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Sherry Eniola Adesina Bello July 16, 2015

The Role of Mitochondrial Reactive Oxygen Species in the Development of Hypoxia-induced Pulmonary Hypertension

By

Sherry Eniola Adesina Bello

Doctor of Philosophy

Graduate Division Biological and Biomedical Sciences,

Molecular & Systems Pharmacology

C. Michael Hart, M.D.
Advisor

Roy L. Sutliff, Ph.D.
Advisor

Lou Ann Brown, Ph. D.
Committee Member

Kathy Griendling, Ph.D.
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

Date

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Sherry Eniola Adesina Bello
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An Abstract of
A dissertation submitted to the Faculty of the
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Abstract

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Pulmonary hypertension (PH) is characterized by increased pulmonary vascular resistance, pulmonary vascular remodeling, and increased pulmonary pressures that result in right ventricular hypertrophy and if untreated, right heart failure and death. Pathogenic derangements in PH include an imbalance in the production of vasodilating and vasoconstricting mediators and enhanced proliferation of pulmonary vascular wall cells.

Numerous studies have noted increased reactive oxygen species (ROS) in patients and models of PH. ROS are produced as intermediates in the redox reactions leading from O_2 to H_2O and comprise both free radicals (superoxide, $O_2^{\bullet -}$) and non-radical derivatives of oxygen (e.g. hydrogen peroxide, H_2O_2). ROS in the form of $O_2^{\bullet -}$ and H_2O_2 play a vital role in vascular cell signaling, and regulate cellular proliferation, differentiation, and apoptosis. Current evidence suggests that ROS generated by both mitochondrial respiration and NADPH oxidases (Noxes) may contribute to PH pathogenesis by altering vascular cell proliferation and apoptotic signaling pathways. **If hypoxia increases mitochondrial ROS generation (mtROS) to stimulate Nox expression, then targeted reduction of mtROS will prevent pulmonary vascular wall proliferation, remodeling, and PH pathogenesis.**

The overall goal of this project was to assess if reducing mtROS levels by increasing mitochondrial antioxidants could mitigate hypoxia-induced aberrations in Nox expression, vascular remodeling, and molecular signaling. To our knowledge, the direct assessment of the contribution of mtROS to Nox expression and activity in hypoxia-induced PH has not been previously reported. Using three transgenic models (MCAT, Tg^{hSOD2}, and Tg^{hTrx2}), studies emphasize: A) the importance of mtROS in hypoxia-induced PH pathogenesis, and B) that targeted therapies directed at lowering mtH_2O_2 may be uniquely effective in reducing pulmonary vascular cell proliferation, remodeling, and PH.

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TABLE OF CONTENTS

Index of Tables

Index of Figures

List of Abbreviations

Ang-II	angiotensin II
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAPI	4',6-diamidino-2-phenylindole
DCF	dichlorofluorescein
DPI	diphenyliodonium chloride
ET-1	endothelin-1
GPx	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
$HO^{\bullet}$	hydroxyl
HPAEC	human pulmonary arterial endothelial cells
HPASMC	human pulmonary arterial smooth muscle cells
HPLC	high-performance liquid chromatography
HPV	hypoxic pulmonary vasoconstriction
MCAT	transgenic mice with human catalase targeted to mitochondria
mtH_2O_2	mitochondria- derived hydrogen peroxide
$mtO_2^{\bullet-}$	mitochondria-derived superoxide

mtROS	mitochondria- derived reactive oxygen species
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOS	nitric oxide synthase
Nox	NADPH oxidase
Nox2	NADPH oxidase 2
Nox4	NADPH oxidase 4
$O_2^{\bullet-}$	superoxide
ONOO-	peroxynitrite
PAB	pulmonary artery banding
PAH	pulmonary arterial hypertension
PDGF	platelet derived growth factor
PGI_2	prostaglandin I_2 (Prostacyclin)
PH	pulmonary hypertension
PPH	primary pulmonary hypertension
PRX	peroxiredoxins
ROS	reactive oxygen species
RVH	right ventricular hypertrophy
RVSPs	right ventricular systolic pressures
SDS	sodium dodecyl sulfate
sGC	soluble guanylyl cyclase
SMC	smooth muscle cells

SOD	superoxide dismutase
Tg^{hSOD2}	transgenic mice expressing human SOD2 targeted to mitochondria
Tg^{hTrx2}	transgenic mice expressing human Trx2
TGF-β	tumor growth factor β
TNF-α	tumor necrosis factor-α
Trx	thioredoxin
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells
XO	xanthine oxidase

Chapter 1: Introduction

Pulmonary Hypertension

Pulmonary hypertension (PH) is defined as an elevation of pulmonary arterial pressures and is characterized by a narrowing of the pulmonary vasculature and is associated with dysregulated proliferation of vascular wall cells, pulmonary vascular remodeling, and inhibition of apoptosis of vascular cells (Barst, 2008; Rabinovitch, 2012). This increase in pulmonary vascular pressure and resistance increases right ventricular afterload and the pressure required to force blood through the narrowed pulmonary vascular bed. The progressive nature of PH is characterized by an elevation of pulmonary artery pressure and pulmonary vascular resistance, right ventricle hypertrophy leading to right ventricular failure and death. The diagnosis of PH is based on findings of mean pulmonary arterial pressure greater than 25 mmHg at rest or greater than 30 mmHg during exercise (Sutliff et al., 2010; Zuo et al., 2014). In the US, the decline in PH-associated death rates among men from 1980 to 2005 has recently reversed, now showing a significant increasing trend (George et al., 2014). In addition, the death rates for women with PH have significantly increased during the past decade (George et al., 2014). PH-associated mortality rates for those aged 85 years and older have also accelerated, and PH hospitalization rates for those aged 85 years and older (men and women) have nearly doubled (George et al., 2014).

