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The Role of G-Protein Coupled Receptor 75 in Pulmonary Hypertension

Catherine Ann D'Addario

A Doctoral Dissertation in the Program in Pharmacology

Submitted to the Faculty of the

Graduate School of Basic Medical Sciences

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

at New York Medical College

2022

The Role of G-Protein Coupled Receptor 75 in Pulmonary Hypertension

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Abbreviations

20-HETE – 20 Hydroxyeicosatetranoic acid

AAA – 6(Z),15(Z)-hydroxyeicoas-6,15-dienamido-diencoic acid

Bmpr1 – Bone Morphogenic Protein Receptor Type 1

cAMP – Cyclic adenosine monophosphate

CCB – Calcium Channel Blocker

CCL2 – Chemokine Ligand 2

CCL5 – Chemokine Ligand 5

CCR –Chemokine C-C motif receptors

cGMP – Cyclic guanosine monophosphate

Cnn1 – Calponin 1

COX – Cytochrome c oxidase

CREB – cAMP Response Element Binding Protein

CXCL12 – C-X-C Motif Chemokine Ligand 12

DAG – Diacylglycerol

DCM – Department of Comparative Medicine

DDMS – Dibromo-dodecenylmethylsulfimide

ELISA – Enzyme-linked immunosorbent assays

ET-1 – Endothelin-1

ERK – Extracellular signal-regulated kinase

GDP – Guanosine diphosphate

GPR – G-protein-coupled receptors

GPR75 – G-protein-coupled receptor 75

Gpr75$^{-/-}$ – G-protein-coupled receptor 75 knockout

GTP – Guanosine Triphosphate

H&E – Hematoxylin and eosin

HCN – Hyperpolarization cation channel

hPAECs – Human pulmonary arterial endothelial cells

hPASMCs – Human pulmonary arterial smooth muscle cells

HPH – Hypoxia-induced pulmonary hypertension

HPV – Hypoxic pulmonary vasoconstriction

Hx – Hypoxic

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

IP3 – Inositol 1,4,5-trisphosphate

IPA – Intra-lobar pulmonary artery

iPAH – Idiopathic pulmonary arterial hypertension

KCl – Potassium Chloride

MAPK – Mitogen-activated protein

MLCK – Myosin light chain kinase

mRNA – Micro ribonucleic acid

Myh11 – Myosin heavy chain 11

NIH – National Institutes of Health

Nx – Normoxic

PA – Pulmonary artery

PAEC – Pulmonary artery endothelial cells

PAH – Pulmonary arterial hypertension

PAP – Pulmonary artery pressure

PASMC – Pulmonary arterial smooth muscle cell

PCR – Polymerase chain reaction

PDE5 – Phosphodiesterase type 5

PFA – Paraformaldehyde

PH – Pulmonary hypertension

PKA – Protein kinase A

Prom1 – Prominin 1

PVR – Pulmonary vascular resistance

qRT-PCR – Quantitative real-time polymerase chain reaction

RANTES-Ab – Antibodies against RANTES

Rock2 – Rho-kinase 2

RVDP – Right ventricular diastolic pressure

RVSP – Right ventricular systolic pressure

TNF-α – Tumor necrosis factor- α

WBC – White blood cells

WHO – World Health Organization

WT – Wild type

Abstract

Pulmonary Hypertension (PH) is a cardiopulmonary disease estimated to affect between 20 million to 70 million individuals globally, with poor prognosis and inadequate treatment. Recent studies show the orphan G Protein Coupled Receptor 75 (GPR75) is upregulated in the pulmonary vessels of PH patients, especially in females. Therefore, we hypothesized that *Gpr75* knock down (*Gpr75*$^{-/-}$) mice will be protected from developing PH. To test our hypothesis, we challenged isolated intra-lobar pulmonary artery (IPA) from wild-type (WT; N=40) and *Gpr75*$^{-/-}$ (N=19) mice with hypoxia to examine their hypoxic vasoconstrictive (HPV) response, which is a physiological response of pulmonary circulation to balance ventilation-to-perfusion ratio that becomes maladaptive in chronic hypoxia leading to the development of PH. IPA of *Gpr75*$^{-/-}$ mice developed very little or no HPV response as compared to the WT mice. Next, *Gpr75*$^{-/-}$ (N=8) as compared with WT (N=18) kept in hypoxic (10% O_2) conditions for 5-weeks developed less PH as indicated by significantly less increase in right ventricular systolic pressure (RVSP) and diastolic pressure (RVDP). *Gpr75*$^{-/-}$ mice also expressed less inflammatory gene (*Ccl2, Ccl5, Cxcl12,* and *Prom1*) and gene encoding rho associated coiled-coil containing protein kinase 2 (*Rock2*), which are often overexpressed in lungs and pulmonary arteries of PH mice and patients, but expressed more genes that encode bone morphogenetic protein receptor 1a (*Bmpr1a*) and myosin heavy chain 11 (*Myh11*), a smooth muscle specific protein gene that is often reduced in lungs and pulmonary arteries of PH mice and patients. Vascular reactivity of WT and *Gpr75*$^{-/-}$ mice was also examined by challenging isolated intra-lobar pulmonary arteries with vasoconstricting agents such as potassium chloride (KCl), thromboxane mimetic (U46619), cyclic nucleotide channel inhibitor (ZD7288), and

endothelin-1 (ET-1). The *Gpr75*$^{-/-}$ as compared with wild-type pulmonary arteries contracted less to all the contractile agents. Since GPR75 is a G-protein coupled receptor, to determine second messengers that potentially contribute to regulating contractile function of pulmonary arteries we measured cAMP and IP3 in lungs of wild-type and *Gpr75*$^{-/-}$ mice. While we found no difference in the IP3 levels between the WT and *Gpr75*$^{-/-}$ groups, cAMP levels were significantly higher in *Gpr75*$^{-/-}$ compared to levels in WT mice. These results suggest GPR75 plays a major role in the development of PH by potentially attenuating cAMP-dependent signaling and concomitantly augmenting pulmonary constriction in response to hypoxia.

1. BACKGROUND

1.1 Pulmonary Hypertension

Pulmonary hypertension (PH) is a progressive and deadly cardiopulmonary disease that is estimated to affect between 20 million to 70 million individuals globally and has a 1-year mortality rate of 46% (Schmitt & Stork, 2001). PH is defined as having a resting mean pulmonary artery pressure (PAP) of ≥25mmHg, which is due to remodeling of pulmonary arteries (PAs) via increased proliferation of apoptosis resistant pulmonary arterial smooth muscle cells (PASMCs) which leads to pulmonary arterial narrowing and increased vascular resistance (Nie et al., 2018; Remy-Jardin et al., 2021; Tello et al., 2021). PH is defined as mild with a mean PAP of >25 mm Hg, moderate with a mean PAP is between 41-55 mm Hg, and severe if it's >55 mm Hg (Lumb & Slinger, 2015). To reach this diagnosis, patients first undergo echocardiography to establish the probability of having PH, then right heart catheterization follows for an accurate diagnosis (Augustine et al., 2018; Moreira et al., 2015)

There are five major classifications of PH as described by the World Health Organization (WHO), determined by origin of the disease which are summarized in Table 1. Group 1 corresponds to pulmonary arterial hypertension (PAH) which can develop due to idiopathic causes, heritability of gene mutations, drugs or toxins, and is commonly associated with connective tissue disease, HIV infection, portal hypertension, and congenital heart disease. Group 2 is PH due to left heart disease, which can develop because of left ventricular systolic dysfunction, left ventricular diastolic dysfunction, valvular disease, congenital cardiomyopathies, and congenital or acquired pulmonary vein stenosis. Group 3, the one we focus on in the following experiments, is PH due to lung diseases and/or hypoxia. This form of PH can develop from chronic obstructive pulmonary disease, interstitial lung disease, sleep

apnea, alveolar hypoventilation syndromes, and chronic exposure to high altitudes and low oxygen levels. Group 4 describes chronic thromboembolic pulmonary hypertension and other pulmonary artery obstructions, which can be developed because of pulmonary artery obstructions, angiosarcoma, intravascular tumors, arteritis, and congenital pulmonary artery stenosis. Group 5 describes PH with unclear and/or multifactorial mechanisms, such as that associated with hematological disorders such as anemia, systemic disorders such as sarcoidosis, metabolic disorders, such as glycogen storage disease, Gaucher disease, or thyroid disorders (Kovacs et al., 2018). My thesis focuses on Group 3 PH.

The Five Groups of Pulmonary Hypertension	
Group	**Causes**
Group 1: Pulmonary Arterial Hypertension	Idiopathic, genetics, drug/toxin exposure, scleroderma, lupus, HIV, portal hypertension, and congenital heart disease
Group 2: Left Heart Disease	Coronary artery disease, high blood pressure, damage to the heart muscle, heart valve disease, and age
Group 3: Lung Disease	Chronic obstructive pulmonary disease (COPD), interstitial lung disease, hypoxia, and sleep apnea
Group 4: Chronic Thromboembolic Pulmonary Hypertension	Old blood clots within the lungs – creates barriers for blood flow within pulmonary arteries
Group 5: Pulmonary Hypertension resulting from unclear mechanisms	Sarcoidosis, sickle cell anemia, chronic hemolytic anemia, spleen removal, Gaucher disease, and thyroid disease

Table 1. The five classifications of pulmonary hypertension and some of their known causes, according to the World Health Organization.

1.2 Treatments for Pulmonary Hypertension

There are no cures for PH, only treatments to alleviate the symptoms (Table 2), such as calcium channel blockers (CCBs), endothelin receptor antagonists, phosphodiesterase-5 inhibitors, guanylate cyclase stimulators, prostacyclin analogues, and prostacyclin receptor agonists, which is why research into more effective treatments for PH is so important (Pesto et al., 2016). A summary of these drug classes with examples from each group is provided in Table 2.

1.2.1 Calcium Channel Blockers

Dihydropyridine CCBs, such as Amlodipine, help to alleviate symptoms in patients suffering from PH by acting on vascular smooth muscle to reduce vascular resistance and reduce PAP. Not all PH patients respond well to CCBs, but those who do can elicit an acute 15% decrease in mean PAP and a 26% reduction in pulmonary vascular resistance (PVR) (Liu et al., 2013). Studies have also shown that the responding patients treated long-term with the highest tolerable dose of CCBs had a mortality of 6% after 5 years, compared to those unresponsive to CCBs that had a 45% mortality rate after 5 years (Liu et al., 2013).

1.2.2 Endothelin Receptor Antagonists

For those patients who do not respond well to CCBs, there are other treatments available, for example ET-1 antagonists, such as Ambrisentan. ET-1 is a peptide, that is induced by hypoxia, shear stress, cytokines, and thrombin, and is primarily produced in the vascular endothelium, as well as in PASMCs and lung fibroblasts (Ould Amer & Hebert-Chatelain, 2018). There are two ET-1 receptors, ET_AR, which is expressed primarily in the large PAs, and ET_BR, which predominates in the airway smooth muscle, alveolar walls, and

capillaries (Galie et al., 2004). ET-1-ET_AR and ET-1-ET_BR coupling in PASMCs triggers PA vasoconstriction through activation of phospholipase C, which increases IP3 and diacylglycerol (DAG), and intracellular Ca^{2+} levels (Galie et al., 2004). ET-1-ET_AR coupling stimulates proliferation of HPASMCs and HPAECs, which contributes to remodeling of PAs and causes an increase in PVR (Galie et al., 2004; Ould Amer & Hebert-Chatelain, 2018). ET-1-ET_AR coupling also causes lung fibroblasts to proliferate, which leads to further increases in PVR (Ould Amer & Hebert-Chatelain, 2018). ET-1-ET_BR coupling stimulates the production of endothelium-derived NO, via endothelial nitric oxide synthetase (eNOS) and potassium channel activation (Maron et al., 2012). The vasodilatory effects of ET-1-ET_BR coupling becomes attenuated in PH due to the high levels of reactive oxygen species present within the PAs (Maron et al., 2012).

ET-1 receptor antagonists can either inhibit ET_AR specifically or both ET_AR and ET_BR (dual antagonists). Ambrisentan is a selective ET_AR antagonist, while Bosentan and Macitentan are dual ET-1R antagonists. PH patients treated with dual ET-1R antagonists showed significant decreases in PVR as well as an increase in their 6-minute walking distances, lower mortality rates, and a slowing in disease progression (Dupuis & Hoeper, 2008; Galie et al., 2004; Ould Amer & Hebert-Chatelain, 2018). However, clinical trials did not find ET_AR antagonists to give better prognoses to PH patients, compared to dual ET-1R antagonists, even though ET_AR antagonists do not affect ET_BR (Dupuis & Hoeper, 2008; Galie et al., 2004; Wilkins et al., 2008).

1.2.3 Phosphodiesterase Inhibitors

Phosphodiesterase type 5 (PDE5) inhibitors, such as Sildenafil, are another treatment available to PH patients. This class of drugs inhibits PDE5, the main enzyme responsible for

degrading cyclic guanosine monophosphate (cGMP), to the inactive guanosine monophosphate (Wilkins et al., 2008). cGMP, which is generated by NO-sensitive guanylyl cyclase and transmembrane guanylyl cyclases that are activated by natriuretic peptides, is responsible for regulating vascular smooth muscle cell proliferation and vascular tone. cGMP activates cGMP-dependent protein kinase, which phosphorylates myosin phosphatase, which controls the calcium-dependent phosphorylation of myosin light chain by myosin light chain kinase, leading to smooth muscle contraction, and the IP3 receptor-associated cGMP kinase substrate, which inhibits calcium release from the sarcoplasmic reticulum (Krawutschke et al., 2015; Masuda et al., 2010; Nie et al., 2018). By inhibiting the degradation of cGMP through PDE5, cGMP levels increase, allowing for smooth muscle relaxation to occur and PVR to decrease.

1.2.4 Guanylate Cyclase Stimulators

Guanylate cyclase stimulators, such as Riociguat, can also be prescribed to PH patients. These drugs work to increase cGMP through a different mechanism than PDE5 inhibitors. Instead of preventing the breakdown of cGMP, guanylate cyclase stimulators enhance the production of cGMP by enhancing the response of soluble guanylate cyclase (sGC) to endogenous NO, allowing for the reduction of PVR through mechanisms previously described (Ghofrani et al., 2017; McLaughlin et al., 2017). Riociguat has a dual mode of action. It increases the activity of sGC up to 73-fold (direct action) and up to 112-fold when acting in synergy with NO (Ghofrani et al., 2017). Riociguat has been shown to improve exercise mobility in PH patients and delay clinical worsening ("Riociguat (Adempas) for pulmonary hypertension," 2014).

1.2.5 Prostacyclin Analogues

Prostacyclin I_2 (PGI_2) analogues, such as Epoprostenol, can be prescribed to PH patients. PGI_2 was first isolated from rabbit and pig aortas and found to relax arterial smooth muscle cells and since then its analogs have been utilized to treat PH (Pluchart et al., 2017). PGI_2 is a major metabolite of arachidonic acid, produced by endothelial cells, and elicits potent vasodilatory effects, antiproliferative, and anti-inflammatory effects on vascular smooth muscle (Pluchart et al., 2017). PGI_2 binds to the PGI_2 receptor, which is a GPR that activates G_s-coupled receptors on vascular smooth muscle cells to exert its vasodilatory effects on PAs through cAMP signaling(Pluchart et al., 2017). PGI_2 can also activate the peroxisome proliferator-activated receptors (PPARs) which contribute to the vasodilatory effects of PGI_2 (Pluchart et al., 2017). PPARs activate endothelial NO synthase (eNOS) which promotes vasodilation through activation of sGC, and controls endothelial cell apoptosis through numerous pathways (Pluchart et al., 2017). Infusion of PGI_2 analogs promotes a decrease in PVR and PAP while prolonging survival of PH patients (Pluchart et al., 2017).

