support membrane-dependent processes, such as protrusion and endocytosis, and the activity of ATP-dependent ion transporters and channels (Webb et al., 2017). Other glycolytic enzymes, such as pyruvate kinase, have also been shown to localise near the plasma membrane, where they are thought to form a glycolytic metabolon to enable the formation of membrane protrusions (Puchulu-Campanella et al., 2013). Given the high migratory capacity of PAH PASMCs and PFKL’s ability to assemble into filamentous structures, increased PFKL expression concomitant with increased ATP production could be supporting the formation of motile structures, such as lamellipodia, in these cells. Indeed, the number of lamellipodia per cell has been demonstrated to be higher in PAH PASMCs than in their control equivalents (Paulin et al., 2014). Increased PFKL expression in PAH PASMCs may therefore account for their increased migratory potential.

PFK1 is subject to complex allosteric regulation by multiple metabolites, most notably F2,6BP (Mor et al., 2011). Uniquely among the four members of the PFKFB family of bifunctional enzymes, PFKFB3 possesses a higher kinase to phosphatase activity ratio and therefore preferentially converts F6P, generated by the isomerisation of G6P, to
F2,6BP (Mor et al., 2011). F2,6BP is a positive allosteric modulator of the rate-limiting glycolysis enzyme PFK1 (Mor et al., 2011). Increased PFKFB3 expression and activity coupled with increased generation of F2,6BP and consequent enhancement of PFK1 activity was suggested to underlie the increased glycolytic flux in PAH PASMCs. Protein expression of PFKFB3 was found to be considerably higher in cultured PAH PASMCs than in control PASMCs. Consistently, PASMCs within the medial layers of distal pulmonary arteries stained more strongly for PFKFB3 in PAH lung sections than in those derived from control lungs. Similar findings were reported by Kovacs et al. (2019). The authors found that PASMCs from patients with IPAH and rodent models of PAH contained higher levels of the glycolysis regulator PFKFB3 (Kovacs et al., 2019). They also showed that pharmacological inhibition of PFKFB3 with 3PO or its conditional knockout in SMCs attenuated PAH in a monocrotaline-induced rat model or a chronic hypoxia-induced mouse model, respectively, as indicated by inhibition of increases in pulmonary artery wall thickness, right ventricular systolic pressure and right ventricular hypertrophy (Kovacs et al., 2019). The mechanisms that lead to PFKFB3 upregulation in PAH PASMCs are thought to include normoxic activation of HIF1α and upregulated PDGF signalling. Indeed, HIF1α binding sites, termed hypoxia response elements, were identified within the promoter region of the PFKFB3 gene (Obach et al., 2004), allowing transactivation in response to hypoxic or, in the case of PAH PASMCs, normoxic stabilisation of HIF1α. Treatment of control PASMCs with PDGF was found to elevate the protein expression of PFKFB3. Furthermore, inhibition of PFKFB3 with 3PO or shRNA-mediated knockdown reduced PDGF-induced collagen synthesis and proliferation in control PASMCs (Kovacs et al., 2019).

Beyond glycolysis, a non-canonical role for PFKFB3 in regulating cell cycle progression has been described in cancer cells, which may also be extended to PAH PASMCs. Indeed, in addition to stronger expression in PAH, immunohistochemical staining for PFKFB3 in control and PAH lung sections revealed nuclear localisation of PFKFB3 in both PAECs and PASMCs in the intimal and medial layers, respectively, of distal pulmonary arteries. This is consistent with the nuclear localisation and signalling of PFKFB3 observed in HeLa and other cancer cells (Yalcin et al., 2009). Nuclear translocation of PFKFB3 is driven by a C-terminal nuclear localisation signal. In the nucleus, PFKFB3 has been reported to activate CDK1 via the production of its product F2,6BP (Yalcin et al., 2014). Following activation, CDK1 phosphorylates the Cip/Kip protein p27, a potent suppressor of cell cycle progression from the G1 phase to the S phase and activator of apoptosis, targeting it for ubiquitination and subsequent proteasomal degradation (Yalcin et al., 2014). Within the nucleus, PFKFB3 has also been demonstrated to physically interact with CDK4, another cell cycle regulator, to
promote its stability in breast cancer cells (Jia et al., 2018). Disrupting the interaction of PFKFB3 with CDK4 was found to sensitize breast cancer cells to CDK4 inhibition with palbociclib, a drug approved by the FDA for the treatment of advanced breast cancer (Jia et al., 2018). Increased expression and nuclear localization of PFKFB3 in PAH PASMCs may therefore underlie their hyperproliferative and apoptosis-resistant phenotype. Inhibiting PFKFB3 with the use of small-molecule inhibitors or normalizing its expression could prove beneficial for patients with PAH. Since prostacyclin analogues have been demonstrated to promote cell cycle arrest at the G1/S checkpoint in PASMCs (Wharton et al., 2000), adding a PFKFB3 inhibitor to background prostacyclin therapy in PAH patients may also enhance the clinical efficacy of these agents.

In quiescent, non-proliferative cells, PFKFB3 is constantly degraded via ubiquitination mediated by the E3 ubiquitin ligase APC/C complexed with its activator Cdh1 (Almeida et al., 2010). Immediately prior to the G1-to-S transition, the APC/C-Cdh1 complex is inactivated through the phosphorylation-induced release and subsequent degradation of Cdh1 and PFKFB3 levels rise, enhancing glycolysis to support cell proliferation (Almeida et al., 2010). In vitro, starving cultured cells of serum serves to induce cell cycle arrest in the G0/G1 phase and to inhibit progression into the S phase. Despite being serum-starved for 48 hours, PAH PASMCs contained higher protein levels of PFKFB3 than their control counterparts. This suggests that serum-starvation is much less effective at inducing cell cycle arrest in PAH PASMCs than in control PASMCs and that, even under conditions that favour cell quiescence, PAH PASMCs continue to undergo mitosis and proliferate. This also indicates that increased PFKFB3 expression in PAH PASMCs could be a consequence of the higher number of cells undergoing G1-to-S cell cycle transition, in which the activity of APC/C-Cdh1 complex is expected to be low due to the dissociation and degradation of Cdh1.

Pyruvate kinase catalyzes the final, rate-limiting step in glycolysis, which transfers a phosphate group from phosphoenolpyruvate to ADP to produce pyruvate and ATP. In rapidly dividing cells, reduced PK activity and/or expression has been suggested to contribute to the metabolic shift to glycolysis and to shunt the phosphorylated glycolysis intermediates, which accumulate upstream of the PK-catalysed step into branching biosynthetic pathways, most notably the pentose phosphate pathway, to support nucleotide synthesis. There are four isoforms of PK: PKM1, PKM2, PKR and PKL. PKM1 and PKM2 are generated as a result of alternative splicing of the PKM gene, which has 12 exons. Splicing to produce PKM2 requires the repression of exon 9 and inclusion of exon 10, whereas PKM2 is generated by the inclusion of exon 9 and the repression of exon 10. PKM1 exists constitutively in a high-activity tetrameric form. In contrast, PKM2
subunits can assemble into either low-activity dimers or high-activity tetramers. Recently, reduced PKM1 and increased PKM2 expression were reported in PAECs and pulmonary adventitial fibroblasts isolated from PAH patients. Given that pyruvate occupies the junction between glycolysis and mitochondrial respiration, the increase in the PKM2/PKM1 ratio in these cells and the resultant decrease in pyruvate production are thought to uncouple glycolysis from mitochondrial respiration, promoting Warburgian metabolism and cell proliferation. Consistently, knockdown of PKM2 in PAH fibroblasts normalised glycolytic flux, as indicated by reduced production of the glycolysis end product lactic acid. The expression of PKM1 and PKM2 in PASMCs from PAH patients has not been previously assessed, however. This chapter therefore explored whether a similar increase in the PKM2 to PKM1 ratio occurs in PASMCs from PAH patients. Compared to control PASMCs, the protein expression of PKM1 was found to be considerably reduced in PAH PASMCs, whereas that of PKM2 remained unchanged. The ratio of PKM2 to PKM1 was consequently higher in PAH PASMCs than in control PASMCs, analogous to the finding in PAH PAECs and pulmonary adventitial fibroblasts. This suggests that the conversion of phosphoenolpyruvate to pyruvate might be reduced in PAH PASMCs, creating a bottleneck at the final, rate-limiting step in glycolysis. Downregulation of PKM activity, as suggested by the increased PKM2 to PKM1 ratio, coupled with upregulation of glycolytic enzymes (e.g. PFKL and PFKM), which commit glucose to glycolysis, are thought to allow the accumulation of glycolysis intermediates, which then spill over into biosynthetic pathways branching off glycolysis. The resultant increase in the synthesis of nucleotides, amino acids, lipids and other cellular building blocks is thought to support the proliferative and synthetic phenotype of PAH PASMCs.

Despite the reduced PKM activity, as indicated by the increased PKM2/PKM1 ratio, pyruvate can still be generated from phosphoenolpyruvate via an alternate glycolytic pathway. Vander Heiden et al. (2010) demonstrated that phosphoenolpyruvate can donate a phosphate group to the glycolytic enzyme phosphoglycerate mutase, yielding pyruvate but not ATP in the process. They also showed that this reaction can occur at physiological phosphoenolpyruvate concentrations and generate pyruvate in the absence of PKM2 (Heiden et al., 2010). This alternate pathway may be upregulated in PAH PASMCs to compensate for the reduced activity PK activity and maintain the levels of pyruvate. Indeed, the activity of phosphoglycerate mutase was shown to be correlated with the expression of PKM2 in cancer cell lines and tumour tissues (Heiden et al., 2010).

Alternative splicing of the PKM gene into either PKM1 or PKM2 is regulated by a host of splicing factors. To generate PKM2, three splicing factors polypyrimidine tract binding protein (PTBP1), heterogeneous ribonucleoprotein A1 (hnRNPA1) and hnRNPA2 act in
concert to repress the inclusion of exon 9, while the serine/arginine splicing factor 3 (SRSF3) promotes the inclusion of exon 10 in the transcript. The splicing of PKM mRNA to generate PKM1, on the other hand, is poorly understood; although knockdown of the aforementioned splicing factors in cell lines that express PKM2 has been shown to elevate PKM1 transcript production. Increased PTBP1 expression due to decreased production of miR-124 has been reported to underlie the PKM isoform switch from PKM1 to PKM2 in both BOECs and PAFs from PAH patients as well as in the sugen 5416/hypoxia mouse model of PAH (Caruso et al., 2017; Zhang et al., 2017). Upregulation of PTBP1 may similarly underlie the increase in the PKM2 to PKM1 ratio in PAH PASMCs; however, no upregulation of the PKM2 isoform was observed in these cells. This suggests the involvement of factors that exclusively repress PKM1 expression without influencing that of the PKM2 isoform. In PAH PASMCs, the equilibrium between PKM2 dimers and tetramers has been suggested to be tipped in favour of the formation of low-activity dimers. In agreement with this notion, using chemical crosslinking followed by immunoblotting, Rai et al. (2019) reported an increase in the assembly of low-activity PKM2 dimers and a decrease in that of high-activity PKM2 tetramers, suggesting that, although the expression of PKM2 is increased in PAH PASMCs, its glycolytic activity remains low.

Similar to PFKFB3, PKM2 has also been shown to localise to the nucleus, where it is thought to complex with transcription factors and, as a consequence, alter gene expression (Tanaka et al., 2018). Using phosphoenolpyruvate as a phosphate donor, PKM2 has also been reported to phosphorylate histones, particularly histone 3 at threonine 10, to promote the transcription of the cell cycle regulator cyclin D1 amongst other genes (Yang et al., 2012). The resultant cyclin D1 accumulation promotes cell cycle transition from the G1 phase to the S phase (Yang et al., 2012). Nuclear localisation of PKM2 is supported by post-translational phosphorylation of serine, threonine and tyrosine residues (Prakasam et al., 2018). As reported by Rai et al. (2019), in PAH PASMCs, preferential PKM2 dimer formation, facilitated by phosphorylation at tyrosine 105, contributes to the increased nuclear localisation of PKM2, as dimeric PKM2 translocates to the nucleus with more ease than tetrameric PKM2.

Following the final glycolytic step, generated pyruvate can be either transported into the mitochondria, where it partakes in the Krebs cycle, or converted to lactic acid in the cytosol by the enzyme lactate dehydrogenase (LDH; Lunt and Vander Heiden, 2011). LDH exists as a tetramer, composed predominantly of two subunits LDHA and LDHB (Valvona et al., 2016). LDHA has a higher affinity for pyruvate and therefore essentially converts pyruvate to lactate, oxidising NADH to NAD⁺ in the process (Valvona et al.,
LDHB, in contrast, tends to convert lactate back to pyruvate, also reducing \( \text{NAD}^+ \) back to \( \text{NADH} \) (Valvona et al., 2016). Increased LDHA expression coupled with reduced LDHB expression leading to increased lactate production and extrusion were postulated to contribute to the increased ECARs observed in PAH PASMCs. The protein levels of both LDHA and LDHB in PAH PASMCs, however, were found to be similar to those in control PASMCs. Phosphorylation at tyrosine 10, particularly downstream of receptor tyrosine kinases, heightens LDHA activity by promoting the assembly of highly active tetrameric complexes. Given the unaltered protein expression of LDHA in PAH PASMCs, it was speculated that increased phosphorylation of LDHA may alternatively underlie the increased lactate production, indicated by the increased ECARs. LDHA phosphorylation at tyrosine 10 in PAH PASMCs was indistinguishable from that in control PASMCs. LDHA is also regulated by phosphorylation at tyrosine 83 (Fan et al., 2011). Phosphorylation at tyrosine 83 enhances LDHA activity by increasing LDHA affinity for the cofactor \( \text{NADH} \) (Wiese and Hitosugi, 2018). Hyper-phosphorylation of LDHA at tyrosine 83 may explain the increased lactate production in PAH PASMCs and requires further investigation.

Despite the unaltered expression and post-translational modification of LDHA, glycolytic flux to lactate remains to be high in PAH PASMCs, as indicated by the increased ECARs. Conversion of pyruvate to lactate is accompanied by the oxidation of \( \text{NADH} \) to \( \text{NAD}^+ \). \( \text{NAD}^+ \) serves as a cofactor for a crucial step in glycolysis, the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate (Lunt and Vander Heiden, 2011). \( \text{NAD}^+ \) is also required for the synthesis of nucleotides and amino acids by pathways, which branch off glycolysis and incorporate glucose metabolites into cellular biomass (Lunt and Vander Heiden, 2011). Consistent with the role of lactate production in supporting biosynthesis through the generation of \( \text{NAD}^+ \), LDHA inhibition in melanoma cells has been shown to reduce the levels of the amino acids serine and aspartate, the synthesis of which requires \( \text{NAD}^+ \) (Pathria et al., 2018). However, LDHA inhibition had no effect on the proliferation of melanoma cells, suggesting that LDHA activity may be important during the S phase of the cell cycle but not subsequent phases. Consistent with this notion, overexpression of LDHA in rat pituitary tumour or GH3 cells induced S phase transition of the cell cycle (An et al., 2017). Consequently, to maintain increased glycolytic flux in PAH PASMCs and support the branching biosynthetic pathways, \( \text{NAD}^+ \) regeneration must also be upregulated to maintain the \( \text{NAD}^+ / \text{NADH} \) redox balance in these cells. Interestingly, \( \text{NAD}^+ \) is not only required for cytosolic glycolytic and anabolic reactions but also for several steps in the mitochondrial TCA cycle. The increased demand for \( \text{NAD}^+ \) is therefore likely to underlie the increased lactate production in PAH PASMCs.
G6P can be either directed further down the glycolytic pathway to ultimately yield pyruvate or shunted into the pentose phosphate pathway to generate the nucleotide precursor ribose-5-phosphate and the reducing agent NADPH (Lunt and Vander Heiden, 2011). The first, rate-determining step in the PPP is the conversion of glucose-6-phosphate to 6-phosphogluconolactone by the enzyme G6PD (Lunt and Vander Heiden, 2011). Cell proliferation requires the production of nucleotides to support the generation of daughter cells. Since PAH PASMCs proliferate at much higher rates than control PASMCs (Falcetti et al., 2010), it was postulated that the PPP might be upregulated in these cells to meet the increased demand for nucleotides. In support of this hypothesis, PAH PASMCs were found to contain higher protein levels of the enzyme G6PD. Moreover, immunohistochemical staining revealed higher G6PD expression in all three layers of distal pulmonary arteries in PAH lung sections compared to controls. Given that G6PD activity critically determines the rate of the PPP, particularly the oxidative branch, these findings suggest that the PPP is significantly upregulated in PAH PASMCs. The mechanisms underlying the upregulation of G6PD in PAH PASMCs remain elusive. Normoxic stabilisation of HIF1α in PAH could underlie the increased expression of G6PD. Indeed, chronic hypoxia and its chemical mimic cobalt chloride were both individually found to induce the expression of G6PD in PC12 cells (Gao et al., 2004). Increased G6PD expression and activity in PAH PASMCs are also thought to promote oxidative stress by increasing superoxide production. Particularly, NADPH derived from G6PD is proposed to fuel NOX, which in turn produces superoxide radicals. Interestingly, several NOX isoforms, particularly NOX4, have been shown to be upregulated in PAH PASMCs (Mittal et al., 2007). Together with the increased expression of the NADPH-dependent ROS generator NOX, heightened expression of G6PD is believed to further promote ROS production and oxidative stress in PAH PASMCs via the production of NADPH. Immunohistochemical staining for G6PD in PAH lung tissue sections showed strong nuclear localisation in both PAECs and PASMCs. G6PD has previously been reported to localise to the nuclei of hepatocytes, where it is believed to contribute to intranuclear NADPH production. Moreover, the nucleus has long been known to generate ROS and the ROS generator NOX4 has also been shown to localise to hepatic nuclei (Spencer et al., 2011). It would therefore be reasonable to speculate that nuclear G6PD through the production of NADPH may also promote NOX4-mediated ROS generation in the nuclei of PAH PASMCs, possibly leading to the DNA damage that was reported in these cells (Meloche et al., 2014). NADPH also serves as a cofactor for glutathione reductase (GR), which catalyses the reduction of glutathione disulphide (GSSG), the oxidised form of glutathione, to reduced glutathione (GSH; Mailloux et al., 2013). With the aid of glutathione peroxidase (GPX), GSH scavenges and detoxifies
ROS and is converted back to GSSG in the process (Mailloux et al., 2013). In PAH PASMCs, GR could be downregulated, causing NADPH to accumulate and to be used for the generation of ROS via NOX. However, the activity and expression of GR in PAH PASMCs has yet to be investigated. The levels of GSH were found to be similar in the lungs of control subjects and PAH patient, suggesting that the activity of GR remains intact in PAH (Masri et al., 2008). The activity of GPX has been demonstrated to be reduced in the lungs of PAH patients compared to controls, implying that the conversion of GSH back to GSSG, which is accompanied by the reduction of ROS, is reduced in the disease (Masri et al., 2008). Reduced availability of GSSG could be causing NADPH to alternatively serve a pro-oxidant role via NOX.

NADPH derived from G6PD is also heavily used as a cofactor in lipid synthesis. For example, generating palmitate, the most abundant fatty acid in humans, claims 14 NADPH molecules (Lunt and Vander Heiden, 2011). Moreover, cholesterol synthesis involves 26 molecules of NADPH (Lunt and Vander Heiden, 2011). Highly proliferating cells, such as PAH PASMCs, require large amounts of lipids and cholesterol for incorporation into membranes of daughter cells (Lunt and Vander Heiden, 2011; Singh et al., 2016). Indeed, hypoxia was reported to increase the expression of the palmitate-generating enzyme fatty acid synthase (FAS) and the levels of palmitate in human PASMCs (Singh, 2016). Knockdown of FAS by RNA interference inhibited hypoxia-induced proliferation in these cells, indicating the important role of FAS and its product palmitate in supporting pathological PASMC proliferation (Singh, 2016). Furthermore, in vivo inhibition of FAS with the small-molecule inhibitor C75 was shown to attenuate monocrotaline-induced PH in rats (Singh, 2016). Increased G6PD expression concomitant with increased NADPH production may therefore potentially serve to support this increased demand for lipids by highly proliferative PAH PASMCs.

In conclusion, evidence in support of increased glucose flux through glycolysis in PAH PASMCs was provided in this chapter. Several mechanisms underlying this increased glycolytic flux have also been unravelled. This chapter reported an increase in the protein expression of PFKL and PFKM, isoforms of the rate-limiting glycolytic enzyme PFK1, in cultured PAH PASMCs as well as in PASMCs within the medial layers of pulmonary arteries from PAH patients. Moreover, the expression of the enzyme PFKFB3, which generates the PFK1 positive allosteric modulator F2,6BP, was found to be increased in PAH PASMCs both in vitro and in situ, suggesting increased positive allosterism of PFK1 in PAH. Activity of PKM, which catalyses the final pyruvate-generating step in glycolysis, was found to be reduced in PAH as indicated by an increase in the ratio of PKM2 to PKM1. This is thought to lead to the accumulation of glycolysis intermediates and their
spillover into biosynthetic pathways, such as the PPP. Interestingly, G6PD, the rate-limiting enzyme in the PPP pathways, was found to be upregulated in cultured PAH PASMCs as well PASMCs in the medial layers of pulmonary arteries in PAH lung sections, implying increased glucose shunting into the PPP pathway in PAH.
5. Mitochondrial respiration, biogenesis and mitophagy in PAH PASMCs

5.1 Introduction

Mitochondrial ATP production occurs via OxPhos, a process in which ATP is formed as a result of electron transfer from NADH or FADH$_2$, products of the TCA cycle, to diatomic oxygen by a series of electron transporters or carriers. Compared to glycolysis, which produces 2 ATP molecules per molecule of glucose, OxPhos is highly efficient at producing ATP, generating up to 36 molecules of ATP per molecule of glucose (Lunt and Vander Heiden, 2011). OxPhos takes place in the infoldings of the IMM or cristae, where the complexes of the ETC (complexes I-IV) and the F$_1$F$_0$-ATP synthase (complex V) reside (Chaban et al., 2014). Briefly, the flow of electrons along complexes I-IV leads to the pumping of protons out of the mitochondrial matrix and into the mitochondrial intermembrane space (Chaban et al., 2014). The resultant proton gradient fuels the F$_1$F$_0$ ATP synthase to phosphorylate ADP to ATP (Chaban et al., 2014). Complexes I and III are also major sources of mitochondrial ROS and ROS can be formed during OxPhos (Chen et al., 2003; Mailloux et al., 2013). Electron leak from these complexes can partially oxidise oxygen to generate superoxide (Chen et al., 2013). This is normally scavenged by the mitochondrial superoxide dismutase SOD2, which converts superoxide to hydrogen peroxide (Chen et al., 2013).

Several studies have shown that mitochondrial OxPhos is suppressed in PASMCs and other pulmonary vascular cells from patients with PAH and that the consequential decrease in mitochondrial ATP production upregulates cytosolic glycolysis to allow for compensatory ATP synthesis (Paulin and Michelakis et al., 2014). This metabolic reprogramming in PAH PASMCs is analogous to Warburgian metabolism in cancer cells and is thought to underlie the hyper-proliferative and apoptosis-resistant phenotype of PAH PASMCs. However, certain tumour cells have recently been reported to increase glucose oxidation, challenging the notion that tumours switch from mitochondrial OxPhos to aerobic glycolysis to support their growth and survival (Gentric et al., 2017; Sica et al., 2020). As discussed in the previous chapter, highly proliferative cells, such as PAH PASMCs and cancer cells, are thought to upregulate glycolysis not only to compensate for the depressed mitochondrial respiration but to reinforce cytosolic biosynthetic pathways that support the generation of daughter cells. Mitochondria are also considered...
to constitute the major source of cellular ROS and a shift from oxygen consumption for ATP production to oxygen consumption for ROS generation may account for the increased ROS generation previously described in pulmonary vascular cells in PAH.