PH was originally classified as either “primary PH” (PPH) or, if there was a known risk factor or cause, “secondary PH”. PH is currently classified into 5 major categories of disorders based on etiology, pathological findings, and hemodynamic characteristics: Group 1 - pulmonary arterial hypertension (PAH), Group 2 – Pulmonary hypertension due to left heart disease, Group 3 – PH due to lung diseases and hypoxia, Group 4 – Chronic thromboembolic pulmonary hypertension (CTEPH), and Group 5 – PH of unclear multifactorial mechanisms (Simonneau et al., 2013). PH is the overall disease, whereas Group 1 PAH is a major subset of disorders (idiopathic development, heritable, or toxin-induced) that lead to PH (Simonneau et al., 2013). Group 3 PH associated with disorders of the respiratory system or hypoxemia describes the disease mainly caused by inadequate oxygenation of arterial blood due to lung disease, impaired breathing, or high altitude. Though the prevalence of PH in patients with COPD remains unknown because PH is secondary and not screened for (Barbera and Blanco, 2009; Gologanu et al., 2012), the prevalence of hypoxia-induced PH has been found to be as high as 37% of high altitude dwellers (>2,500 meters) (Norboo et al., 2015). Though people and animal models of PH are genetically influenced in their response to hypoxia (Tuder et al., 2013a), hypoxia-induced PH models are often used to study PH pathogenesis.

Several factors are associated with the increase in pulmonary vascular resistance including: vasoconstriction, pulmonary vascular wall cell remodeling, and thrombosis (Humbert et al., 2004). As of yet, the precise molecular signaling

cascades activated and dysregulated in PH development are not fully understood, but endothelial cell dysregulation is thought to be a significant contributor. Identification and assessment of treatment endpoints sufficient to confirm treatment efficacy has also remained limited since cardiovascular hemodynamics only improve marginally, even in cases where clinical responses have shown significant improvements (Humbert et al., 2004). From 1980-2000, PH death rates were higher for men than women; however, by 2002, no difference in rate was observed because of increasing death rates among women and declining death rates among men. These reports of increasing PH mortality rates indicate the urgent need for improved understanding of PH pathogenesis and for better therapies to address the altered molecular and signaling pathways that cause PH (Hyduk et al., 2005).

PAH Etiology

While the treatment and survival outcomes for patients with other forms of PH have been defined, Group 1 PAH remains less characterized (McLaughlin et al., 2004). PAH is rare with a prevalence estimated from 5-50 cases per million in the U.S. (Peacock et al., 2007; Rich and Rich, 2014). Group 1 PAH is subdivided into categories including: idiopathic PAH (IPAH), heritable PAH, drug/toxin-induced PAH, disease-associated PAH, and persistent pulmonary hypertension of the newborn (PPHN) (Simonneau et al., 2013). IPAH arises from unknown origin and occurs at a rate of 1-2 cases per million in industrialized nations

(Humbert et al., 2006; Taichman and Mandel, 2013). In the US and Europe, IPAH is 2-4 times (1.7:1 ratio) as common in women as in men (Badesch et al., 2010; Rich and Rich, 2014; Taichman and Mandel, 2013). A French registry found that current practices for detection and measurement of PAH, detects PAH late in the course of the disease, at which point the majority of patients display severe functional and hemodynamic compromise (Humbert et al., 2006). Prior to the development of targeted PAH therapies, IPAH subjects had a median survival of approximately 2.8 years emphasizing the dismal prognosis for this disorder (Badesch et al., 2010; Benza et al., 2012; McLaughlin et al., 2004; Taichman and Mandel, 2013). More recently, 1-, 3-, and 5-year survival rates among subjects with IPAH, familial, and anorexigen-related PAH were reported to be 67%, 45%, and 37% respectively (D'Alonzo et al., 1991; Rich and Rich, 2014; Rich et al., 2000). However, those rates likely underestimate current survival as the course of the disease has been favorably altered by therapeutic advances since reports from the 1980s (Barst, 2008). As research on PH has increased, awareness and diagnosis of the disorder has increased and survival has increased (Barst, 2008; George et al., 2014; Hyduk et al., 2005).

Within Group 1 PH, other subcategories include heritable PAH in which 80% of cases are attributable to mutations of bone morphogenetic protein receptor type 2 (BMPR2, a member of the TGFβ super family), and 5% have rare mutations to other genes in the TGFβ super family (Machado et al., 2009). Drug/toxin-induced PAH is related to ingestion or exposure to drugs or toxins that

have been identified as risk factors for PAH development. Drugs like benfluorex, dasatinib, or interferon-α or -β have all been associated with PAH development (Savale et al., 2012). Multiple epidemiological studies have indicated that xenobiotics, including anorexigens, are positively associated with PAH development (Barst, 2008; Rich et al., 2000). Exposure to fenfluramine, a derivative of phentermine which induces the release of the vasoconstrictor serotonin to create a feeling of fullness and loss of appetite, increased the odds ratio of PAH diagnosis by 7.5 times. Though only 2% of fenfluramine users developed PAH, the risk to fenfluramine users compared to nonusers was 52:1 (Rich et al., 2000; Taichman and Mandel, 2013). Patients that were exposed to fenfluramine during the 5 years preceding hypertension diagnosis, were more likely to have PAH as opposed to chronic thromboembolic PH (CTEPH), which is characterized by intraluminal thrombus organization and fibrous stenosis or complete obliteration of pulmonary arteries (Hoeper et al., 2006). This indicates that anorexigen use increased likelihood of PAH development in patients that had underlying conditions that could lead to PAH development (Barst, 2008; Rich et al., 2000; Simonneau et al., 2004).