Epoprostenol is a synthetic and unstable analogue of PGI_2 that requires infusion to exert its effects (Pluchart et al., 2017). When treating a patient with Epoprostenol, PVR decreases due to vasodilation in the pulmonary arteries by mimicking PGI_2 and by inhibiting the actions of ET-1 (Sitbon & Vonk Noordegraaf, 2017). Clinical trials showed patients treated with Epoprostenol experienced lowered PVR and increased exercise capacity, while also slowing the progression of PH (Sitbon & Vonk Noordegraaf, 2017). Epoprostenol is also the only therapy within the PGI_2 analog class to reduce mortality (Coghlan et al., 2019; Ould Amer & Hebert-Chatelain, 2018).

Selexipag is unique amongst the PGI_2 agonist class as it is a prodrug that is structurally unique from the PGI_2 analogs and therefore more stable, allowing for it to be administered orally (Simonneau et al., 2012). Selexipag is cleaved and converted to its active form in the liver (Simonneau et al., 2012). The active metabolite of Selexipag is specific for the PGI_2 receptor and is not cross-reactive with other prostaglandin receptors (Simonneau et al., 2012). Selexipag's active metabolite was shown to relax rat PAs contracted with ET-1 or phenylephrine (Coghlan et al., 2019). While Selexipag is not as effective in reducing mortality as the PGI_2 analogs that require infusion, its benefit lies in its oral administration, allowing for better quality of life for PH patients (Coghlan et al., 2019).

Drugs used for the treatment of pulmonary hypertension	
Drug Class	**Examples of Drug**
Calcium channel blockers	Amlodipine, Nifedipine, Diltiazem
Endothelin receptor antagonists	Ambrisentan, Bosentan, Macitentan
Phosphodiesterase type 5 inhibitors	Sildenafil, Tadalafil
Guanylate cyclase stimulators	Riociguat
Prostacyclin analogs/agonists	Epoprostenol, Iloprist, Treprostinil, Selexipag

Table 2. The five classes of drugs used to treat pulmonary hypertension with examples from each category.

1.3 Hypoxia-induced Pulmonary Hypertension (Group 3 PH)

1.3.1 Hypoxic Pulmonary Vasoconstrictive Response

The lungs have a unique response when exposed to inhaled hypoxia. The PAs in the lungs contract in response to hypoxic conditions, while the systemic circulation dilates (Dunham-Snary et al., 2017). The hypoxic pulmonary vasoconstriction (HPV) response is an intrinsic reflex contraction of vascular smooth muscle in the PAs in response to a low partial pressure of oxygen (Lumb & Slinger, 2015) and is theorized to be triggered by a mitochondrial sensor of low oxygen, which causes a shift in the levels of reactive oxygen species in the PASMCs, which inhibits potassium channels, depolarizes PASMCs, activates voltage-gated calcium channels, increases intracellular calcium, and increases Ca^{2+} sensitivity to the myofilament, which leads to constriction of the PAs (Dunham-Snary et al., 2017). This reflex occurs within seconds of hypoxia exposure and consists of two phases. Phase 1 is a brief transient increase in PAP, which will quickly return to baseline, however, if the hypoxia exposure is prolonged, phase 2 begins and is a maintained increase in PAP (Lumb & Slinger, 2015) (Figure 1). PAs contract to divert blood from hypoxic regions of the lungs to regions with more oxygen to maintain a perfusion-to-ventilation ratio (Dunham-Snary et al., 2017). Persistent hypoxia eventually leads to the development of PH.

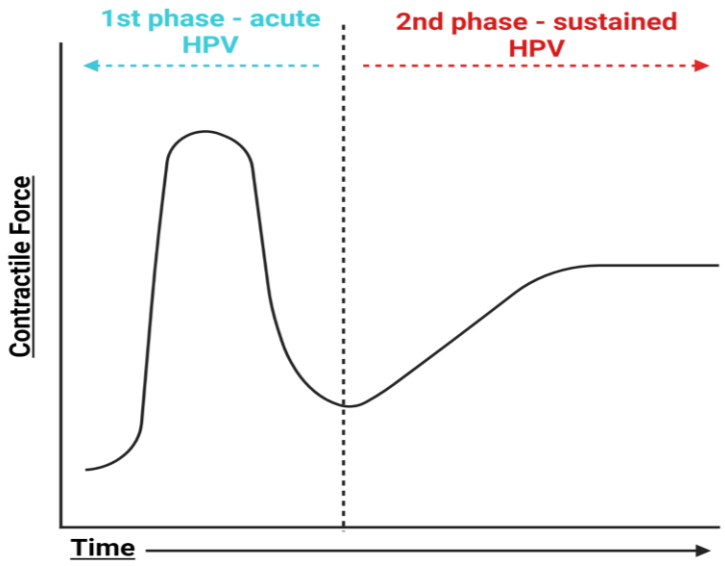

Figure 1. A graph depicting the hypoxic pulmonary vasoconstriction response in intra-lobar PAs. Created with BioRender.

1.3.2 Chronic Hypoxia Induced PA Constriction and Remodeling

Individuals exposed to chronic hypoxic conditions do not elicit the same HPV response compared to individuals exposed to acute hypoxia. Chronic exposure to hypoxia can occur when living in high altitudes or with chronic lung diseases such as COPD, cystic fibrosis, asthma, and sleep apnea (Stenmark & McMurtry, 2005). Chronic hypoxia exposure leads to the development of PH, which is caused by PA constriction and remodeling (Figure 2). There are two structural changes that contribute to the increased PVR seen in chronic hypoxia-induced pulmonary hypertension (HPH), remodeling of the PA wall and a reduction in the number of small peripheral PAs (aka pruning) (Stenmark & McMurtry, 2005). The PA remodeling is characterized by increased muscularization and hypertrophy in vessel walls, causing increased PA resistance (Fagan et al., 2004; Stenmark & McMurtry, 2005; Swenson, 2013).

1.3.2.1 Potential Mechanism of Pulmonary Artery Constriction to Acute and Chronic Hypoxia

The HPV response elicited by PAs is a reflex contraction of the PASMCs in response to hypoxia in order to maintain a constant perfusion-to-ventilation ratio in the lungs (Dunham-Snary et al., 2017; Lumb & Slinger, 2015). The oxygen sensing capabilities of the pulmonary system is multifactorial, as mitochondria, carbon dioxide and pH, K^+ channel modulation, hypoxia-inducible factor (HIF), and the cellular energy state can all contribute to oxygen sensing. Mitochondria are postulated to assist with oxygen sensing through their electron transport chain (Dunham-Snary et al., 2017). Hypoxia exposure increases the electron transport chain's production of reactive oxygen species, which ultimately become hydrogen peroxide via superoxide dismutase 2, a redox mediator, which alters redox-sensitive ion channels and

enzymes (Dunham-Snary et al., 2017). Hypoxia also causes lower oxygen levels within the blood, resulting in a high concentration of H^+ in the blood, causing PA vasoconstriction (Lumb & Slinger, 2015). K^+ channel modulation within the PASMCs is also hypothesized to be used for oxygen sensing. Oxygen can bind reversibly to the sulfur-containing proteins within the channel, altering their function, and therefore when oxygen content is low, so is the amount of K^+ channel function (Lumb & Slinger, 2015). Another potential oxygen sensor within the pulmonary circulation is HIF, which is an enzyme that initiates transcription of hypoxia-induced genes (Lumb & Slinger, 2015). When in a hypoxic environment, HIF has a long half-life due decreased oxygen content, which limits the function of prolyl hydroxylase, which is a key factor in the breakdown of the HIF enzyme (Andrew B. Lumb, 2015). Lastly, cellular energy state can also contribute to the oxygen sensing capabilities of the pulmonary system. Hypoxia enhances glycolysis within the PASMCs, which leads to the production of low energy molecules such as adenosine monophosphate, which activate the enzyme adenosine monophosphate-activated kinase, which acts to decrease ATP consumption and may release Ca^{2+} from the sarcoplasmic reticulum, increasing the intracellular Ca^{2+} concentration and causing PA contraction (Lumb & Slinger, 2015). Upon exposure to hypoxic conditions, the PAs will elicit an HPV response.

The HPV response within the PAs consists of two phases, a potent and transient increase in PA contraction followed by sustained PA contraction. The first phase of the HPV response is due to the increase in intracellular Ca^{2+} in PASMCs triggered by voltage-dependent and voltage independent Ca^{2+} entry and release of Ca^{2+} from intracellular stores, such as the sarcoplasmic reticulum (Dumas et al., 1999; Dunham-Snary et al., 2017; Ward & McMurtry, 2009). The second phase of the HPV response, which consists of a sustained contraction of

PAs, is dependent on the sensitization of the PAs to Ca^{2+} through Rho-kinase (ROCK) mediated signaling (Ward & McMurtry, 2009). ROCK activation inhibits myosin light chain phosphatase by phosphorylating the myosin-binding subunit, increasing the amount of phosphorylation of myosin light chain, which augments PA contractility (Fagan et al., 2004). While acute exposures to hypoxia are not deleterious, chronic hypoxia exposure leads to sustained PA constriction and remodeling and increased PAP, thereby leading to PH.

1.3.2.2 Pulmonary Artery Remodeling

PA remodeling is in part, due to ROCK signaling, which regulates actin polymerization, gene transcription, differentiation, growth, migration, and contraction through inactivation of myosin light-chain phosphorylation (Fagan et al., 2004; Stenmark & McMurtry, 2005) . ROCK activation also causes a reduction of eNOS within the PA endothelial cells (PAECs) (Fagan et al., 2004). eNOS is a key factor in the production of NO within pulmonary vasculature, which allows for vasodilation through the activation of guanylate cyclase. When eNOS levels are reduced because of ROCK activation, less NO is produced, meaning the vasoconstrictive effects elicited by hypoxia are not offset through guanylate cyclase activation. PASMC growth and hypertrophy, which are present with prolonged hypoxia exposure, are also regulated by ROCK signaling (Fagan et al., 2004). PASMCs also undergo phenotypic switching, where they shift from a contractile to synthetic phenotype, meaning the PASMCs have decreased contractile protein expression and increased elastolytic enzymes such as matric metalloproteinases, ultimately increasing PA stiffness (Pappalardo et al., 2017). The increase in PASMC growth and hypertrophy is a major cause of PA remodeling, which increases PVR, ultimately leading to the development of PH.

Another form of PA remodeling is the pruning of small peripheral PAs, which leads to an increase in resistance because it decreases the number of parallel vascular pathways (Stenmark & McMurtry, 2005). Reducing the number of parallel vascular pathways in the pulmonary vasculature, causes a further increase in PVR. Another cause of PA remodeling is chronic inflammation, which is present in PH.

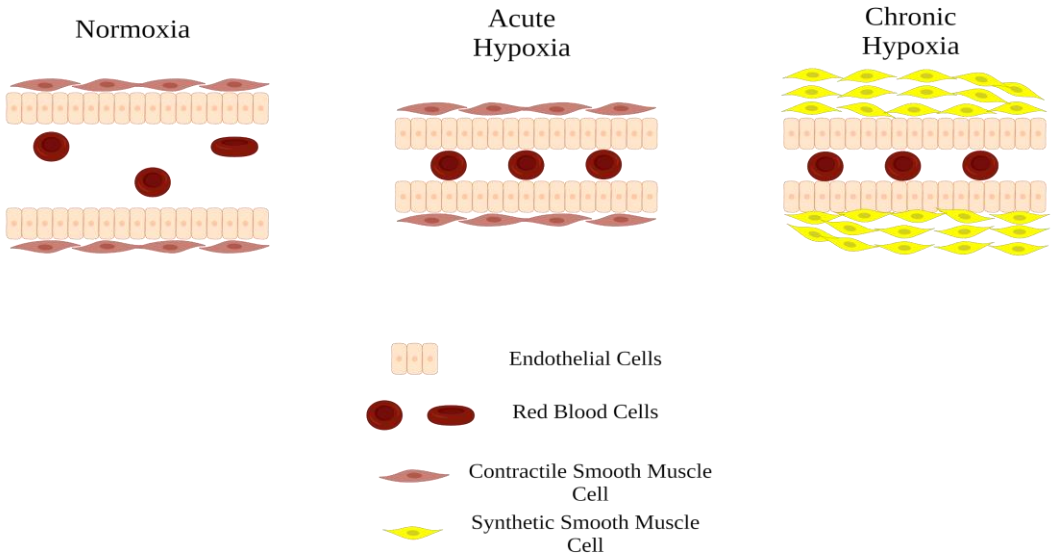

Figure 2. A depiction of the pulmonary artery response when exposed to normoxic, acute hypoxic, and chronic hypoxic conditions. Created with Biorender.

1.4 Inflammation and Chemokines/Cytokines

PH is a multifactorial disease that is caused by vasoconstriction, which is caused by increased intracellular calcium signaling and inflammation of vasculature within in the lungs and decreased second messenger (cGMP and cAMP) levels, as well as chronic inflammation.

There are two main theories on how inflammation begins in PH: Inside-out theory and Outside-in theory. In Inside-out theory, chemokines form a gradient on the apical side of endothelial cells, which provides an environment ideal for inflammatory cell recruitment and invasion in blood vessels. In contrast, Outside-in theory suggests immune and inflammatory macrophages accumulate in the adventitia and produce chemokines leading to inflammation (Lakhkar et al., 2016).

Inflammation can be caused by T- and B-lymphocytes and macrophage infiltration, which upon binding to inflammatory markers such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and various other cytokines, release chemokines (Alexander et al., 2019; Bordenave et al., 2020; Mamazhakypov et al., 2021). Chemokines, also known as chemotactic cytokines, are primarily, except for CXCL16 and CX3CL1, small soluble proteins that regulate immune cell recruitment and functions (Chen et al., 2018; Lumb & Slinger, 2015). Chemokines can bind to a wide variety of receptors, most of which are classified as G-protein-coupled receptors (GPR), which have different subgroups such as G_q, G_s, and G_i (Alexander et al., 2019; Amsellem et al., 2017). While there are almost 800 GPRs, many of them remain orphan receptors, of which their ligand is unknown (Alexander et al., 2019).

1.5 G-protein Coupled Receptors

GPRs are one of the most abundant protein families in humans and are what 30-40% of pharmaceuticals target (Ahmad & Dalziel, 2020). All GPRs contain a characteristic seven transmembrane helices that span the intracellular, transmembrane, and extracellular domains seen in this receptor family (Atanes et al., 2021). The primary function of these receptors is the transduction of extracellular stimuli into intracellular signals through second messengers such as cyclic adenosine monophosphate (cAMP) or inositol triphosphate (IP3). Upon binding to its ligand, an intracellular change occurs on the receptor's heterotrimeric G protein, where the guanosine diphosphate (GDP) alpha subunit becomes activated to guanosine triphosphate (GTP) and separates from the receptor and the other beta and gamma subunits of the heterotrimer. Next, the heterotrimer is broken into two portions, the alpha and beta-gamma subunits (Moreira, 2014). GPRs are classified as G_q, G_i, or G_s based on their downstream pathways. G_q receptors stimulate adenylate cyclase (AC), which produces cAMP. cAMP activates protein kinase A (PKA), leading to an increase in protein phosphorylation (Mizuno & Itoh, 2009). G_i signaling inhibit AC and prevent the formation of cAMP (Moreira, 2014). G_s signaling initiates phospholipase C activity, cleaving phosphatidylinositol to IP3 and diacylglycerol (DAG), which promote the release of Ca^{2+} from intracellular stores and activates Ca^{2+}-binding proteins (Mizuno & Itoh, 2009). One GPR that is gaining a lot of attention recently is G protein coupled receptor 75 (GPR75).