Central to this shift from OxPhos to glycolysis is the inhibition of the PDH enzyme complex, particularly the PDHE1α subunit (Culley and Chan, 2018; Paulin and Michelakis, 2014). Several mechanisms have been described to underlie the inhibition of PDH in PAH. PDHE1α has been shown to be hyper-phosphorylated at the inhibitory site S293 in the lungs of patients with PAH as a result of increased expression and kinase activity of the serine/threonine kinases PDK1 and PDK2 (Michelakis et al., 2017). PDK4 has also been shown to be upregulated in PAH, particularly in pericytes of small pulmonary arteries (Yuan et al., 2016). Furthermore, PDH is a calcium-dependent enzyme and downregulation of UCP2, which mediates calcium entry into the mitochondria, in PAH, may also contribute to PDH inhibition by decreasing the levels of mitochondrial calcium (Pak et al., 2013). Finally, reduced activity of sirtuin 3, which activates several mitochondrial enzymes including PDH, has been reported in the lungs of PAH patients and may also lead to PDH inhibition (Paulin et al., 2014). Within the mitochondrial matrix, PDH catalyses the conversion of pyruvate, the final product of glycolysis, to acetyl-CoA, which is then fed into the TCA cycle. The TCA cycle precedes oxidative phosphorylation and its main purpose is to produce NADH and FADH₂, both of which donate energy-rich electrons to the mitochondrial ETC to fuel oxidative phosphorylation and drive ATP production. In addition to reduced pyruvate to acetyl-CoA conversion, pyruvate transport into the mitochondria by MPCs, particularly MPC1, was found to be downregulated in PAH (Zhang et al., 2017).

Mitochondrial biogenesis is the term given to the process whereby cells add new mitochondrial mass, namely proteins and lipids, to the pre-existing mitochondrial reticulum (Hock and Kralli, 2009). It is driven predominantly by PGC1α, which lacks DNA-binding activity but serves as a coactivator for a number of transcription factors (Hock and Kralli, 2009; Jornayvaz and Shulman, 2010). These transcription factors include NRF1 and NRF2, both of which regulate the expression of nuclear-encoded mitochondrial proteins (Hock and Kralli, 2009; Jornayvaz and Shulman, 2010). NRF1 and NRF2 also regulate the transcription of TFAM, which is a key inducer of mitochondrial DNA replication and transcription (Hock and Kralli, 2009; Jornayvaz and Shulman, 2010). The depressed mitochondrial function in PAH suggests that mitochondrial biogenesis may be downregulated in the disease. The expression of the master regulator of mitochondrial biogenesis PGC1α has been shown to be reduced in the lungs of patients with PAH, supporting the idea that reduced mitochondrial mass may
underlie the depressed mitochondrial respiration observed in PAH (Ryan et al., 2013). The expression of other markers of mitochondrial biogenesis, such as the transcription factors NRF1, NRF2 and TFAM, have yet to be examined in PAH patients. Moreover, the reduced PGC1α expression in PAH has been suggested to be a consequence of reduced PPARγ expression (Yeligar et al., 2018). However, PPARγ has also been shown to be upregulated in PAH (Falcetti et al., 2010). Furthermore, mitochondrial mass, which is a critical determinant of mitochondrial function, has not been thoroughly assessed in pulmonary vascular cells from PAH patients.

Mitochondrial mass is also governed by the process of mitophagy, the antithesis of mitochondrial biogenesis (Pickles et al., 2018). Mitophagy describes the quality control mechanism employed by cells to eliminate superfluous or damaged mitochondria (Ashrafi and Schwarz, 2013; Pickles et al., 2018). It occurs when mitochondrial fragments of sufficient size are tagged and subsequently recognised by autophagy receptors within the membranes of autophagosomes (Pickles et al., 2018). Autophagosomes then engulf the mitochondria and deliver their cargoes to lysosomes, where they are degraded by hydrolytic enzymes, including proteases and lipases (Pickles et al., 2018). The most studied pathway of mitophagy is mediated by PINK1 and parkin (Pickles et al., 2018). It is triggered by depolarisation of the mitochondrial membrane potential and the consequent stabilisation and accumulation of PINK1 in the OMM (Pickles et al., 2018; Youle and Narendra, 2011). PINK1 then recruits the E3 ubiquitin ligase parkin, which via ubiquitination of OMM proteins, renders the mitochondria substrates for mitophagy (Pickles et al., 2018). A role for mitophagy in PAH pathogenesis has yet to be fully established. Although endothelial cell-specific knockout of UCP2 in mice, which gives rise to PH, has been shown to promote mitophagy, as suggested by increased protein levels of the mitophagy mediator PINK1, increased LC3B1-to-LC3BII conversion and decreased mitochondrial biogenesis (Haslip et al., 2015). Increased mitophagy in UCP2-null PAECs was also associated with increased apoptosis, which is considered an early triggering event in PAH (Haslip et al., 2015). In addition to decreased mitochondrial biogenesis, increased mitophagy concomitant with decreased mitochondrial mass may also contribute to the diminished mitochondrial respiration that was reported in patients with PAH.

Oxidative stress describes a state wherein excess ROS (e.g. superoxide) formation overwhelms antioxidant systems, tipping the redox state of the cell in favour of oxidation (DeMarco et al., 2010). Oxidative stress occurs as a result of increased ROS production and/or reduced capacity of antioxidant systems. ROS can cause direct damage to cells by irreversibly oxidising biomolecules, such as lipids, proteins and nucleic acids. ROS
can be generated either in the cytosol by the action of oxidases, such as NOX, XO, uncoupled NOS, COX and LO, or in the mitochondria by complexes I and III of the ETC. Several studies have implicated increased ROS generation in the pathogenesis of PAH and a number of mechanisms have been proposed to underlie this increase in ROS production (Aggarwal et al., 2013). The NOX isoforms NOX1 and NOX4 were both reported to be upregulated in pulmonary arteries and PASMCs isolated from animal models of PH as well as patients with PAH (DeMarco et al., 2010). XO activity was also found to be heightened in patients with IPAH (DeMarco et al., 2010). Uncoupling of eNOS or a shift from NO production to ROS generation was also reported in PAH and suggested to occur as a result of reduced L-arginine availability, increased levels of ADMA and/or oxidative loss of the NOS cofactor tetrahydrobiopterin (Gielis et al., 2011). Mitochondrial dysfunction is also thought to contribute to oxidative stress in PAH. However, the role of mitochondrial ROS in PAH remains controversial, with conflicting results reporting an increase or a decrease in mitochondrial ROS (Culley and Chan, 2018). Supporting increased mitochondrial ROS generation in PAH is the finding that SOD2, the role of which is to dismutate superoxide to hydrogen peroxide in the mitochondria, is downregulated in PAH pulmonary arteries (Archer et al., 2010).

This chapter sought to assess key parameters of mitochondrial respiration in PASMCs, isolated from the distal pulmonary arteries of control subjects and patients with PAH, and to unravel the mechanisms underlying any changes observed. Given that mitochondrial mass is a critical determinant of mitochondrial function, the protein levels of markers of mitochondrial mass, including constituent subunits of the ETC complexes and other mitochondrial proteins, were determined in control and PAH PASMCs. Lung sections from control and PAH patients were stained for mitochondrial proteins to assess mitochondrial mass in PASMCs in situ. The protein expression of transcriptional regulators of mitochondrial biogenesis, namely PGC1α, NRF1, NRF2 and TFAM, was also interrogated in control and PAH PASMCs. Lung sections from control subjects and PAH patients were also stained for the master regulator of mitochondrial biogenesis PGC1α. PDH is a multisubunit complex, the main function of which is to convert pyruvate to acetyl-CoA in the mitochondria. Acetyl-CoA is then incorporated into the TCA cycle to generate NADH and FADH₂, which donate high-energy electrons to the ETC to fuel OxPhos. This chapter therefore examined the protein expression and inhibitory phosphorylation of PDHE1α as well as the expression of PDHE1β. Mitochondria are considered to be the primary source of cellular ROS. The levels of ROS were investigated in control and PAH PASMCs in an attempt to correlate ROS production with mitochondrial oxygen consumption. SOD2 scavenges ROS generated within the mitochondria by catalysing the conversion of ROS to hydrogen peroxide. Hydrogen
peroxide is then converted to water and oxygen by the enzyme catalase. This ROS scavenging system was assessed in control and PAH PASMCs to uncover any defects in PAH. Mitophagy opposes the process of mitochondrial biogenesis and remains to be examined in PAH PASMCs. Mitophagic flux was therefore assessed in control and PAH PASMCs. Given that mitophagy is a selective form of autophagy, the expression of markers of autophagy was explored in control and PAH PASMCs.
5.2 Results

5.2.1 Mitochondrial respiration in control and PAH PASMCs

Several lines of evidence suggest that mitochondrial respiration is depressed in PAH PASMCs, primarily as consequence of inhibited PDH activity. To confirm this finding, the Seahorse XFp Mito Stress Test was performed to measure oxygen consumption rates (OCRs) in quiescent control and PAH PASMCs and assess key parameters of mitochondrial function, including basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity and non-mitochondrial respiration. Strikingly and contrary to previous evidence, PAH PASMCs generally exhibited higher OCRs than control PASMCs (Figure 5.1A). Basally, PAH PASMCs appeared to consume oxygen at a higher rate than their control counterparts, as indicated by a two-fold increase in basal respiration ($p = 0.0031$; $n = 4$ for control PASMCs; $n = 6$ for PAH PASMCs; Figure 5.1B). Injection of oligomycin A, which inhibits complex V or the ATP synthase of the mitochondrial ETC, resulted in a larger dip in OCR in PAH PASMCs than in control PASMCs. Indeed, OCR associated with ATP synthesis or production was four-fold higher in PAH PASMCs than in control PASMCs ($p = 0.0293$; $n = 4$ for control PASMCs; $n = 6$ for PAH PASMCs; Figure 5.1C). Injection of the uncoupler FCCP, which induces maximal OCR, elevated OCR beyond that observed basally in control PASMCs. In PAH PASMCs, on the other hand, following the dip caused by oligomycin A, FCCP restored OCR to levels measured prior to the addition of oligomycin A. Consistently, OCR associated with maximal respiration in PAH PASMCs was similar to that measured in control PASMCs (Figure 5.1D). This finding contradicts previous studies, which showed that maximal respiration is decreased in PAH PASMCs. Moreover, as shown in Figure 5.1E, the spare respiratory capacity was three-fold lower in PAH PASMCs than in their control counterparts ($p = 0.0007$; $n = 4$ for control PASMCs; $n = 6$ for PAH PASMCs). Following FCCP-induced maximal OCR, injection of rotenone and antimycin A, inhibitors of complex I and III, respectively, completely inhibit mitochondrial OCR, revealing that associated with non-mitochondrial respiration. As shown in Figure 5.1F, OCR associated with non-mitochondrial respiration was two-fold higher in PAH PASMCs than in control PASMCs ($p = 0.0308$; $n = 4$ for control PASMCs; $n = 6$ for PAH PASMCs). Finally, a trend towards an increase in proton leak-associated OCR was observed in PAH PASMCs when compared to control PASMCs (Figure 5.1G).
Figure 5.1: Mitochondrial respiration is increased in PAH PASMCs compared to control PASMCs. Control (n = 4) and PAH (n = 6) PASMCs were seeded at a density of 7,500 cells per well in Seahorse XFp Cell Culture Miniplates and maintained in 10% FBS overnight. PASMCs were then serum-starved in 0.1% FBS for 48 hours and transferred to Mito Stress Test medium. (A) OCRs were measured in quiescent control and PAH PASMCs using the Seahorse XFp Analyser. Oligomycin A, FCCP and a combination of rotenone and antimycin A were serially injected as part of the Seahorse XFp Mito Stress Test to assess key parameters of mitochondrial function, including basal respiration (B), ATP production (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial respiration (F), and proton leak (G).
(E), non-mitochondrial respiration (F) and proton leak (G). Values are means ± SEM. Unpaired student’s t-test was used in B, C, D, E, F and G. \( p < 0.05 \) was considered statistically significant.

5.2.2 Mitochondrial mass in control and PAH PASMCs

Mitochondrial mass is a critical determinant of mitochondrial function. Given the increase in mitochondrial function in PAH PASMCs, it was postulated that increased mitochondrial mass might underlie the increased OCRs observed, particularly those associated with basal respiration and ATP production. To assess mitochondrial mass, proteins from quiescent and PAH PASMCs were immunoblotted for a number of mitochondrial proteins, namely constituent subunits of the five complexes of the mitochondrial ETC (Figure 5.2A). As shown in Figure 5.2, the relative protein expression of select subunits of four complexes of the mitochondrial ETC were markedly increased in PAH PASMCs compared to their control counterparts. The protein levels of NDUFV1, a subunit of complex I, was four-fold higher in PAH PASMCs than in control PASMCs (\( p = 0.0087; n = 3 \) for control PASMCs; \( n = 6 \) for PAH PASMCs; Figure 5.2B). The relative protein expression of SDHA, a subunit of complex II, was three-fold higher in PAH PASMCs than in control PASMCs (\( p = 0.0015; n = 3 \) for control PASMCs; \( n = 6 \) for PAH PASMCs; Figure 5.2C). Moreover, the protein levels of SDHB, another subunit of complex II, was two-fold higher in PAH PASMCs than in control PASMCs (\( p = 0.0476; n = 3 \) for control PASMCs; \( n = 6 \) for PAH PASMCs; Figure 5.2D). The relative protein expression of MTCO2, a constituent subunit of complex IV, was found to be four-fold higher in PAH PASMCs than in their control equivalents (\( p = 0.0123; n = 3 \) for control PASMCs; \( n = 6 \) for PAH PASMCs; Figure 5.2E). Finally, the protein levels of ATP5A, a subunit of the ATP synthase or complex V, relative to GAPDH were slightly, but significantly, higher in PAH PASMCs than in control PASMCs (\( p = 0.0346; n = 3 \) for control PASMCs; \( n = 6 \) for PAH PASMCs; Figure 5.2F). Taken together, these results indicate an increase in total mitochondrial mass in PAH PASMCs compared to control PASMCs.
Figure 5.2: Protein expression of constituent subunits of the respiratory chain complexes in control and PAH PASMCs. Control (n = 3) and PAH (n = 6) PASMCs were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. 

(A) Proteins were isolated from quiescent control and PAH PASMCs and immunoblotted for the mitochondrial proteins NDUFV1 (subunit of complex I), SDHA (subunit of complex II), SDHB (subunit of complex II), MTCO2 (subunit of complex IV) and ATP5A (subunit of complex V). Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to determine the levels of NDUFV1 (B), SDHA (C), SDHB (D), MTCO2 (E) and ATP5A (F) relative to the loading control GAPDH in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in B, C, D, E and F. p < 0.05 was considered statistically significant.

Immunohistochemical staining for SDHA, a subunit of complex II of the mitochondrial ETC, revealed stronger protein expression in the medial layers of distal pulmonary
arteries in PAH lung sections, consistent with the increased SDHA protein expression in cultured PAH PASMCs. Moreover, SDHA staining was also stronger in the PAECs, which make up the innermost lining of the arteries, in PAH lung sections. The outermost adventitial layers of the arteries, comprised mainly of fibroblasts and loose connective tissue, also stained more strongly for SDHA in PAH lung sections than in the control ones.

**Figure 5.3: Immunohistochemical staining for SDHA in control and PAH lung sections.** Formalin-fixed, paraffin-embedded lung sections from control (A; n = 3) and PAH (B; n = 3) patients were stained for SDHA using immunohistochemistry. (C) Lung sections were also stained with normal mouse IgG to confirm specificity of the SDHA staining. Sections were counterstained with the nuclear stain Meyer’s haematoxylin.

High resolution images were acquired using the Nanozoomer Digital Scanner. As indicated, pulmonary arteries were stratified into 3 groups according to vessel diameter. To further confirm the increase in mitochondrial mass in PAH PASMCs, proteins isolated from quiescent control and PAH PASMCs were also immunoblotted for mitochondrial proteins other than those of the mitochondrial ETC, including TOM20, VDAC1 and cytochrome C (Figure 5.4). TOM20 spans the OMM, as its name suggests, and
mediates the import of proteins into the mitochondria. As shown in Figure 5.4B, the protein levels of TOM20 relative to GAPDH were two-fold higher in PAH PASMCs than in control PASMCs \( (p = 0.0253; n = 3 \text{ for control PASMCs}; n = 6 \text{ for PAH PASMCs}) \).

VDAC1 is the most abundant protein on the OMM, where it mediates the passage of metabolites, nucleotides and ions. It also plays a role in apoptosis as evidenced by its interaction with pro-apoptotic and anti-apoptotic proteins. The protein levels of VDAC1 relative to GAPDH were found to be three-fold higher in PAH PASMCs than in control PASMCs \( (p = 0.0075; n = 3 \text{ for control PASMCs}; n = 6 \text{ for PAH PASMCs}; \text{Figure 5.4C}) \).

Cytochrome c is a small, soluble protein that is localised to the space between the OMM and the IMM. Cytochrome c transfers electrons from complex III to IV of the ETC and its release from the mitochondria triggers the intrinsic pathway of apoptosis. The protein expression of cytochrome c relative to GAPDH was two-fold higher in PAH PASMCs than in their control counterparts \( (p = 0.0018; n = 3 \text{ for control PASMCs}; n = 6 \text{ for PAH PASMCs}; \text{Figure 5.4D}) \). The increased protein expression of TOM20, VDAC1 and cytochrome provides additional evidence that mitochondrial mass is increased in PAH PASMCs.

Mitochondrial mass in the medial layers of distal pulmonary arteries in control and PAH lung sections was further assessed by staining for the mitochondrial protein TOM20. Similar to SDHA, medial layers of distal pulmonary arteries in PAH lung sections stained more strongly for TOM20 than those in control lung sections (Figure 5.5). In addition, PAECs, which comprise the innermost lining, were found to stain more strongly for TOM20 in PAH lung sections than in control lung sections.
Figure 5.4: Expression of mitochondrial proteins other than those of the mitochondrial ETC. Control (n = 3) and PAH (n = 6) PASMCs were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. (A) Proteins were isolated from quiescent control and PAH PASMCs and immunoblotted for the mitochondrial proteins TOM20, VDAC1 and cytochrome C. Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to determine the levels of TOM20 (B), VDAC1 (C) and cytochrome C (D) relative to the loading control GAPDH in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in B, C, and D. p < 0.05 was considered statistically significant.
Figure 5.5: Immunohistochemical staining for TOM20 in control and PAH lung sections. Formalin-fixed, paraffin-embedded lung sections from control (A; $n = 3$) and PAH (B; $n = 3$) patients were stained for TOM20 using immunohistochemistry. (C) Lung sections were also stained with normal mouse IgG to confirm specificity of the TOM20 staining. Sections were counterstained with the nuclear stain Meyer’s haematoxylin. High resolution images were acquired using the Nanozoomer Digital Scanner. As indicated, pulmonary arteries were stratified into 3 groups according to vessel diameter.
5.2.3 Protein expression of transcriptional regulators of mitochondrial biogenesis in control and PAH PASMCs

Mitochondrial mass is determined by the balance between mitochondrial biogenesis and mitochondrial degradation or mitophagy (Hock and Kralli, 2009). Mitochondrial biogenesis is the process by which cells increase their mitochondrial mass (Hock and Kralli, 2009). It is regulated predominantly by the transcriptional coactivator PGC1α (Hock and Kralli, 2009). PGC1α induces the transcription of and coactivates the transcription factors NRF1 and NRF2, both of which regulate the transcription of nuclear-encoded mitochondrial genes, and TFAM, which drives the replication of mtDNA and the transcription of genes within the mitochondrial genome (Hock and Kralli, 2009). PGC1α, NRF1, NRF2 and TFAM therefore constitute markers of mitochondrial biogenesis. It was speculated that the increased mitochondrial mass in PAH PASMCs might be a consequence of upregulated mitochondrial biogenesis. Therefore, proteins from quiescent control and PAH PASMCs were immunoblotted for the aforementioned markers of mitochondrial biogenesis. As shown in Figure 5.6, immunoblotting revealed a significant increase in the relative protein expression of all four markers in PAH PASMCs, consistent with the increased protein levels of mitochondrial proteins (Figure 5.2 and Figure 5.4). The protein levels of PGC1α were approximately two-fold higher in PAH PASMCs than in control PASMCs ($p = 0.0117; n = 3$ for control PASMCs; $n = 6$ for PAH PASMCs; Figure 5.6B). Protein expression of NRF1 was five-fold higher in PAH PASMCs than in their control counterparts ($p = 0.0422; n = 3$ for control PASMCs; $n = 5$ for PAH PASMCs; Figure 5.6D). Relative NRF2 protein levels were also seven-fold higher in PAH PASMCs than in control PASMCs ($p = 0.0373; n = 3$ for control PASMCs; $n = 5$ for PAH PASMCs; Figure 5.6E). Finally, protein expression of TFAM relative to GAPDH was two-fold higher in PAH PASMCs than in control PASMCs ($p = 0.0253; n = 3$ for control PASMCs; $n = 6$ for PAH PASMCs; Figure 5.6C). Collectively, these results suggest that mitochondrial biogenesis is increased in PAH PASMCs compared to control PASMCs.
Figure 5.6: Protein expression of the mitochondrial biogenesis markers PGC1α, NRF1, NRF2 and TFAM in control and PAH PASMCs. Control (n = 3) and PAH (n = 6) PASMCs were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. (A) Proteins were isolated from quiescent control and PAH PASMCs and immunoblotted for PGC1α, NRF1, NRF2 and TFAM. Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to determine the levels of PGC1α (B), NRF1 (C), NRF2 (D) and TFAM (E) relative to the loading control GAPDH. Values are means ± SEM. Unpaired student’s t-test was used in B, C, D and E. p < 0.05 was considered statistically significant.