Although the incidence of PAH in patients with other illnesses is not known with certainty, from various reports it appears that 2-4% of patients with portal hypertension and 0.1-0.6% of HIV patients have PAH (Barst, 2008). PAH associated with connective tissue diseases, such as scleroderma, occurs in 7-12% of cases and is associated with the worst PAH prognosis. The incidence of

PAH that occurs in patients with connective tissue disease is extremely variable and the reported prevalence ranges from 2 to 35% in patients, though the scleroderma spectrum of disease may reach as high as 50% of patients with limited scleroderma. PAH has also been reported to occur in 10-45% of patients with mixed connective tissue disease and the prognosis for these patients is worse than for those with IPAH. Estimates for 2-year survival in scleroderma patients with associated PAH are 40% compared with 48% for 3-year survival in patients with IPAH. HIV-associated PAH has remained stable within the last decade, with prognosis improving since the advent of highly active antiretroviral therapy (HAART). Optimistically, survival in patients with HIV-associated PAH is similar to patients with IPAH. With current HIV therapies, most of the deaths in patients with HIV and associated PAH are now attributed to PAH (Barst, 2008).

A 1991 prospective study by the National Heart, Lung, Blood Institute of 194 PAH patients found that median survival was 2.8 years with an estimated 1 year survival rate of 68% (D'Alonzo et al., 1991; Rich and Rich, 2014). Elevated end-expiratory mean pulmonary artery pressure (PAP), decreased cardiac output, elevated pulmonary vascular resistance, and low mixed venous oxygen saturation are all characteristic of right heart failure and PAH (Humbert et al., 2014; Humbert et al., 2004). Furthermore, vasoconstriction, remodeling of the pulmonary vessel wall, and thrombosis *in situ* are thought to cause the increased pulmonary vascular resistance that characterizes PAH (Humbert et al., 2004). Morbidity and mortality are most closely associated with right ventricular

hemodynamic function (D'Alonzo et al., 1991). The Registry to Evaluate Early And Long-term PAH disease management (REVEAL Registry) had fifty-four US centers screen patients with Group I PAH who met expanded hemodynamic criteria consisting of mPAP > 25 mmHg at rest or 30 mmHg with exercise, pulmonary capillary wedge pressure (PCWP) ≤ 15 mmHg, and pulmonary vascular resistance ≥ 240 dynes· s · cm^{--5}. Of the 2,967 patients enrolled in the study, the majority (2,525 adults) met traditional hemodynamic criteria. The remaining patients fit the expanded criteria with a PCWP of 16 – 18 mmHg. In addition, they differed in a number of important respects from those meeting the traditional hemodynamic definition of PAH. Patients with the expanded PCWP displayed elevated: age, obesity, lower 6-min walk distance, sleep apnea, renal insufficiency, and a higher incidence of systemic hypertension and diabetes (Badesch et al., 2010; Benza et al., 2012).

PH Pathobiology

The pulmonary artery consists of 3 layers. The inner layer, tunica intima, consists of a monolayer of endothelial cells. The middle layer, tunica media, contains smooth muscle cells in a complex extracellular matrix. The outer layer, tunica adventitia, is made up of mast cells, fibroblasts, monocytes, nerve endings and microvessels (Libby et al., 2011; Stenmark et al., 2011; Tang et al., 2014). Paracrine signaling allows for molecular changes experienced by blood-exposed endothelial cells to affect signaling and remodeling in the vascular smooth

muscle cells. To maintain O_2 supply in the respiratory and circulatory systems, smooth muscle and endothelial cells have evolved specialized O_2-sensing components. The extreme sensitivity of these cells to changes in O_2 partial pressure drives hypoxic pulmonary vasoconstriction (HPV) (Schumacker, 2011; Voelkel et al., 2013).

As pathology is not necessary for clinical diagnosis, only recently has knowledge involving the pathobiology and pathophysiology of PH accelerated. Several factors are associated with the increase in pulmonary vessel resistance associated with PH including: vasoconstriction, pulmonary wall cell remodeling, and thrombosis (Humbert et al., 2004). The Pulmonary Hypertension Breakthrough Initiative (PHBI) sponsored by the Cardiovascular Medical Research and Education Fund (CMREF) has collected, phenotyped, and studied more than 100 PAH and 30 control lungs. Normal pulmonary arteries branch approximately 15 – 17 times, contain 108 vascular segments, and precapillary artery diameter of 20 μm (Tuder et al., 2013b). The critical segment responsible for vascular tone is around 200 μm in diameter and is affected by medial and intimal lesions in PH. There is also significant pruning of the pulmonary vasculature branches in PH (Tuder et al., 2013a; Tuder et al., 2013b). Along with these derangements, in PAH, the pulmonary arterial luminal area is reduced, which likely further increases pulmonary vascular resistance (PVR). In addition, plexiform lesions composed of endothelial cells and myofibroblasts can further reduce luminal area (Tuder et al., 2013a; Tuder et al., 2013b). The PHBI study

detected increases in perivascular inflammatory cells including mast cells, macrophages, T cells, and B cells. Higher numbers of immune cells (macrophages, mast cells, and T-cells) are detected in PAH samples, indicating dysregulated autoimmunity in PAH as well (Mouthon et al., 2005; Tuder et al., 2013b). The increase in perivascular inflammation correlated to detrimental pulmonary hemodynamics and intimal, medial, and adventitial remodeling (Tuder et al., 2013b). Surprisingly, the PHBI study was unable to detect differences between controls and PAH lungs in adventitial thickening or pulmonary vein remodeling. It should be noted that patients with mutations in BMRP2 also displayed intimal remodeling similar to plexiform lesion development (Tuder et al., 2013a; Tuder et al., 1994; Tuder et al., 2013b).