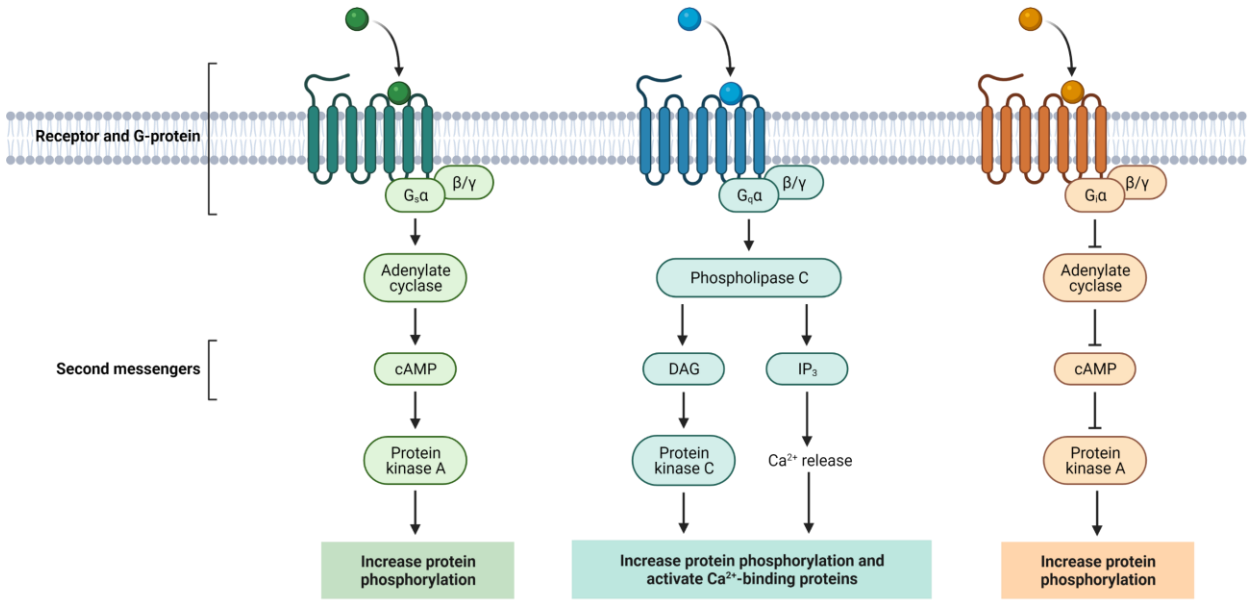

Figure 3. Depiction of the three classes of G-coupled protein receptors: G_s, G_q, G_i. Created with BioRender.

1.6 G-protein Coupled Receptor 75

GPR75 is a 540 amino acid orphan G-protein-coupled receptor that is expressed in many human tissues such as the brain, lungs, and endocrine tissues (Akbari et al., 2021; Uhlen et al., 2015). While not much is known about GPR75's mechanism of function, it may play an important role in the development of diseases, such as macular degeneration, diabetes, obesity, and potentially PH (McDonald, 2013; Mizuno & Itoh, 2009; Pappalardo et al., 2017; Sauer et al., 2001). Recent studies have demonstrated a link between PH and GPR75 using PASMCs derived from patients diagnosed with idiopathic PH (iPAH) and secondary PAH, in which the PASMCs had a higher expression level of GPR75 compared to control groups (Iyinikkel, 2019; McDonald, 2013). These studies show GPR75 is elevated in PH patient pulmonary vessels, especially in females (Iyinikkel, 2019; McDonald, 2013). While groups agree that GPR75 is a contributing factor in the development of the previously mentioned diseases, the classification of GPR based on the second messenger signaling is debated. Some studies indicate GPR75 as a G_q receptor which utilizes the second messengers DAG and IP3 (Ignatov et al., 2006; Sumin Lu, 2021), while others suggest it is maybe a G_i-coupled receptor which reduces cAMP (Iyinikkel, 2019; McDonald, 2013). As mentioned before, GPR75 is classified as an orphan receptor, indicating that its endogenous ligand remains unknown. While its ligand remains unknown, there are two contenders that are documented: 20 Hydroxyeicosatetraenoic acid (20-HETE) and chemokine ligand 5 (CCL5) (Garcia et al., 2017; Ignatov et al., 2006; Paul et al., 1999).

1.7 Ligands of GPR75

1.7.1 20-HETE

20-HETE is an eicosanoid derived from the metabolism of arachidonic acid, by the cytochrome P450 4A and 4F isozymes, which are present in many tissues including endothelial cells and vascular smooth muscle cells (Garcia et al., 2017; Mizuno & Itoh, 2009). 20-HETE, an autocrine and paracrine mediator of cellular processes, has been identified as a contributor to many disease states, such as hypertension, ischemic and hemorrhagic strokes, acute renal failure, vascular restenosis, cardiac hypertrophy, myocardial infarction, renal ischemia-reperfusion injury, and shock (Roman & Fan, 2018). Endothelial dysfunction is a cause of hypertension and vascular disease, promoted through 20-HETE uncoupling eNOS and increasing formation of reactive oxygen species (Roman & Fan, 2018). 20-HETE has been shown to bind to GPR75 and activates a Gq-signaling in endothelial cells and SMCs (Garcia et al., 2017; Pascale et al., 2021). 20-HETE contributes to systemic hypertension by sensitizing vessels to phenylephrine, increased oxidative stress, and endothelial dysfunction, as well as activating the renin-angiotensin system through increased expression of angiotensin-converting enzyme, contributing to further vasoconstriction (Roman & Fan, 2018).

However, while 20-HETE is deleterious and stimulates vasoconstriction in the systemic circulation, previous studies show 20-HETE has conflicting effects within the pulmonary vasculature.

One study found 20-HETE causes transient contraction of bovine PA rings followed by dilation and induces a potent vasodilatory response in human and rabbit pulmonary vasculature (Kizub et al., 2016). 20-HETE was also shown to increase survival of pulmonary

vascular endothelial cells (PMVECs) which underwent hypoxia reoxygenation injury, prevented mitochondrial depolarization, and enhanced the antioxidant capacity in PMVECs, which protected ATP production capacity (Sugumaran et al., 2020). These studies indicate 20-HETE is protective in the pulmonary vasculature.

Other studies, however, found 20-HETE to be deleterious within the pulmonary vasculature. One group found 20-HETE acted as a vasoconstrictor in smaller PAs isolated from rats by blocking Ca^{2+}-activated K^{+} channels and inhibiting the formation of the vasodilator NO (Elshenawy et al., 2017). 20-HETE was also found to promote PASMC proliferation and reactive oxygen species production in another study (Wang et al., 2020). The findings from these studies indicate 20-HETE is deleterious in the pulmonary vasculature and can aid in the pathogenesis of PH.

1.7.2 Chemokine Ligand 5

The other potential ligand for GPR75, CCL5 is a proinflammatory chemokine ligand that has three different chemokine C-C motif receptors (CCRs): CCR1, CCR3, and CCR5 (Culley et al., 2006; Dorfmuller et al., 2002; Singh et al., 2018). CCR1 and CCR3 are highly expressed in the lungs after injury or perturbation, but CCR5 expression increased only once CCL5 levels are increased (Nie et al., 2018). CCL5 has been previously shown to contribute to the pathogenesis of PH, and CCL5-deficient mice develop less PH (Dorfmuller et al., 2002; Nie et al., 2018). In the lungs, CCL5 is strongly expressed on the primary cells involved in PH, vascular endothelial cells, SMCs, T-cells, and macrophages (Nie et al., 2018). Once CCL5 binds to one of its receptors, it recruits monocytes, neutrophils, T cells, and eosinophils, initiating integrin activation, lipid mediator biosynthesis, and degranulation, all of which are

proinflammatory (Culley et al., 2006; Remy-Jardin et al., 2021). CCL5 can also induce endothelin-converting enzyme-1, which cleaves big endothelin to its active form, ET-1, a very potent vasoconstrictor (Uhlen et al., 2015). However, in $CCL5^{-/-}$ mice, PASMC proliferation and migration was suppressed, which are key contributors to PH development through PA remodeling (Nie et al., 2018).

Based on this information, we believe GPR75 has a role in the development of chronic HPH. Using a novel GPR75 knockdown ($GPR75^{-/-}$) mouse model we aim to uncover the role of GPR75 in the development of HPH.

2 **Central Hypothesis:** We hypothesize that GPR75 plays a critical role in the development of HPH and silencing of GPR75 can prevent HPH.

3 **Specific Aims:**

1. **Does GPR75 knockdown reduce hypoxic pulmonary vasoconstriction?**
2. **Does GPR75 knockdown reduce chronic hypoxia induced PH?**
3. **Does GPR75 regulate cAMP or IP3 and associated signaling in lungs?**

3.1 Specific Aim 1:

Does GPR75 knockdown reduce hypoxic pulmonary vasoconstriction?

1. **Are there changes in the contraction of *Gpr75*$^{-/-}$ vs WT mice pulmonary arteries exposed to hypoxia *ex vivo*?** In PH, pulmonary arteries constrict and remodel in response to hypoxic conditions and in previously mentioned studies (Lu et al., 2021; Mamazhakypov et al., 2021), PH patients experienced elevated expression levels of GPR75, and therefore we hypothesize that GPR75 aids in controlling hypoxic pulmonary vasoconstriction (HPV), which is a physiological response to maintain the alveolar ventilation capillary perfusion ratio. However, persistent HPV becomes maladaptive and evokes HPH. To test this hypothesis, intra-lobar pulmonary arteries (IPAs) were harvested from BL6/J (WT) and *Gpr75*$^{-/-}$ mice, both 12 weeks of age, and their vascular reactivity tested. The vessels were briefly exposed to hypoxia (95% N_2 – 5% CO_2) that lowered the oxygen tension in the tissue bath to 40 Torr, and we compared the contractions of the two groups of mice to see if there is a reduced HPV in the *Gpr75*$^{-/-}$ mice.
2. **Can the HPV be altered by putative GPR75 ligands?** CCL5 and 20-HETE are two putative ligands of GPR75, therefore we tested their effects on HPV response. Briefly, IPAs from WT and *Gpr75*$^{-/-}$ mice were pretreated with CCL5 or 20-HETE before exposure to hypoxia, and the HPV response was determined.

3.2 Specific Aim 2:

Does GPR75 knockdown reduce chronic HPH?

1. **Are *Gpr75*$^{-/-}$ mice protected from developing HPH?** PH can be induced by a variety of conditions, including chronic hypoxia. In hypoxic conditions, pulmonary arteries constrict to maintain ventilation-to-perfusion quotient and when exposed to hypoxia for a prolonged period of time, the vessels begin to remodel, which increases pulmonary blood pressure and afterload on the right heart. This leads to right heart hypertrophy and failure. If our hypothesis in Aim 1 is correct and GPR75 does lead to an increase in HPV, then we hypothesize that *Gpr75*$^{-/-}$ mice are resistant against the development of chronic HPH. To test this, we will have four groups of mice: Normoxic and hypoxic WT and *Gpr75*$^{-/-}$ mice. We kept mice in normoxic (21% O_2) or normobaric hypoxic (10% O_2) conditions for 5 weeks. At the end of the 5 weeks, we performed echocardiography and right ventricular catheterization to establish if they developed HPH (pulmonary arterial blood pressure >25mmHg and significant right heart hypertrophy).
2. **Are genes involved in the pathogenesis of HPH up or down regulated by GPR75 knockdown?** Further, to determine whether GPR75 signaling affects the development of HPH, the expression of genes encoding smooth muscle markers, *Myh11* and *Cnn1*, inflammatory proteins, *Ccl2, Ccl5, Cxcl12*, and *Prom1*, and contractile proteins, *Bmpr1a* and *Rock2*, which are implicated in the pathogenesis of HPH, was measured via qRT-PCR in RNA from lungs of WT and *Gpr75*$^{-/-}$ mice exposed to normoxic and hypoxic conditions.
3. **Are GPR75 and its ligands up or down regulated in humans with PH?** While we are studying how GPR75 affects HPH in mice, it is also important to test if *GPR75* expression is affected in humans with PH. To study this, blood obtained by Dr. Lanier at Westchester

Medical Center, from patients with scleroderma-associated and idiopathic PH was utilized. We will determine the gene expression in leukocytes of these two groups to see if *GPR75* is more highly expressed in PH patients than non-PH patients. Doing this will allow us to test if our study in mice holds the potential to be translational.

3.3 Specific Aim 3:

Does GPR75 regulate cAMP or IP3 and associated signaling in lungs?

1. **Determining the 2nd messenger of GPR75.** Since GPR75 is a G-protein-coupled receptor, it affects second messengers, such as inositol 1,4,5-trisphosphate (IP3) and Cyclic adenosine monophosphate (cAMP). However, the signaling molecules/pathways are unclear. While some groups have suggested GPR75 functions as a G_q receptor, with its signaling associated IP3 signaling (Ignatov et al., 2006), others have suggested GPR75 to function as a G_i receptor, as overexpression and knockdown respectively decrease and increase cAMP levels in smooth muscle cells (Lu et al., 2021; Mamazhakypov et al., 2021). The previously mentioned studies indicate that GPR75 signaling is still controversial. To determine which of these second messengers (cAMP and IP3) are affected by GPR75, we utilized enzyme-linked immunosorbent assays (ELISAs) in lungs of WT and *Gpr75*$^{-/-}$ mice.
2. **Determining the 2nd messenger signaling and pulmonary artery function.** Wire myography, an *ex vivo* approach, was utilized to determine which 2nd messenger is responsible for regulating GPR75 in PA contraction-relaxation function. IPAs were isolated from WT and *Gpr75*$^{-/-}$ mice, pretreated with a PKA agonist and antagonist, and challenged with vasoconstrictors. Altogether, these experiments will provide insight into signaling downstream of GPR75.

4. Materials and Methods:

4.1 Animals

Gpr75$^{-/-}$ mice were originally generated by Dr. Artiom Gruzdev and were housed and bred in New York Medical College's Department of Comparative Medicine (DCM). Littermates and/or C57BL/6 (Taconic Biosciences) were eugenic controls. All procedures were approved by the New York Medical College Institutional Animal Care and Use Committee.

4.2 Generation of the *Gpr75*$^{-/-}$ Mouse Model

The *Gpr75*$^{-/-}$ mice were created through a CRE/LOX system, allowing for the gene of interest, *Gpr75* to be excised nonspecifically, generating a global knockdown model. The conditional null ("Flox") Gpr75 locus was generated by CRISPR/Cas9-mediated targeting in G4 (B6129F1) ES cells. The LoxP sites flank 3133 bp, which contains 184 bp of Intron 1-2, the single coding exon of Gpr75 (exon 2), the entire 3' UTR, and 208 bp of 3' extragenic sequence (floxed sequence chr11:30,890,805-30,893,937 [GRCm38/mm10]). CAS9 double stranded breaks were targeted to CAGGAATACGACCTCTCCATNGG (5') and CCAAAATCCTATACTAGTAGNGG (3') using the PX459v2 SgRNA/CAS9/Puro delivery plasmid, a gift from Feng Zhang (Addgene #62988, PMID: 24157548). Two circular repair templates, each with 600 bp total homology, were used to insert the LoxP sites. To aid clonal screening, a unique restriction endonuclease site was inserted with each LoxP site: BamHI (5' LoxP) and ClaI (3' LoxP). ES clonal screening was done with high fidelity PCR, followed by restriction digest, and confirmed by direct sequencing of the PCR amplicon sequencing verification. PCR Primers: 5'LoxP-Fwd: GTACATTGTGCACCTCTTCACAC; 5'LoxP-Rev: CTTCCTGAAGGATGGGTCAAAGA; 3'LoxP-Fwd:

ACCAAGCTGTTACAAATGTGCTG; 3' LoxP- Rev: TTGGTTGCTTAATATGCATGACCC). To confirm that no aberrant recombination has occurred within the Gpr75 coding exon, the entire targeted locus was PCR amplified and sequenced using the distal screening primers (5'LoxP-Fwd and 3' LoxP-Rev). Neither of the two CAS9 target sequences were predicted to have any genetically linked off-target sites, therefore the ES clones were not screened for any off-target CAS9-mediated mutations. Homozygously targeted ES clones were microinjected into wildtype blastocyst for standard chimeric founder generation. The primary Gpr75 flox allele was maintained as a perpetual backcross to wildtype C57BL/6J mice. To generate the Gpr75-deficient allele, the Gpr75 flox allele was crossed to B6 CMV-Cre transgenic mice (Jackson Labs Strain 006054; PMID: 8559668). Cre-mediated recombination/excision of the floxed genomic region was confirmed by direct amplicon sequencing. Genotyping of the flox and null mouse colonies was done by Transnetyx using allele-specific primer/probe assays.