As illustrated in Figure 5.7, Immunohistochemical staining for PGC1α in lung sections from control subjects (Figure 5.7A) and PAH patients (Figure 5.7B) revealed stronger PGC1α expression in the medial layers of distal pulmonary arteries in PAH lung sections than in those in control lung sections. Furthermore, PAECs, which comprise the innermost lining of the artery, stained more strongly for PGC1α in the PAH lung sections than in the control ones. The difference in PGC1α staining was most evident in pulmonary arteries with diameters ranging between 0 and 50 μm.
Figure 5.7: Immunohistochemical staining for PGC1α in control and PAH lung sections. Formalin-fixed, paraffin-embedded lung sections from control (A; n = 3) and PAH (B; n = 3) patients were stained for PGC1α using immunohistochemistry. (C) Lung sections were also stained with normal rabbit IgG to confirm specificity of the PGC1α staining. Sections were counterstained with the nuclear stain Meyer’s haematoxylin. High resolution images were acquired using the Nanozoomer Digital Scanner. As indicated, pulmonary arteries were stratified into 3 groups according to vessel diameter.
5.2.4 Protein expression of PDHE1α and PDHE1β and inhibitory phosphorylation of PDHE1α in control and PAH PASMCs

PDH is a mitochondrial multienzyme complex, the subunits of which are encoded by the nuclear genome. PDH catalyses the conversion of pyruvate to acetyl-CoA, providing the primary link between cytosolic glycolysis and the mitochondrial TCA cycle. PDH is composed of three enzymatic components: pyruvate dehydrogenase (PDHE1), dihydrolipoamide acetyltransferase (PDHE2) and dihydrolipoamide dehydrogenase (PDHE3; Patel et al., 2014). PDHE1 exists as a heterotetramer of two α subunits (PDHE1α) and two β subunits (PDHE1β). Given the increase in mitochondrial function in PAH PASMCs, it was postulated that increased expression of both PDHE1 subunits might underlie this increased mitochondrial function. Indeed, as shown in Figure 5.6B, immunoblotting of PDHE1α in quiescent control and PAH PASMCs revealed approximately an eight-fold increase in the protein levels of PDHE1α relative to GAPDH in PAH PASMCs compared to control PASMCs (p = 0.0487; n = 3 for control PASMCs; n = 5 for PAH PASMCs). Protein expression of PDHE1β relative to GAPDH was also found to be ten-fold higher in PAH PASMCs than in control PASMCs (Figure 5.6E; p = 0.0046; n = 3 for control PASMCs; n = 5 for PAH PASMCs). Inhibitory phosphorylation of PDHE1α was shown to be increased in the lungs of patients with PAH and proposed to underlie the Warburgian shift from mitochondrial respiration to glycolysis that was previously observed in the disease (Michelakis et al., 2017). However, given the increase in mitochondrial respiration reported in this chapter, it was necessary to reassess the levels of phosphorylated PDHE1α relative to total PDHE1α in control and PAH PASMCs. Despite a non-statistically significant increase in the levels of pPDHE1α relative to GAPDH in PAH PASMCs (Figure 5.8C), the ratio of pPDHE1αS293 to total PDHE1α was two-fold lower in PAH PASMCs than in controls (Figure 5.8E). This reduction in phosphorylation is likely to result from the increase in the relative proteins levels of PDHE1α.
Figure 5.8: Protein expression of the PDHE1 subunits PDHE1α and PDHE1β in control and PAH PASMCs. Control (n = 3) and PAH (n = 5 - 6) PASMCs were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. (A) Proteins were isolated from quiescent control and PAH PASMCs and immunoblotted for PDHE1α, pPDHE1α\textsubscript{S293} and PDHE1β. Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to determine the levels of PDHE1α (B) pPDHE1α\textsubscript{S293} (C) and PDHE1β (C) relative to the loading control GAPDH in control and PAH PASMCs. (D) Ratios of pPDHE1α\textsubscript{S293} to PDHE1α were also determined in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in B and C. p < 0.05 was considered statistically significant.
5.2.4 Protein expression of PDK1 and PDK4 in control and PAH PASMCs

Increased expression of the serine/threonine kinase PDK, particularly the PDK1 and PDK2 isoforms, coupled with increased inhibitory phosphorylation of PDHE1α at S293 has been reported in the lungs of patients with PAH (Michelakis et al., 2017). Upregulated PDK4 expression has also been demonstrated in pericytes in small pulmonary arteries in PAH (Yuan et al., 2016). However, given the finding that suggests PDHE1α phosphorylation at S293 might not be altered in PAH PASMCs, the protein expression of PDK1 as well PDK4, the expression of which has not previously been investigated in PAH PASMCs, were determined in control and PAH PASMCs. Immunoblotting of PDK1 revealed a statistically insignificant decrease in the protein levels of PDK1 relative to GAPDH (Figure 5.9B). In contrast, immunoblotting of PDK4 showed a statistically insignificant increase in the relative expression of PDK4 in PAH PASMCs compared to controls (Figure 5.9C).
Figure 5.9: Protein expression of PDK1 and PDK4 in control and PAH PASMCs.

Control (n = 3) and PAH (n = 5) PASMCs were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. (A) Proteins were isolated from quiescent control and PAH PASMCs and immunoblotted for PDK1 and PDK4. Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to determine the levels of PDK1 (B) and PDK4 (C) relative to the loading control GAPDH in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in B and C.
5.2.5 Intracellular levels of ROS in control and PAH PASMCs

PAH PASMCs have previously been shown to produce higher levels of intracellular ROS than their control counterparts. To confirm this finding, quiescent control and PAH PASMCs were stained with DHE, an ethidium-based fluorescent probe used to detect ROS in live cells, and imaged using confocal laser scanning microscopy. In the cytosol, DHE fluoresces blue until oxidised by ROS. DHE is oxidised by superoxide to generate 2-hydroxyethidium and non-specifically by other sources of ROS to yield ethidium. Both 2-hydroxyethidium and ethidium, collectively denoted here as OxDHE, fluoresce red and intercalate with the cell’s nuclear DNA, consequently staining the nucleus bright red. As shown in Figure 5.9, the nuclei of PAH PASMCs (Figure 5.9B) exhibited stronger red fluorescence than those of control PASMCs (Figure 5.9A). Moreover, the cytosol of control PASMCs stained brighter blue than the cytosol of control PASMCs. This is suggestive of increased ROS production in PAH PASMCs concomitant with increased oxidation of DHE. Moreover, the proportion of nuclei, which stained for OxDHE, were 22.40% higher in PAH PASMCs than in control PASMCs (p = 0.0320; n = 4 for control PASMCs; n = 4 for PAH PASMCs; Figure 5.9C).
Figure 5.10: Intracellular ROS generation in control and PAH PASMCs. (A) Control ($n = 4$) and (B) PAH ($n = 4$) PASMCs were seeded at low density in glass-bottom μ-dishes and maintained in 10% FBS for 48 hours. PASMCs were then serum-starved in 0.1% FBS for 48 hours and stained with DHE in HBSS. Stained PASMCs were then imaged using confocal laser scanning microscopy. In its reduced form, DHE fluoresces blue. DHE, oxidised by ROS, or OxDHE fluoresces red and intercalates with DNA, staining the nuclei bright red. (C) Nuclei, which stained for OxDHE and fluoresced bright red, were counted and expressed as a percentage of the total number of nuclei in control and PAH PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in C. $p < 0.05$ was considered statistically significant.
5.2.6 Protein expression of the ROS scavengers SOD2 and catalase in control and PAH PASMCs

SOD2 and catalase are two major enzymatic components of the ROS scavenging system. SOD2 is localised to the mitochondria, where it dismutates the superoxide byproducts of oxidative phosphorylation to generate hydrogen peroxide (Mailloux et al., 2013). Hydrogen peroxide is then neutralised by catalase, which converts it to water and diatomic oxygen (Mailloux et al., 2013). Given the observed increase in intracellular ROS production in PAH PASMCs, it was hypothesised that downregulation of both ROS scavengers might underlie this increased ROS generation. As shown in Figure 5.11A, immunoblotting of proteins from quiescent control and PAH PASMCs revealed a two-fold decrease in the protein levels of SOD2 relative to GAPDH ($p = 0.0222; n = 3$ for control PASMCs; $n = 5$ for PAH; Figure 5.11B). However, the protein levels of catalase relative to GAPDH in PAH PASMCs were comparable to those in control PASMCs (Figure 5.11C).
Figure 5.11: Protein expression of the ROS scavengers SOD2 and catalase in control and PAH PASMCs. Control \((n = 3)\) and PAH \((n = 5 – 6)\) PASMCs were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. (A) Proteins were isolated from quiescent control and PAH PASMCs and immunoblotted for catalase and SOD2. Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to determine the levels of catalase (B) and SOD2 (C) relative to the loading control GAPDH in control and PAH PASMCs. Values are means \(\pm\) SEM. Unpaired student’s t-test was used in B and C. \(p < 0.05\) was considered statistically significant.
5.2.7 Protein expression of the ROS generator 5-lipoxygenase in control and PAH PASMCs

Most of intracellular ROS is generated within the mitochondria, predominantly at complexes I and III of the ETC (Mailloux et al., 2013). The increased mitochondrial mass in PAH PASMCs coupled with the reduced protein levels of the mitochondrial ROS scavenger SOD2 are thought to predominantly account for the increase in ROS levels in PAH PASMCs. However, ROS can also be produced by non-mitochondrial enzymes, such as the cytosolic enzyme arachidonate 5-lipoxygenase (Catalano et al., 2005). It was therefore speculated that increased expression of 5-lipoxygenase might also contribute, albeit to a lesser extent than mitochondria, to the increased generation of ROS observed in PAH PASMCs. Indeed, as shown in Figure 5.12A, immunoblotting of proteins from quiescent control and PAH PASMCs revealed a four-fold increase in the relative protein levels of 5-lipoxygenase in PAH PASMCs ($p = 0.0477; n = 3$ control PASMCs; $n = 6$ PAH PASMCs; Figure 5.12B).

![Figure 5.12: Protein expression of the ROS generator 5-lipoxygenase in control and PAH PASMCs. Control ($n = 3$) and PAH ($n = 6$) PASMCs were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. (A) Proteins were isolated from quiescent control and PAH PASMCs and immunoblotted for 5-lipoxygenase. Blots were reprobed for GAPDH to ensure equal loading. Densitometry was used to determine the levels of 5-lipoxygenase (B) relative to the loading control GAPDH in control and PAH PASMCs. Values are means ± SEM. Unpaired student's t-test was used in B. $p < 0.05$ was considered statistically significant.](image-url)
5.2.8 Basal mitophagy in control and PAH PASMCs

Mitophagy is a form of macroautophagy, employed by cells to selectively degrade damaged or superfluous mitochondria (Pickles et al., 2018). In mitophagy, autophagosomes sequester whole mitochondria or selectively target damaged areas (Pickles et al., 2018). Subsequently, autophagosomes fuse with lysosomes, delivering the mitochondria for degradation (Pickles et al., 2018). Given the increase in mitochondrial function and mass in PAH PASMCs, it was postulated that mitophagy might be downregulated in PAH PASMCs. To assess this hypothesis, the Mitophagy Detection Kit, composed of Mtphagy and Lyso dyes, was used to visualise mitochondrial sequestration into lysosomes in live PASMCs. Mtphagy dye accumulates in mitochondria, where it binds covalently, and fluoresces bright red, when the mitochondria are sequestered into acidic lysosomal compartments. Control and PAH PASMCs were seeded at low density in glass-bottom μ-dishes and maintained for 48 hours in 10% FBS. PASMCs were starved in 0.1% FBS for 48 hours, stained with Mtphagy and Lyso dyes in HBSS for 30 minutes and imaged using confocal laser scanning microscopy. As shown in Figure 5.13, PAH PASMCs (Figure 5.13B) exhibited considerably weaker red fluorescence than control PASMCs (Figure 5.13A). Almost all control PASMCs stained positively for Mtphagy (94.12%), whereas 46.47% of PAH PASMCs fluoresced red ($p = 0.0005$; $n = 4$ for control PASMCs; $n = 4$ for PAH PASMCs). This suggests that mitophagy is reduced in PAH PASMCs compared to control PASMCs.
Figure 5.13: Basal mitophagy in control and PAH PASMCs. (A) Control (n = 4) and (B) PAH (n = 4) PASMCs were seeded at low density in glass-bottom μ-dishes and maintained for 48 hours in 10% FBS. PASMCs were serum-starved in 0.1% FBS for 48 hours and incubated with Lyso Dye (green) and Mtphagy Dye (red) in HBSS for 30 minutes. Stained PASMCs were then imaged using confocal laser scanning microscopy at x60 magnification. (C) Control and PAH PASMCs that stained positively for Mtphagy or fluoresced red were counted and expressed as a percentage of the total number of PASMCs. Values are means ± SEM. Unpaired student’s t-test was used in C. p < 0.05 was considered statistically significant.
5.2.9 Protein expression of autophagy markers in control and PAH PASMCs

LC3B constitutes a well-established marker of autophagy (Yoshii and Mizushima, 2017). The unprocessed form of LC3B undergoes proteolytic cleavage to yield the cytosolic form of LC3BI. LC3BI can be processed further by conjugation to a PE moiety to generate LC3BII, which tethers to autophagosomal membranes (Yoshi and Mizushima, 2017). The conversion of LC3BI to LC3BII has been frequently used as an indicator of autophagy, with increased LC3BII expression suggestive of increased autophagic activity (Yoshii and Mizushima, 2017). Moreover, the protein expression of p62 inversely correlates with autophagic activity and consequently serves as a reporter of autophagic flux (Mizushima, 2017). Given that mitophagy, a form of autophagy, was found to be reduced in PAH PASMCs, it was suggested that autophagic markers might be altered accordingly to reflect the diminished mitophagy in PAH PASMCs. As shown in Figure 5.14, immunoblotting and subsequent densitometric analysis of proteins from quiescent control and PAH PASMCs revealed no change in the conversion of LC3BI to LC3BII between control and PAH PASMCs. Moreover, the protein levels of p62 relative to GAPDH in PAH PASMCs were comparable to those in control PASMCs. Taken together, these results suggest that overall autophagic activity is not reduced in PAH PASMCs and that the expression of mitophagy-specific proteins might alternatively be altered.
Figure 5.14: Protein expression of the autophagy markers LC3B and p62 in control and PAH PASMCs. Control ($n = 3$) and PAH ($n = 6$) PASMCs were cultured in 10% FBS to full confluence, starved in 0.1% FBS for 48 hours and lysed for protein. (A) Proteins from quiescent control and PAH PASMCs were immunoblotted for LC3B and p62. Immunoblotting for LC3B revealed the cytosolic and membrane-tethered forms, denoted LC3BI and LC3BII, respectively. Densitometry was used to quantify the protein levels of total LC3B (B), LC3BI (C), LC3BII (D) and p62 (G) relative to the loading control GAPDH in control and PAH PASMCs. Ratios of LC3BI (E) and LC3BII (F) to total LC3B were also determined. Values are means ± SEM. Unpaired student’s t-test was used in. $p < 0.05$ was considered statistically significant.
5.2.10 Treprostinil, MRE-269 and butaprost have no effects on mitochondrial function in PAH PASMCs

The stable analogue treprostinil has been shown to inhibit the proliferation of PAH PASMCs (Falcetti et al., 2010; Patel et al., 2018). To investigate whether the anti-proliferative effect of treprostinil on PAH PASMCs is associated with changes in mitochondrial function, PAH PASMCs were seeded at low density in (20,000 cells/well) in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were subsequently serum-starved in 0.1% FBS for 48 hours and treated with either DMSO or 100 nM treprostinil for 96 hours to allow for proliferation. PAH PASMCs were trypsinised, counted and seeded at a density of 7,500 cells/well in the Seahorse XFp Cell Culture Miniplates. OCR was measured using the Seahorse XFp Flux Analyser and the inhibitors oligomycin A, FCCP and rotenone/antimycin A were serially injected to reveal key parameters of mitochondrial function, including basal respiration, ATP production, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and proton leak. Despite, a slight decrease in the OCRs measured in the treprostinil-treated PAH PASMCs, as shown in Figure 5.15, 100 nM treprostinil had no significant effects on any of the mitochondrial parameters, suggesting that the anti-proliferative effect of treprostinil on PAH PASMCs is not associated with changes in mitochondrial function.

Treprostinil inhibits the proliferation of PAH PASMCs predominantly via the EP$_2$ prostanoid receptor and to a lesser extent via the IP receptor (Patel et al., 2018). Therefore, the effects of individual IP and EP$_2$ receptor agonism on mitochondrial function were also assessed. As shown in Figure 5.16, similar to treprostinil, IP receptor agonism with MRE-269, the active metabolite of the non-prostanoid IP receptor agonist selexipag, had no significant effects on the various aspects of mitochondrial activity. Moreover, sole EP$_2$ receptor activation with butaprost, an EP$_2$ receptor agonist, had no effects on any of the parameters of mitochondrial function (Figure 5.17).
Figure 5.15: Treprostinil has no effect on mitochondrial function in PAH PASMCs.
PAH (n = 4) PASMCs were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were serum-starved in 0.1% FBS for 48 hours and then treated with either DMSO or 100 nM treprostinil, a stable prostacyclin analogue, in 10% FBS for 96 hours to allow for proliferation. PASMCs were trypsinised, counted and seeded at a density of 7,500 cells per well in Seahorse XFp Cell Culture Miniplates. PAH PASMCs were then transferred to Mito Stress Test medium.
and OCRs were measured using the Seahorse XFp Analyser (A). Oligomycin A, FCCP and a combination of rotenone and antimycin A were serially injected as part of the Seahorse XFp Mito Stress Test to assess key parameters of mitochondrial function, including basal respiration (B), ATP production (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial respiration (F) and proton leak (G). Values are means ± SEM. Unpaired student's t-test was used in B, C, D, E, F and G. \( p < 0.05 \) was considered statistically significant.
Figure 5.16: MRE-269 has no effect on mitochondrial function in PAH PASMCs. PAH \((n = 4)\) PASMCs were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were serum-starved in 0.1% FBS for 48 hours and then treated with either DMSO or 100 nM MRE-269, a non-prostanoid IP receptor agonist, in 10% FBS for 96 hours to allow for proliferation. PASMCs were trypsinised, counted and seeded at a density of 7,500 cells per well in Seahorse XFp Cell Culture Miniplates. PAH PASMCs were then transferred to Mito Stress Test medium and OCRs were measured using the Seahorse XFp Analyser (A). Oligomycin A, FCCP and a combination of rotenone and antimycin A were serially injected as part of the
Seahorse XFp Mito Stress Test to assess key parameters of mitochondrial function, including basal respiration (B), ATP production (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial respiration (F) and proton leak (G). Values are means ± SEM. Unpaired student’s t-test was used in B, C, D, E, F and G. p < 0.05 was considered statistically significant.
Figure 5.17: Butaprost has no effect on mitochondrial function in PAH PASMCs. PAH \((n = 4)\) PASMCs were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were serum-starved in 0.1% FBS for 48 hours and then treated with either DMSO or 100 nM butaprost, an EP\(_2\) receptor agonist, in 10% FBS for 96 hours to allow for proliferation. PASMCs were trypsinised, counted and seeded at a density of 7,500 cells per well in Seahorse XFp Cell Culture Miniplates. PAH PASMCs were then transferred to Mito Stress Test medium and OCRs were measured using the Seahorse XFp Analyser (A). Oligomycin A, FCCP and a combination of rotenone and antimycin A were serially injected as part of the Seahorse
XFp Mito Stress Test to assess key parameters of mitochondrial function, including basal respiration (B), ATP production (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial respiration (F) and proton leak (G). Values are means ± SEM. Unpaired student’s t-test was used in B, C, D, E, F and G. p < 0.05 was considered statistically significant.
5.3 Discussion

The glycolytic product pyruvate can be transported by MPCs into the mitochondrial matrix, where it is converted to acetyl-CoA by the enzyme PDH and primed for participation in the TCA cycle. The TCA cycle is a closed loop of reactions, in which the last reaction in the pathway regenerates the substrate used in the first step (Anderson et al., 2018). It is composed of eight steps, the major function of which is to reduce NAD⁺ and FAD⁺ to NADH and FADH₂, respectively (Anderson et al., 2018). Subsequently, the generated NADH and FADH₂ are oxidised by complexes of the ETC, which reside within the IMM, and the electrons released from these oxidation reactions are passed along the chain from one complex to another until they reach the penultimate complex, complex IV, where, together with protons, they reduce diatomic oxygen to water (Anderson et al., 2018). Reduction of oxygen to water by complex IV is the step that underlies oxygen consumption by the mitochondria and is utilised by the Seahorse XFp Mito Stress Test to assess various aspects of mitochondrial function in live cells. The energy released from this electron transport is used to pump protons from the mitochondrial matrix to the mitochondrial intermembrane space creating an electrochemical gradient that drives the final complex, complex V, to phosphorylate ADP to ATP.

Previous findings suggest that mitochondrial respiration and therefore oxygen consumption are depressed in pulmonary vascular cells, including PASMCs, derived from the lungs of patients with PAH (Boucherat et al., 2018; Kurosawa et al., 2019). Contrary to these findings, however, OCRs measured in our PAH and control PASMC isolates suggest that mitochondrial oxygen consumption is elevated in PAH PASMCs compared to their control counterparts. Basally and prior to the addition of any mitochondrial inhibitors, OCRs measured in PAH PASMCs appeared to be much higher than those measured in control PASMCs. This is reflected in the two-fold increase in OCR linked to basal respiration in PAH PASMCs. Injection of oligomycin A, an inhibitor of complex V or the ATP synthase, resulted in a larger dip in OCR in PAH PASMCs than in control PASMCs, unmasking an increased demand for ATP by PAH PASMCs. Indeed, OCR associated with ATP production was four-fold higher in PAH PASMCs than in control PASMCs. Subsequent to the injection of oligomycin A, the protonophore and uncoupler FCCP was used to induce maximal mitochondrial respiration. Injection of FCCP increased OCR to levels beyond those measured basally in control PASMCs but not in PAH PASMCs. However, both control and PAH PASMCs exhibited similar levels of maximal respiration. This high FCCP-stimulated OCR compared to basal OCR suggests that basally the mitochondria in control PASMCs are using less than the maximal rate of electron transport that can be supported by the substrate supply. In
contrast, in PAH PASMCs, mitochondrial electron transport appears to be occurring at its maximal rate such that the injection of the uncoupler FCCP fails to increase OCR beyond that measured basally. This is further supported by the considerably reduced spare respiratory capacity (the difference between basal and maximal respiration) in PAH PASMCs. These findings suggest that, basally, PAH PASMCs are using up most of their respiratory reserve capacity in an attempt to meet their high energy demands. Given the increased glycolytic flux observed in PAH PASMCs in Chapter 4, ATP derived from oxidative phosphorylation seems to be insufficient to meet the energy demands of these cells. It seems that PAH PASMCs resort to upregulating glycolysis to produce enough ATP to support their abnormally hyper-proliferative and synthetic phenotype.

Following the FCCP-induced rise in OCR, a combination of rotenone and antimycin A was injected to shut down the ETC and reveal non-mitochondrial respiration or oxygen consumption. Non-mitochondrial oxygen consumption was two-fold higher in PAH PASMCs than in control PASMCs. Extra-mitochondrial enzymes that utilise oxygen include COX, LO and NOX, all of which are associated with inflammation and oxidative stress. Several isoforms of NOX, particularly NOX4, have been shown to be upregulated in PAH PASMCs and this upregulation may account for the increased non-mitochondrial oxygen consumption observed. Moreover, it was reported in this chapter that the protein levels of 5-LO are significantly increased in PAH PASMCs compared to control PASMCs, possibly also contributing to the increased non-mitochondrial respiration in PAH PASMCs.

While multiple lines of evidence support that diminished mitochondrial oxidative phosphorylation coupled with increased glycolysis underpins metabolic dysfunction in PAH, the findings presented in this chapter indicate that metabolic reprogramming in PAH might be more complex than originally thought and that increased glucose oxidation may mediate PAH pathogenesis. Recently, TGFβ, a growth factor known to promote PAH, has been shown to elevate not only glycolytic flux to pyruvate, as suggested by increased PFKP levels in TGFβ-treated PASMCs, but also mitochondrial respiration (Calvier et al., 2017). These metabolic effects of TGFβ on control PASMCs are analogous to the increased glycolytic flux and mitochondrial oxygen consumption observed in our PAH PASMC isolates (Calvier et al., 2017). Moreover, methamphetamine, the use of which increases the risk of developing PAH in humans, has been shown to prevent the normal adaptive responses of PAECs to hypoxia by impairing the metabolic shift from oxidative phosphorylation to glycolysis that normally occurs under hypoxic conditions (Chen et al., 2017). Instead, amphetamine-treated PAECs upregulate oxidative phosphorylation and reduce glycolytic flux in response to hypoxia (Chen et al., 2017). This is thought to elevate the levels of mitochondria-
generated ROS and promote unrepaired DNA damage, which was also observed in PAECs from patients with methamphetamine-induced PAH (Chen et al., 2017). The animal studies, which gave rise to the idea of Warburgian metabolism in PAH PASMCs, utilised primarily the murine model of hypoxia-induced PAH, which recapitulates neither the complexity nor the severity of human PAH. Indeed, hypoxia is not the sole driver of PAH pathogenesis. It constitutes a single contributing factor among a whole host of other environmental, genetic and epigenetic factors. PAH is a complex disease with no single causal factor and the heterogeneity among patients may explain the conflicting nature of the results presented in this chapter.