Unfortunately, current PH therapies do not have great efficacy, and many animal models do not accurately reproduce all the structural and molecular pathways involved in human PH. While new PH treatments have been implemented in the past 10-20 years, these treatments largely fail to reverse the pulmonary vascular remodeling PH-associated pathological derangements in the pulmonary vasculature. As a result, new long-term pharmacological treatments that more effectively inhibit the pulmonary vascular remodeling in PH hold promise for more effective therapeutic strategies for PH.

Current PH Therapy

Aside from therapies that address underlying derangements associated with PH (e.g. diuretics for patients with left sided heart failure and Group 2 PH, supplemental oxygen for patients with Group 3 PH, or anti-coagulation therapy for patients with Group 4 PH), targeted therapy is largely limited to reducing pulmonary vasoconstriction and increasing vasodilation (Humbert et al., 2004). Since PH patients display compromised blood flow through the pulmonary circulation due, in part, to vasoconstriction, vasodilating agents such as calcium channel blockers, prostacyclin analogues, endothelin receptor antagonists, or agents that promote nitric oxide signaling have been useful. Prostacyclin, or prostaglandin I_2 (PGI_2), is a major product of arachidonic acid metabolism in the vascular endothelium (Freund-Michel et al., 2013; McLaughlin, 2006). PGI_2 promotes smooth muscle relaxation and vasodilation through production of cyclic adenosine monophosphate (cAMP) (Freund-Michel et al., 2013; Humbert et al., 2004). Endothelin receptor antagonists prevent binding of the vasoconstrictor, endothelin-1 (ET-1), to its receptor thereby inhibiting ET-1-induced vascular smooth muscle cell proliferation and vasoconstriction (Humbert et al., 2004). While ET-1 antagonists may also reduce metabolic derangements in endothelial cells associated with PH (Sun et al., 2014), they may have reduced efficacy over time and cause peripheral edema and hepatotoxicity (Mielniczuk et al., 2014; Shanmugam et al., 2015). Nitric oxide (NO) therapy promotes vasodilation by directly relaxing vascular smooth muscle cells through stimulation of cyclic guanosine monophosphate (cGMP) (Humbert et al., 2004; Pauvert et al., 2003).

Riociguat is a first-in-class drug aimed at PH treatment by activating soluble guanylate cyclase. Riociguat has a two prong impact on vasodilation and anti-proliferation by stimulating cGMP independent of NO and increasing cGMP sensitivity to NO. Currently Riociguat has been approved by the European Union and Health Canada, the equivalent of the US Food and Drug Administration (Mielniczuk et al., 2014; Shanmugam et al., 2015). Similarly, phosphodiesterase-5 inhibitors are employed in PH to prevent the degradation of cGMP thereby prolonging NO-mediated vasodilation.

In addition to these vasodilating drugs that are currently used in the clinical management of PH patients, many additional agents have been shown to improve experimental PH in animal models. For example, selective serotonin reuptake inhibitors have been examined in PH therapy since serotonin has been established as a key molecule in both human and experimental models of PH (Humbert et al., 2004). Inflammation has also been identified as a central mediator in PH progression. 10-Nitro-Oleic acid attenuated hypoxia-induced elevations in right ventricular systolic pressures (RVSP), right ventricular hypertrophy (RVH), fibrosis, vascular remodeling, inflammation, and superoxide production in activated macrophages (Klinke et al., 2014). Though these examples attenuate experimental PH, it is important to recognize that there are numerous other agents that have shown similar efficacy.

Evolving work has suggested that targeting broader biological processes involved in PH pathogenesis may produce additional benefits that cannot be achieved by vasodilator therapies alone. For example, ROS have been implicated in the pathogenesis of multiple derangements in PH. The efficacy of targeting ROS generation to treat PH remains an area of active investigation, and is extensively explored in this proposal. Though not PH, in spontaneous hypertensive rat (SHR) models, antioxidant vitamin treatment improved vasodilation, decreased NADPH oxidases (Noxes) activity, and improved endogenous SOD activity (Chen et al., 2001; Zuo et al., 2014). Since the study of dietary antioxidants studies in PH remains extremely limited, few inferences can be made. It is likely that dietary antioxidant studies are limited because systemic distribution of antioxidants may be hampered by an inability of the antioxidants to reach specific cellular or subcellular compartments to target specific sources of ROS. The current studies begin to address these limitations in part by examining PH pathogenesis in models that permit targeted manipulation of mitochondrial ROS (mtROS) generation.

Experimental Animal Models of PH

Rodent models of PH such as those used for the current studies have several limitations. The following section does not provide an exhaustive review of all models of PH; rather, this section highlights some of the most commonly

utilized rodent models to study PH. Benefits and limitations of these *in vivo* models of PH are discussed.