4.3 Wire Myography

Wire myography was performed on intralobar pulmonary arteries collected from *Gpr75*$^{-/-}$ and WT mice in order to examine pulmonary arterial function in response to hypoxic conditions as well as various treatments (20-HETE, 20-SOLA, CCL5, CCL5 antibody, KCl, U46619, cAMP-rp, ZD7288, 8-bromo-cAMP, and ET-1). Animals were sacrificed the day of surgery so freshly harvested intralobar pulmonary arteries can be used for the experiments. The vessels are mounted in tissue baths filled with 5mL of 1x Krebs Buffer (NaCl: 6.9g/L KCl: 0.35g/L, KH_2PO_4: 0.163g/L, $MgSO_4$: 0.144g/L, $CaCl_2$: 0.28g/L, $NaHCO_3^-$: 2.1g/L, D-Glucose: 1.98g/L, and EDTA: 0.0011g/L in DI water) in order to provide the tissue with an isotonic solution similar to what would be in the organism, bubbled with an air mixture (21% O_2 -

5%CO_2) that mimics room air, and warmed to 37ºC. A depiction of the vessel isolation and mounting are located in Figure 3. After normalization, vessels rest for 60 minutes in normoxia before undergoing treatment with hypoxia (95%N_2 – 5%O_2; 40 Torr) and/or drug treatments. Measurements are taken via the programs Myodaq (Myodaq, Danish Myo Technology) and LabChart (Dunedin, Otago) and analyzed on GraphPad Prism 8.

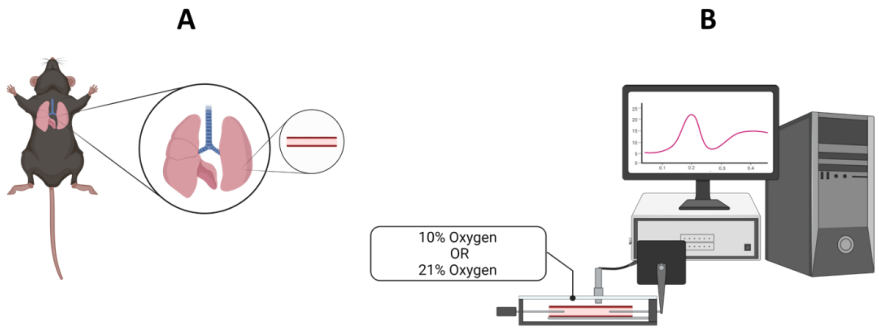

Figure 4. A depiction of the wire myography set up. (A) Either a WT or *Gpr75*$^{-/-}$ mouse is sacrificed and its lungs quickly arvested and placed in a Kreb's buffer solution. Intra-lobar pulmonary arteries are dissected out and placed in a wire myograph bath filled with Kreb's buffer solution. (B) The wire myograph bath was connected to the transducer, warmed to 37ºC, and bubbled with either a normoxic gas mixture or a 95% N_2 – 5% CO_2 gas mixture to lower the O_2 partial pressure in the bath to 40 Torr. Vessel tensions were measured through MyoDaq and analyzed in LabChart8 and MyoData.

4.4 Hypoxia Treatment

Both WT and *Gpr75*$^{-/-}$ mice were born and allowed to reach 3 months of age before undergoing echocardiography to determine baseline measurements of the heart and pulmonary artery. Afterwards, mice were either placed in a normobaric hypoxic chamber that was connected to a regulator set to 10% O_2 or left in an animal bay at normoxic conditions for 5 weeks (Figure 5). After 5 weeks, the mice were removed from their conditions and underwent echocardiography again to determine end point measurements before undergoing terminal right heart catheterization and having their organs collected, snap frozen, and stored in -80ºC until needed.

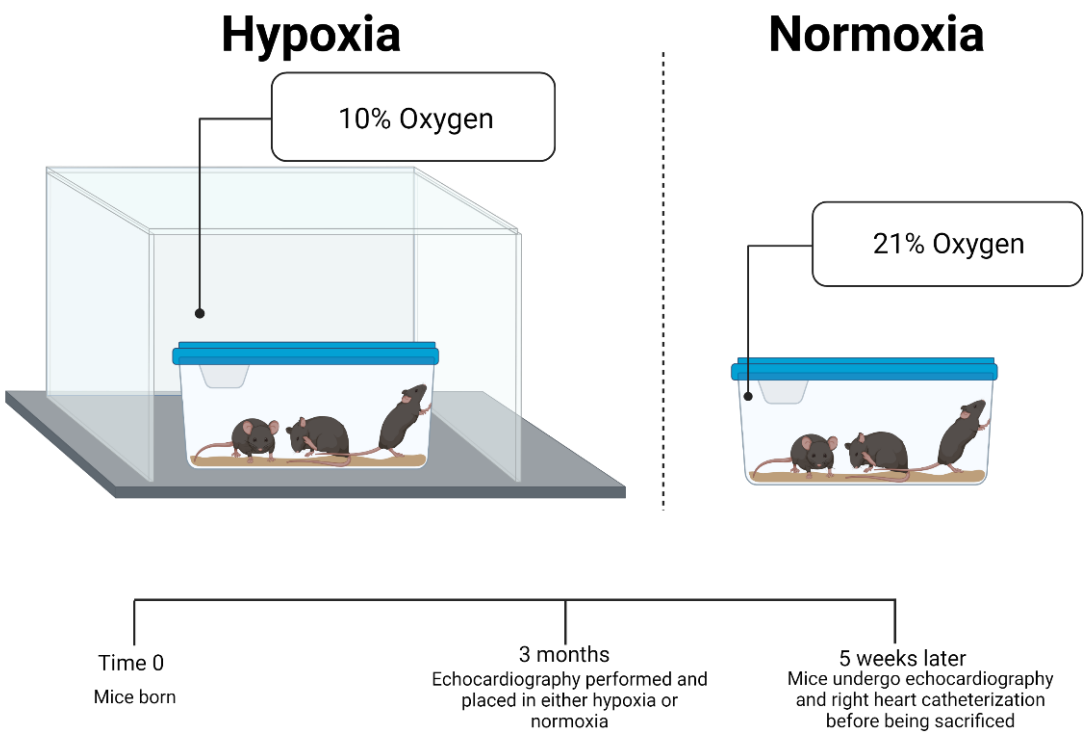

Figure 5. Summary of the timeline followed for the mouse experiments and conditions the mice were kept in.

4.5 Echocardiography and Right Ventricular Catheterization

Mice were allowed to reach 3 months of age before undergoing echocardiography under isoflurane anesthesia (4% induction; 2% maintenance) to determine baseline reads for their heart and lung function using the Vevo 770 imaging system (VisualSonics, Toronto, ON, Canada), before being placed in normobaric normoxic (21% O_2 – 5% CO_2) or hypoxic (10% O_2 – 5%CO_2) for 5 weeks. At the end of five weeks, mice underwent echocardiography under anesthesia again (4% induction; 2% maintenance) to determine if they developed PH by comparing their endpoint measurements to their baselines before they underwent cardiac catheterization while under anesthesia (4% induction; 2% maintenance). After the catheterization was completed, the animals were sacrificed and their heart, lungs, and aortas were collected and snap frozen in liquid nitrogen for later biochemical analysis. Detailed protocols for the echocardiography and the catheterization are referenced in a previously published paper by our lab (Joshi et al., 2020). A summary of the group set up and timeline are outlined in Figure 5.

4.6 Human Blood Samples

Blood samples from healthy individuals and patients that diagnosed with scleroderma-associated PH or iPAH were generated by Dr. Lanier at Westchester Medical Center. Plasma was isolated by centrifuging for 15 minutes at 2500 RPM at 4°C. The upper layer containing plasma was removed and frozen, while the remaining lower layer containing red blood cells was treated with a red blood cell lysing solution for 30 minutes in the dark. The samples were once again spun for 15 minutes at 2500 RPM to separate the white blood cells from the lysing solution. The top layer was removed and discarded while the remaining layer containing white blood cells was stored at -80°C until needed for analysis through qRT-PCR and ELISA.

4.7 ELISAs

Three ELISA kits are used during the course of these experiments. A Cyclic Adenosine Monophosphate (cAMP) ELISA, purchased from Cayman Chemicals (#501040) and the protocol provided by the company for tissues and plasma was utilized. A DuoSet ELISA kit to measure Chemokine Ligand 5 (CCL5) levels was purchased from R&D Systems (#DY478-05). The protocol provided by R&D Systems was utilized for the measurement of CCL5 in tissues and plasma. An ELISA kit to measure Mouse inositol 1,3,5 Triphosphate (IP3) was purchased from MyBioSource (#MBS2515928) and the protocol provided by this company was used to analyze the content of IP3 in tissues and plasma.

4.8 Histology

Lungs were harvested, perfused with 10% paraformaldehyde (PFA) through the main IPA via drip, and stored in PFA for a week before being given to the New York Medical College Histology Core for slicing, mounting, and staining. Tissue sections were cut 7μm thick and stained with Hematoxylin and eosin (H&E), Elastin, or Trichrome. Images were taken on a Zeiss microscope (Jena, Germany) and wall thickness and vessel size calculated by measuring the diameter of outer layers of the vessel and the diameter of the vessel lumen, respectively, using the measurement tools in the program Zen Blue.

4.9 qRT-PCR

RNA was extracted using the QIAGEN miRNeasy kits, the RNA content and quality was analyzed in a Tek3 plate in the BioTek plate reader. The RNA was transformed into cDNA using the Thermofisher SuperScript™ IV VILO™ Master Mix kit (Cat# 11766050) and stored at -80ºC until necessary. qRT-PCR was performed using Taqman FAM primers and the

Taqman fast advanced master mix (Cat#: 4444556), following the protocol recommended by ThermoFisher. *Tuba1a* is the control gene for these experiments.

4.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. Values are presented as mean ± standard error (SE). Statistical comparisons of samples were made between two groups with Student's t-test and to make comparisons among more than two groups, two-way ANOVA followed by Sidak's post hoc test for multiple comparisons was used. Values of $P \leq 0.05$ were considered significant.

5 Results

5.1 Aim 1: Does GPR75 knockdown reduce hypoxic pulmonary vasoconstriction?

5.1.1 *Gpr75*$^{-/-}$ mice HPV response is less as compared to WT mice.

IPAs from WT and *Gpr75*$^{-/-}$ mice were mounted on a wire myograph and pretreated with 1 μmol/L of phenylephrine and exposed to hypoxia (P_{O2} = 40 Torr). IPAs from *Gpr75*$^{-/-}$ mice as compared to WT mice contracted less to hypoxia (*Gpr75*$^{-/-;}$ 0.042±0.01 mN/mm^2 N=19 and WT: 0.448±0.049 mN/mm^2 N=40) and 2nd phase HPV response of IPAs was less in *Gpr75*$^{-/-}$ compared to WT mice (Figure 6).

As previously mentioned, GPR75 has two putative ligands, CCL5 and 20-HETE. How the two putative ligands and their inhibitors affected the WT and *Gpr75*$^{-/-}$ mouse IPA HPV response were assessed via wire myography.

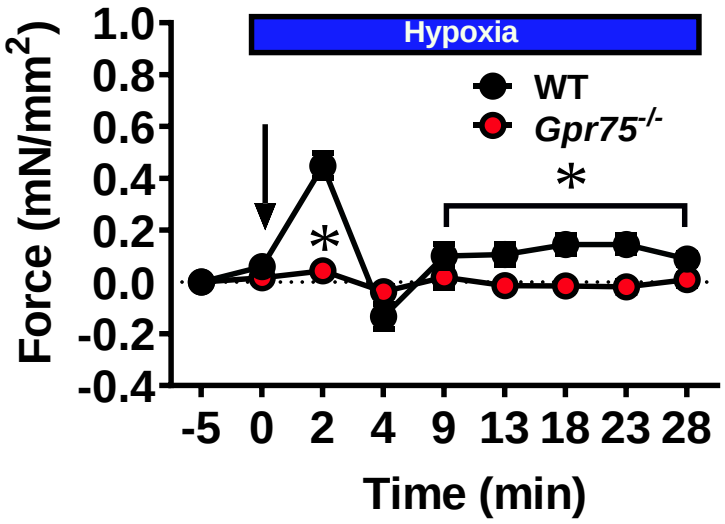

Figure 6: *Gpr75*$^{-/-}$ mice had a significantly decreased HPV response in comparison to WT mice. The arrow indicates when the hypoxic gas mixture was added to the bath. Both 1st and 2nd phase of HPV is suppressed in *Gpr75*$^{-/-}$ mice in comparison to WT mice. *P<0.05 vs WT, N=40 in WT group, and N=19 in *Gpr75*$^{-/-}$ group. Statistical comparisons of samples were performed with Student's t-test for comparing the two groups, and two-way ANOVA followed by Sidak's post-hoc test was performed to compare different time points.

5.1.2 Pretreatment with CCL5 elicited a stronger 2nd phase of HPV in WT IPAs

To test if CCL5, a putative ligand of GPR75 alters the HPV response in mouse IPAs, IPAs were isolate from WT mice and pretreated with CCL5 and CCL5-binding antibodies before exposure to hypoxia. Pretreatment of WT IPAs with CCL5 augmented 2nd phase of HPV response (CCL5: 0.465±0.234 mN/mm^2; N=4; WT: 0.144±0.037 mN/mm^2; N=40; $p<0.05$), while pretreatment with functional blocking CCL5 antibodies (CCL5-Ab) had no effect on HPV response as compared to controls (Figure 7A). Interestingly, while CCL5 altered the 2nd phase HPV response in WT IPAs, CCL5 did not augment the HPV response of *Gpr75*$^{-/=}$ IPAs (Figure 7B).

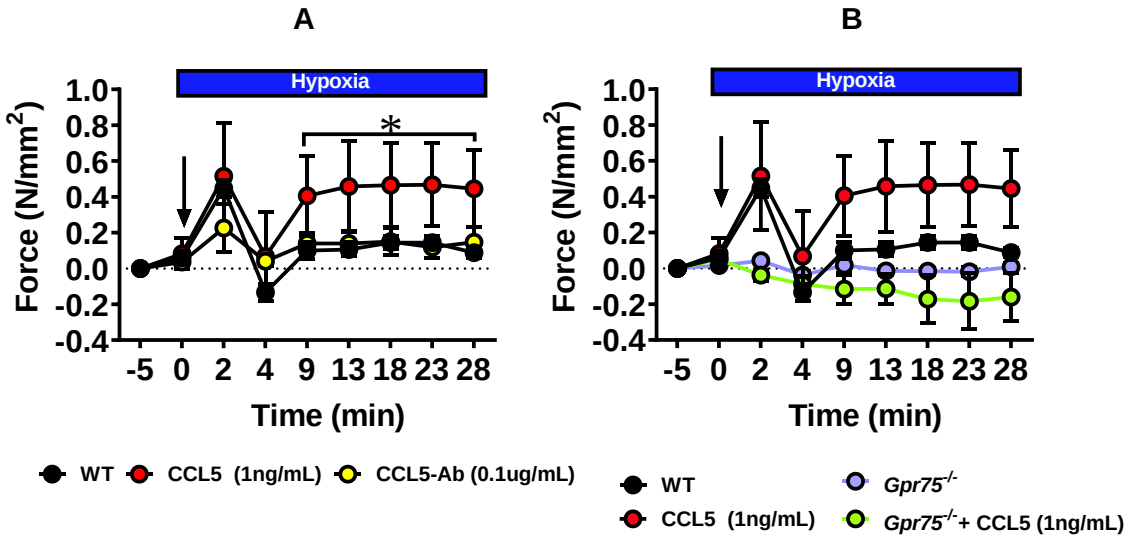

Figure 7: Pretreatment of WT IPAs with CCL5-Ab before exposure to hypoxia (P_{O2} = 40 Torr) elicited an effect on HPV response similar to what was seen with *Gpr75*$^{-/-}$ mouse IPAs exposed to hypoxia. (A) Pretreatment of WT IPAs with CCL5 before hypoxia exposure led to an elevated 2nd phase HPV response. (B) Pretreatment of *Gpr75*$^{-/-}$ mouse IPAs with CCL5 did not elicit any changes in their HPV response. Statistical comparisons of samples were performed with Student's t-test for comparing the two groups, and two-way ANOVA followed by Sidak's post-hoc test was performed to compare different time points. WT N=40; CCL5-Ab N=5; CCL5=4; *Gpr75*$^{-/-}$ N=19; *Gpr75*$^{-/-}$+CCL5 N= 6.