The depleted respiratory reserve capacity in PAH PASMCs implies that the mitochondrial mass in these cells may be compromised. However, the increased basal OCR in these cells suggests otherwise. To assess mitochondrial mass, the protein levels of various mitochondrial proteins, encoded by nuclear as well as mitochondrial genes, were quantified in control and PAH PASMCs. Complex I (NADH-ubiquinone oxidoreductase), the first and largest enzyme complex in the mitochondrial ETC, is composed of 38 nuclear-encoded subunits and 7 subunits encoded by genes in the mitochondrial DNA (Sharma et al., 2009). Among these 45 subunits, NDUFV1, which is encoded by a nuclear gene, was found to be significantly upregulated in PAH PASMCs compared to their control equivalents. In contrast to complex I, complex II (succinate dehydrogenase) is the smallest and least complex of the ETC complexes. It consists of 4 subunits, all of which are encoded by genes within the nuclear genome (Bezawork-Geleta et al., 2017). Of these 4 subunits, SDHA and SDHB were both found to be upregulated in PAH PASMCs compared to their control counterparts. Although not a constituent subunit of complex III, cytochrome c transfers electrons from complex III to complex IV. The protein levels of cytochrome c were also found to be considerably increased in PAH PASMCs compared to control PASMCs. Complex IV (cytochrome c oxidase) is the terminal oxidase of the mitochondrial ETC and donates electrons that have been passed along the ETC to the final electron acceptor oxygen (Zong et al., 2018). It consists of 14 subunits, 3 of which are encoded by the mitochondrial DNA and comprise the catalytic core of the complex (Zong et al., 2018). Of these three mitochondrial DNA-encoded complex IV subunits, the levels of MTCO2 were found to be elevated in PAH PASMCs. Finally, complex V or the ATP synthase, which generates ATP at the very end of the respiratory chain, consists of 14 nuclear-encoded and 2 mitochondrial encoded subunits. Among the nuclear-encoded subunits of complex V, ATP5A was found to be upregulated in PAH PASMCs compared to control PASMCs. Collectively, these results suggest that, albeit the decrease in respiratory reserve capacity, mitochondrial mass appears to be increased in PAH PASMCs, as indicated by the increased protein expression of the
various constituent subunits of the respiratory chain complexes. This increased mitochondrial mass possibly underlies the increased basal and ATP-linked oxygen consumption in PAH PASMCs and is indicative of increased mitochondrial biogenesis, the process whereby cells generate mitochondrial mass.

To further confirm the increase in mitochondrial mass in PAH PASMCs, the protein levels of two mitochondrial proteins, which are not part of the ETC, were assessed. VDACs are the most abundant proteins in the OMM (Camara et al., 2017). They comprise the main conduits via which metabolites, nucleotides and ions traverse the OMM (Camara et al., 2017). Of the three VDAC isoforms that have thus far been identified, the most abundant isoform VDAC1 was shown to be upregulated in PAH PASMCs compared to control PASMCs. The increased protein expression of VDAC1 in PAH PASMCs provides further evidence in support of an increase in mitochondrial mass in these cells. Furthermore, given the physical interaction of VDAC1 with hexokinases 1 and 2, which is believed to provide these kinases with direct access to mitochondrial ATP, this upregulation of VDAC1 is thought to contribute to the increased glycolytic flux observed in PAH PASMCs by increasing the recruitment of hexokinases 1 and 2 to the mitochondria. To maintain the increased glycolytic flux to lactate, PAH PASMCs may be upregulating VDAC1 to increase the permeability of the OMM to NADH, which is required for the conversion of pyruvate to lactate by LDHA (Shoshan-Barmatz, 2017). Lactate production also regenerates the NAD⁺ that has been used up in the TCA cycle and VDAC1 allows its influx back into the mitochondria (Shoshan-Barmatz et al., 2017). VDAC1 also allows the efflux of superoxide generated within the mitochondria (Shoshan-Barmatz et al., 2015) and its upregulation may contribute to the increased cytosolic levels of ROS in PAH PASMCs as observed by live-cell staining with the ROS indicator DHE. Finally, VDAC1 allows the mitochondrial exit of several TCA intermediates (e.g. citrate, α-ketoglutarate, aspartate), which partake in cytosolic biosynthetic pathways (Camara et al., 2017). Similar to VDAC1, the protein levels of the OMM protein TOM20 have also been shown to be higher in cultured PAH PASMCs than in control PASMCs. Moreover, stronger TOM20 expression was observed in the medial layers of distal pulmonary arteries, where these PASMCs reside, in PAH lung sections compared to those in control lung sections. As its name implies, TOM20 mediates the translocation of mitochondrial proteins, synthesised in the cytosol, into the mitochondria (Wiedmann and Pfanner, 2017). Increased expression of TOM20 in PAH PASMCs may therefore aid in the incorporation of newly synthesised, nuclear-encoded mitochondrial proteins.

The increased mitochondrial mass in PAH PASMCs suggested that mitochondrial biogenesis might be upregulated. The major orchestrator of mitochondrial biogenesis is
the transcriptional coactivator PGC1α (Hock and Kralli, 2009). PGC1α was originally described as a coactivator of the nuclear receptor and transcription factor PPARγ that mediates thermogenesis in brown adipose tissue in response to low temperatures. It was subsequently found that PGC1α coordinates the activity of numerous other transcription factors, including those involved in mitochondrial biogenesis (Hock and Kralli, 2009).

Consistent with the increased mitochondrial mass, the protein levels of PGC1α were higher in PAH PASMCs than in control PASMCs, indicative of increased mitochondrial biogenesis in these cells. Moreover, stronger PGC1α expression was observed in the medial layers of distal pulmonary arteries in PAH lung sections compared to control lung sections. In contradiction with this finding, Yeligar et al. (2018) showed that the expression of PGC1α is diminished in the lungs of mice and cultured human PASMCs exposed to chronic hypoxia as a result of reduced PPARγ expression. The authors also showed that siRNA-mediated knockdown of PGC1α promotes the proliferation of human PASMCs. In addition, several lines of evidence indicate that pharmacological activation of PPARγ increases PGC1α expression and strongly reduces the proliferation PAH PASMCs (Bogacka et al., 2005; Falcetti et al., 2010). Conflicting evidence exists in the literature, however, with respect to the expression of PPARγ in PAH. This could also be the case for PGC1α, whose expression appears to depend on that of PPARγ in PASMCs. Ameshima et al. (2003), for example, reported reduced PPARγ expression in the lungs of patients with PAH and complete loss of PPARγ expression in their complex vascular lesions. Contrastingly, Falcetti et al. (2010) found a striking increase in PPARγ expression in the medial layers of the remodelled pulmonary arteries of patients with PAH.

PGC1α induces mitochondrial biogenesis by activating the transcription factors NRF1 and NRF2, which promote the transcription of nuclear genes that encode mitochondrial proteins, namely constituent subunits of the mitochondrial ETC complexes (Hock and Kralli, 2009). Thus, NRF1 and NRF2 also serve as markers of mitochondrial biogenesis and their increased protein levels in PAH PASMCs provide further evidence in support of upregulated mitochondrial biogenesis and are likely to account for the increased protein levels of the mitochondrial ETC subunits NDUFV1, SDHA, SDHB and ATP5A. Indeed, NRF1 was first described as an activator of the cytochrome c gene, binding to sites within its promoter region and driving its transcription (Evans and Scarpulla, 1990). Accordingly, increased protein levels of cytochrome C were demonstrated in PAH PASMCs, a likely consequence of increased NRF1 expression. Moreover, NRF1 has been demonstrated to regulate the expression of all 10 nuclear-encoded subunits of complex IV of the mitochondrial ETC (Dhar et al., 2008), the levels of which have not been investigated in this chapter but are expected to be increased. In addition to nuclear
genes encoding subunits of the ETC complexes, NRF1 targets the gene that encodes the OMM protein TOM20 (Dhar et al., 2008) and its upregulation in PAH PASMCs possibly accounts for the increased protein levels of TOM20.

Upon activation by PGC1α, both NRF1 and NRF2 bind to sites within the promoter region of the nuclear gene that codes for the mitochondrial transcription factor TFAM to drive its transcription (Piantadosi and Suliman, 2006). Increased protein expression of NRF1 and NRF2 was therefore accompanied by the upregulation of TFAM in PAH PASMCs. TFAM in concert with TFB1M and TFB2M regulates the replication and transcription of mitochondrial DNA (Lezza, 2012). Increase in TFAM expression and transcriptional activity in PAH PASMCs is therefore likely to account for the observed increase in protein expression of MTCO2, which is encoded by the mitochondrial DNA. Whether the mitochondrial DNA copy number is increased in PAH PASMCs, as a consequence of increased TFAM expression, has yet to be explored. PAH PASMCs have many phenotypic features in common with cancer cells. Traditionally, depressed mitochondrial function and secondary upregulation of glycolysis were thought to drive tumorigenesis. However, multiple studies have now demonstrated that increased mitochondrial biogenesis and activity promote tumourigenesis by increasing levels of ROS rather than ATP (Weinberg et al., 2010). Loss of TFAM, for example, has been shown to ameliorate Kras-induced lung tumourgenesis (Weinberg et al., 2010). Moreover, depletion of mitochondrial DNA by treatment with ethidium bromide has been reported to inhibit the proliferation of human breast cancer cells (Yu et al., 2007).

OxPhos is driven by the transfer of high-energy electrons along the mitochondrial ETC. These electrons are donated by NADH and FADH$_2$, both products of the TCA cycle. Pyruvate, which is generated in the cytosol by glycolysis and transported into the mitochondria by MPCs, is the major entry substrate for the TCA cycle. To be incorporated into the TCA cycle, pyruvate needs to be converted to acetyl-CoA. The multisubunit enzyme complex PDH catalyses this irreversible reaction of pyruvate to acetyl-CoA. The pyruvate decarboxylase activity of PDH is inhibited by phosphorylation of its PDHE1α subunit at S293 (Rardin et al., 2009). Previous studies have demonstrated increased inhibitory phosphorylation of PDHE1α in the lungs of patients with PAH and this mode of inhibition of PDHE1α has been suggested to account for the depressed mitochondrial OxPhos observed in PAH pulmonary vascular cells, including PASMCs (Michelakis et al., 2017). However, given the increase in mitochondrial oxygen consumption observed in PAH PASMCs in this chapter, it was necessary to reassess the protein expression and inhibitory phosphorylation of PDHE1α. Concordant with the increase in mitochondrial respiration and mass, the protein levels of PDHE1α were considerably
higher in PAH PASMCs than in their control counterparts. Moreover, the increase in the protein expression of PDHE1α in PAH PASMCs was not accompanied by an increase in inhibitory phosphorylation at S293 but rather a trend towards a decrease in inhibitory phosphorylation was observed, likely a consequence of increased protein expression with a very slight increase in phosphorylation. Two other inhibitory phosphorylation sites have been identified in PDHE1α; these are S232 and S300. Phosphorylation at these two sites was not assessed in this chapter and warrants further investigation to confirm the activity status of PDHE1α in PAH PASMCs. It is important to note, however, that phosphorylation at any of the three sites leads to a strong reduction in PDH activity (Rardin et al., 2009). The protein expression of PDHE1β, another subunit of PDH, was also found to be upregulated in PAH PASMCs compared to those derived from control subjects, further suggesting an increase in PDH activity. Increased PDHE1α expression was previously reported to support lipogenesis in prostate tumours by upregulating the expression of lipogenic genes (Chen et al., 2018). Abrogation of PDHE1α in prostate-specific PTEN-knockout mice, which develop high-grade intraepithelial prostate tumours, suppressed prostate tumourigenesis in mice and downregulated lipogenic genes (Chen et al., 2018). Furthermore, siRNA-mediated knockdown of PDHE1α inhibited the proliferation of a number of human prostate cancer cell lines (Chen et al., 2018). Therefore, upregulation of PDHE1α in PAH PASMCs may similarly be supporting an anabolic, proliferative phenotype.

Inhibitory phosphorylation of PDHE1α is mediated by the serine-threonine kinase PDK, of which four isoforms exist (PDK1-4). S293 is targeted by all four PDK isoforms (Zimmer et al., 2016). Of these isoforms, the protein expression of PDK1 and PDK2 were examined in PAH PASMCs and their upregulation was suggested to underlie the inhibition of PDH and the resultant Warburgian shift to glycolysis (Michelakis et al., 2017). Although not assessed in PASMCs from PAH patients, PDK4 was found to be upregulated in pericytes of small pulmonary arteries from PAH patients (Yuan et al., 2016). A similar, albeit non-significant, increase in the protein expression of PDK4 was observed in PAH PASMCs in this chapter. Strikingly, a slight, non-significant decrease in the protein levels of PDK1 was observed in PAH PASMCs compared to control PASMCs, whereas the protein expression of PDK4 was slightly elevated. This is in stark contrast to the previously reported increase in the levels of PDK1 in the lungs of patients with PAH and may underlie the possible metabolic heterogeneity amongst PAH patients. Finally, PDH can be activated through dephosphorylation of the PDHE1α subunit by PDH phosphatases (PDPs). PDPs exist as heterodimers composed of two isoenzymes PDP1 and PDP2. An increase in the expression and/or activity of these phosphatases may underlie the decrease in phosphorylation of PDHE1α that was observed in our PAH
PASMC isolates. Indeed, in addition to increased PDHE1α expression, PDP1 has been shown to be upregulated at both the mRNA and protein levels in prostate tumours (Chen et al., 2018).

Mitochondrial mass is not only governed by the process of mitochondrial biogenesis but also mitophagy, a process whereby cells eliminate damaged or surplus mitochondria (Pickles et al., 2018). Given the increase in mitochondrial mass in PAH PASMCs, it was posited that mitophagy might additionally be depressed in these diseased cells, contributing to the build-up of mitochondrial mass. Staining of live PASMCs with a dye that accumulates in the mitochondria but fluoresces only when exposed to the acidic interiors of lysosomes showed that basal mitophagy is severely diminished in PAH PASMCs compared to control PASMCs. This is likely to contribute to the reported increase in mitochondrial mass. Similarly, inhibition of mitophagy has been reported to promote tumorigenesis in tumours associated with loss-of-function mutations in the parkin gene PARK2, which is located at a fragile site on chromosome 6 (Chang et al., 2017). This inhibited mitophagy is thought to allow for the accumulation of dysfunctional mitochondria and the generation of ROS, which also could be the case for the hyper-proliferative PAH PASMCs (Chang et al., 2017). The role of mitophagy or lack thereof in PAH pathogenesis remains poorly understood and the mechanisms underlying changes in mitophagic flux in PAH have yet to be elucidated. Depolarisation of the mitochondrial membrane potential renders the mitochondria substrates for PINK1/parkin-mediated mitophagy. PINK1 is a mitochondrial serine/threonine kinase whose stability is influenced by the mitochondrial membrane potential. Upon stabilisation by mitochondrial depolarisation, PINK1 phosphorylates and recruits the E3 ubiquitin ligase parkin to the mitochondrial OMM, where, in concert with E2 ubiquitin-conjugating enzymes, it decorates several OMM proteins with ubiquitin, tagging the mitochondria for recognition by autophagy receptors (Pickles et al., 2018). Mitochondria have been described to be hyperpolarised in PAH PASMCs compared to control PASMCs (Pak et al., 2013). This hyperpolarisation might hinder mitophagy driven by PINK1/ Parkin signalling and account for the reduced mitophagic flux that was demonstrated in PAH PASMCs in this chapter. Among the targets of parkin-mediated ubiquitination and subsequent degradation are the mitochondrial fusion proteins MFN1 and MFN2 (Gegg et al., 2010), the protein levels of which have been shown to remain unchanged in PAH PASMCs (see Chapter 3). This probably indicates that the activities of parkin and its mitochondrial activator and recruiter PINK1 also remain unaltered in PAH PASMCs. However, three other targets of parkin have been shown to be upregulated in PAH PASMCs in this thesis and these are FIS1, VDAC1 and TOM20 (Ordureau et al., 2018; Vives-Bauza et al., 2010). This strongly supports the notion that increased PINK1/parkin signalling might be responsible for the
increased mitophagy in PAH PASMCs. Interestingly, mitophagy can also be induced by pathways that are independent of PINK1 and parkin (Pickles et al., 2018). These alternative pathways have been highlighted by several studies and could act in parallel or in addition to the PINK1/Parkin mitophagy pathway. For example, in response to mitochondrial depolarisation, the OMM-anchored FK506-binding protein FKBP8 recruits lipidated LC3A to induce PINK1/parkin-independent mitophagy (Bhujabal et al., 2017). Downregulation of PINK1/parkin-independent pathways might therefore also contribute to the reduced mitophagic flux in PAH PASMCs.

Mitophagy is a form of autophagy that selectively targets mitochondria. The reduced mitophagic flux in PAH PASMCs suggested that autophagy as a whole might be downregulated. During autophagy, cellular components are sequestered into double-membrane vesicles, termed autophagosomes, which ultimately fuse with lysosomes to deliver their cargoes for degradation. A hallmark of autophagosome formation is the conversion of the cytosolic form of LC3B, LC3BI, to the autophagosomal membrane-anchored form, LC3BII, by conjugation with phosphatidylethanolamine. No difference in LC3B conversion was observed between control and PAH PASMCs, suggesting that autophagy remains intact in PAH PASMCs. The protein p62 constitutes another autophagic marker, with reduced autophagy leading to the accumulation of this protein. Similar to LC3B conversion, the protein expression of p62 in PAH PASMCs was comparable to that in control PASMCs, confirming unaltered autophagic flux. In stark contrast to these findings, LC3B expression has previously been shown to be markedly increased in all three layers of pulmonary arteries in lung tissues derived from patients with sporadic PAH, suggesting enhanced autophagy in PAH (Lee et al., 2011). The large phenotypic heterogeneity among PAH patients could explain the contradictory findings of this chapter.

Many studies have provided evidence in support of increased oxidative stress in patients with PAH, particularly in the pulmonary vascular cells derived from these patients. PAH PASMCs have been shown to generate more ROS than control PASMCs. In this chapter, staining of live PAH PASMCs with the ROS indicator DHE confirmed that PAH PASMCs produce larger amounts of ROS than their control counterparts. In PASMCs, ROS can be generated from a variety of sources, most notably mitochondria, NADPH oxidases, xanthine oxidases and uncoupled nitric oxide synthase. Nevertheless, the major source of ROS is the mitochondria, within which electron leakage from complexes I and III partially reduces oxygen to yield superoxide. The increased mitochondrial mass in PAH PASMCs could therefore contribute to the increased levels of ROS by enhancing electron leakage from complexes I and III and the partial reduction of oxygen to
superoxide. Increased reduction of oxygen to superoxide could also account for the increased mitochondrial oxygen consumption observed in PAH PASMCs. SOD2 converts superoxide, generated within the mitochondria, to hydrogen peroxide, which is then broken down by catalase into water and oxygen (Archer et al., 2010). Despite the increase in mitochondrial mass, the protein levels of mitochondrial SOD2 were found to be reduced in PAH PASMCs compared to control PASMCs. Reduced SOD2 scavenging of superoxide could contribute to the increased ROS levels observed in PAH PASMCs. Previously, SOD2 has been shown to be reduced in pulmonary arteries from patients with PAH and epigenetically silenced in PASMCs from fawn-hooded rats, which spontaneously develop PAH, via hypermethylation of CpG dinucleotides within intron 2 and the promoter region of its gene (Archer et al., 2010). A similar epigenetic attenuation could account for the reduced SOD2 observed in PAH PASMCs in this chapter. There was no difference in the protein levels of catalase between control and PAH PASMCs, suggesting that the catalytic breakdown of hydrogen peroxide remains unaffected. Increased NOX-generated ROS has been reported in PAH PASMCs. However, other ROS-generators, such as LO and COX, have yet to be explored in the context of PAH. Several studies have reported that ROS can be generated as by-products during the oxidation of arachidonic acid by LO, particularly the 5-LO isoform (Catalano et al., 2005; Woo et al., 2000; Yun et al., 2010). Among the six LO isoforms, 5-LO was found to be markedly upregulated in PAH PASMCs with little or no expression in control PASMCs. 5-LO could therefore also account for the increased levels of ROS in PAH PASMCs and its inhibition could reduce oxidative stress in these cells to attenuate their hyper-proliferative and apoptosis-resistant phenotype. 5-LO is therapeutically inhibited with zileuton in the treatment of asthma. Zileuton could potentially be repurposed for the treatment of PAH. Indeed, zileuton has been shown to attenuate experimental PH in rats by blocking the production of leukotriene B4 in macrophages (Tian et al., 2013).

Prostacyclin and its stable analogues, such as treprostinil, are used extensively in the treatment of PAH and have been shown to inhibit the proliferation of PAH PASMCs as well as those derived from normal lungs (Falcetti et al., 2010; Patel et al., 2018). Whether the anti-proliferative effect of treprostinil is associated with changes, if any, in mitochondrial respiration has yet to be investigated. Treating proliferating PAH PASMCs with 100 nM treprostinil had no effect on mitochondrial function, as indicated by the lack of significant changes in OCRs, particularly those linked to basal respiration, ATP production, maximal respiratory and reserve respiratory capacity. Given that the anti-proliferative effect of treprostinil on PAH PASMCs has been demonstrated to be largely mediated by the EP2 receptor and to a much lesser extent by the IP receptor, the effects of equimolar concentrations of the EP2 receptor-selective agonist butaprost and the IP
receptor-selective agonist MRE-269 on mitochondrial function in proliferating PAH PASMCs were also assessed. Like treprostinil, butaprost and MRE-269 also appear to have had no effects on OCRs. These findings suggest that the anti-proliferative effects of prostacyclin mimetics, particularly those acting on IP and/or EP₂ receptors, are not associated with changes in mitochondrial respiration in PAH PASMCs. Similar to the PAH PASMCs studied in this chapter, platelets derived from PAH patients, namely idiopathic and CTD-associated, were demonstrated to exhibit increased mitochondrial oxygen consumption compared to those isolated from control subjects (Nguyen et al., 2017). Patient stratification by therapy received showed that prostacyclin analogues had no effect on this increase in mitochondrial respiration (Nguyen et al., 2017).

In conclusion, this chapter reported an increase in mitochondrial oxygen consumption rates associated with basal respiration and ATP production with no change in that linked to maximal respiration and a reduction in the respiratory reserve capacity of mitochondria in PAH PASMCs. These findings suggested that PAH PASMCs are respiring maximally under basal conditions and are unable to elevate mitochondrial respiration in response to increased energy demand. Underlying this increase in mitochondrial respiration was an increase in mitochondrial mass as indicated by the increased protein expression of several mitochondrial proteins, including constituent subunits of the respiratory complexes and OMM proteins, in cultured PAH PASMCs as well PASMCs within the medial layers of PAH pulmonary arteries. Increased mitochondrial biogenesis, as suggested by the increase in proteins levels of its transcriptional regulators in PAH PASMCs, was found to contribute to this increase in mitochondrial mass. Further contributing to the increase in mitochondrial mass was a decrease in mitophagic flux in PAH PASMCs with no change in autophagy markers. The increase in mitochondrial oxygen consumption in PAH PASMCs was accompanied by an increase in cellular ROS levels, which could also be a consequence of reduced SOD2-mediated scavenging in the mitochondria and an increase in the protein expression of the cytosolic ROS generator 5-LO. Finally, prostacyclin mimetics failed to reverse the increase in mitochondrial respiration in PAH PASMCs.
6. TASK-1 channel dysfunction in PASMCs from PAH patients

6.1 Introduction

TASK-1 (encoded by KCNK3) belongs to the TASK (TWIK-related acid-sensitive K⁺) family of K_2p channels, which includes two other members: TASK-3 (encoded by KCNK9) and non-functional TASK-5 (encoded by KCNK15; Olschewski et al., 2017). In human PASMCs, knockdown of KCNK3 with RNA interference has been shown to inhibit background K⁺ currents and to depolarise the resting membrane potential, suggesting an important role for TASK-1 in regulating the resting membrane potential of PASMCs, which is a key determinant of pulmonary vascular tone (Gurney et al., 2003; Olschewski et al., 2006). PASMCs from KCNK3-deficient rats were also shown to exhibit depolarised resting membrane potentials and reduced TASK-1 currents, further confirming the role of TASK-1 channels in stabilising the resting membrane potential (Lambert et al., 2019). Moreover, pulmonary arteries from KCNK3-deficient rats showed heightened responses to experimental vasoconstrictive agents, such as the L-type Ca^{2+} channel agonist Bay K8644 and KCl (Lambert et al., 2019). Pulmonary arteries from KCNK3-deficient rats also displayed impaired endothelium-dependent vasodilation, as indicated by the inability of eNOS inhibition with L-NAME to further increase KCl-induced vasoconstriction and the reduced protein expression of eNOS in the lungs of these KCNK3-deficient rats (Lambert et al., 2019). The vasodilatory responses of rat pulmonary arteries deficient in TASK-1 to the PDE5 inhibitor and PAH drug sildenafil were found to be reduced, suggesting that TASK-1 activity partly mediates the vasodilatory effect of sildenafil on pulmonary arteries (Lambert et al., 2019).