Monocrotaline

Monocrotaline (MCT) treatment is a common model of PH. MCT is an alkaloid derived from the *Crotalaria spectabilis* plant and was established in 1967 as a model of PH. A single treatment with MCT provides a pathological insult that can cause experimental PH. MCT is most commonly used on rats (Maarman et al., 2013) due to its ability to mirror many of the fundamental features of human PH. A single subcutaneous or intraperitoneal injection of 60 – 100 mg/kg in the rat produces PH. MCT is metabolized to dehydromonocrotaline in the liver by cytochrome P450 CYP3A4 into pyrrolic derivatives that induce endothelial injury and pulmonary smooth muscle proliferation and resistance to apoptosis. MCT also damages endothelial cells which results in medial hypertrophy in small pulmonary arterioles, vascular inflammation, RVH, and right ventricle (RV) failure. Furthermore, the MCT model in the rat results in the development of complex vascular lesions and pulmonary vasoconstriction that more closely mirrors PH found in patients that die from right heart failure (Gomez-Arroyo et al., 2012; Ryan et al., 2013). The MCT model has been particularly useful due to its high reproducibility, low cost, and lack of technical skills needed to implement the model (Gomez-Arroyo et al., 2012; Maarman et al., 2013; Ryan et al., 2013).

The MCT model has been integral to research exploring the development of PH. For example, research using MCT-induced PAH has helped to identify the mutation of bone morphogenetic protein receptor-2 (BMPR-2) in PH development and the role of inflammatory macrophages in vascular remodeling (Atkinson et al., 2002; Maarman et al., 2013; Morrell et al., 2001). In addition, MCT-induced PH causes mitochondrial swelling and increased mtROS in the lung (Ryan et al., 2013). PH models that consist of multiple pathological insults are often employed to better mimic human PH. For example, pneumonectomy alone does not cause PAH, but MCT plus pneumonectomy in young (12 week) rats causes a more severe PAH phenotype including exacerbated right ventricular hypertrophy and further elevated mPAP (Cantin, 2004; Ryan et al., 2013). In rats, MCT mimics all the derangements found in the human disease, but MCT has limited efficacy in inducing PH in mice. Furthermore, the model can also be limited by concomitant myocarditis, obstructive pulmonary vein thrombosis, and severe right heart failure resulting in death (Gomez-Arroyo et al., 2012; Ryan et al., 2013).

Hypoxia

Hypoxia-induced PH is commonly used to model Group 3 PH *in vivo* (Nisbet et al., 2010). In these models, animals are exposed to hypoxic gas mixtures rather than normoxic atmospheric oxygen levels of 21% (160 mmHg). Due to tissue specificity, as well as varying redox gradients between cell types, some controversy surrounds what constitutes physiologically-relevant oxygen

levels. For example, cardiac tissue has an oxygen tension of 20 – 25 mmHg at rest that decreases to less than 10 mmHg during exercise. Alveoli in the adult lung rarely reach oxygenation greater than 110 mmHg O_2, and the cortex of the kidney has oxygen tensions of over 60 mmHg (McKeown, 2014). Regardless of tissue specificity, physiological hypoxia can be broadly defined as the O_2 level that forces cells and tissues to physiologically respond to maintain homeostasis (generally below 3 – 7% O_2 or 1.6 – 4.8 mmHg) (McKeown, 2014; Winslow, 2013). Despite a wide range of oxygen tension requirements, the majority of cell types behave similarly when placed in conditions with suboptimal oxygen concentrations. Since ATP production is not limited in cells until a critically low level of O_2, and mitochondria are the largest O_2 consuming organelle within the cell, mitochondrial molecular mechanisms may contribute to the cellular response to hypoxia (Schumacker, 2011).To address this, a new system to detect mitochondrial O_2 tension, protoporphyrin IX-triplet state lifetime technique (PpIX-TSLT), in living cells and tissue has been developed. This system, which relies on oxygen-dependent quenching of delayed fluorescence of 5-aminolevulinic acid, has been developed to detect O_2 tension in living cells and tissues (Mik, 2013).

A common chronic hypoxia model of PH involves exposing rodents to 10% O_2 (simulating altitude of 18, 000 feet) for 3 – 5 weeks. Chronic hypoxia causes PH and right ventricular hypertrophy associated with pulmonary vascular remodeling. Hypoxia induces proliferation of vascular smooth muscle cells

(VSMCs) and adventitial fibroblasts to cause muscularization of small pulmonary arterioles with endothelial cells failing to significantly proliferate under hypoxic conditions in rodent models. It is believed that remodeling of pulmonary vascular smooth muscle is mediated in part by closure of potassium channels through a process regulated by mitochondria (Sato et al., 2000; Voelkel and Tuder, 2000). Hypoxia-induced PH is characterized by medial hypertrophy of small pulmonary arteries, adventitial thickening, mild RVH, and inflammation (Ryan et al., 2013). Hypoxia exposure increases production of platelet-activating factor, which plays a role in the development of pulmonary vascular remodeling (Hoshikawa et al., 2001; Ono and Voelkel, 1991). Importantly, hypoxia alone fails to induce the severe pulmonary vascular remodeling and complex vascular lesions observed in Group 1 human PAH. There is little evidence of vascular obstruction or the development of complex/plexiform lesions in the pulmonary vasculature of hypoxic rodents (Green et al., 2012; Nisbet et al., 2010; Ryan et al., 2013). In addition, chronic hypoxia also causes polycythemia which can further exacerbate PH (Ryan et al., 2013).

The reasons hypoxia-induced rodent models of PH fail to develop the proliferative, plexiform arteriopathy seen in patients with severe IPAH remain speculative. Hypoxia in rodent models may not activate all of the signaling pathways that are active in human PAH pathobiology (Stenmark et al., 2009). Despite these limitations, the current studies employed the hypoxia-induced mouse model of PH which more accurately models Group 3 PH (PH caused by

hypoxia or associated with lung disease) (Ryan et al., 2011; Simonneau et al., 2013). This model has provided new insights into PH pathobiology and treatment (Bauer et al., 2007; Stenmark et al., 2009) and provides the added advantage of being able to be employed reproducibly in knockout or transgenic mice.