5.1.3 Pretreatment with 20-HETE and 20-HETE inhibitors/antagonists did not alter WT IPAs HPV response

To test if 20-HETE, the other putative ligand of GPR75, altered the HPV response in mouse IPAs, IPAs were isolated and pretreated with 20-HETE and 20-HETE antagonists/inhibitors before exposure to hypoxia. Pretreatment with a low dose of 20-HETE (10 nM; N=4) did not cause any changes in HPV response and a high dose of 20-HETE (30 Nm; N=5) trended to reduce 1st phase HPV response (20-HETE: 0.092±0.028 mN/mm^2 N=5; WT: 0.448±0.049 mN/mm^2 N=40) (Figure 8A). WT IPAs were also pretreated with antagonists and inhibitors of 20-HETE, 6(Z),15(Z)-hydroxyeicoas-6,15-dienamido-diencoic acid (AAA: antagonist of 20-HETE; N=4), dibromo-dodecenylmethylsulfimide (DDMS; an inhibitor of 20-HETE synthesis; N=4), and 3-oxa-20-6 15-HEDE (20-HETE antagonist; N=3). None of these agents affected the HPV response (Figure 8B).

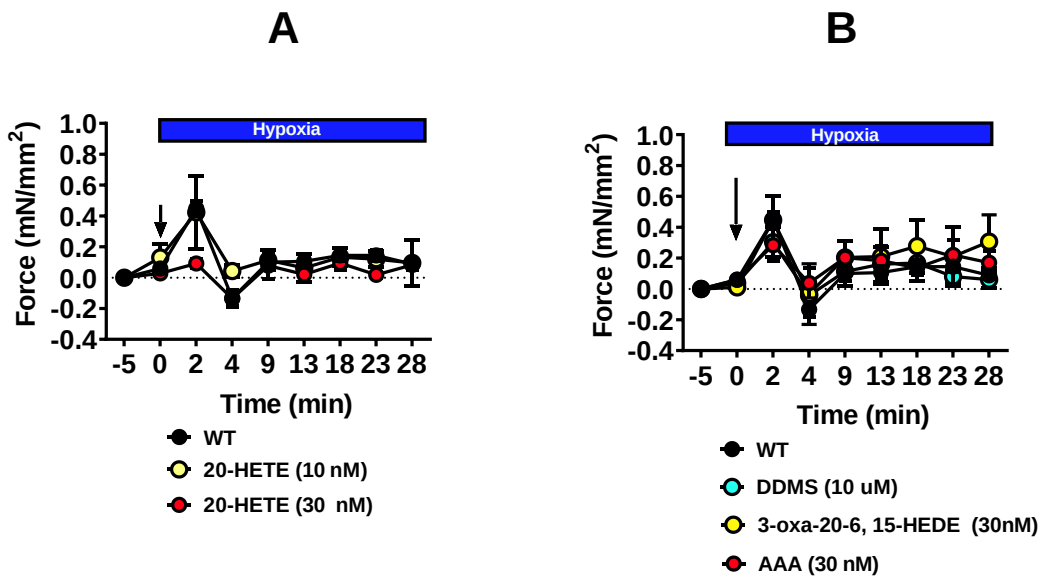

Figure 8: Effect of 20-HETE or its inhibitors/antagonists on HPV response. (A) Pretreatment with 20-HETE did not alter the HPV response of WT mouse IPAs. (B) Pretreatment with AAA, DDMS, nor 3-oxa-20-6, 15-HEDE elicited changes in WT mouse HPV responses. Statistical comparisons of samples were performed with Student's t-test for comparing the two groups, and two-way ANOVA followed by Sidak's post-hoc test was performed to compare different time points. WT N=40; 20-HETE 10nM N=4; 20-HETE 30nM N=5; AAA N=4; 3-oxa-20-6, 15-HEDE N=3; DDMS N=4.

5.2 Aim 2: Does GPR75 knockdown reduce chronic HPH?

5.2.1 Hypoxic *Gpr75*$^{-/-}$ mice developed less right ventricular pressure but not PA remodeling

Upon seeing the differences in the HPV of IPAs of *Gpr75*$^{-/-}$ and WT mice, we tested whether *Gpr75*$^{-/-}$ mice were protected from developing HPH by exposing WT and *Gpr75*$^{-/-}$ mice to normoxic (21% O_2) and normobaric hypoxic (10% O_2) conditions for five weeks. *Gpr75*$^{-/-}$ mice exposed to hypoxic conditions for five weeks developed less HPH and RV hypertrophy (Figure 9). Right heart catheterization showed there was no significant increase in right ventricular systolic pressure (RVSP) between the normoxic (24.33±1.20 mmHg; N=3) and hypoxic (36.53±3/97 mmHg; N=8) *Gpr75*$^{-/-}$ mice, but the RVSP increased ($P<0.05$) in hypoxic (56.63±3.46 mmHg; N=11) WT mice (Figure 9A). Right ventricular diastolic pressure (RVDP) was significantly lower in *Gpr75*$^{-/-}$ (2.00±0.0 mmHg; N=3) than WT (3.82±0.38 mmHg; N=11) mice in normoxic conditions (Figure 9B). RVDP did not change for *Gpr75*$^{-/-}$ mice exposed to hypoxia as compared to normoxia (2.00±00 mmHg; N=3), but RVDP increased significantly after 5 weeks of hypoxia (9.793±0.907 mmHg; N=18) compared to normoxic (3.818±0.377 mmHg; N=11) WT mice (Figure 9B). Another indicator of HPH is hypertrophy of the right ventricle (RV), which was measured using Fulton's Index (mass of right ventricle/mass of LV and septum). While there was no RV hypertrophy in the hypoxic *Gpr75*$^{-/-}$ mice, WT mice in hypoxia (0.3515±0.015; N=5) developed significant RV hypertrophy compared to the normoxic (0.2233±0.0118; N=5) mice (Figure 9C). These results reveal that *Gpr75*$^{-/-}$ mice are protected from developing HPH after exposure to chronic hypoxia, suggesting GPR75 signaling is critical for the development HPH.

In HPH, remodeling of distal and proximal PAs occurs, causing them to be hypertrophic and narrowed, which contributes to increase PA resistance that leads to the higher RVDP and RVSP seen in patients with PH. Having seen the *Gpr75*$^{-/-}$ mice were protected from developing increased RVDP and RVSP, as well as right heart hypertrophy, next we determine the morphology of the PAs within the lungs harvested from WT and *Gpr75*$^{-/-}$ mice exposed to normoxia and hypoxia. Lung sections were stained with H&E, Van Gieson's Stain (EVG), and Trichrome-mason. EVG and Trichrome-mason staining revealed remodeling of PAs >100µm was not different between hypoxia WT and *Gpr75*$^{-/-}$ groups (Figures 10A and B). However, morphometric analysis of measuring wall thickness to lumen revealed that both WT and *Gpr75*$^{-/-}$ mice exposed to hypoxia developed hypertrophy of 100µm PAs as compared to the normoxic groups (Figure 11).

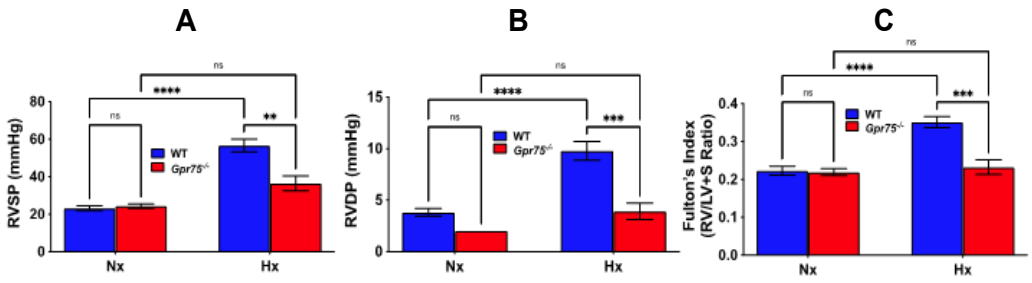

Figure 9. *Gpr75*$^{-/-}$ prevented the development of HPH. Wild type (WT) and *Gpr75* knockout (*Gpr75*$^{-/-}$) mice were exposed to normobaric hypoxia (10% O_2) or kept in normoxia (21% O_2) for 5 weeks. (A, B) Right ventricular systolic (RVSP) and diastolic (RVDP) pressure increased in hypoxic WT but not *Gpr75*$^{-/-}$ mice. (C) Right ventricular hypertrophy (Fulton's Index) increased in hypoxic WT but not *Gpr75*$^{-/-}$ mice. *P<0.05 vs normoxia (Nx) and #P<0.05 vs wild type (WT), N = 11 in wild-type Nx group, N = 18 in wild-type Hx group, N = 5 in *Gpr75*$^{-/-}$ group, and N = 8 in *Gpr75*$^{-/-}$ Hx group. Statistical comparisons of samples were performed with two-way ANOVA followed by Sidak's *post-hoc* test.

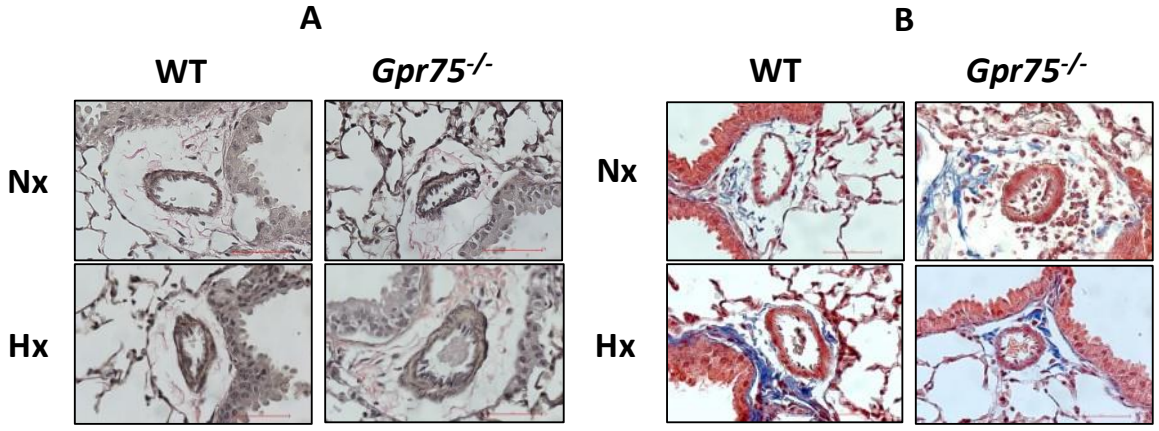

Figure 10. WT and *Gpr75*$^{-/-}$ mice exposed to prolonged hypoxia appear to undergo PA remodeling. Elastin and Trichrome-mason staining was performed on lungs isolated from WT and Gpr75$^{-/-}$ normoxia- and hypoxia-treated mice. Elastin staining indicated that pulmonary arteries (>100µm) had media thickening (hypertrophy) in both WT and Gpr75$^{-/-}$ mice exposed to hypoxia for 5-weeks compared to their normoxic-treated counterparts. Trichrome staining indicated that both Gpr75$^{-/-}$ and WT mice exposed to hypoxia *appeared to have* more collagen deposition than WT normoxic mice. N = 11 in wild-type Nx group, N = 18 in wild-type Hx group, N = 5 in Gpr75$^{-/-}$ group, and N = 8 in Gpr75$^{-/-}$ Hx group.

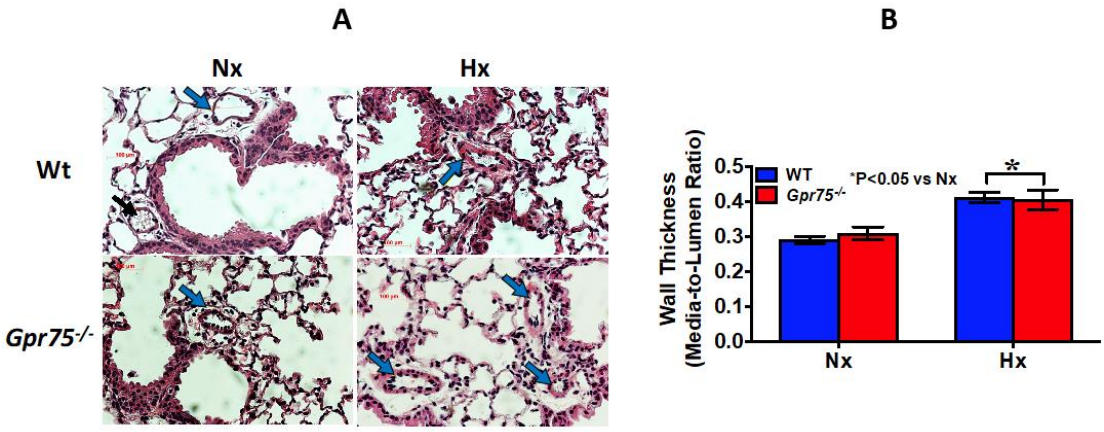

Figure 11. **Both WT and *Gpr75*$^{-/-}$ experienced vascular remodeling after 5 weeks of hypoxia.** (A, B) Pulmonary arteries less than 100μm in diameter in lung slices derived from WT and *Gpr75*$^{-/-}$ mice exposed to hypoxia for 5-weeks increased in media-to-lumen ratio. **P<0.05* vs normoxia (Nx) and N=6 in wild-type group, and N=4 in *Gpr75*$^{-/-}$ group. Statistical comparisons of samples were performed with two-way ANOVA followed by Sidak's *post-hoc* test.

5.2.2 *Gpr75* increases in lungs of WT mice exposed to hypoxia and is it primarily expressed in the pulmonary arteries

After determining the *Gpr75*$^{-/-}$ mice were protected from developing HPH, it was important to examine whether *Gpr75* expression changed in the mice exposed to hypoxia. *Gpr75* expression significantly increased in the lungs of WT mice exposed to hypoxia for 5 weeks compared to their normoxia exposed counterparts (Figure 12A). However, as expected, lung *Gpr75* expression did not increase in the *Gpr75*$^{-/-}$ hypoxic group compared to their normoxic counterparts (Figure 12A). This indicates that *Gpr75* levels increase under hypoxic conditions in WT mice, potentially contributing to the development of HPH.

Next, to determine if *Gpr75*$^{-/-}$ expression was within PAs or the surrounding soft tissues, qRT-PCR was performed on lungs with and without intra-lobar and 2nd order PAs. Lungs of WT mice with their PAs as compared to PAs removed had significantly more *Gpr75* mRNA (Figure 12B). These results indicate that a majority of *Gpr75* is expressed in the PAs of mice.

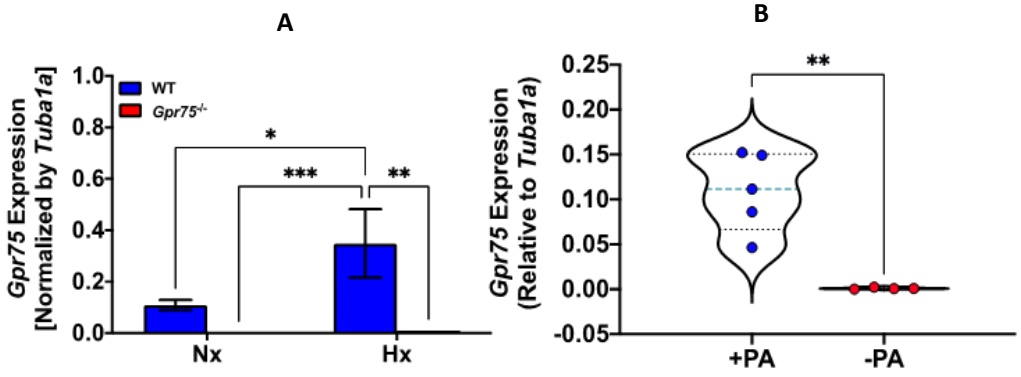

Figure 12. Wild-type mice treated with hypoxia had elevated *Gpr75* expression. (A) *Gpr75* mRNA expression was elevated in wild-type mice kept in hypoxic conditions for 5-weeks, compared to their normoxic counterparts. There was no change in *Gpr75* mRNA expression in the *Gpr75* knockout mice in normoxic and hypoxic conditions. (B) mRNA *Gpr75* expression was measured in isolated lungs of wild-type mice with PAs then without PAs. *P<0.05 vs normoxia; N= 7 for WT normoxic mice; N= 5 for WT hypoxic mice; N= 7 for *Gpr75*$^{-/-}$ normoxic mice, and N=5 for *Gpr75*$^{-/-}$ hypoxic mice. Statistical comparisons of samples were performed with Student's t-test for comparing the two groups, and two-way ANOVA followed by Sidak's post-hoc test for comparing the four groups.