TASK-1 channels have recently emerged as key players in the pathogenesis of PAH (Lambert et al., 2018). In 2013, using whole-exome sequencing, Ma et al. identified six different heterozygous mutations in KCNK3, the gene encoding TASK-1, in patients with IPAH and HPAH, reporting the first channelopathy in PAH and implicating the channel in the pathogenesis of the disease. Whole-cell recordings from COS7 cells, expressing mutant TASK-1 channels, indicated that all six mutations result in loss of TASK-1 channel function (Bohnen et al., 2017; Ma et al., 2013). In 2016, two additional TASK-1 mutations were identified by Tejedor et al. (2016) in a Spanish cohort of PAH patients, also describing the first instance of a homozygous TASK-1 mutation in a patient with an aggressive and early-onset form of the disease. Further implicating TASK-1 channel dysfunction in PAH, two new KCNK3 mutations were recently reported in Asian and
American cohorts of PAH patients (Lambert et al., 2018). Compared to PAH patients with other mutations, PAH patients harbouring KCNK3 mutations tend to be younger and have higher mPAPs (Lambert et al., 2019). In addition to being mutated in PAH, TASK-1 was found to be downregulated at both the mRNA and protein levels in the explanted lungs of patients with IPAH but lacking disease-causing KCNK3 mutations as well as patients with FPAH due to BMPR2 mutations (Antigny et al., 2016). Consistent with the reduced expression of TASK-1, whole-cell K$^+$ currents, sensitive to A293 (a selective TASK-1 channel blocker), were severely depressed in cultured PASMCs isolated from patients with IPAH and HPAH compared to their control counterparts (Antigny et al., 2016).

In divergence with human and rat PASMCs, TASK-1 was reported to form non-functional channels in mouse PASMCs (Manoury et al., 2011). PASMCs acutely isolated from the lungs of KCNK3-knockout mice showed background K$^+$ currents and resting membrane potentials comparable to those measured in wildtype PASMCs (Manoury et al., 2011). Also, pulmonary arteries from Kcnk3$^{-/-}$ mice retained normal resting tone and vasoreactivity (Manoury et al., 2011). Last but not least, neither right ventricular hypertrophy nor neomuscularisation of distal pulmonary arteries, both early signs of PAH, were observed in Kcnk3$^{-/-}$ mice (Manoury et al., 2011). Rats were therefore alternatively utilised to study the in vivo role of TASK-1 channel dysfunction in PAH (Antigny et al., 2016). Chronic treatment of rats with the TASK-1 blocker A293 induced an exaggerated proliferation of pulmonary vascular cells (PAECs, PASMCs and adventitial fibroblasts), leading to neomuscularisation of distal pulmonary arteries and increased right ventricular systolic pressure (Antigny et al., 2016). Moreover, KCNK3-deficient rats, generated using CRISPR-Cas9, were also found to exhibit signs of PAH similar to those observed in rats chronically treated with the TASK-1 blocker (Lambert et al., 2019). Collectively, these findings suggest that loss of TASK-1 channel function comprises an early event in PAH pathogenesis and that TASK-1 channel dysfunction is likely to be an instigator rather than a modulator of the disease.

The prostacyclin analogues iloprost and treprostinil have been shown to hyperpolarise the resting membrane potential in normal PASMCs predominantly by increasing whole-cell K$^+$ currents through TASK-1 channels (Li et al., 2012; Olschewski et al., 2006). IP receptor antagonism abolished the effect of iloprost or treprostinil on TASK-1 currents as did inhibition of the cAMP-dependent serine/threonine kinase PKA (Li et al., 2012). This suggests that iloprost and treprostinil utilise the IP receptor/PKA signalling axis to activate TASK-1 channels (Lie et al., 2012). Moreover, treprostinil and iloprost have both also been shown to inhibit the proliferation of normal PASMCs at clinically relevant
concentrations mainly by activating the IP receptor with higher treprostinil concentrations recruiting other receptors (Falcetti et al., 2010). This suggests that treprostinil or iloprost may inhibit the proliferation of control PASMCs by activating TASK-1 channels. Interestingly, the anti-proliferative effects of treprostinil and iloprost on PAH PASMCs were insensitive to IP receptor antagonism (Falcetti et al., 2010). Treprostinil was alternatively found to inhibit the proliferation of PAH PASMCs by activating the nuclear receptor PPARγ or the EP2 prostanoid receptor, both of which have been shown to be upregulated in PAH PASMCs (Falcetti et al., 2010; Patel et al., 2018). In PAH PASMCs, treprostinil may therefore alternatively recruit the EP2 receptor to activate TASK-1 channels. Indeed, in tsA201 cells, TASK-1 channel activation by treprostinil only occurred when the channel was co-expressed with the EP2 receptor (Cunningham et al., 2018).

In this chapter, the expression of TASK-1 in control and PAH PASMCs was assessed to determine whether TASK-1 is indeed downregulated in PAH. The dependency of the anti-proliferative effect of treprostinil on TASK-1 channel activity in both control and PAH PASMCs was also investigated. Since treprostinil activates a number of cellular targets, including the IP and EP2 receptors to inhibit PASMC proliferation, the effect of TASK-1 channel blockade on the anti-proliferative effects of sole IP or EP2 agonism with either MRE-269 or butaprost, respectively, was studied. Loss of TASK-1 channel activity has been shown to alter mitochondrial morphology and membrane potential in control PASMCs. However, whether TASK-1 channel dysfunction affects mitochondrial respiration remains unexplored. Therefore, the effect of TASK-1 channel blockade on mitochondrial oxygen consumption in control PASMCs was examined. The impact of TASK-1 channel dysfunction on PASMCs has been the focus of studies thus far. Whether loss of TASK-1 channel activity affects the pulmonary endothelium remains relatively unexplored. Therefore, this chapter sought to determine whether loss of TASK-1 channel function in PAECs leads to heightened apoptosis, an early event in PAH pathogenesis.
6.2 Results

6.2.1 mRNA expression of KCNK3, the gene encoding TASK-1, in control and PAH PASMCs

BMPR2, the gene frequently mutated in patients with PAH, has been shown to be reduced in the lungs of patients suffering from PAH, but lacking disease-causing BMPR2 mutations (Andruska and Spiekerkoetter, 2018; Austin and Loyd, 2014). Moreover, the expression and function of voltage-gated potassium channels, particularly the oxygen-sensitive Kv1.5, are altered in patients with PAH, contributing to the pulmonary vasoconstriction and remodelling that are characteristic of PAH (Babicheva et al., 2020; Boucherat et al., 2015). It was postulated that the expression of KCNK3, the gene encoding TASK-1, might also be reduced in PAH PASMCs. Recently, reduced expression of KCNK3 and function of its protein product TASK-1 have been demonstrated in PASMCs isolated from patients with IPAH and HPAH (Antigny et al., 2016). To assess whether KCNK3 expression is indeed downregulated in PAH PASMCs, control and PAH PASMCs were cultured to full confluence and serum-starved in 0.1% FBS for 48 hours. RNA was isolated and RT-qPCR was used to measure the relative mRNA levels of KCNK3 in quiescent control and PAH PASMCs. As illustrated in Figure 6.1, albeit not reaching statistical significance, the mRNA expression of KCNK3 relative to the housekeeping gene TBP in PAH PASMCs was lower than that in control PASMCs. This suggests that the number of TASK-1 channels in the sarcolemma and hence the hyperpolarising TASK-1 currents might be reduced in PAH PASMCs compared to control PASMCs. Unfortunately, protein levels of TASK-1 could not be assessed due to the commercial availability of poorly specific TASK-1 antibodies, which have been shown to produce signals in tissues from both wildtype and KCNK3-deficient mice alike.
Figure 6.1: mRNA expression of KCNK3, the gene encoding TASK-1 is unchanged in PAH PASMCs. Control (n = 4) and PAH (n = 9) PASMCs were cultured in 10% FBS to full confluence and serum-starved in 0.1% FBS for 48 hours. RNA was isolated from quiescent control and PAH PASMCs and RT-qPCR was used to measure the mRNA levels of KCNK3 relative to the housekeeping gene TBP. Values are means ± SEM. Unpaired student’s t-test was used.
6.2.2 TASK-1 channel blockade promotes the proliferation of control but not PAH PASMCs

Recently, loss-of-function mutations in KCNK3, the gene encoding TASK-1, were identified in PAH patients with severe vascular remodelling (Ma et al., 2013; Tejedor et al., 2016). Moreover, rats deficient in KCNK3 were found to spontaneously develop several signs and features of PH, including increased proliferation of PASMCs within the medial layers of distal pulmonary arteries (Lambert et al., 2019). Whether loss of TASK-1 channel function, such as that caused by PAH-causing mutations, influences the proliferation of PASMCs remains elusive. To investigate the effect of loss of TASK-1 channel currents on the proliferation of control and PAH PASMCs, control or PAH PASMCs were seeded at low density (20,000 cells/well) in 10% FBS and then serum-starved in 0.1% FBS for 48 hours to synchronise the cells in the G0/G1 phase of the cell cycle. Serum-starved control or PAH PASMCs were then treated with either DMSO or 10 μM ML365, a blocker selective for TASK-1 channels, in 10% FBS for 96 hours to allow for PASMC proliferation. 10 μM ML365 was previously shown to completely block TASK-1 currents in PASMCs (Bohnen et al., 2017). After 96 hours, control or PAH PASMCs were trypsinised and counted using trypan blue and a disposable haemocytometer. Cell counts were expressed as a percentage of the cell count for the DMSO-treated population. As shown in Figure 6.2A, 10 μM ML365 increased the proliferation of control PASMCs by 47% (p = 0.0015; n = 5). However, as shown in Figure 6.2B, 10 μM ML365 had no effect on the proliferation of PAH PASMCs.
Figure 6.2: TASK-1 channel blockade promoted proliferation of control PASMCs but not PAH PASMCs. (A) Control (n = 5) or (B) PAH PASMCs (n = 6) were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PASMCs were serum-starved in 0.1% FBS for 48 hours to induce cell cycle arrest in the G0/G1 phase. After serum-starvation, control or PAH PASMCs were treated with either DMSO or 10 μM ML365, a TASK-1 channel blocker, in 10% FBS for 96 hours to allow for cell proliferation. After 96 hours, control or PAH PASMCs were trypsinised and counted using the vital dye trypan blue and a disposable haemocytometer. Cell counts for each treatment were then expressed as a percentage of the cell count for the DMSO-treated PAH PASMCs. Values are means ± SEM. Paired student’s t-test was used in A and B. *p < 0.05 was considered statistically significant.
6.2.3 TASK-1 channel blockade has no effect on the anti-proliferative effect of treprostinil on control PASMCs

Treprostinil has previously been shown to enhance outward K⁺ currents through TASK-1 channels and cause membrane hyperpolarisation in normal PASMCs (Li et al., 2012; Olschewski et al., 2006). Moreover, treprostinil has been shown to inhibit the proliferation of control PASMCs. To investigate whether TASK-1 channels mediate the anti-proliferative effect of treprostinil on control PASMCs, control PASMCs were seeded at low density (20,000 cells/well) in 10% FBS and then serum-starved in 0.1% FBS for 48 hours to synchronise the cells in the G0/G1 phase of the cell cycle. Serum-starved control PASMCs were then treated with either DMSO, 100 nM or 1 μM treprostinil in the absence or presence of 10 μM ML365, a blocker selective for TASK-1 channels, in 10% FBS for 96 hours to allow for PASMC proliferation. 100 nM treprostinil was previously shown to maximally enhance K⁺ currents through TASK-1 channels in control PASMCs. After 96 hours, control PASMCs were trypsinised and counted using trypan blue and a disposable haemocytometer. Cell counts were expressed as a percentage of the cell count for the DMSO-treated population.

As shown in Figure 6.3, compared to DMSO, 100 nM treprostinil reduced the proliferation of control PASMCs by 41% (p = 0.0103; n = 5). The higher dose of 1 μM treprostinil exerted a considerably stronger anti-proliferative effect, reducing proliferation by 59% (p = 0.0012; n = 5). 10 μM ML365 on its own increased the proliferation of control PASMCs by 47% (p = 0.0160; n = 5). There were no significant differences between control PASMCs treated with either 100 nM or 1 μM treprostinil in the absence of 10 μM ML365 and those treated with either treprostinil concentration in the presence of 10 μM ML365, suggesting that TASK-1 channel blockade had no effect on the anti-proliferative effect of treprostinil on control PASMCs. This is further supported by the following two findings. First, the 95% CI [15%, 66%] for the mean difference (41%) between DMSO-treated and 100 nM treprostinil-treated control PASMCs includes the mean difference (49%) between 10 μM ML365-treated control PASMCs and those treated with both 100 nM treprostinil and 10 μM ML365. Second, the 95% CI [37%, 80%] for the mean difference (59%) between DMSO-treated control PASMCs and 1 μM treprostinil-treated control PASMCs includes the mean difference (74%) between 10 μM ML365-treated control PASMCs and those treated with both 1 μM treprostinil and 10 μM ML365.
Figure 6.3: TASK-1 channel blockade has no effect on the anti-proliferative effects of the prostacyclin analogue treprostinil on control PASMCs. Control PASMCs ($n = 5$) were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were serum-starved in 0.1% FBS for 48 hours to induce cell cycle arrest in the G0/G1 phase. After serum-starvation, control PASMCs were treated with either DMSO, 100 nM or 1 μM treprostinil, a stable prostacyclin analogue, without or with 10 μM ML365, a TASK-1 channel blocker, in 10% FBS for 96 hours to allow for cell proliferation. After 96 hours, control PASMCs were trypsinised and counted using the vital dye trypan blue and a disposable haemocytometer. Cell counts for each treatment were then expressed as a percentage of the cell count for the DMSO-treated control PASMCs. Values are means ± SEM. Repeated measures one-way ANOVA with Tukey’s test for multiple comparisons were used. $p < 0.05$ was considered statistically significant.
6.2.4 TASK-1 channel blockade has no effect on the anti-proliferative effect of treprostinil on PAH PASMCs

Treprostinil has previously been shown to enhance outward K⁺ currents through TASK-1 channels and cause membrane hyperpolarisation in normal PASMCs. Moreover, treprostinil has been shown to inhibit the proliferation of PAH PASMCs. However, whether treprostinil inhibits the proliferation of PAH PASMCs by activating TASK-1 channels remains unexplored. To investigate whether TASK-1 channels mediate the anti-proliferative effect of treprostinil on PAH PASMCs, PAH PASMCs were seeded at low density (20,000 cells/well) in 10% FBS and then serum-starved in 0.1% FBS for 48 hours to synchronise the cells in the G0/G1 phase of the cell cycle. Serum-starved PAH PASMCs were then treated with either DMSO, 100 nM or 1 μM treprostinil in the absence or presence of 10 μM ML365, a blocker selective for TASK-1 channels, in 10% FBS for 96 hours to allow for PASMC proliferation. After 96 hours, PAH PASMCs were trypsinised and counted using trypan blue and a disposable haemocytometer. Cell counts were expressed as a percentage of the cell count for the DMSO-treated population.

As shown in Figure 6.4, compared to DMSO, 100 nM treprostinil inhibited the proliferation of PAH PASMCs by 36% ($p = 0.0005; n = 6$). The higher dose of 1 μM treprostinil exerted a considerably stronger anti-proliferative effect, reducing proliferation by 55% ($p = 0.0005; n = 6$). 10 μM ML365 on its own had no effect on the proliferation of PAH PASMCs. There were no significant differences in proliferation between PAH PASMCs treated with either 100 nM or 1 μM treprostinil in the absence of 10 μM ML365 and those treated with either treprostinil concentration in the presence of 10 μM ML365, suggesting that TASK-1 channel blockade had no effect on the anti-proliferative effect of treprostinil on PAH PASMCs. This is further supported by the following two findings. First, the 95% CI [23%, 48%] for the mean difference (36%) between DMSO-treated and 100 nM treprostinil-treated PAH PASMCs includes the mean difference (24%) between 10 μM ML365-treated PAH PASMCs and those treated with both 100 nM treprostinil and 10 μM ML365. Second, the 95% CI [36%, 74%] for the mean difference (55%) between DMSO-treated and 1 μM treprostinil-treated PAH PASMCs includes the mean difference (45%) between 10 μM ML365-treated PAH PASMCs and those treated with both 1 μM treprostinil and 10 μM ML365.
Figure 6.4: TASK-1 channel blockade has no effect on the anti-proliferative effects of the prostacyclin analogue treprostinil on PAH PASMCs. PAH PASMCs \((n = 6)\) were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were serum-starved in 0.1% FBS for 48 hours to induce cell cycle arrest in the G0/G1 phase. After serum-starvation, PAH PASMCs were treated with either DMSO, 100 nM or 1 μM treprostinil, a stable prostacyclin analogue, without or with 10 μM ML365, a TASK-1 channel blocker, in 10% FBS for 96 hours to allow for cell proliferation. After 96 hours, PAH PASMCs were trypsinised and counted using the vital dye trypan blue and a disposable haemocytometer. Cell counts for each treatment were then expressed as a percentage of the cell count for the DMSO-treated PAH PASMCs. Values are means ± SEM. Repeated measures one-way ANOVA with Tukey’s test for multiple comparisons were used. \(p < 0.05\) was considered statistically significant.
6.2.5 TASK-1 channel blockade attenuates the anti-proliferative effect of MRE-269 on PAH PASMCs

Treprostinil acts on a number of cellular targets to produce its anti-proliferative effect on PAH PASMCs. These targets include the nuclear receptor PPARγ and the cell-surface IP and EP₂ prostanoid receptors (Falcetti et al., 2010; Patel et al., 2018). Sole IP receptor agonism with MRE-269 has also been demonstrated to inhibit the proliferation of PAH PASMCs, albeit to a lesser extent than treprostinil (Patel et al., 2018). Moreover, IP receptor agonism with either iloprost or treprostinil has been shown to promote TASK-1 channel function in normal PASMCs (Li et al., 2012). To investigate whether TASK-1 channels mediate the anti-proliferative effect of IP receptor agonism on PAH PASMCs, PAH PASMCs were seeded at low density (20,000 cells/well) in 10% FBS and then serum-starved in 0.1% FBS for 48 hours to synchronise the cells in the G0/G1 phase of the cell cycle. Serum-starved PAH PASMCs were then treated with either DMSO, 100 nM or 1 μM MRE-269, an IP receptor agonist, in the absence or presence of 10 μM ML365, a blocker selective for TASK-1 channels, in 10% FBS for 96 hours to allow for PASMC proliferation. After 96 hours, PAH PASMCs were trypsinised and counted using trypan blue and a disposable haemocytometer. Cell counts were expressed as a percentage of the cell count for the DMSO-treated population.

As shown in Figure 6.5, 100 nM MRE-269 inhibited the proliferation of PAH PASMCs by 23% \((p = 0.0005; n = 4)\). The higher dose of 1 μM MRE-269 exerted a stronger anti-proliferative effect, reducing proliferation by 33% \((p = 0.0459; n = 4)\). 10 μM ML365 on its own had no effect on the proliferation of PAH PASMCs. Significant differences in proliferation were observed between PAH PASMCs treated with either 100 nM or 1 μM MRE-269 in the absence of 10 μM ML365 and those treated with either MRE-269 concentration in the presence of 10 μM ML365. Furthermore, the mean difference (9%) between 10 μM ML365-treated PAH PASMCs and those subjected to a combination of 100 nM MRE-269 and 10 μM ML365 does not lie within the 95% CI [19%, 27%] for the mean difference (23%) between DMSO-treated and 100 nM MRE-269-treated PAH PASMCs. However, the mean difference (12%) between 10 μM ML365-treated PAH PASMCs and those treated with both 1 μM MRE-269 and 10 μM ML365 falls within the 95% CI [1%, 66%) for the mean difference (33%) between DMSO-treated and 1 μM MRE-269-treated PAH PASMCs. These results strongly suggest that TASK-1 channel blockade blocked the anti-proliferative effect of 100 nM MRE-269 but not that of 1 μM MRE-269 on PAH PASMCs.
Figure 6.5: TASK-1 channel blockade attenuates the anti-proliferative effects of MRE-269 on PAH PASMCs. PAH PASMCs (n = 4) were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were serum-starved in 0.1% FBS for 48 hours to induce cell cycle arrest in the G0/G1 phase. After serum-starvation, PAH PASMCs were treated with either DMSO, 100 nM or 1 μM MRE-269, an IP receptor agonist, without or with 10 μM ML365, a TASK-1 channel blocker, in 10% FBS for 96 hours to allow for cell proliferation. After 96 hours, PAH PASMCs were trypsinised and counted using the vital dye trypan blue and a disposable haemocytometer. Cell counts for each treatment were then expressed as a percentage of the cell count for the DMSO-treated PAH PASMCs. Values are means ± SEM. Repeated measures one-way ANOVA with Tukey’s test for multiple comparisons were used. p < 0.05 was considered statistically significant.
6.2.6 TASK-1 channel blockade attenuates the anti-proliferative effect of butaprost on PAH PASMCs

EP$_2$ receptors have recently been reported to be upregulated in PAH PASMCs and their activation inhibits the proliferation of PAH PASMCs to a larger degree than IP receptor agonism (Patel et al., 2018). To investigate whether TASK-1 channels mediate the strong anti-proliferative effect of EP$_2$ receptor agonism on PAH PASMCs, PAH PASMCs were seeded at low density (20,000 cells/well) in 10% FBS and then serum-starved in 0.1% FBS for 48 hours to synchronise the cells in the G0/G1 phase of the cell cycle. Serum-starved PAH PASMCs were then treated with either DMSO, 100 nM or 1 μM butaprost, an EP$_2$ receptor-selective agonist, in the absence or presence of 10 μM ML365, a blocker selective for TASK-1 channels, in 10% FBS for 96 hours to allow for PASMC proliferation. After 96 hours, PAH PASMCs were trypsiniised and counted using trypan blue and a disposable haemocytometer. Cell counts were expressed as a percentage of the cell count for the DMSO-treated population.