Hypoxia + Sugen

Hypoxia combined with a single dose of VEGFR2 antagonist (SUGEN, SU5416) has been shown to lead to a progressive form of PH in rat and mouse models of PH. This progressive form of PH promotes angioproliferative vascular lesions similar to those seen in patients with severe PH (Voelkel et al., 2013). SU5416 was initially developed as the anticancer agent, Semaxinib. In rats, the model typically involves subcutaneous injections of SU5416 (20 mg/kg) combined with 3 weeks of exposure to hypoxic conditions (10% O_2)(Ryan et al., 2013). While well established in the rat, only recently has this model been employed in mice (Ciuclan et al., 2011; Lu and McLoughlin, 2014; Ryan et al., 2013). This refinement has the potential to induce a more pulmonary vasculopathy that more accurately models the more severe PAH observed in Group 1 patients. Since suppression of VEGF with SU5416 induces more severe PH and vascular remodeling than hypoxia alone, these findings suggest that disordered angiogenesis may play a critical role in causing complex vascular lesions (Lu and McLoughlin, 2014; Partovian et al., 2000; West and Hemnes, 2011). Very recent reports have emphasized the importance of the vehicle used

in SU5416 administration. When SU5416 dissolved in carboxymethylcellulose (CMC) was compared to SU5416 dissolved in DMSO, hypoxia + SU5416 in DMSO caused a significantly greater increase in PVR than that observed in the hypoxia-DMSO alone or hypoxia + SU5416 in CMC. Furthermore, a lower dose of SU5416 (20 mg/kg) in DMSO was able to cause the same increase in PVR as SU5416 (40 mg/kg) in CMC (Lu and McLoughlin, 2014).

Model Differences

Major differences between the models (MCT, hypoxia, or hypoxia + SU5416) involve the form of PH addressed. MCT and hypoxia + SU5416 are better suited to experimentally mimic Group 1 PAH, whereas hypoxia alone more accurately models Group 3 PH. Unfortunately, no single animal model can perfectly recapitulate the many pathophysiological derangements found in all forms of PH. MCT is generally used in rat models of PAH, whereas hypoxia can be used in both rat and mouse models of PH. Initially, the hypoxia + SU5416 model was optimized as a rat PAH model, though subsequent modifications to the protocol have extended the model's efficacy to studies using transgenic and knockout mice (Table 1.1). Chronic hypoxia alone mimics Group 3 hypoxic PH, but chronic hypoxia plus SU5416 in the rat recapitulates many features of human PAH (Ryan et al., 2013). Finally, PAH induced by MCT or chronic hypoxia + SU5416 (CH+SU) in rats display endothelial dysfunction, proliferation/apoptosis

imbalance, and develop the glycolytic metabolic profile of human PAH (Ryan et al., 2013).

Model	Method	Effects	Limitations
Monocrotaline	• 60 – 100mh/kg subcutaneous or intraperitoneal • Can be combined with pneumonectomy	• Models Group 1: PAH • Endothelial dysfunction • Proliferation • Apoptosis resistance • Glycolytic metabolic profile • Plexiform/complex lesions • RVH • mPAP = 40 – 60 mmHg	• myocarditis • obstructive pulmonary vein thrombosis • severe right heart failure resulting in death
Hypoxia	• O_2 10% for 3 – 4 weeks	• Models Group 3 PH • Muscularization • Proliferation • RVH • mPAP = 30 – 40 mmHg	• lacks plexiform/complex lesions
Hypoxia + SU5416	• O_2 10% for 3 weeks • SU5416 (20 - 40mg/kg)	• Models Group 1: PAH • Endothelial dysfunction • Proliferation • Apoptosis resistance • Glycolytic metabolic profile • Plexiform/complex lesions • RVH • mPAP = 40 – 60 mmHg	

Table 1.1: Common PH Animal Models

Hypoxia-induced ROS

ROS occur in many forms due the reduction of molecular oxygen during normal cellular respiration. ROS are chemically active oxygen-based molecules that participate in cellular responses and signaling cascades. Oxygen can be converted to superoxide, which can be converted to hydrogen peroxide, which can be converted to a hydroxyl radical or water plus oxygen each through the sequential addition of an electron (Freund-Michel et al., 2013; Koskenkorva-Frank et al., 2013; Mailloux, 2015). ROS are classified as free radicals if they contain unpaired valence electrons. For example, superoxide ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), peroxyl ($RO_2^{\bullet}$), and hydroperoxyl ($HO_2^{\bullet}$) radicals have unpaired electrons that make them extremely reactive with intracellular molecules (Perez-Vizcaino et al., 2010). More stable ROS like hydrogen peroxide (H_2O_2), and ozone lack unpaired electrons, but are still able to exchange electrons with other molecules (Perez-Vizcaino et al., 2010). $O_2^{\bullet -}$ and H_2O_2 play an important role as signaling molecules for normal cellular functions in vascular wall cells (Griendling et al., 2000; Lyle and Griendling, 2006; Perez-Vizcaino et al., 2010) and as modulators of vascular tone (Perez-Vizcaino et al., 2010).