5.2.3 *Gpr75*$^{-/-}$ mice do not experience expression changes in many of the genes involved in the pathogenesis of HPH

After realizing the *Gpr75*$^{-/-}$ mice are protected from developing HPH we wanted to determine whether the gene expression of proteins that contribute to the pathogenesis of HPH are altered in the lungs of *Gpr75*$^{-/-}$ mice.

5.2.3.1 Vascular Smooth Muscle Cell Genes

First, we determined the expression of smooth muscle restricted genes myosin heavy chain 11 (*Myh11*) and calponin 1 (*Cnn1*). While expression of *Myh11* expression was not significantly different between the WT and *Gpr75*$^{-/-}$ mice, there was a significant increase in *Myh11* expression in the lungs of *Gpr75*$^{-/-}$ hypoxic mice compared to their normoxic counterparts (Figure 13A). There were no significant changes in *Cnn1* expression between the normoxic and hypoxic WT and *Gpr75*$^{-/-}$ mice (Figure 13B).

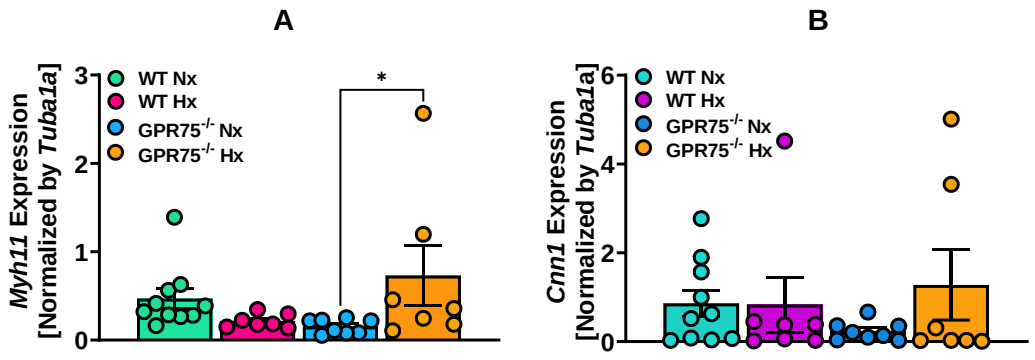

Figure 13. *Myh11* expression was elevated in *Gpr75*$^{-/-}$ mice exposed to prolonged hypoxia. (A) *Myh11* mRNA expression was measured in the lungs of WT and *Gpr75*$^{-/-}$ mice exposed to normoxia and hypoxia. A decreasing trend in *Myh11* expression was seen between the Nx and Hx WT groups, but *Gpr75*$^{-/-}$ mice exposed to hypoxia had higher *Myh11* expression compared to their normoxic counterparts. (B) *Cnn1* mRNA expression was measured in the lungs of WT and *Gpr75*$^{-/-}$ mice exposed to normoxia and hypoxia. No changes in *Cnn1* expression was seen in any of the groups. Statistical comparisons of samples were performed with two-way ANOVA followed by Sidak's post-hoc test for comparing the four groups. *P<0.05 compared to Nx, N = 10 for WT-Nx, N = 8 for WT-Hx, N = 8 *Gpr75*$^{-/-}$-Nx (*Myh11*), N = 10 *Gpr75*$^{-/-}$-Nx (*Cnn*), and N = 7 for *Gpr75*$^{-/-}$-Hx.

5.2.3 Bone Morphogenic Protein Receptor and Rho-kinase 2 Genes

Another set of genes that are typically dysregulated in HPH are the bone morphogenic protein receptor genes, which aid in the regulation of cellular growth and division. Consistently, *Bmpr1a* gene expression decreased in lungs from WT hypoxia mice as compared to the WT normoxia group but not in the lungs of *Gpr75*$^{-/-}$ normoxia and hypoxia groups (Figures 14A).

Rho-kinase 2 (*Rock2*) gene encodes for ROCK2 protein, which increases the Ca^{2+} sensitivity to myofilament and enhances VSMC contraction, is increased in HPH and aids in the thickening of the smooth muscle in PAs (Shimizu et al., 2013). *Rock2* expression increased in the WT hypoxic group compared to their normoxic counterparts, but there was no change in gene expression found in the *Gpr75*$^{-/-}$ groups (Figure 14B).

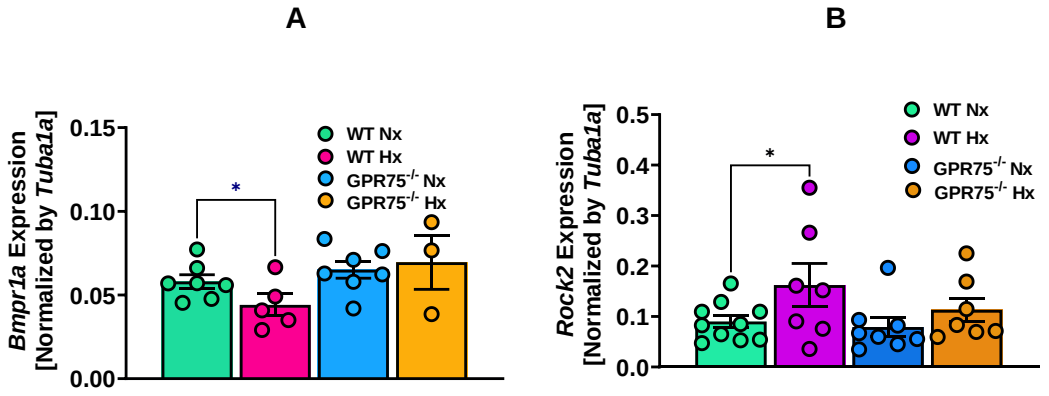

Figure 14. *Bmpr1a* and *Rock2* gene expression was not altered in *Gpr75*$^{-/-}$ mice exposed to prolonged hypoxia. *Bmpr1a* and *Rock2* expression was measured in the lungs of WT and *Gpr75*$^{-/-}$ mice exposed to normoxia or hypoxia. (A) *Bmpr1a* expression decreased and (B) *Rock2* expression was elevated in WT mice exposed to hypoxia as compared to their normoxic counterparts, but no changes were seen between the *Gpr75*$^{-/-}$ groups. *P<0.05 compared to Nx, N = 7 for WT-Nx (*Bmpr1a)*, N = 8 for WT-Nx (*Rock2*), N = 5 for WT-Hx (*Bmpr1a*), N = 5 for WT-Hx (*Rock2*), N = 7 *Gpr75*$^{-/-}$-Nx (*Bmpr1a*), N = 7 *Gpr75*$^{-/-}$-Nx (*Rock2*), and N =3 for *Gpr75*$^{-/-}$-Hx (*Bmpr1a*), N = 3 for *Gpr75*$^{-/-}$-Hx (*Rock2*) . Statistical comparisons of samples were performed with two-way ANOVA followed by Sidak's post-hoc test for comparing the four groups.

5.2.3.3 Inflammatory genes

Since inflammation is one of the factors for the pathogenesis of HPH, inflammatory gene expression was also measured in both the WT and *Gpr75*$^{-/-}$ mice (Stenmark & McMurtry, 2005). The first group examined consisted of C-C Motif Chemokine Ligand 2 (*Ccl2*), *Ccl5*, and C-X-C Motif Chemokine Ligand 12 (*Cxcl12*), which are chemokines that aid in chemotaxis and immune cell activation. *Ccl2* expression did not change between the normoxic and hypoxic WT nor the *Gpr75*$^{-/-}$ groups. However, *Ccl2* expression was lower in the *Gpr75*$^{-/-}$ normoxic group compared to the WT normoxic group (Figure 15A). *Ccl5* and *Cxcl12* gene expression increased in the WT mice exposed to hypoxia as compared to the WT normoxic mice but no changes were seen within the *Gpr75*$^{-/-}$ groups (Figure 15B-C). Lastly, Prominin 1 (*Prom1*), which is expressed on hematopoietic stem cells, which are myeloid cell precursors, (Foris et al., 2016), expression increased in lungs of hypoxic as compared to normoxic WT mice but did not increase in the lungs of hypoxic *Gpr75*$^{-/-}$ mice (Figure 15D). Most of the inflammatory markers increased in the WT mice exposed to hypoxia, but none increased in the *Gpr75*$^{-/-}$ hypoxia-exposed mice, which indicates perhaps *Gpr75*$^{-/-}$ mice have less inflammation than the WT mice under hypoxic conditions.

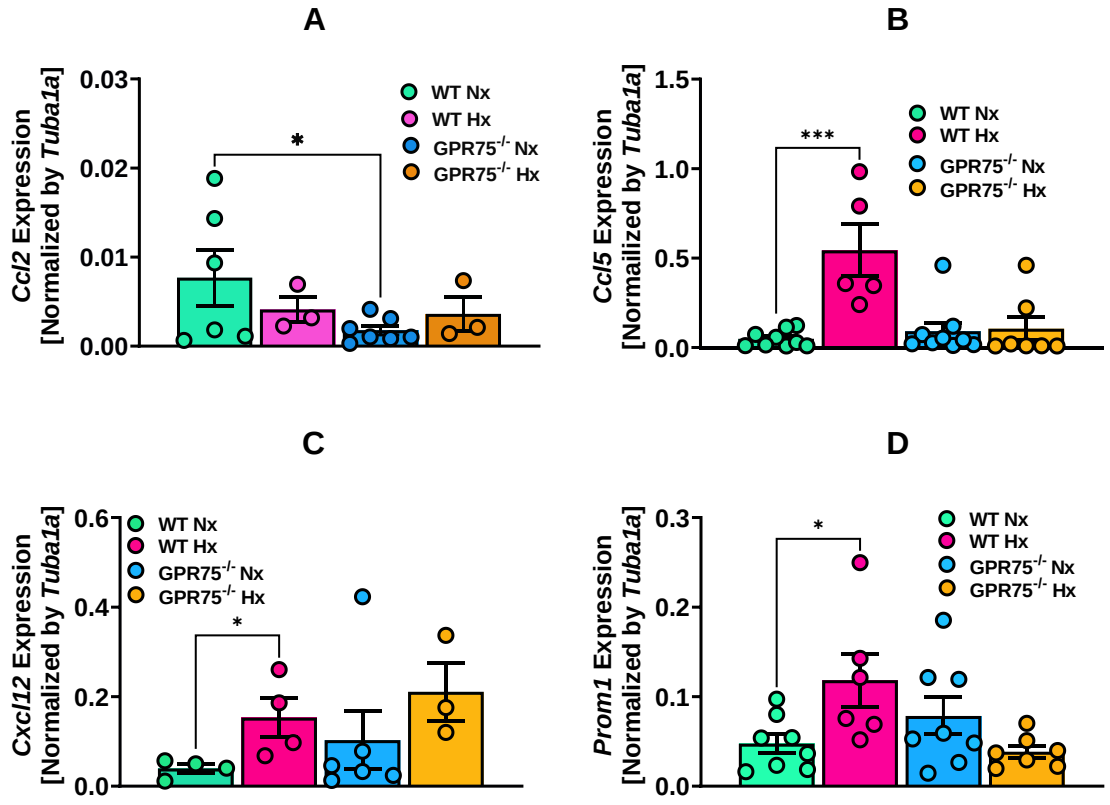

Figure 15. Inflammatory markers did not change in *Gpr75*$^{-/-}$ mouse lungs after prolonged hypoxia exposure. (A) *Ccl2* expression did not change between the hypoxic and normoxic WT mice nor the *Gpr75*$^{-/-}$ mice, but less *Ccl2* expression was lower in lungs of *Gpr75*$^{-/-}$ normoxia mice than the WT normoxic mice. (B,C,D) *Ccl5*, *Cxcl12*, and *Prom1* expression was significantly elevated in the lungs of WT mice exposed to hypoxia as compared to normoxia. No changes were seen in the gene expression levels in the *Gpr75*$^{-/-}$ mouse groups. *P<0.05 compared to Nx, *Ccl2:* WT-Nx N=6; WT-Hx N=3; *Gpr75*$^{-/-}$-Nx N=8; *Gpr75*$^{-/-}$-Hx N=3; *Ccl5:* WT-Nx N=8; WT-Hx N=3; *Gpr75*$^{-/-}$-Nx N=7; *Gpr75*$^{-/-}$-Hx N=5; *Cxcl12:* WT-Nx N=6; WT-Hx N=4; *Gpr75*$^{-/-}$-Nx N=8; *Gpr75*$^{-/-}$-Hx N=3; *Prom1:* WT-Nx N=6; WT-Hx N=5; *Gpr75*$^{-/-}$-Nx N=6; *Gpr75*$^{-/-}$-Hx N=3. Statistical comparisons of samples were performed with two-way ANOVA followed by Sidak's post-hoc test for comparing the four groups.

5.2.4 *GPR75* as well as its potential ligand CCL5 increases in leukocytes from pulmonary arterial hypertension patients

To determine if this study was translational to humans, we measured *GPR75* and *CCL5* mRNA as well as CCL5 and 20-HETE levels in white blood cells (WBC) and plasma, respectively. WBCs (mononuclear cells) were recently used as surrogates for lungs/PAs to measure epigenetic enzymes (Potus et al., 2020). Further, bone marrow derived stem cells, which are precursory for myeloid cells, are upregulated in PAH (Sahara et al., 2007). Therefore, we used WBC to determine *GPR75* and *CCL5* mRNA levels. PAH patients, compared to the control group, have elevated levels of a significant *GPR75* in their WBC compared to the control group, similar to how mice with PH had a significant increase in *Gpr75* expression compared to their healthy counterparts (Figures 16A and E). *Ccl5* showed an increasing trend in both mice (Figure 15B) and humans (Figures 16B). Both PH patients and mice had higher levels of CCL5 in their plasma and lungs respectively (Figure 16C and F). Levels of 20-HETE, the other proposed ligand to GPR75, was also measured in human plasma and mouse lungs. Interestingly, 20-HETE levels did not change between the PAH and control groups in humans, but there was a decrease in 20-HETE levels in the hypoxia exposed mouse lungs (Figures 16D and G). These results indicate that the study in our mouse model is translational, as humans experience similar trends in *GPR75* gene expression, as well as expression levels and concentrations of the receptor's putative ligands.