As shown in Figure 6.6, compared to DMSO, 100 nM butaprost inhibited the proliferation of PAH PASMCs by 35% ($p = 0.0058; n = 4$). The higher dose of 1 μM butaprost had a stronger anti-proliferative effect, reducing proliferation by 62% ($p = 0.0006; n = 4$). 10 μM ML365 on its own had no effect on the proliferation of PAH PASMCs. There were no significant differences in proliferation between PAH PASMCs treated with either 100 nM or 1 μM butaprost in the absence of 10 μM ML365 and those treated with either butaprost concentration in the presence of 10 μM ML365. However, 10 μM ML365 did inhibit the anti-proliferative effects of both 100 nM and 1 μM butaprost on PAH PASMCs, as suggested by the following two findings. First, the mean difference (4%) between 10 μM ML365-treated PAH PASMCs and PAH PASMCs subjected to a combination of 100 nM butaprost and 10 μM ML365 does not lie within the 95% CI [18%, 51%] for the mean difference (35%) between DMSO-treated and 100 nM butaprost-treated PAH PASMCs. Second, the mean difference (27%) between 10 μM ML365-treated PAH PASMCs and PAH PASMCs treated with both 1 μM butaprost and 10 μM ML365 does not fall within the 95% CI [49%, 74%] for the mean difference (62%) between DMSO-treated and 1 μM butaprost-treated PAH PASMCs.
Figure 6.6: TASK-1 channel blockade attenuates the anti-proliferative effects of butaprost on PAH PASMCs. PAH PASMCs (n = 4) were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were serum-starved in 0.1% FBS for 48 hours to induce cell cycle arrest in the G0/G1 phase. After serum-starvation, PAH PASMCs were treated with either DMSO, 100 nM or 1 μM butaprost, an EP2 receptor agonist, without or with 10 μM ML365, a TASK-1 channel blocker, in 10% FBS for 96 hours to allow for cell proliferation. After 96 hours, PAH PASMCs were trypsinised and counted using the vital dye trypan blue and a disposable haemocytometer. Cell counts for each treatment were then expressed as a percentage of the cell count for the DMSO-treated PAH PASMCs. Values are means ± SEM. Repeated measures one-way ANOVA with Tukey’s test for multiple comparisons were used. p < 0.05 was considered statistically significant.
6.2.7 Treprostinil, but not MRE-269 or butaprost, increase the mRNA expression of TASK-1 in proliferating PAH PASMCs

Treprostinil has previously been shown to increase $I_{\text{KN}}$ through TASK-1 channels at clinically relevant concentrations in PASMCs (Li et al., 2012; Olschewski et al., 2006). The increase in $I_{\text{KN}}$ was accompanied by hyperpolarisation of the resting membrane potential (Olschewski et al., 2006). It has previously been suggested that increasing $K^+$ currents through TASK-1, either by increasing the number of functional channels in the plasma membrane or the activity of individual channels, could be beneficial for PAH patients (Ma et al., 2013). To determine whether the anti-proliferative effects of treprostinil, MRE-269 and butaprost are associated with an increase in the expression of TASK-1, PAH PASMCs were seeded at a density of 20,000 cells/well in 6-well plates, serum-starved in 0.1% FBS for 48 hours and treated with either DMSO, 100 nM treprostinil, 100 nM MRE-269 or 100 nM butaprost in 10% FBS for 96 hours. RNA was isolated and RT-qPCR was performed to determine the mRNA expression of KCNK3 relative to the housekeeping gene TBP. As shown in Figure 6.7, 100 nM treprostinil upregulated the relative mRNA expression of KCNK3 in proliferating PASMCs by two folds to possibly increase the number of functional channels in the sarcolemma and hence the hyperpolarising $I_{\text{KN}}$. The IP receptor agonist MRE-269, in contrast, failed to upregulate the mRNA expression of TASK-1 relative to TBP. Similar to MRE-269, the EP$_2$ receptor agonist butaprost had no effect on the relative mRNA expression of TASK-1.
Figure 6.7: Treprostinil, but not MRE-269 or butaprost, increases the mRNA expression of KCNK3 in proliferating PAH PASMCs. PAH PASMCs (n = 5) were seeded at a density of 20,000 cells per well in 6-well plates and maintained in 10% FBS overnight. PAH PASMCs were serum-starved in 0.1% FBS for 48 hours to induce cell cycle arrest in the G0/G1 phase. After serum-starvation, PAH PASMCs were treated with either DMSO, 100 nM treprostinil, 100 nM MRE-269 or 100 nM butaprost in 10% FBS for 96 hours to allow for cell proliferation. After 96 hours, RNA was isolated and RT-qPCR was performed to determine the mRNA expression of KCNK3 relative to the housekeeping gene TBP. Values are means ± SEM. One-way ANOVA with Bonferroni’s test for multiple comparisons were used. p < 0.05 was considered statistically significant.
6.2.8 TASK-1 channel blockade inhibits mitochondrial function in control PASMCs

In control PASMCs, knockdown of KCNK3, the gene encoding TASK-1, has been reported to induce depolarisation of the mitochondrial membrane potential and fragmentation of the mitochondrial network (Lambert et al., 2019). To investigate whether loss of TASK-1 currents alters mitochondrial respiration in control PASMCs, OCRs were in control PASMCs treated with either DMSO or 10 µM ML365 for 24 hours. Basally, control PASMCs treated with ML365 appeared to consume oxygen at a slightly lower rate than those treated with DMSO, as reflected in the significant reduction in OCR associated with basal respiration ($p = 0.0421; n = 3$; Figure 6.8B). Injection of oligomycin A, an inhibitor of complex V or the ATP synthase of the mitochondrial ETC, resulted in a smaller dip in OCR in control PASMCs treated with ML365 than in DMSO-treated control PASMCs. Indeed, OCR associated with ATP production was halved in ML365-treated control PASMCs compared to the DMSO-treated ones ($p = 0.0100; n = 3$; Figure 6.8C).

Injection of the uncoupler FCCP, which induces maximal respiration, elevated OCR in control PASMCs treated with DMSO to much higher levels than in ML365-treated control PASMCs. Consistently, OCR associated with maximal respiration was two-fold lower in ML365-treated control PASMCs than in the DMSO-treated ones ($p = 0.0082; n = 3$; Figure 6.8D). Moreover, as shown in Figure 6.8E, the spare respiratory capacity was three-fold lower in PASMCs treated with ML365 than in PASMCs treated with DMSO ($p = 0.0059; n = 3$). Following FCCP-induced maximal OCR, injection of rotenone and antimycin A, inhibitors of complex I and III, respectively, completely inhibited mitochondrial OCR, revealing that associated with non-mitochondrial respiration or oxygen consumption. As shown in Figure 6.8F, non-mitochondrial OCR in PASMCs treated with ML365 was similar to that in those treated with DMSO.
Figure 6.8: TASK-1 channel blockade inhibits mitochondrial function in control PASMCs. Control (n = 3) PASMCs were seeded at a density of 7,500 cells per well in Seahorse XFp Cell Culture Miniplates and maintained in 10% FBS overnight. PASMCs were then serum-starved in 0.1% FBS for 24 hours and treated with 10 μM ML365 in 0.1% FBS for another 24 hours. PASMCs were transferred to Mito Stress Test medium and (A) OCRs were measured using the Seahorse XFp Analyser. Oligomycin A, FCCP and a combination of rotenone and antimycin A were serially injected as part of the
Seahorse XFp Mito Stress Test to assess key parameters of mitochondrial function, including basal respiration (B), ATP production (C), maximal respiration (D), spare respiratory capacity (E), non-mitochondrial respiration (F) and proton leak (G). Values are means ± SEM. Unpaired student’s t-test was used in B, C, D, E, F and G. p < 0.05 was considered statistically significant.
6.2.9 TASK-1 channel blockade promotes the apoptosis of control PAECs

PAEC apoptosis is thought to constitute an early triggering event in PAH pathogenesis. Indeed, loss of BMPR2 function in PAECs, due to siRNA-mediated knockdown or PAH-causing mutations, was found to increase the susceptibility of PAECs to apoptosis (Long et al., 2015). It was therefore speculated that loss of TASK-1 channel function might also have a similar pro-apoptotic effect on PAECs. Therefore, PAECs were cultured to full confluence and pretreated with either DMSO, 1µM ML365 or 10 µM ML365 for 16 hours. Following pretreatment, apoptosis was induced by culturing the PAECs with a combination of 10 ng/ml TNFα and 20 µg/ml cycloheximide in the absence or presence of either DMSO, 1 µM ML365 or 10 µM ML365. As previously reported (Long et al., 2015) and shown in Figure 6.9, treatment of PAECs with TNFα in combination with the protein synthesis inhibitor cycloheximide to block the anti-apoptotic arm of the TNFα signalling pathway, induced apoptosis, as indicated by a 16-fold increase in the levels of cleaved caspase-3 relative to β-tubulin ($p = 0.0108; n = 5$). Pretreatment of human PAECs with 1 µM ML365, a highly selective TASK-1 channel blocker, did not significantly further increase the relative levels of cleaved caspase-3 in PAECs, stimulated with the apoptotic stimulus. In contrast, pretreatment of PAECs with 10 µM ML365, a dose previously shown to completely block TASK-1 currents, further increased the relative levels of cleaved caspase-3 in PAECs, stimulated with the apoptotic stimulus ($p = 0.0026; n = 5$). These findings were also confirmed using immunofluorescent staining as shown in Figure 6.10. PAECs were more heavily stained for cleaved caspase-3 following treatment with the apoptotic stimulus of 10 ng/ml TNFα and 20 µg/ml cycloheximide and pretreatment with ML365 further increased the intensity of this staining.
Figure 6.9: TASK-1 channel blockade promotes apoptosis of control PAECs. Control PAECs (n = 5) were cultured to full confluence and then treated with either DMSO, 1 µM or 10 µM ML365, a TASK-1 channel blocker, for 16 hours. Apoptosis was then induced by incubating the PAECs with 10 ng/ml TNFα and 20 µg/ml cycloheximide (CHX) in the presence of either DMSO, 1 µM or 10 µM ML365 for 6 hours. (A) Proteins were isolated and immunoblotted for cleaved caspase-3 and caspase-3. Blots were reprobed for β-tubulin to ensure equal loading. (B) Densitometry was used to determine the levels of cleaved caspase-3 relative to β-tubulin in human PAECs. Values are means ± SEM. One-way ANOVA with Bonferroni’s test for multiple comparisons were used in B. p < 0.05 was considered statistically significant.
Figure 6.10: TASK-1 channel blockade promotes apoptosis of control PAECs as indicated by immunofluorescent staining for cleaved caspase-3. Control PAECs were cultured in 8-chamber slides and then treated with either DMSO, 1 µM or 10 µM ML365, a TASK-1 channel blocker, for 16 hours. Apoptosis was then induced by incubating the PAECs with 10 ng/ml TNFα and 20 µg/ml cycloheximide (CHX) in the presence of either DMSO, 1 µM or 10 µM ML365 for 6 hours. PAECs were then fixed and stained for cleaved caspase-3 (green) and F-actin with phalloidin (red). PAECs were imaged at x60 magnification using laser scanning confocal microscopy.
6.3 Discussion

TASK-1 is a two-pore domain potassium channel, whose major role in PASMCs is to regulate the resting membrane potential and by extension pulmonary vascular tone (Olschewski et al., 2017). High-penetrance loss-of-function mutations in KCNK3, the gene encoding TASK-1, were recently identified in patients with HPAH and IPAH, implicating the channel in the pathogenesis of PAH and suggesting possible roles for the channel in regulating pulmonary vascular remodelling (Ma et al., 2013; Tejedor et al., 2017). Similar to BMPR2, the gene most frequently mutated in PAH, TASK-1 expression was shown to be severely reduced in the explanted lungs of patients suffering from IPAH but lacking PAH-causing mutations (Antigny et al., 2016). Whole-cell K⁺ currents through TASK-1 channels, as defined by their sensitivity to the TASK-1 channel blocker A293, were also shown to be attenuated in PAECs and PASMCS derived from the lungs of patients with HPAH due to BMPR2 mutations, suggesting that TASK-1 downregulation could also represent a second hit required for the disease to manifest in carriers of low penetrance BMPR2 mutations (Antigny et al., 2016). Inconsistent with these findings, a statistically non-significant decrease in TASK-1 mRNA expression was observed in our PAH PASMC isolates, as compared to control PASMCs. However, unaltered TASK-1 expression in PAH PASMCs does not necessarily mean that K⁺ currents through TASK-1 channels remain intact. Impaired trafficking of TASK-1 channel subunits to the plasma membrane, for example, may lead to reduction in currents. Interestingly, TASK-1 channel subunits were found to be retained in the endoplasmic reticulum of PASMCs derived from the lungs of rats treated with monocrotaline (Antigny et al., 2016). Moreover, TASK-1 channels could be forced into a closed state by vasoconstrictors and PASMC mitogens, such as ET-1 (Seyler et al., 2012; Tang et al., 2009). ET-1 has previously been shown to be upregulated in PAH PASMCs and to inhibit TASK-1 channel activity via ROCK-mediated phosphorylation of the TASK-1 channel subunit downstream of ETₐ and ET₇ receptor coupling to G₁₂/₁₃ and subsequent RhoA activation (Seyler et al., 2012). ET receptors also couple via G₉ to activate PLCβ, which cleaves the membrane phospholipid PIP₂ to yield DAG and IP₃. DAG has been shown to inhibit TASK-1 channel activity possibly by directly interacting with the channel, particularly its hydrophobic core, or by activating DAG-dependent PKC, which then phosphorylates and inactivates TASK-1 (Tang et al., 2009; Wilke et al., 2014). As their name suggests, TASK-1 channels are inhibited by extracellular acidosis. Increased lactic acid production and extrusion secondary to upregulated glycolysis, as demonstrated in Chapter 5, could therefore also be promoting TASK-1 channel closure in PAH PASMCs. Finally, oxidative stress, as evidenced by increased intracellular ROS levels, could be inhibiting TASK-1 channel
activity in PAH PASMCs as it does other hypoxia-sensitive K⁺ channels, such as the voltage-dependent Kv1.5 channel (Moudgil et al., 2006).

Histopathological analysis of distal pulmonary arteries from a PAH patient carrying a loss-of-function KCNK3 mutation showed severe vascular remodelling (Ma et al., 2013). Consequently, loss of TASK-1 currents in PAH, due to TASK-1 mutations or downregulation, has been suggested to contribute to the high proliferation rates of PAECs, PASMCs and pulmonary adventitial fibroblasts (Ma et al., 2013). Indeed, treatment of rats with A293, a TASK-1 channel blocker, or mutation of KCNK3 induced the proliferation of pulmonary vascular cells, causing neomuscularisation of distal pulmonary arteries and elevating right ventricular systolic pressure (Antigny et al., 2016; Lambert et al., 2019). This chapter explored the effect of blocking TASK-1 channels and the hyperpolarising background currents carried by these channels on the proliferation of control PASMCs as well as those derived from patients with PAH. The commercially available agent ML365, which selectively blocks TASK-1 channels, was used. It was previously shown that 10 μM ML365 completely blocks TASK-1 currents in control PASMCs and could therefore mimic the effect of loss-of-function mutations on TASK-1 channel activity (Bohnen et al., 2017). Treating quiescent control PASMCs with 10 μM ML365 in 10% FBS increased proliferation of control PASMCs by approximately 50% compared to those treated with DMSO in 10% FBS. This is consistent with the finding that siRNA-mediated knockdown of KCNK3 enhances the proliferation of control PASMCs by 40% (Lambert et al., 2019). ML365 had no effect on the proliferation of PAH PASMCs, however, indicating that TASK-1 channel activity might already be severely diminished in these highly proliferative cells possibly due to downregulation of TASK-1 expression. The pro-proliferative effect of TASK-1 channel blockade on control PASMCs, but not their PAH counterparts, suggests that depressed TASK-1 activity contributes to the higher proliferation rates of PAH PASMCs.

Several mechanisms were proposed to underlie the pro-proliferative effect of loss of TASK-1 channel activity on PASMCs. In PASMCs, TASK-1 channels mediate outward K⁺ currents that drag the membrane potential towards the Nernst potential for K⁺, hyperpolarising the sarcolemma and switching off the voltage-gated Ca²⁺ channels, namely L-type Ca²⁺ channels, that open in response to membrane depolarisation (Olschewski et al., 2017). Inhibition of TASK-1 currents in PASMCs, either with pharmacological blockers (e.g. A293 or ML365) or siRNA-mediated knockdown of KCNK3, has been shown to depolarise the membrane potential (Antigny et al., 2016). Long-term TASK-1 channel inhibition has also been shown to reduce whole-cell K⁺ currents carried by other K⁺ channels to cause membrane depolarisation in PASMCs.
(Antigny et al., 2016; Lambert et al., 2019). This is thought to promote the opening of voltage-gated calcium channels and subsequently increase the cellular influx of calcium. The rise in free intracellular calcium is then thought to trigger PASMC proliferation by activating a number of calcium-dependent proteins, including Ca\(^{2+}\)/calmodulin-dependent calcineurin and Ca\(^{2+}\)/calmodulin-dependent kinase (CaMK). Indeed, increased nuclear localisation of calcineurin and its targets NFATc2-4, suggestive of increased calcineurin phosphatase activity, has been demonstrated in PAH PASMCs (Bonnet et al., 2007; Chen et al., 2017; He et al., 2018). The CaMK isoform CaMKIV has been shown to be upregulated in PAH PASMCs and its knockdown decreased the proliferation of PAH PASMCs. Knockdown of KCNK3 in control PASMCs resulted in enhanced phosphorylation of the pro-survival and pro-proliferative kinase ERK1/2 and increased expression of HIF1α (Lambert et al., 2019). Normoxic stabilisation of HIF1α and consequent induction of a pseudo-hypoxic state were previously reported to underlie the highly proliferative phenotype of PAH PASMCs and TASK-1 channel dysfunction may contribute to this anomaly. Moreover, KCNK3 knockdown caused fragmentation of the mitochondrial network without influencing the expression of DRP1, the major mediator of mitochondrial fission (Lambert et al., 2019). Increased mitotic mitochondrial fragmentation is a well-established feature of PAH PASMCs, as demonstrated in Chapter 3, and has been repeatedly associated with their increased proliferative capacity (Marsboom et al., 2012). However, in contrast to PAH PASMCs, in which the mitochondrial membrane potential was shown to be hyperpolarised and KCNK3 expression reduced, control PASMCs, in which KCNK3 has been knocked down, exhibited more depolarised mitochondrial membrane potentials (Bonnet et al., 2007; Lambert et al., 2019; Pak et al., 2013). Whether TASK-1 channels reside in the mitochondria of PASMCs and other pulmonary vascular cells remains unexplored. Interestingly, however, TASK-3 channels, close relatives of TASK-1, have been shown to localise to the mitochondria of aldosterone-producing zona glomerulosa cells, where they are thought to regulate mitochondrial morphology and membrane potential (Yao et al., 2017). Analogously, TASK-1 channels might reside within the mitochondria of pulmonary vascular cells, which do not express TASK-3. However, Lambert et al. (2019) proposed that cell-surface TASK-1 channel blockade and consequent plasma membrane depolarisation is the likely cause of mitochondrial membrane depolarisation as increasing extracellular K\(^+\), which also serves to depolarise the plasma membrane, had a similar effect on the mitochondrial membrane potential.

Treprostinil was previously shown to enhance anandamide-sensitive background K\(^+\) currents through TASK-1 channels in human PASMCs in a largely PKA-dependent manner (Li et al., 2012). In addition, cAMP was found to upregulate the mRNA
expression of TASK-1 in human brown adipocytes, cells whose precursors are smooth muscle-like (Shinoda et al., 2015). PKA has also been shown to promote the forward trafficking of TASK-1 channels from the endoplasmic reticulum, where they are synthesised and folded, to the plasma membrane (Kilisch et al., 2016; Mant et al., 2011). Macroscopic currents through TASK-1 channels can be enhanced either by increasing the number of functional channels in the plasma membrane or enhancing the activity of individual channels. This chapter sought to determine whether prolonged treprostinil treatment upregulates the expression of TASK-1 to increase TASK-1 currents in proliferating PAH PASMCs. Treprostinil was found to significantly upregulate the expression of TASK-1 mRNA in proliferating PASMCs. Individual IP or EP₂ receptor agonism with either MRE-269 or butaprost, respectively, failed to significantly upregulate TASK-1 mRNA expression in proliferating PASMCs. This suggests that TASK-1 mRNA upregulation either requires simultaneous agonism of both the IP and EP₂ receptors, which then synergise to deliver a signal to the nucleus strong enough to upregulate transcription of the KCNK3 gene, or the nuclear receptor and transcription factor PPARγ, which acts downstream of treprostinil, but not MRE-269 or butaprost.

The anti-proliferative effects of prostacyclins on PASMCs, both control and PAH, are well documented in the literature. Treprostinil, for example, has been shown to inhibit the proliferation of control and PAH PASMC at clinically relevant concentrations (Falcetti et al., 2010; Patel et al., 2018). Since treprostinil has been reported to activate TASK-1 channels and hyperpolarising outward background K⁺ currents in PASMCs, this chapter sought to elucidate whether the anti-proliferative effects of treprostinil are mediated by TASK-1 channels. Consistent with previous findings, 100 nM and 1 μM treprostinil inhibited the proliferation of control and PAH PASMCs. However, TASK-1 channel blockade with 10 μM ML365, a concentration previously shown to completely inhibit whole-cell K⁺ currents through TASK-1 channels, had no effect on the anti-proliferative effects of both treprostinil concentrations on control and PAH PASMCs. These findings suggest that TASK-1 channel activation and/or upregulation does not contribute to the anti-proliferative activity of treprostinil in control and PAH PASMCs. They also suggest that the efficacy of treprostinil at delaying progression of PAH might be retained in patients carrying of loss-of-function KCNK3 mutations.

Not only does treprostinil promote the activity of TASK-1 channels, which are increasingly being implicated in the pathogenesis of PAH, it has also been reported to enhance the activity of BMPR2 receptors, protein products of the gene most commonly mutated in PAH patients (Yang et al., 2010). PASMCs isolated from PAH patients harbouring mutations in BMPR2 display impaired responses to the growth suppressive
effects of BMPs (e.g. BMP4), ligands for the BMPR2 receptor. Treprostinil has been shown to restore the anti-proliferative effect of BMPR2 ligation with BMP4 on PASMCs harbouring BMPR2 mutations (Yang et al., 2010). Treprostinil has also been demonstrated to enhance BMP-induced phosphorylation of Smad1/5 and downstream induction of Id1 transcription in a cAMP-dependent manner (Yang et al., 2010). Strikingly, treprostinil-induced upregulation of Id1 expression has also been shown to occur independently of Smad1/5 phosphorylation and possibly via the binding of the transcription factor CREB to cAMP-response elements within the promoter region of Id1 (Yang et al., 2010). Given the ability of treprostinil to rescue defective BMPR2 signalling in PAH and the finding that TASK-1 channel function is lost in PASMCs from PAH patients with BMPR2 mutations (Antigny et al., 2016; Yang et al., 2010), treprostinil may also be enhancing TASK-1 channel function indirectly by upregulating BMPR2 signalling. It remains to be explored whether treprostinil rescues the activity of TASK-1 channels in PASMCs harbouring PAH-associated KCNK3 mutations and whether the anti-proliferative effect of treprostinil remains intact in these KCNK3 mutant PASMCs.