Many studies confirm elevated cellular ROS production in response to hypoxic stimuli. Most importantly, hypoxia increases plasma levels of glutathione disulfide (GSSG) by 60%, and this increase in oxidized glutathione is indicative that hypoxia induces oxidative stress *in vivo* (Chang et al., 1989; Voelkel et al.,

2013). It has been suggested that hypoxia causes vascular remodeling by stimulating vasoconstriction and increases in PVR (Sato et al., 2000; Voelkel and Tuder, 2000). Hypoxia-induced increases in PVR are also related to upregulation of the vasoconstrictors, ET-1 and serotonin, in the lung. In addition to their vasoconstrictive properties, these agents serve as VSMC growth factors and can promote vascular proliferation and remodeling.

In the chronic hypoxic lung, xanthine oxidase (XO), cyclooxygenases, lipoxygenases, endothelial NO synthase (eNOS), and activated macrophages produce ROS and inflammation (Demiryurek and Wadsworth, 1999; Tuder et al., 1994; Voelkel and Tuder, 2000). ROS can be generated enzymatically from a variety of cellular sources. Mitochondria are a non-enzymatic source of ROS; however, XO, uncoupled nitric oxide synthase (NOS), and Noxes are all enzymatic sources of ROS. XO levels are elevated in vascular endothelial cells in hypoxia-exposed male Sprague-Dawley rats (Hoshikawa et al., 2001). Activated XO in the beginning phase of hypoxic exposure has been shown to contribute to PH (Hoshikawa et al., 2001; Wind et al., 2010). Noxes, produce superoxide as a byproduct of single electron donation, and have been confirmed to be elevated in multiple PH models (Fike et al., 2008; Liu et al., 2006; Mittal et al., 2007b). Uncoupled NOS leads to the formation of $O_2^{\bullet -}$ rather than NO, and can therefore impact vascular tone. ROS produced by inflammatory and vascular wall cells cause oxidative stress which contributes to endothelial dysfunction (Blanquicett et al., 2010).

$O_2^{\bullet-}$ can be measured with redox sensitive probes (dihydroethidine, DHE; dichlorofluorescein, DCF; or MitoSOX), electron spin resonance (ESR), and fluorescence microscopy. In addition, H_2O_2 can be measured using Amplex Red, fluorescent probes Hyper, MitoPy1, and other techniques (Dikalov and Harrison, 2014; Nauseef, 2014; Schmitt et al., 2014; Woolley et al., 2013). These methodologies have been used to demonstrate hypoxia-induced ROS. For example, hypoxia increases oxidation of DCF in live cell imaging and increased oxidation of protein cysteine thiols by HSP-33 fluorescence resonance energy transfer detection (Dikalov and Harrison, 2014). Hypoxia appears to cause an increase in ROS production in the intermembrane space of mitochondria, and these ROS can diffuse to the cytosol (Schumacker, 2011). Furthermore, functional mitochondrial complex III is necessary for hypoxia-induced ROS production by the mitochondria, and blockade of complex III inhibits vasoconstriction in response to hypoxia (Schumacker, 2011). During hypoxic exposure, the mitochondria of O_2-sensing cells, including vascular wall cells, inhibit oxidative phosphorylation. This metabolic switch is evidence of altered mitochondrial metabolism in response to the hypoxic stimulus (Jung et al., 2010; Schumacker, 2011). Mitochondria sense hypoxia, increasing the release of ROS from the intermembrane space leading to activation of transcription factors and signaling cascades (Waypa et al., 2010). The studies discussed later identify the form of ROS within the mitochondria required for the molecular and physiological derangements that occur within vascular wall cells during PH.

ROS can function to mediate multiple functions in the cardiovascular system. Imbalance in ROS and downstream signaling leads to various pathological disorders. ROS production is elevated in multiple animal models of PH, and these ROS have been directly linked to pulmonary vascular remodeling, endothelial dysfunction, inflammation, and vasoconstrictive responses (Jones, 2006). Elevations in ROS are associated with activation of various growth factors and signaling pathways (Griendling et al., 1994; Ismail et al., 2009). The effects of ROS elevation can be seen in the differential expression of proteins that regulate important functions in PH development. For example, hormones angiotensin II (Ang-II), platelet derived growth factor (PDGF), and tumor necrosis factor-α (TNF-α) can alter the expression of antioxidants enzymes (Fukai et al., 1999; Lyle and Griendling, 2006; Shaffer et al., 1990; Visner et al., 1992). Furthermore, Ang-II increases the expression of Nox2 and Nox4 (Lyle and Griendling, 2006; Rajagopalan et al., 1996). The two major forms of ROS that have been found to be upregulated in PH are $O_2^{\bullet -}$ and H_2O_2. In addition, both $O_2^{\bullet -}$ and H_2O_2 have been suggested to promote dysregulated vascular remodeling, hypertrophy, and muscularization of vessel media (Perez-Vizcaino et al., 2010; Wedgwood et al., 2001). It is the distinct characteristics of $O_2^{\bullet -}$ and H_2O_2 that allow for them to modulate divergent functional responses.

Superoxide

Many studies have shown that $O_2^{\bullet-}$ plays a vital role in PH pathology. The $O_2^{\bullet-}$ anion is formed by a one electron reduction of molecular oxygen by various systems: mitochondrial complex I and III, XO, Noxes, lipoxygenases, and cytochrome p450s (Goeptar et al., 1995). $O_2^{\bullet-}$ has limited lipid solubility which limits its diffusion. $O_2^{\bullet-}$, normally found in picomolar to nanomolar concentrations, is electronically charged and highly reactive so it is rapidly converted to H_2O_2 and water by superoxide dismutase (Cu-Zn SOD in the cytosol and MnSOD2 in the mitochondria) (Dromparis and Michelakis, 2013; Perez-Vizcaino et al., 2010). SOD is present in micromolar concentrations and functions as the major enzyme that removes $O_2^{\bullet-}$ (Beckman and Koppenol, 1996). Cells are extremely efficient, with only 1 – 5% of total O_2 consumption reduced to $O_2^{\bullet-}$ (Beckman and Koppenol, 1996; Imlay and Fridovich, 1991), and mitochondrial complex I and complex III only lose 1 – 3% of O_2 during the electron transport chain to generate $O_2^{\bullet-}$ (Valko et al., 2007).