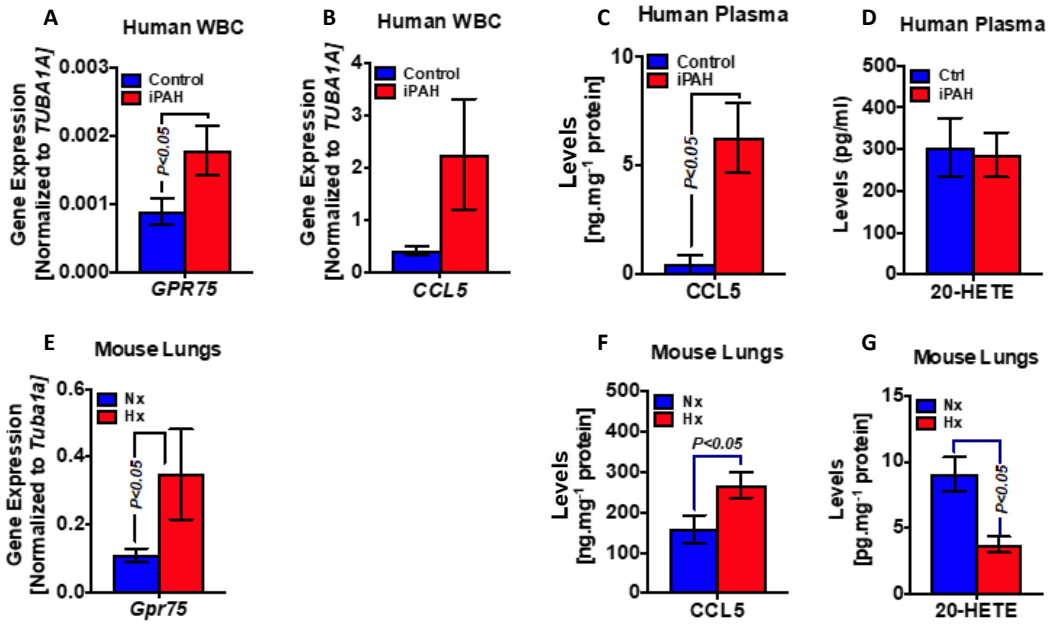

Figure 16. *GPR75* and *CCL5* mRNAS and CCL5 protein are increased in pulmonary hypertension. (A-D) mRNA of *GPR75* and *CCL5* and CCL5 protein levels are increased, while 20-HETE did not change in white blood cells and plasma from iPAH patients (N=5; sex: Female) as compared with control individuals (N=5; Sex: Female). (E-G) mRNA of *Gpr75* and CCL5 protein expression are increased, and 20-HETE is decreased in lungs of female wild-type mice exposed to hypoxia as compared to normoxia. *P<0.05 compared to Nx or iPAH. N=5 in normoxia and hypoxia. Statistical comparisons of samples were performed with Student's t-test.

5.3 Aim 3: Does GPR75 regulate cAMP or IP3 and associated signaling in lungs?

5.3.1 Determining the 2nd messenger of GPR75 in mouse lungs

After learning that a lack of GPR75 in the PAs lead to a dampened HPV response and HPH, we set out to determine whether this was a result of the *Gpr75*$^{-/-}$ mice having less IP3 or more cAMP present, and if this would alter how the *Gpr75*$^{-/-}$ mouse PAs reacted to common vasoconstrictors. The concentration of IP3 and cAMP was determined using an ELISA kit in lungs of WT and *Gpr75*$^{-/-}$ mice. Surprisingly, IP3 did not change between the two groups (Figure 17B), but the *Gpr75*$^{-/-}$ mice had more cAMP in their lungs compared to the WTs (Figure 17A). This increase in cAMP when *Gpr75* is knocked out, indicates that GPR75 potentially functions as a G_i-coupled receptor within the lungs, allowing for increased relaxation in the PAs.

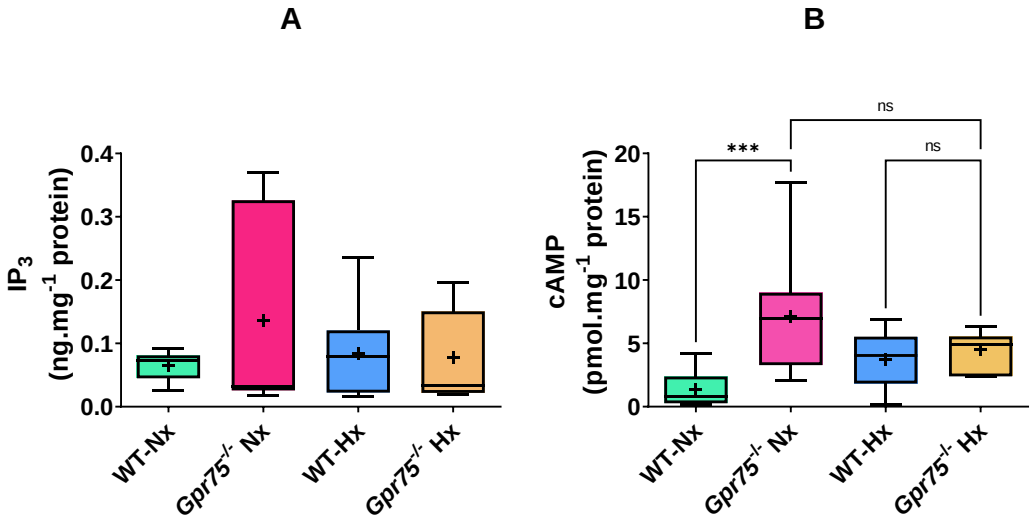

Figure 17. Cyclic Adenosine Monophosphate (cAMP), not Inositol Triphosphate (IP_3) was elevated in *Gpr75*$^{-/-}$ mouse lungs. (A, B) Camp, but no IP3, levels were elevated significantly in the normoxic *Gpr75*$^{-/-}$ mouse lungs compared to WT lungs. *P<0.05 compared to Nx. IP3: N=8 WT-Nx, N=8 *Gpr75*$^{-/-}$, N=9 WT-Hx, N=8 *Gpr75*$^{-/-}$-Hx. cAMP: N = 9 WT-Nx, N = 9 WT-Hx, N = 15 *Gpr75*$^{-/-}$-Nx, and N = 7 *Gpr75*$^{-/-}$-Hx. Statistical comparisons of samples were performed with two-way ANOVA followed by Sidak's post-hoc test for comparing the four groups.

5.3.2 Determining the 2nd messenger signaling and pulmonary artery function

As there was a higher concentration of cAMP present within the lungs of *Gpr75*$^{-/-}$ mice, and as cAMP is a potent vasodilator (Rybalkin et al., 2003), we hypothesized that PAs from these animals would respond differently than WTs when challenged with various vasoconstrictors, such as KCl, a membrane depolarizing agent that activates Rho-kinase signaling in SMCs, U46619, ET-1, and ZD7288. Therefore, to determine force generation by the IPAs when challenged with KCl, U46619, an agonist for the Thromboxane A_2 receptor, ZD7288, an antagonist of the hyperpolarization activated cation channels (HCN channels), and ET-1, which is elevated in PH and couples with ET_BR in PASMCs triggers PA vasoconstriction through an increase in intracellular Ca^{2+} levels (Galie et al., 2004). PAs of *Gpr75*$^{-/-}$ mice generated less tension than the PAs of WT mice to KCl, U46619, ZD7288, and ET-1 (Figures 18A-D). Therefore, these results suggested the decrease in contractile response after challenging with vasoconstrictors is potentially due to the higher levels of cAMP within the PAs of the *Gpr75*$^{-/-}$ mice.

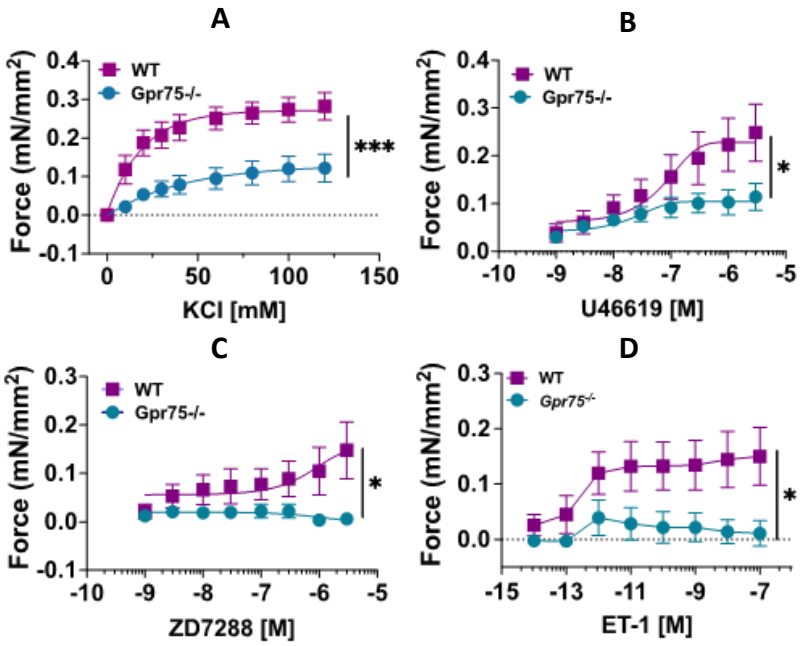

Figure 18. *Gpr75*$^{-/-}$ mice PAs contracted less than WT PAs when treated with vasoconstrictors. (A, B, C, D) KCl, U46619, ZD7288, and ET-1 treatment elicited less contraction in *Gpr75*$^{-/-}$ than in WT IPAs. *P<0.05 compared to WT. N = 5 for WT KCl, N = 12 for *Gpr75*$^{-/-}$ KCl, N = 5 for WT U46619, N = 5 for *Gpr75*$^{-/-}$ U46619, N = 5 for WT ZD7288, N = 5 for *Gpr75*$^{-/-}$ ZD7288, N = 6 for WT ET-1, and N = 5 for *Gpr75*$^{-/-}$ ET-1. Statistical comparisons of samples were performed with two-way ANOVA followed by Sidak's post-hoc test.

Next, to determine if the elevated cAMP concentration in the *Gpr75*$^{-/-}$ IPAs was responsible for the blunted contractile response seen with the addition of KCl, U46619, ZD7288, and ET-1, *Gpr75*$^{-/-}$ IPAs were pretreated with protein kinase A (PKA) signaling inhibitor Rp-cAMPs. Pretreatment with Rp-cAMPs caused *Gpr75*$^{-/-}$ IPAs to elicit stronger contractions with addition of KCl and ET-1 compared to the untreated IPAs (Fig 19). However, application of PKA activator 8-Bromo-cAMPs to *Gpr75*$^{-/-}$ IPAs was refractory or did not change force generation by ET-1 but increased force generation by KCl (Fig 19). The same experiment was also performed on WT IPAs, where pretreatment with Rp-cAMPs caused a rapid increase in force generation with addition of KCl and ET-1 (Fig 20). When WT IPAs were pretreated with 8-Bromo-cAMPs addition of KCl and ET-1 generated less forced than the control vessels (Fig 20).

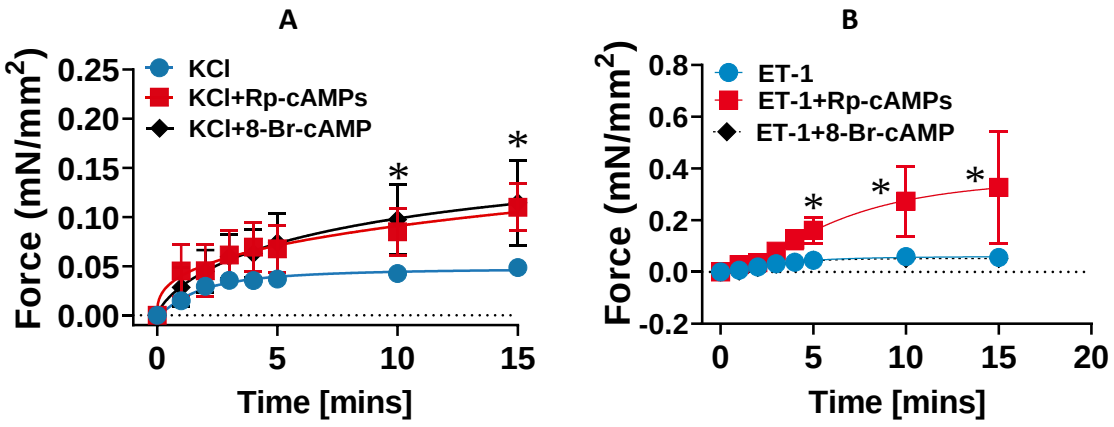

Figure 19. *Gpr75*$^{-/-}$ mouse IPAs contracted more strongly in response to KCl and ET-1 addition with PKA inhibition. (A, B) Pretreatment of *Gpr75*$^{-/-}$ IPAs with PKA inhibitor, Rp-cAMPs caused the IPAs to contract more in response to KCl (A) and ET-1 (B). *P<0.05 compared to KCl/ET-1. N = 11 KCl, N = 7 KCl+Rp-cAMPs, N = 5 KCl+8-Br-cAMPs, N = 13 ET-1, N = 6 ET-1+RpcAMPS, and N = 5 ET-1+8-Br-cAMP. Statistical comparisons of samples were performed with Student's t-test for comparing the two groups, and two-way ANOVA followed by Sidak's post-hoc test was performed to compare different time points.

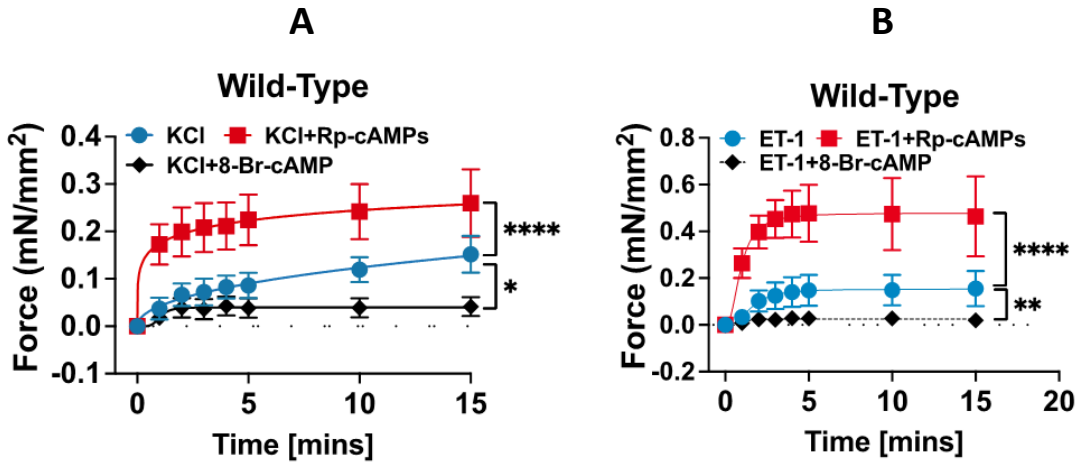

Figure 20. WT mouse IPAs contracted more strongly in response to KCl and ET-1 addition with PKA inhibition. (A, B) Pretreatment of WT IPAs with PKA inhibitor, Rp-cAMPs caused the IPAs to contract more in response to KCl (A) and ET-1 (B). *P<0.05 compared to KCl/ET-1. N = 8 KCl, N = 5 KCl+Rp-cAMPs, N = 4 KCl+8-Br-cAMPs, N = 8 ET-1, N = 4 ET-1+RpcAMPS, and N = 4 ET-1+8-Br-cAMP. Statistical comparisons of samples were performed with Student's t-test for comparing the two groups, and two-way ANOVA followed by Sidak's post-hoc test was performed to compare different time points.

6 Discussion

The results of this study provide the first evidence that GPR75 signaling is an important contributor in the pathogenesis of HPH in mice as the *Gpr75* gene knockdown mitigated HPV response and HPH.

HPH consists of two components: vasoconstriction and remodeling. However, in different animal models of PH, such as rats and mice, these two components have varying importance (Cahill et al., 2012). PH in rats is caused primarily by sustained ROCK2 mediated vasoconstriction, and PA remodeling, such as narrowing of the vascular lumen, does not occur (Cahill et al., 2012). In mice, however, ROCK2 mediated vasoconstriction and remodeling, such as narrowing of the vascular lumen, of PAs contributed equally to the development of PH (Cahill et al., 2012). Since both vasoconstriction and remodeling are equally important in the development of PH in mice, it was interesting to see that our *Gpr75*$^{-/-}$ mice did not develop PH, as they experience limited vasoconstriction, but still underwent PA remodeling.