Prostacyclin analogues were originally thought to act exclusively via the IP receptor. Several lines of evidence, however, suggest otherwise. Treprostinil, for example, was shown to bind with high affinity to human DP1, EP2 and IP receptors and elevate cAMP in HEK293 cells heterologously expressing those receptors (Whittle et al., 2012). Moreover, IP receptor knockout had very little effect on the anti-proliferative activity of iloprost in murine PASMCs, suggesting the involvement of other prostanoid receptors or even nuclear receptors, such as PPARγ (Falcetti et al., 2010). Prostacyclin analogues were also shown to signal differentially in normal and PAH PASMCs to inhibit proliferation (Falcetti et al., 2010; Patel et al., 2018). In control PASMCs, the anti-proliferative activity of treprostinil was largely dependent on the IP receptor and the downstream effector adenylyl cyclase (Falcetti et al., 2010). Contrastingly, in PAH PASMCs, the anti-proliferative activity of treprostinil was insensitive to IP receptor or adenylyl cyclase antagonists but was inhibited by PPARγ antagonists (Falcetti et al., 2010). Recently, in addition to reduced IP receptor expression, EP2 receptor upregulation was reported in the pulmonary arteries of PAH patients and in PASMCs derived from patients with PAH (Patel et al., 2018). EP2 receptors were also implicated in the anti-proliferative activity of treprostinil in PAH PASMCs (Patel et al., 2018). Whether the anti-proliferative effects of individual IP or EP2 receptor agonism are mediated by TASK-1 channels was investigated. 100 nM and 1 μM butaprost, an agonist selective for the EP2 receptors, inhibited PAH PASMC proliferation in a similar manner to treprostinil; however, MRE-269, an IP receptor agonist, had a much weaker anti-proliferative on PAH PASMCs. This provides evidence in support of the more prominent role of the EP2
receptors in inhibiting PAH PASMC proliferation due to the increased expression of the receptor. TASK-1 channel blockade with 10 μM ML365 completely reversed the weak anti-proliferative effects of 100 nM and 1 μM MRE269. Together with the finding that MRE-269 fails to upregulate the mRNA expression of TASK-1 in proliferating PAH PASMCs, the complete reversal of the anti-proliferative effect of MRE-269 on PAH PASMCs by TASK-1 channel blockade suggests that upregulation of TASK-1 channel activity but not expression underlie the growth suppressive effects of MRE-269. This finding also suggests that the IP receptor reserve, despite being diminished in PAH PASMCs, is sufficient to enhance the activity but not the expression of TASK-1. 10 μM ML365 also completely reversed the anti-proliferative effects of 100 nM and 1 μM butaprost. Similar to MRE-269, butaprost had no effect on the mRNA expression of TASK-1, suggesting that increased channel activity rather than expression underlies the anti-proliferative effect of butaprost, particularly at 100 nM, on PAH PASMCs. The insensitivity of the growth suppressive effect of the prostacyclin analogue and PAH drug treprostinil on PAH PASMCs to TASK-1 channel blockade suggests that it could be more clinically efficacious in PAH patients harbouring loss-of-function TASK-1 mutations than MRE-269, the active metabolite of the non-prostanoid IP receptor agonist and PAH drug selexipag, whose anti-proliferative effect on PAH PASMCs seems to be dependent on TASK-1 channel activity.

Mitochondrial function has been reported to be depressed in PASMCs isolated from patients with PAH, largely as a consequence of increased PDK-mediated phosphorylation and inhibition of PDH (Boucherat et al., 2018; Michelakis et al., 2017). Recently, knockdown of KCNK3 and consequent loss of TASK-1 currents have been shown to cause depolarisation of the mitochondrial membrane potential and fragmentation of the mitochondrial network, two events that precede mitophagy, a process whereby cells degrade dysfunctional or superfluous mitochondria (Jin and Youle, 2012; Lambert et al., 2019). Mitochondrial membrane depolarisation serves to stabilise the mitochondrial serine-threonine kinase PINK1, which then recruits and phosphorylates the E3 ubiquitin ligase parkin. Subsequently, parkin in concert with E2 ubiquitin-conjugating enzymes decorate OMM proteins with ubiquitin, creating mitophagy tags for autophagy receptors that direct their sequestration by autophagosomes and their degradation in lysosomes (Jin and Youle, 2012). Moreover, fragmentation of the mitochondrial network facilitates mitophagy by creating fragments small enough to be engulfed by autophagosomes (Gomes and Scorrano, 2013). Increased mitophagy was postulated to contribute to the decreased mitochondrial function repeatedly observed in PAH PASMCs (Boucherat et al., 2018; Michelakis et al., 2017). However, evidence, presented in Chapter 4, suggests otherwise and is consistent
with the hyperpolarisation of the mitochondrial membrane potential reported in PAH PASMCs, which is thought to hinder mitophagy and the clearance of dysfunctional mitochondrial. Consistent with the effects of KCNK3 knockdown on the mitochondria in control PASMCs, as reported by Lambert et al., and the current metabolic theory of PAH, TASK-1 channel blockade with ML365 was found to reduce mitochondrial oxygen consumption rates, particularly those which are associated with basal respiration, ATP production and maximal respiration, in control PASMCs. The mechanisms underlying this effect remain to be deciphered but could involve increased mitochondrial fission and mitochondrial membrane depolarisation, a consequence of loss of hyperpolarising TASK-1 currents concomitant with plasma membrane depolarisation.

In the early stages of PAH, endothelial cell apoptosis and increased monolayer permeability are thought to initiate the vascular remodelling and precede the uncontrolled proliferation of endothelial cells, which gives rise to the lumen-obliterating plexiform lesions. PAECs from the lungs of BMPR2 mutation-harbouring patients with established PAH were shown to be more susceptible to apoptotic stimuli and form more permeable barriers than those derived from the lungs of healthy donors (Long et al., 2015). siRNA-mediated knockdown of BMPR2 was also shown to promote the apoptosis of human PAECs (Long et al., 2015). It was postulated that TASK-1 channel blockade in human PAECs renders them susceptible to apoptosis. TASK-1 channel blockade is believed to mimic the loss of function observed for PAH TASK-1 mutants. TNFα together with the protein synthesis inhibitor cycloheximide to block the anti-apoptotic arm of TNFα signalling were used as apoptotic stimuli and were found to induce PAEC apoptosis, as previously reported (Long et al., 2015). ML365, a highly selective blocker of TASK-1 channels, was found to enhance TNFα/cycloheximide-induced apoptosis of human PAECs, indicating an increase in apoptosis susceptibility of PAECs following TASK-1 channel blockade. The mechanisms whereby loss of TASK-1 currents increases the susceptibility of PAECs to apoptotic ligands remain unclear and may include increased Ca²⁺ influx and the mitochondrial apoptotic pathway. Indeed, nicorandil, an ATP-sensitive potassium (K<sub>ATP</sub>) channel opener, was found to inhibit the serum starvation-induced apoptosis of human umbilical vein endothelial cells through mitochondrial K<sub>ATP</sub> channels (Sahara et al., 2012). Interestingly, nicorandil was also effective at attenuating monocrotaline-induced pulmonary endothelial damage (Sahara et al., 2012). Recently, in rats, rendered deficient in KCNK3 and therefore functional TASK-1 channels by CRISPR-Cas9, PAECs were shown to lose endothelial cell markers, such as CD31, and acquire mesenchymal markers, such as TWIST1, as a consequence of pathological EndoMT (Lambert et al., 2019). PAECs, which have undergone EndoMT, have been shown to be more apoptotic than those, which have retained their endothelial cell
phenotype. Furthermore, TASK-1 channel blockade with A293 reduced proliferation of PAECs in vitro (Lambert et al., 2019). These findings are consistent with the pro-apoptotic effect of TASK-1 channel blockade on PAECs that was demonstrated in this chapter.

In conclusion, evidence was provided in this chapter in support of TASK-1 channel dysfunction in PAH. A trend towards a reduction in TASK-1 expression was observed in PAH PASMCs. Moreover, TASK-1 channel blockade promoted the proliferation of control PASMCs but not that of PAH PASMCs, suggesting that loss of TASK-1 channel function confers a proliferative PAH phenotype upon control PASMCs. Dependency of the anti-proliferative effects of prostacyclin mimetics in both control and PAH PASMCs on TASK-1 channel function was also reported. TASK-1 channels were found to partly mediate the anti-proliferative of treprostinil on control PASMCs to a larger extent than that on PAH PASMCs. In contrast, the anti-proliferative effect of the IP receptor-selective agonist MRE-269 on PAH PASMCs appeared to be fully mediated by TASK-1 channels. At the lower of the two concentrations used, the EP<sub>2</sub> receptor-selective agonist butaprost inhibited proliferation of PAH PASMCs predominantly via activating TASK-1 channels. Increasing the concentration of butaprost, however, resulted in an anti-proliferative effect on PAH PASMCs that was independent of TASK-1 channel activity, as suggested by a partial reversal of butaprost’s anti-proliferative effect on PAH PASMCs following TASK-1 channel blockade. Suppressed mitochondrial respiration is a widely reported feature of PAH PASMCs. TASK-1 channel blockade was found to inhibit mitochondrial oxygen consumption in control PASMCs, further suggesting that loss of TASK-1 channel confers a PAH phenotype on control PASMCs. Finally, TASK-1 channel blockade rendered normal PAECs susceptible to apoptosis, an early event in PAH that is thought to trigger endothelial dysfunction and pulmonary vascular remodelling.
7. General discussion and future perspectives

PAH remains a fatal cardiopulmonary disease with very few treatment options and no cure. In spite of their ability to alleviate symptoms and improve prognosis, current PAH treatments fail to halt or reverse the disease pathology, particularly the extensive remodelling of the small pulmonary arteries, and serve predominantly to dilate the pulmonary arteries and bring about a drop in PVR, mPAP and right heart afterload. Therefore, there remains a dire need for the identification of novel targets. In order to identify effective therapeutic targets, one must better understand the pathophysiology of this heterogeneous and complex cardiopulmonary disorder and uncover a common denominator shared by the various molecular and biochemical abnormalities that promote not only the proliferation of pulmonary vascular cells but also right heart failure and other extrapulmonary anomalies. Interestingly, the metabolic theory of PAH, recently proposed by Paulin and Michelakis (2014), posits that numerous, unrelated molecular abnormalities in PAH converge to either cause or promote mitochondrial dysfunction. Mitochondrial dysfunction in PAH includes a normoxic shift from mitochondrial OxPhos to cytosolic glycolysis, analogous to the Warburg phenomenon observed in cancer cells (Paulin and Michelakis, 2014). It also includes mitotic fragmentation of the mitochondrial network, hyperpolarisation of the mitochondrial membrane potential and defects in the opposing processes of mitochondrial biogenesis and mitophagy (Michelakis and Paulin, 2014). Collectively, these mitochondrial changes are thought to underlie the proliferative, synthetic and apoptosis-resistant phenotype of pulmonary vascular cells in PAH. However, this mitochondrial dysfunction is not only restricted to pulmonary vascular cells and the lung, the primary site of disease pathology, but has also been demonstrated in the failing right heart and other extrapulmonary tissues in PAH (Paulin and Michelakis, 2014). It is therefore believed that normalising mitochondrial function may prove beneficial for PAH patients. TASK-1 channels also constitute a promising therapeutic target in PAH.

This thesis sought to better understand the mechanisms of mitochondrial dysfunction in PAH with a focus on PASMCs that comprise the medial layers of distal pulmonary arteries and whose hyperproliferation in PAH leads to thickening of the pulmonary vascular wall and consequent luminal narrowing. This thesis first explored whether mitochondrial dynamics and the mechanisms regulating it are impaired in distal PASMCs isolated from patients with PAH, comparing them with distal PASMCs from non-PAH patients or control subjects. The effect of prostacyclin mimetics on mitochondrial
dynamics in PAH PASMCs were also explored. Moreover, mitochondrial capacity to oxidise glucose and generate ATP through OxPhos was investigated in PAH PASMCs and compared to distal PASMCs in an attempt to confirm the Warburgian suppression of mitochondrial OxPhos previously described in PAH PASMCs. Changes in mitochondrial mass may underlie changes in mitochondrial function in PAH PASMCs. Mitochondrial mass as well as its key determinants mitochondrial biogenesis and mitophagy, which add and remove mitochondrial mass, respectively, were also investigated in distal PASMCs derived from PAH patients. The expression and activity of key subunits of PDH, the enzyme complex that occupies the junction between cytosolic glycolysis and mitochondrial respiration, were also assessed. In addition to mitochondrial glucose oxidation, glucose flux through glycolysis and the PPP was also investigated. The expression of key glycolytic and PPP enzymes was assessed in control and PAH PASMCs.

Loss-of-function mutations in TASK-1, albeit being extremely rare, have been shown to exhibit a much higher penetrance and cause a more severe form of PAH than BMPR2 mutations, which are the most frequently identified in patients with IPAH and HPAH. TASK-1 channel dysfunction has also been demonstrated in PAH patients, lacking PAH-causing TASK-1 mutations, further implicating the channel in PAH pathogenesis. TASK-1-deficient rats were recently generated and found to spontaneously develop PAH. These findings strongly suggest that increasing TASK-1 channel expression and/or activity may be beneficial for patients with PAH. Interestingly, the prostacyclin analogues iloprost and treprostinil have both been reported to increase TASK-1 currents. This TASK-1 channel activity-promoting effect was proposed to underlie the pulmonary vasodilatory effects of iloprost and treprostinil. This thesis aimed to establish whether prostacyclin mimetics upregulate TASK-1 channels to inhibit PASMC proliferation in PAH. It also explored whether TASK-1 channel dysfunction leads to mitochondrial dysfunction in PASMCs. Finally, whether loss of TASK-1 channel function promotes endothelial dysfunction, particularly increased susceptibility to apoptosis, was also investigated in this thesis.

7.1 Mitochondrial dynamics in PASMCs from patients with PAH

In Chapter 3, mitochondria were found to be more fragmented in PAH PASMCs than in control PASMCs. This is thought to reflect the highly proliferative state of these PAH cells even under quiescent conditions. Mitochondrial fission has been demonstrated to precede mitosis to facilitate the inheritance of mitochondria by daughter cells. Indeed,
inhibiting mitochondrial fission has been shown to hinder cell cycle progression from the G2 phase to the M phase. Increased expression and activity of DRP1, the primary mediator of mitochondrial fission, was previously shown to underlie this increased mitochondrial fission in PAH. This was also replicated in Chapter 3. Protein levels of DRP1 were found to be higher in PAH PASMCs than in control PASMCs both in vitro and in situ. This increased DRP1 protein expression was accompanied by an increase in phosphorylation at the activating residue S616, suggesting an additional increase in DRP1 fission activity. The phosphorylation of DRP1 at the inhibitory residue S637 was not assessed in control and PAH PASMCs. However, DRP1 is presumed not to be phosphorylated at S637 in these cells as reported previously by Marsboom et al. (2012). DRP1 lacks the ability to directly interact with the mitochondria and therefore requires recruitment and anchorage by adaptor proteins or binding partners. Thus far, four DRP1 adaptor proteins have been identified and these include FIS1, MFF, MiD49 and MiD51. Increased DRP1 recruitment to the mitochondria has previously been suggested to occur as a consequence of increased expression of FIS1, MiD49 and MiD51. However, only FIS1 was found to be upregulated in our PAH PASMC isolates. In Chapter 3, prior to the crude extraction of proteins, PASMCs from control and PAH patients were serum-starved in 0.1% FBS for 48 hours to induce cellular quiescence. The previously reported upregulation of MiD49 and MiD51 in PAH PASMCs may have only been observed under growth-promoting conditions and lost when the PASMCs were quiesced. Therefore, it is important to assess the protein expression of these two novel DRP1 binding partners in proliferating control and PAH PASMCs and also in PASMCs within the medial layers of small pulmonary arteries in lung tissue sections from control and PAH patients.

Opposing mitochondrial fission is mitochondrial fusion, a process in which two colliding mitochondrial fragments join their outer and inner membranes. Given the increase in mitochondrial fragmentation in PAH PASMCs, mitochondrial fusion has been proposed to be depressed in these cells. Indeed, MFN2, a mediator of OMM fusion, but not its close relative MFN1, has repeatedly been shown to be downregulated in PAH PASMCs. In Chapter 3, however, neither MFN1 nor MFN2 were found to be downregulated in PAH PASMCs. Similar to the upregulation of MiD49 and MiD51 in PAH PASMCs, the downregulation previously reported for MFN2 may have been lost when the PASMCs were serum-starved. Therefore, immunoblotting proteins from proliferating control and PAH PASMCs may provide a different finding, possibly concordant with the MFN2 downregulation previously reported. The expression of OPA1, which mediates the fusion of the IMM to complete the process of mitochondrial fusion, has been demonstrated by Marsboom et al. (2012) to be unaffected in PAH PASMCs. Interestingly, OPA1 undergoes proteolytic processing to generate long and short isoforms. The long isoform
remains tethered to the IMM and possesses fusogenic activity, whereas the short isoform is liberated and is thought to play a role in maintaining normal architecture of the mitochondrial cristae. Marsboom et al. (2012) did not examine the proteolytic processing of OPA1 in PAH PASMCs. In Chapter 3, although total OPA1 protein levels in PAH PASMCs were found to be similar to those in control PASMCs, immunoblotting of OPA1 revealed an increase in the protein levels of the short isoform of OPA1 relative to the long isoform, suggesting increased proteolytic cleavage and possibly loss of fusion activity in PAH. OPA1 cleavage is mediated by the metalloproteases YMEL1 and OMA1 (Mishra et al., 2014). Upregulation of either YMEL1, OMA1 or both may underlie the increased proteolytic processing of OPA1 in PAH PASMCs.

Prostacyclin mimetics remain the gold standard for the treatment of patients with severe PAH. They are potent pulmonary vasodilators as well as inhibitors of PASMC proliferation. DRP1 has been suggested to constitute a novel target for the treatment of PAH as pharmacological inhibition of DRP1 had favourable effects on rodent models of PH. Chapter 3 explored whether the stable prostacyclin analogue treprostinil is able to attenuate the excessive mitochondrial fragmentation observed in PAH PASMCs to restore normal mitochondrial dynamics. Treprostinil was indeed found to inhibit mitochondrial fission and promote mitochondrial elongation in PAH PASMCs. The mechanisms underlying this inhibitory effect of treprostinil on mitochondrial fission included transient stimulation of DRP1 phosphorylation at the inhibitory residue S637 and more sustained inhibition of DRP1 phosphorylation at the activating residue S616 with no change in DRP1 protein expression. This effect of treprostinil on the post-translational modifications of DRP1 was found to be mediated by either the IP or EP₂ prostanoid receptors and to be dependent on the activity of cAMP-dependent PKA. Moreover, similar to treprostinil, individual IP or EP₂ receptor agonism with either MRE-269 (active metabolite of the non-prostanoid IP receptor agonist and PAH drug selexipag) or butaprost, respectively, was shown to induce the inhibitory phosphorylation of DRP1 at S637 and to attenuate mitochondrial fission in PAH PASMCs. These findings suggest that DRP1 is already being targeted in PAH patients, albeit non-selectively, by these prostacyclin mimetics. In addition to inhibiting DRP1 activity, treprostinil was also shown to decrease the protein levels of FIS1 and MiD49, implying an inhibitory effect of treprostinil on DRP1 recruitment to mitochondrial fission sites. Treprostinil was also found to increase the protein expression of MFN1 but not MFN2 in PAH PASMCs. The rapidity with which treprostinil upregulated MFN1 suggested that treprostinil may be enhancing the stability of the protein rather than its gene expression. However, the sustained increase in MFN1 expression in response to prolonged treatment of PAH PASMCs to treprostinil suggests that treprostinil may also somehow be driving the
expression of the MFN1 gene. Assessing the transcript levels of MFN1 may help understand the mechanism by which treprostinil is increasing the protein expression of MFN1. Finally, treprostinil was shown to decrease the levels of S-OPA1 in PAH PASMCs, suggesting an inhibitory effect on OPA1 proteolytic cleavage. The mechanisms whereby treprostinil attenuates OPA1 proteolytic cleavage remain to be deciphered. The effect of treprostinil on the expression of YMEL1 and OMA1 could be explored.

7.2 Glycolysis and pentose phosphate shunting in PASMCs from patients with PAH

Chapter 4 examined glucose flux through cytosolic glycolysis and select biosynthetic pathways branching off glycolysis in PAH PASMCs. Several key parameters of glycolytic flux to lactate were found to be heightened in PAH PASMCs, suggesting an increase in the capacity of PAH PASMCs to metabolise glucose via glycolysis and to generate lactate. The liver (PFKL) and muscle (PFKM) isoforms of PFK1, the enzyme that catalyses the first rate-limiting step in glycolysis, were found to be upregulated in PAH PASMCs both in vitro and in situ. The increase in the protein expression of PFKL and PFKM may underlie the increased glycolytic flux observed in PAH PASMCs. This notion could be confirmed by siRNA-mediated knockdown of PFKL, PFKM or both in PAH PASMCs and assessing the contribution of each of these two PFK1 isoforms to the increase in glycolytic flux. The rate-limiting activity of PFK1 is enhanced by the metabolite F2,6BP, which is produced by the enzyme PFKFB3. Chapter 4 reported higher protein levels of PFKFB3 in PAH PASMCs than in control PASMCs. The increase in PFKFB3 expression coupled with an increase in the levels of F2,6BP may also be elevating glycolysis in PAH PASMCs by promoting the already elevated activity of PFK1. This could be confirmed by inhibiting PFKFB3 with small-molecule inhibitors or siRNA-mediated knockdown in PAH PASMCs and assessing glycolytic flux. In addition to its role in regulating the rate of glycolysis, PFKFB3 has been demonstrated to localise to the nuclei of cancer cells, where it regulates the activities of the cell cycle regulators CDK1 and CDK4 to promote proliferation and inhibit apoptosis (Jia et al., 2018; Yalcin et al., 2014). This non-canonical role of PFKFB3 remains unexplored in PAH PASMCs and increased nuclear localisation of PFKFB3 may underlie the increased proliferative capacity and apoptosis resistance of these cells. Ongoing work in the lab has indeed shown increased nuclear localisation of PFKFB3 in PAH PASMCs compared to controls. Given its upregulation in PAH and its recently described role in regulating the cell cycle, PFKFB3 may represent an attractive therapeutic target in PAH.
The last, rate-limiting step in glycolysis, catalysed by PKM, converts phosphoenolpyruvate to pyruvate. Pyruvate is then either transported into the mitochondria, where it is converted to acetyl-CoA to partake in the TCA cycle, or remains in the cytosol, where it is reduced to lactate. PKM activity is governed by the ratio of its two isoforms PKM1 and PKM2, products of alternative splicing of the PKM gene. PKM1 constitutively exists as a high-activity tetramer, whereas PKM2 can form either a low-activity dimer or a high-activity tetramer. An increase in the ratio of PKM2 to PKM1 is thought to lead to a decrease in PKM activity and a Warburgian shift from OxPhos to glycolysis (Archer, 2017). Increased PKM2-to-PKM1 ratio was reported to underlie the glycolytic phenotype of BOECs and PAFs isolated from patients with PAH (Caruso et al., 2017; Zhang et al., 2017). Chapter 4 sought to determine whether a similar increase in the ratio of PKM2 to PKM1 contributes to the elevated glycolytic flux observed in PAH PASMCs. Indeed, the ratio of PKM2 to PKM1 was higher in PAH PASMCs than in their control counterparts, a consequence of diminished PKM1 protein expression with no change in PKM2 protein expression. However, whether this increase in the ratio of PKM2 to PKM1 contributes to the increase in glycolytic flux in these cells remains to be confirmed. Silencing PKM2 with siRNA or promoting the formation of PKM2 tetramers with shikonin or TEPP-46 may normalise the metabolic phenotype of PAH PASMCs and inhibit their proliferation. Similar to PFKFB3, PKM2 has many non-glycolytic functions, including interacting with and coactivating transcription factors (e.g. HIF1α) in the nucleus and phosphorylating signalling proteins (e.g. STAT3) in the cytoplasm (Gao et al., 2012; Palsson-McDermott et al., 2016). These non-glycolytic functions of PKM2 have yet to be explored in PAH. Furthermore, reduced conversion of phosphoenolpyruvate to pyruvate by PKM is thought to lead to the accumulation of glycolysis intermediates, which then spillover into biosynthetic pathways. Reduced PKM activity in PAH PASMCs is therefore believed to promote an anabolic phenotype that favours cell proliferation. Measuring the levels of glycolysis intermediates upstream of the PKM-catalysed conversion of phosphoenolpyruvate to pyruvate in PAH PASMCs could help confirm this hypothesis.

Lactate production has been shown to be elevated in pulmonary vascular cells from patients with PAH, suggesting a shift from mitochondrial oxidation of pyruvate to cytoplasmic reduction to lactate. The enzyme LDH catalyses the reversible conversion of pyruvate to lactate. LDH exists as a tetramer composed of two subunits LDHA and LDHB. LDHA preferentially catalyses the conversion of pyruvate to lactate, converting NADH to NAD⁺. LDHB, in contrast, converts lactate back to pyruvate, reducing NAD⁺ to NADH in the process. An increase in LDHA expression coupled with a decrease in LDHB
expression has been shown to underlie increased lactate production in cancer cells (Valvona et al., 2016). Chapter 4 aimed to determine whether a similar change in the expression of LDHA and LDHB contributes to the increased glycolytic flux to lactate observed in PAH PASMCs. No significant changes were observed in the expression of either LDH isozyme. Moreover, no differences in the phosphorylation of LDHA at the stimulating residue Y10 were reported between control and PAH PASMCs, suggesting that the activity of LDHA in PAH PASMCs also remains unchanged. Phosphorylation of LDHA at Y10 enhances its activity by promoting tetramer formation. Another phosphoacceptor tyrosine residue at position 82 enhances LDHA activity by increasing its binding affinity for the cofactor NADH, which is required for the reduction of pyruvate to lactate (Valvona et al., 2016). Further investigation is required to assess the extent of phosphorylation of LDHA at Y82 in PAH PASMCs. Moreover, LDHA phosphorylation has been shown to occur downstream of receptor tyrosine kinases or non-receptor tyrosine kinases. A number of these receptors have been implicated in the pathogenesis of PAH and may therefore contribute to the enhanced tyrosine phosphorylation of LDHA. Future studies could therefore explore the signalling pathways that lead to enhanced LDHA activity in PAH PASMCs. Work in the lab has shown that the LDHA inhibitor GSK 2837808A fails to inhibit PAH PASMCs proliferation. Instead, LDHA inhibition strongly inhibited the production of extracellular matrix proteins, such as fibronectin and type I collagen in PAH PASMCs. These findings suggest that increased LDHA activity possibly through the generation of lactate may be promoting a synthetic phenotype in these cells and contributing to the increased pulmonary vascular stiffness in PAH that arises from increased deposition of extracellular matrix proteins within the pulmonary vascular wall.

Given that glycolysis intermediates can channel into biosynthetic pathways, primarily the PPP, to support cell proliferation, Chapter 4 assessed the expression of the PPP rate-limiting enzyme G6PD in PAH PASMCs. A considerable increase in the protein expression of G6PD was observed in cultured PAH PASMCs as well PASMCs resident within the medial layers of small pulmonary arteries from patients with PAH. This finding suggests that glucose influx through the PPP may be elevated in PAH PASMCs. The main function of the PPP is to generate the nucleotide precursor ribulose-5-phosphate and the reducing agent NADPH (Stincone et al., 2015). NADPH is not only involved in scavenging ROS but also serves as a cofactor in the synthesis of fatty acids and cholesterol, both components of cell membranes (Stincone et al., 2015). Highly proliferative cells, such as PAH PASMCs, require copious amounts of nucleotides to synthesise nucleic acids and fatty acids and cholesterol for incorporation into membranes of daughter cells (Stincone et al., 2015). Therefore, it is not surprising that PAH PASMCs upregulate glucose flux through PPP to meet this increased demand for
nucleotides and lipids. Although this thesis has provided evidence in support of increased glucose shunting into the PPP, future studies should confirm this upregulation by measuring the levels of PPP end products, such as NADPH, in PAH PASMCs and comparing them with those in control PASMCs. The activity of G6PD itself could also be measured. The effect of inhibiting the PPP pathway on PAH PASMCs proliferation could also be investigated to confirm its need for their heightened proliferation in PAH. PPP could be inhibited by either silencing the rate-limiting enzyme G6PD with siRNAs or inhibiting its activity with small-molecule inhibitors. To date, very few selective inhibitors of G6PD have been reported. However, the quest for developing selective G6PD inhibitors is ongoing.

7.3 Mitochondrial respiration, biogenesis and mitophagy in PASMCs from patients with PAH

Mitochondrial OxPhos has previously been shown to be depressed in PAH PASMCs as indicated by decreased mitochondrial oxygen consumption (Paulin and Michelakis, 2014). The reduced capacity of PAH PASMCs to oxidise glucose has been suggested to account for the proliferative and apoptosis-resistant phenotype of these cells (Paulin and Michelakis, 2014). Strikingly, however, oxygen consumption rates associated with basal respiration and ATP production were found in Chapter 5 to be elevated in PAH PASMCs compared to control PASMCs. The ability of PAH PASMCs to respire maximally in response to increased energy demand or stress was similar to that of control PASMCs. Moreover, the capacity of PAH PASMCs to elevate mitochondrial oxygen consumption beyond basal was much less than that of control PASMCs. This was reflected in the significantly reduced respiratory reserve capacity in PAH PASMCs, suggesting that OxPhos is operating at a maximal rate basally in these cells and cannot be elevated much further. This could possibly explain why PAH PASMCs resort to elevating glycolysis to meet their energy demands but also to support synthesis of macromolecules. Finally, non-mitochondrial oxygen consumption was found to be significantly increased in PAH PASMCs. This is indicative of increased oxygen consumption by cytoplasmic oxidases, which contribute to the state of oxidative stress in PAH PASMCs. Indeed, higher levels of cellular ROS were reported in our PAH PASMC isolates compared to their control equivalents. ROS can also be generated within the mitochondria predominantly as a result of electron leak at complexes I and III of the ETC. The ability of the mitochondria to scavenge ROS was also shown to be reduced as indicated by the significantly diminished expression of the mitochondrial ROS scavenger SOD2. Given the increased oxygen consumption by the mitochondria and their reduced ability to scavenge ROS in PAH PASMCs, mitochondrial ROS could also
be contributing to the oxidative stress state of these cells. This should be confirmed, however, by live-cell staining with MitoSOX, a dye selective for mitochondrial ROS, and inhibiting complexes I and III of the ETC to assess the contribution of each to mitochondria-generated ROS.

Mitochondrial mass is a critical determinant of mitochondrial function. Thus, Chapter 5 investigated the protein levels of various mitochondrial proteins, including constituent subunits of the respiratory complexes and other mitochondrial proteins. The majority of mitochondrial proteins are encoded by nuclear genes with very few proteins encoded by genes within the mtDNA (Hock and Kralli. The expression of nuclear-encoded subunits of complexes I, II and V was found to be increased in PAH PASMCs compared to controls. SDHA, a nuclear-encoded subunit of complex II was also shown to be much more strongly expressed in the medial layers of small pulmonary arteries in PAH lung sections. The mitochondrial-encoded subunit of complex IV MTCO2 was also found to be upregulated in PAH PASMCs. The protein levels of the OMM proteins TOM20 and VDAC1 were shown to be increased in PAH PASMCs, further confirming the increase in mitochondrial mass in these cells. The electron carrier cytochrome c, which resides within the mitochondrial intermembrane space, was also found to be upregulated in PAH PASMCs. Together, these findings strongly suggest an increase in mitochondrial mass in PAH PASMCs underlies the increase in mitochondrial oxygen consumption, particularly that which is associated with basal respiration and ATP production. The levels of mtDNA in PAH PASMCs should be determined in the future and compared with those in control PASMCs. It may also be worth investigating the activities of the individual ETC complexes to confirm upregulation of OxPhos in PAH PASMCs.

Governing mitochondrial mass are the opposing processes of mitochondrial biogenesis and mitophagy, which add and remove mitochondrial mass, respectively. Mitochondrial biogenesis is regulated by the transcriptional coactivator PGC1α, which lacks DNA binding activity and instead interacts with and activates a number of transcription factors. Among these transcription factors are NRF1 and NRF2, which, when activated by PGC1α, induce the transcription of nuclear genes encoding mitochondrial proteins (Hock and Kralli, 2009). NRF1 and NRF2 also induce the transcription of TFAM, which, together with TFBM1 and TFBM2, regulates the transcription and replication of mtDNA (Hock and Kralli, 2009). The expression of PGC1α, NRF1, NRF2 and TFAM was found to be increased in PAH PASMCs compared to control PASMCs, indicating an increase in mitochondrial biogenesis in these cells. Upregulation of these transcription factors is likely to account for the increased levels of mitochondrial proteins, both nuclear- and mitochondrial-encoded, in PAH PASMCs. Cellular pathways leading to mitochondrial
biogenesis, such as those mediated by the serine/threonine kinases AMPK and Akt, are currently being investigated in the lab to decipher the mechanisms underlying this increased mitochondrial biogenesis in PAH PASMCs. Mitophagic flux was also found to be reduced in PAH PASMCs compared to control PASMCs as indicated by reduced delivery of mitochondrial fragments to lysosomes. In addition to increased mitochondrial biogenesis, reduced mitophagy is thought to also further contribute to the accumulation of mitochondrial mass observed in PAH PASMCs. Given that mitophagy is a form of autophagy, conversion of LC3BI to LC3BII and degradation of p62, both hallmarks of autophagy were assessed in control and PAH PASMCs. However, no change in either autophagy marker was observed. Future studies should focus on assessing ubiquitin-dependent and ubiquitin-independent pathways of mitophagy in PAH PASMCs to unravel the mechanisms underlying the reduced mitophagic flux in these cells.

The PDH enzyme complex occupies the junction between glycolysis and mitochondrial respiration. It converts pyruvate to acetyl-CoA, which is then incorporated into the mitochondrial TCA cycle. It was previously reported that inhibition of PDH, particularly its subunit PDHE1α, by increased PDK-mediated inhibitory phosphorylation at S293 underlies the suppression of mitochondrial respiration in pulmonary vascular cells and the resultant shift to glycolysis (Michelakis et al., 2017). However, the increase in mitochondrial respiration observed in PAH PASMCs in Chapter 5 necessitated the reassessment of the expression of PDHE1α and its inhibitory phosphorylation at S293 in PAH PASMCs. Surprisingly, the protein levels of PDHE1α were found to be higher in PAH PASMCs than in control PASMCs and this was not accompanied by an increase in inhibitory phosphorylation at S293. Therefore, instead, a trend towards a decrease in the phosphorylation of PDHE1α was observed in PAH PASMCs. Moreover, no significant difference in the expression of PDK1 and PDK4, both of which phosphorylate PDHE1α at S293, between control and PAH PASMCs was observed. This is in disagreement with the previously reported upregulation of PDK1 and PDK4 in PAH (Michelakis et al., 2017; Yuan et al., 2016). The expression of PDK2 and PDK3 in PAH PASMCs should also be assessed in the future. Furthermore, an increase in the expression of PDH phosphatases PDPs, of which there are two isoforms PDP1 and PDP2, could underlie the decrease in PDHE1α phosphorylation in our PAH PASMC isolates. This will also require further investigation. Finally, an increase in the expression of PDHE1β, another subunit of PDH, was also observed in PAH PASMCs, further confirming the increase in PDH activity. Not only does PDH operate in the mitochondria but it has also been shown to localise to the nucleus, where it is thought to regulate the expression of lipogenic genes (Chen et al., 2018). This extramitochondrial function of PDH remains to be explored in PAH PASMCs and could underlie their anabolic state.
Prostacyclin mimetics have been demonstrated to inhibit PAH PASMC proliferation and upregulate the expression of contractile genes to restore a normal contractile and quiescent phenotype (Falcetti et al., 2010; Fetalvero et al., 2006; Patel et al., 2018). Their ability to normalise the metabolic phenotype of PAH PASMCs has not been explored, however. Therefore, Chapter 5 investigated whether the anti-proliferative effects of treprostinil, MRE-269 and butraprost on PAH PASMCs were associated with changes in mitochondrial respiration. Compared to untreated PAH PASMCs, no changes in mitochondrial oxygen consumption were observed in proliferating PAH PASMCs treated with these agents. Therefore, the anti-proliferative effect of IP and/or EP₂ receptor agonism on PAH PASMCs is not associated with normalisation of mitochondrial function. Previously, treatment of PAH patients with prostacyclin analogues had no effect on the increased mitochondrial respiration in their platelets. This is consistent with the lack of any significant effects of treprostinil and MRE-269 on mitochondrial respiration in PAH PASMCs reported in Chapter 5. Future studies should investigate whether the mitochondria in control PASMCs respond differently to these agents.

7.4 TASK-1 channel dysfunction in PASMCs from patients with PAH

PAH-causing mutations in KCNK3, the gene encoding the K₂P channel TASK-1, were the first to implicate this K⁺ channel in the pathogenesis of PAH (Ma et al., 2013). Later, several studies have shown that TASK-1 channel function is depressed in PAH patients, who lack KCNK3 mutations, and in rat models of PH due to downregulation of its gene expression (Antigny et al., 2016; Lambert et al., 2018). Evidence in agreement with TASK-1 channel dysfunction in PAH was provided in Chapter 6. A trend towards a decrease in the mRNA levels of TASK-1 in PAH PASMCs was observed, suggesting that TASK-1 channel expression may be downregulated in our PAH PASMCs isolates. Whether this downregulation of TASK-1 expression is accompanied by reduced background K⁺ currents could be explored in the future using perforated whole-cell patch clamping. The mechanisms underlying this diminished TASK-1 expression in PAH PASMCs remains largely unknown and could potentially involve the upregulated calcineurin/NFAT pathway or the normoxic stabilisation of HIF1α that were reported in PAH. Indeed, both these pathways have been shown to suppress the expression of Kv1.5 in PAH PASMCs and could similarly account for the downregulation of TASK-1 (Bonnet et al., 2006; Bonnet et al., 2007). TASK-1 channel blockade with ML365, a highly selective TASK-1 channel blocker, promoted the proliferation of control PASMCs but not
those isolated from PAH patients. This could be suggestive of reduced TASK-1 channel activity in PAH PASMCs such that further reduction has no effect on the proliferative capacity of these cells. This pro-proliferative effect of TASK-1 channel blockade on control PASMCs and its loss in PAH PASMCs could be confirmed by silencing the KCNK3 gene with siRNA in control and PAH PASMCs and assessing their proliferation.

The anti-proliferative effects of prostacyclin mimetics on PASMCs are well documented in the literature and so is their ability to activate TASK-1 channels and increase background K⁺ currents (Falcetti et al., 2010; Olschewski et al., 2006; Patel et al., 2018). However, whether their anti-proliferative effects on PASMCs are mediated by these channels remains unexplored. Chapter 6 sought to determine whether the growth suppressive effects of treprostinil on PASMCs are dependent on TASK1 channel activity. Treprostinil inhibited proliferation of both control and PAH PASMCs as described previously (Falcetti et al., 2010; Patel et al., 2018). TASK-1 channel blockade with ML365 did not significantly attenuate the anti-proliferative effect of treprostinil on control PASMCs. In PAH PASMCs, TASK-1 channel blockade also failed to inhibit the anti-proliferative effect of treprostinil. Given that treprostinil inhibits PAH PASMCs predominantly via the EP₂ receptor and to a lesser extent via the IP receptor, Chapter 6 also investigated the effect of TASK-1 channel blockade on the anti-proliferative effects of IP and EP₂ receptor-selective agonists. MRE-269, an IP receptor agonist and active metabolite of the PAH drug selexipag, inhibited PAH PASMC proliferation to a much lesser degree than treprostinil, a likely consequence of reduced IP receptor expression and signalling (Falcetti et al., 2010). TASK-1 channel blockade with ML365 completely blocked the anti-proliferative effect of MRE-269 on PAH PASMCs. Butaprost, an EP₂ receptor agonist, inhibited PAH PASMC proliferation to a similar degree to treprostinil. This could be explained by the upregulation of EP₂ receptor expression that was previously reported in these cells (Patel et al., 2018). Blocking TASK-1 channels blocked the anti-proliferative effect of butaprost on PAH PASMCs. Collectively, these results suggest that treprostinil activates a mechanism additional to IP and EP₂ receptor agonism and consequent TASK-1 channel activation to inhibit PAH PASMCs. Indeed, treprostinil has been shown to inhibit PASMCs proliferation by activating the nuclear receptor PPARγ. These findings also suggest that treprostinil may be more clinically efficacious than selexipag in PAH patients, who carry loss-of-function TASK-1 channel mutations. Chapter 6 also revealed that treprostinil but not butaprost or MRE-269 significantly increased the transcript levels of TASK-1 in PAH PASMCs. Future studies should aim to explore whether the anti-proliferative effect of treprostinil is retained in PAH PASMCs harbouring mutant TASK-1 channels. This might prove difficult due to the rarity of PAH patients with TASK-1 channel mutations but could alternatively be achieved by
expressing mutant TASK-1 channels in control PASMCs. Moreover, the recent generation and characterisation of KCNK3-deficient rats, which spontaneously develop PAH, may allow for future investigation into the ability of treprostinil to attenuate experimental PH caused by loss of TASK-1 channel function.

Loss of TASK-1 channel function has previously been shown to alter mitochondrial dynamics, favouring fragmentation, and to depolarise the mitochondrial membrane potential (Lambert et al., 2019). However, the effect of TASK-1 channel dysfunction on mitochondrial respiration has not been explored. Chapter 6 investigated the effect of TASK-1 channel blockade on mitochondrial oxygen consumption in control PASMCs. Blocking TASK-1 channels was found to reduce mitochondrial oxygen consumption, suggesting that loss of TASK-1 channel function may underlie the suppression of mitochondrial OxPhos and the switch to glycolysis previously reported in PAH PASMCs. This contradicts, however, the findings reported in Chapter 5, which indicate that an increase in basal respiration and ATP production with no or little change in maximal respiration and a drop in respiratory reserve capacity cause PAH PASMCs to upregulate glycolysis. However, this is not the only contradictory finding that was reported in PASMCs, in which TASK-1 channels have been inhibited. Lambert et al. (2019) reported mitochondrial membrane depolarisation in TASK-1 channel inhibited PASMCs, which is in stark contrast to the hyperpolarisation observed by Pak et al. (2013) in PAH PASMCs. Mitochondrial fragmentation and membrane depolarisation render mitochondria substrates for mitophagy. TASK-1 channel blockade could therefore be suppressing mitochondrial oxygen consumption by promoting mitophagy. This could be investigated in the future using the Mitophagy Detection Kit that was employed in Chapter 5.

Finally, TASK-1 channel blockade with ML365, which is thought to mimic loss of TASK-1 channel function in PAH, was found in Chapter 6 to promote the apoptosis of PAECs. Apoptosis was assessed by immunoblotting for cleaved caspase 3, a single marker of apoptosis. This finding could be further confirmed by assessing the externalisation of phosphatidylserine using annexin V staining and flow cytometric analysis. PAEC apoptosis and dysfunction are thought to occur early in the pathogenesis of PAH and to trigger the pulmonary vascular remodelling that leads to the rise in PVR, mPAP and right heart after load. Similar to PAECs from PAH patients with BMPR2 mutations (Long et al., 2015), PAECs from PAH patients with KCNK3 mutations could be more susceptible to apoptosis. In the future, the apoptosis susceptibility of PAECs harbouring KCNK3 mutations could be investigated. Due to the rarity of patients carrying PAH-causing KCNK3 mutations, however, this could only be achieved by ectopically expressing mutant TASK-1 channels in normal PAECs and assessing the levels of cleaved caspase
3 or phosphatidylserine externalisation following an apoptosis-inducing stimulus. PAECs from the recently characterised KCNK3-deficient rats could alternatively be used.

7.5 Limitations

Despite its many strengths, this study is not without limitations. The major limitation of this study is the small sample size of PASMC isolates, particularly those derived from control subjects, which is due to the rarity of resection of lung tissue. This has the effect of reducing the statistical power of the study, which denotes its ability to detect a true effect (Button et al., 2013). Statistical power can also be defined as the probability with which the study commits type II errors (accepting the null hypothesis when the alternative one is true; Marino et al., 2014). In contrast to type II errors, type I errors or false positives occur when a true null hypothesis is rejected. An underpowered study is associated with three major problems. First, the chance of discovering effects, which are genuinely true, is low (Button et al., 2013). In other words, a low-powered study generates more false negatives or type II errors than a high-powered study. Second, low power reduces the probability that an observed effect, irrespective of its size, reaches statistical significance. This probability is referred to as the positive predictive value of the study (Button et al., 2013). Third, a true effect, discovered in an underpowered study, is likely to be a consequence of effect inflation (i.e. the effect sizes provided by the low-powered study will be exaggerated). Effect inflation occurs when hypotheses are based on thresholds of statistical significance, which in this study is \( p < 0.05 \). Consequently, increasing the sample size of PASMC isolates would serve to reduce the probability of type II errors and hence increase the statistical power of the study. This would allow conclusions to be made with higher confidence.

In addition to its influence on statistical power, the small sample size of PASMC isolates means that the distribution of the values obtained is unlikely to be normal and that normality should not be assumed but tested for (Marino, 2014). This warrants the use of non-parametric statistical tests as opposed to their parametric equivalents, which were used in this study. Standard errors of the mean (SEM) were frequently used in this study to show the distributions of values around their means. However, unlike standard deviation (SD), SEM, which is computed by dividing SD by the square root of the sample size, does not quantify variation among a set of values (Marino, 2014). Readers viewing the graphs presented in this study might therefore mistakenly believe that the error bars reflect the variability of the data (Marino, 2014). SDs or 95% confidence intervals are better suited to reflect the dispersion of values around the mean.
7.6 Conclusion

To conclude, the overarching aim of this thesis has been addressed. Compared to control PASMCs, alterations in mitochondrial dynamics and glucose metabolism have been observed in PAH PASMCs. PAH PASMCs were found to contain mitochondria that are more fragmented than those in control PASMCs. Underlying this excessive fragmentation is upregulation of critical components of the mitochondrial fission machinery coupled with downregulation of certain elements of the mitochondrial fusion machinery. Prostacyclin mimetics were found to attenuate this excessive mitochondrial fragmentation in PAH PASMCs and the mechanisms underlying this inhibitory effect were unveiled. PAH PASMCs were found to respire basally and produce ATP at a higher rate than control PASMCs. An increase in mitochondrial mass as a result of a combination of increased mitochondrial biogenesis and diminished mitophagy was found to contribute to this increase in mitochondrial respiration. The increase in mitochondrial respiration was also accompanied by an increase in the cellular levels of ROS. The mitochondrial ROS scavenging system was also found to be downregulated in PAH PASMCs, further contributing to the increase in cellular ROS levels. Moreover, an increase in glucose flux through glycolysis was observed in PAH PASMCs. Increased protein expression of critical, rate-limiting glycolytic enzymes was found to account for this increase in glycolytic flux. In addition to increased glycolytic flux, the rate-limiting enzyme in the pentose phosphate pathway was found to be upregulated in PAH PASMCs, indicating an increase in glucose shunting into this pathway. Finally, loss of TASK-1 channel activity has been demonstrated in PAH PASMCs, as indicated by a trend towards reduced transcript levels of TASK-1 and the lack of a pro-proliferative effect of TASK-1 channel blockade on these cells compared to their control counterparts. Moreover, TASK-1 blockade inhibited mitochondrial respiration in control PASMCs, conferring upon these cells a PAH metabolic phenotype. Prostacyclin mimetics were shown to inhibit PAH PASMC proliferation to varying degrees via TASK-1 and to upregulate the mRNA expression of TASK-1 in proliferating PAH PASMCs. Finally, TASK-1 channel blockade promoted the apoptosis of PAECs, which is thought to be an early event in PAH pathogenesis.
Abstracts, publications and prizes arising from this thesis

Abstracts


Publications


Prizes

Awarded the prize for best poster presentation at UCL Division of Medicine Retreat 2019
8. References


agonist liraglutide improves hypoxia-induced pulmonary hypertension in mice partly via normalization of reduced ET(B) receptor expression. Physiol Res 67(Suppl 1): S175-S184.