IPAs from *Gpr75*$^{-/-}$ mice did not elicit HPV, a physiological response triggered by low oxygen levels in order to maintain a perfusion-to-ventilation quotient (Dunham-Snary et al., 2017). HPV is regularly seen in PAs when exposed to hypoxic conditions, such as at high altitudes and lung disorders such as chronic obstructive pulmonary disease, interstitial lung disease, and sleep apnea, nor did they contract strongly in response to challenging with vasoconstrictors (Dunham-Snary et al., 2017). Hypoxia exposure activates Ca^{2+} and Rho-kinase-dependent signaling pathways to generate a potent vasoconstrictive response (Knock et al., 2008). In the HPV response, the first phase is associated with a large, but transient, elevation in intracellular Ca^{2+} levels while the second phase of the HPV consists of a small increase in intracellular Ca^{2+} levels but the vasoconstrictive effect on PAs is enhanced due to

a Rho-kinase dependent Ca^{2+} sensitization pathway (Knock et al., 2008). Ca^{2+} sensitization of the PAs is due to Rho-kinase inhibiting myosin light-chain phosphatase, leading to an increase in phosphorylation of myosin light-chain, which causes PA contraction (Knock et al., 2008; Lumb & Slinger, 2015; Ward, 2006). Since the PAs of the *Gpr75*$^{-/-}$ mice did not respond when exposed to hypoxic conditions and did not elicit strong contractions when challenged with various vasoconstrictors, we suggest that *Gpr75* knockdown prevented and reduced intracellular Ca^{2+} and Rho-kinase dependent Ca^{2+} sensitivity to the myofilament, and reduced contraction of IPAs elicited by hypoxia, KCl, U46619, and ET-1. By retaining a relaxed state in hypoxic conditions, the PAs do not undergo the homeostatic response of HPV to divert blood flow in the lungs from low to high oxygenated regions, which when exposed for prolonged periods of time leads to PA remodeling, leading to elevated PAP and eventually HPH. Therefore, we suggest GPR75 signaling has a crucial role in pulmonary/respiratory physiology, especially in the pathogenesis of HPH.

Further, the theory that GPR75 signaling plays an important role in the pathogenesis of HPH, is supported by our observations that *Gpr75* mRNA expression was 3.2-fold higher in the lungs of WT mice exposed to hypoxia and 2.0-fold higher in PAH patients compared to control patients. When *Gpr75* was knocked out in mice, *Gpr75* did not increase, nor did these mice develop HPH and RV hypertrophy, indicating that GPR75 contributes to the development and progression of HPH. While the *Gpr75*$^{-/-}$ mice did not develop HPH, their PAs still underwent wall thickening when exposed to hypoxia, similar to what is seen in the WT mice exposed to hypoxia.

Molecules, such as thromboxane A_2 and ET-1, increase with hypoxia exposure and HPH (Christman et al., 1992). Thromboxane A_2, upon binding to its receptor, and ET-1, when

bound to ET_AR, both elicit G_q-coupled signaling (Horinouchi et al., 2013; Mamazhakypov et al., 2021). Activation of these receptors causes an increase in intracellular Ca^{2+}, which contributes to vasoconstriction and vascular remodeling in PAs (Horinouchi et al., 2013; Mamazhakypov et al., 2021). The *Gpr75*$^{-/-}$ mice exposed to hypoxia for 5-weeks did not develop HPH, but underwent PA remodeling, suggesting *Gpr75*$^{-/-}$ increased cAMP levels, antagonized ET-1 and U46619 (thromboxane A_2 mimic) mediated contraction, but not remodeling of the PAs. This indicates *Gpr75* expression and activation of downstream signaling contributes to constriction, but not the remodeling, of the PAs in HPH mice.

Persistent inflammation and increased cytokines/chemokines are major contributors to the remodeling of PAs in the lungs of those diagnosed with various types of PH. Cytokines such as IL-1β, IL-18, IL-6, IL-8, IL-13, and TNF-α, are elevated in PH and contribute to SMC proliferation and PA muscularization and increase vascular reactivity (Groth et al., 2014). Chemokines, such as CXCl8, CXCL1, CXCL10, CCL2, CCL5, CXCL12, and CX3CL1, are increased in PH, which promote inflammation and therefore PA remodeling (Mamazhakypov et al., 2021). As expected, we saw WT mice exposed to hypoxia express higher levels of *Ccl5* gene and CCL5 protein within the lungs, compared to their normoxia exposed counterparts. Other inflammatory markers and cytokines, such as *Cxcl12*, were expressed more in the lungs of hypoxia exposed WT mice compared to the WT normoxia exposed mice. However, this increase in *Ccl5* and *Cxcl12* was prevented in the *Gpr75*$^{-/-}$ mice exposed to hypoxia. *Ccl2,* another pro-inflammatory cytokine, was decreased in the lungs of *Gpr75*$^{-/-}$ as compared to WT mice. Studies have shown PA endothelial cell CCL2 production is elevated in iPAH patients which contributes to increased monocyte migration (Hu et al., 2020). This reveals the absence of *Gpr75* may reduce chemotaxis within the lungs, preventing the accumulation of immune

and pro-inflammatory cells within the lungs of mice exposed to hypoxia. This is also evident by decreased *prominin* expression, which indicates the myeloid precursor cells, which accumulate around the PA in hypoxic mice and contribute to PA remodeling (Hashimoto et al., 2020). The lack of cytokine elevation, including *Ccl2* at baseline, can also alter PA remodeling, more specifically collagen deposition (Van Linthout et al., 2014). The lack of cytokines, and therefore inflammation, may potentially lead to less collagen deposition around the *Gpr75*$^{-/-}$ mouse PAs. Further, *Gpr75* knockdown reduced CCL5, which reduced HPV and PA pressure, but not remodeling. This indicates CCL5 potentially contributed to increase PA pressure, but not the PA remodeling in the hypoxia exposed animals.

The two putative ligands of GPR75, CCL5 and 20-HETE, can also contribute to the inflammatory and contractile response seen in the systemic vasculature (Chandra et al., 2016; Singh et al., 2018). However, in the pulmonary vasculature, CCL5 is pro-inflammatory and causes contraction of coronary arteries (Dorfmuller et al., 2002; Nakano et al., 2022). During the wire myography experiments performed on WT mice, addition of CCL5 augmented the 2nd phase of the HPV response but had no effect on the *Gpr75*$^{-/-}$ mouse PA's HPV response. These results indicate that CCL5 binds to and activates GPR75 signaling (CCL5-GPR75 coupling) in the pulmonary vasculature and contributes to constriction of PAs which evokes HPV response, which leads to HPH. Meanwhile, pretreatment of the WT mouse IPAs with 20-HETE did not elicit a change from the control WT HPV response, which indicates that 20-HETE-GPR75 coupling does not play a role in the HPV response. It has been shown in previous studies using wire myography, that 20-HETE causes transient contraction followed by prolonged relaxation in bovine PA rings and induces a potent vasodilatory response in human and rabbit pulmonary vasculature (Kizub et al., 2016). In our studies, we found 20-HETE

levels decreased within the lungs of WT mice exposed to hypoxia compared to WT mice kept in normoxic conditions, which potentially indicates that within the mouse pulmonary vasculature 20-HETE acts as a vasodilator, which would decrease in response to hypoxic conditions in order to contract the PAs in order to maintain the perfusion-to-ventilation quotient, worsening HPV and HPH. Consistently, 20-HETE protects the pulmonary vasculature from hypoxia-reoxygenation, dilates PAs in rabbit lungs, and reduces HPV (Jacobs et al., 2012; Kizub et al., 2016; Sugumaran et al., 2020). Therefore, we suggest 20-HETE-GPR75 coupling is unlikely to play a role in regulation of PA function in mice exposed to hypoxia.

Our findings also reveal that cAMP levels are higher in the lungs of *Gpr75*$^{-/-}$ mice in normoxia compared to WT normoxia exposed mice, but there are no differences in IP3 levels between the two groups. This was an unexpected result, as previous studies showed GPR75 as a G_q-coupled receptor in hippocampal cells (Ignatov et al., 2006) and in the systemic vasculature (Garcia et al., 2017), which would imply by knocking out the receptor, you would have lower levels of IP3. Since cAMP levels were higher in the *Gpr75*$^{-/-}$ mice, this suggests GPR75 potentially elicits G_i-coupled signaling in the lungs. We propose the increase in cAMP seen in the *Gpr75*$^{-/-}$ mice due to a lack in G_i-coupled signaling contributed to the dilatory phenotype seen in the PAs of these mice, therefore preventing the development of HPH. These observations are consistent with previous studies (Iyinikkel, 2019; McDonald, 2013). However, it is unclear if any other interaction or crosstalk between GPR75 and other GCPRs is responsible for increasing cAMP in lung and needs to be further evalulated.

cAMP-dependent signaling is not only responsible for the contractile functions of blood vessels, it regulates other pathways including: contraction, cellular proliferation, metabolism, and gene expression.

Contraction

cAMP, a molecule generated by the activation of adenylate cyclase, is one mediator of vasodilation. Within SMCs, cAMP binds to and activates protein kinase A (PKA), which reduces intracellular levels of $Ca^{2+}$and decreases Ca^{2+} sensitivity, imparing myosin light chain phosphorylation, attenuating SMC contraction (Billington et al., 2013). cAMP can also cross-activate protein kinase G (PKG) to a lesser extent, which also causes a decrease in intracellular levels of Ca^{2+}, reducing smooth muscle contraction (Billington et al., 2013; Rybalkin et al., 2003). Elevated levels of cAMP also increases the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which is responsible for sequestering Ca^{2+} in the sarcoplasmic reticulum, and ultimately decreases intracellular Ca^{2+} levels (Billington et al., 2013).

Cellular Proliferation

Depending on the cell type, cAMP can induce or inhibit cellular proliferation through the alteration of the mitogen-activated protein (MAP) kinase or extracellular signal-regulated kinase (ERK) pathways (Schmitt & Stork, 2001; Schmitt & Stork, 2002). In vascular SMCs cAMP inhibits cellular proliferation through protein kinase A (PKA) activating Rap1, allowing for the disruption of Ras/Raf-1 signaling, preventing proliferation (Grader-Beck et al., 2003; Schmitt & Stork, 2001). Cell cycle progression can also be halted via cAMP as it can cause an increase in cell-cycle inhibitor proteins $p21^{cip1}$ and $p27^{kipl}$ while also decreasing the levels of cyclin D1 and cyclin D3 (Fukumoto et al., 1999).

Mitochondrial Function

cAMP also contributes to mitochondrial function by altering complex I, IV, and V activity. Within complex I, subunit NDUFS4, a protein that when deficient can lead to Leigh-like syndrome, a peripheral nervous system pathology, requires PKA phosphorylation for it to function properly (Alexander et al., 2019). Complex IV, also known as cytochrome c oxidase (COX), relies on cAMP-PKA signaling to function properly as well, since in hypoxia PKA phosphorylates subunits I, IV-1, and Vb and induces their degradation, ultimately reducing the ability of COX to perform oxidative phosphorylation (Roman & Fan, 2018). Complex V also relies on cAMP in order to control ATP synthase. The ATPase inhibitory factor AIF1, which binds to complex V to inhibit ATP synthesis, is phosphorylated by PKA in hypoxic conditions, which prevents its binding to complex V, allowing for the promotion of ATP synthesis (Dupuis & Hoeper, 2008). All the functions of cAMP within the mitochondria counteract the effects of hypoxia, allowing for the mitochondria to maintain its ability to produce large amounts of ATP (Figure 20).

Gene Expression

Lastly, cAMP can alter gene expression through cAMP-responsive element binding protein (CREB), which is seen in the lungs of animals with PH. In our study, the *Gpr75*$^{-/-}$ mice with higher levels of cAMP may have increased CREB activity, which activates *Myh11* and *Cnn1* transcription (Kovacs et al., 2018; Qiao et al., 2014; Watson et al., 2002). Increased expression of *Myh11*, a SMC-restricted gene, perhaps contributed to PA hypertrophy which was seen in *Gpr75*$^{-/-}$ mice exposed to hypoxia. This is not unprecedented because *Myh11* has been seen to contribute to media hypertrophy in the previous studies (Qiao et al., 2014). Since expression of cytokine genes, *Cxcl12, Ccl2,* and *Ccl5,* did not increase in lungs of hypoxic

Gpr75$^{-/-}$ mice, this would mean they most likely were turned off by the higher cAMP-CREB/CREM pathway.

PH is a progressive and deadly cardio-pulmonary disease that has no cure at this time. The drugs available for PH patients are only used to alleviate their symptoms, not actually treat the disease itself. Even with treatment PH patients still experience a shortened lifespan. However, based on our findings on GPR75, we propose that an antagonist to this GPCR might be beneficial in treating HPH. By blocking GPR75 early on in the diagnosis, remodeling would still occur within the PAs, but the RV would not experience an increased afterload and hypertrophy. Inhibition of this protein would also allow for relaxation of the PAs and well as prevention of excessive inflammation, both of which lead to worsening of PH.

7 Future Directions

These results reveal some possible actions of GPR75 in PH, however, more experiments need to be performed to further elucidate the signaling pathway of GPR75 in the pulmonary vasculature.

Experiments should be performed to determine which cell type(s), endothelial cells or SMCs, expresses GPR75 in the PAs. Immunofluorescent microscopy could be utilized to see where GPR75 is expressed in PAs, however, there is a lack of readily available GPR75 antibodies, preventing us from performing these experiments. Due to the lack of GPR75 antibodies, in-situ hybridization using RNA sequences for GPR75 would be the best option available for identifying the cell type(s) that express GPR75 in the PAs. Tissue-specific, endothelial cell and SMC, GPR75 knockdown animals should also be generated to determine which cell type plays is most important in the development of HPH.

A time-course experiment would also be ideal to have in future studies in order to determine when certain genes are up or down regulated during hypoxia exposure. Our results only show gene expression and protein levels are a 5-week time course of normoxia or hypoxia, but it would be interesting to see when *Myh11* is elevated in the *Gpr75*$^{-/-}$ mice exposed to hypoxia or when 20-HETE levels decrease in the lungs of hypoxia exposed mice. These studies could help elucidate whether the decrease in 20-HETE is a trigger or a response for some of the gene expression changes seen in the WT mice exposed to hypoxia.

More experiments also need to be performed in order to determine why the *Gpr75*$^{-/-}$ mice had elevated levels of cAMP in their lungs compared to WT mice. As previously mentioned, there are conflicting theories on the signaling pathway activated through GPR75

activation. While our results suggest that GPR75 acts as a G_i-coupled receptor in the pulmonary system, which is supported by some studies (McDonald, 2013), and other studies performed in other organ systems show GPR75 functions as a G_q-coupled receptor (Ignatov et al., 2006). Future experiments should examine why GPR75 acts through different signaling pathways in different organs. These differences could potentially be due to GPR75 interacting with other receptors in different organ systems, which leads to it appearing to act as a G_i- and G_q-coupled receptor depending on its location.

The lack of a clear signaling mechanism for GPR75 also raises the question of whether this receptor affects other signaling pathways that are crucial for PA contraction and relaxation. For example, generation of NO by NOS is important for PA SMC relaxation, as inhibition of eNOS with L-NAME can enhance ROCK-mediated constriction which can cause elevation in RVSP and PVR (Tanaka et al., 2017). While we did not measure NOS activity in the *Gpr75*$^{-/-}$ mice, we did see the HPV response was completely abolished in the PAs, and if NOS was somehow linked to GPR75 function, then the *Gpr75*$^{-/-}$ mouse PAs would have experienced an increased HPV response. This suggests that NOS is not affected in the *Gpr75*$^{-/-}$ mice.

8 Conclusion

In conclusion, deletion of *Gpr75* prevented the development of HPH in mice and we theorize this is through cAMP signaling which allows for PAs to remain relaxed, preventing the increase in PAP, while also preventing increased inflammation in the PAs through the reduction of CCL5-GPR75 coupling in the lungs under hypoxic conditions.

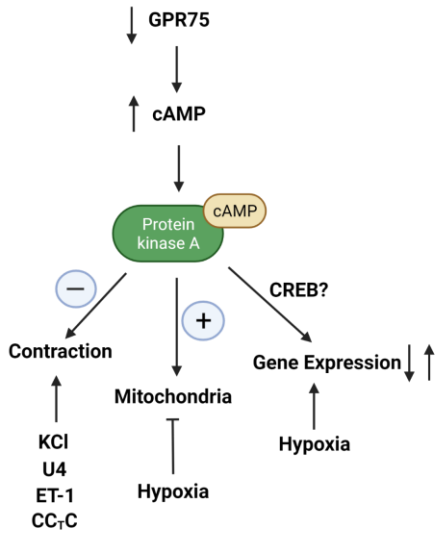

Figure 21. Deletion of *Gpr75* leads to an increase in cAMP, which activates PKA, preventing the effects of hypoxia from taking place within the lungs of mice. Activation of PKA allows for vascular relaxation in PAs, increases mitochondrial activity within the cell, and alters the expression of genes associated with CREB.

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