Elevated $O_2^{\bullet-}$ levels prevent NO from stimulating soluble guanylyl cyclase (sGC). $O_2^{\bullet-}$ directly interacts with NO to form peroxynitrite ($ONOO^-$) and thereby reduces NO bioavailability causing vasoconstriction. Since SODs are in high concentration in both the cytosol and mitochondria and quickly reduce $O_2^{\bullet-}$, the $O_2^{\bullet-}$ molecule rarely interacts with NO at physiologically relevant levels to be converted to toxic $ONOO^-$ (Perez-Vizcaino et al., 2010; Schroder and Eaton, 2008; Sies, 1997). NO has a higher affinity for $O_2^{\bullet-}$ ($6.7 \times 10^9\ M^{-1}\ s^{-1}$), but is

present in 5-10 nM concentration. SOD has a lower reaction rate (2 x 10^9 M^{-1} s^{-1}) with $O_2^{\bullet-}$, but SOD is far more abundant than NO at physiological concentrations. The concentration of NO must rise to micromolar levels to effectively outcompete SOD for $O_2^{\bullet-}$ (Beckman and Koppenol, 1996; Huie and Padmaja, 1993). For these reasons, $O_2^{\bullet-}$ is preferentially reduced by SODs into H_2O_2 (Beckman and Koppenol, 1996; Perez-Vizcaino et al., 2010; Schroder and Eaton, 2008; Sies, 1997).

Hydrogen peroxide

H_2O_2 is implicated in the regulation of various signaling pathways involved with smooth muscle contraction, cellular migration, and inflammation (Lassegue and Clempus, 2003). H_2O_2 is formed from the reduction of $O_2^{\bullet-}$ radical by SOD. The majority of Noxes and mitochondrial respiration produce, $O_2^{\bullet-}$, and that $O_2^{\bullet-}$ can be reduced by SOD to H_2O_2. Alternatively, H_2O_2 is reduced to O_2 and H_2O by catalase, glutathione peroxidase (GPx), or peroxiredoxins (Prxs) (Lyle and Griendling, 2006; Peskin et al., 2007; Rhee et al., 1999). Furthermore, $O_2^{\bullet-}$ produced at mitochondrial complex III is targeted towards the intermembrane space, allowing for rapid conversion to H_2O_2 which is freely diffusible across hydrophobic membranes between cellular compartments. H_2O_2 is less reactive than $O_2^{\bullet-}$ and found in high nM to μM concentrations (Perez-Vizcaino et al., 2010). H_2O_2, which normally serves as a signaling molecule in the vasculature at low physiological concentrations, has a longer half-life than $O_2^{\bullet-}$, and it can diffuse to more distant cellular sites (Ardanaz and Pagano, 2006; Satoh et al.,

2014). Excessive H_2O_2 can deteriorate vascular functions leading to cellular damage, promoting vascular diseases (Satoh et al., 2014; Schroder and Eaton, 2008), and more importantly for PH, H_2O_2 plays a role in endothelial and SMC hypertrophy and proliferation (Bijli et al., 2015; Chettimada et al., 2015; Porter et al., 2014). Since there are multiple forms of ROS that play a role in vascular derangements, targeting the cellular compartment generating the ROS, and not just the ROS themselves, provides novel potential strategies for PH therapy. Exploring the antioxidant systems in place to attenuate ROS will give insights into possible therapeutic targets for PH treatment.

Antioxidants

Antioxidants are molecules that prevent the oxidation of substrates. These substances, at low concentrations compared with that of an oxidizable substrate, delay or inhibit the oxidation of that substrate. Non-enzymatic antioxidants intercept and deactivate ROS and are most efficient when they react with free radicals at a suitable rate and interact with water-soluble compounds to regenerate by themselves. However, traditional descriptions of oxidative stress as the imbalance between pro-oxidant and antioxidant systems likely oversimplifies the complex underlying signaling regulation (Jones, 2006). Multiple failed antioxidant interventions (using vitamin E, beta carotene and retinol) supported the theory that redox signaling occurs through specific signaling pathways and that alterations in one cell compartment did not necessarily impact or regulate redox response in other compartments (Go et al., 2004; Jones, 2006).

These concepts emphasize the importance of compartmentalized cellular response to ROS generation and metabolism. There are three major categories of antioxidant enzymes: glutathione peroxidases (GSH), superoxide dismustase (SOD), and catalase (Sies, 1997), though these studies focus on the latter two (Jones, 2006; Jones et al., 2000).

SODs (cytosolic SOD1, mitochondrial SOD2, and extracellular SOD3) catalyze the reduction of $O_2^{\bullet-}$. In human lung tissue from patients with IPAH, SOD activity is reduced and high expression of the oxidant stress markers, nitrotyrosine and 8-OH-guanosine, are increased (Voelkel et al., 2013). SOD2, which converts $O_2^{\bullet-}$ to H_2O_2, serves to regulate $mtO_2^{\bullet-}$ levels (Schriner and Linford, 2006). Because SOD2 works rapidly to dismute $O_2^{\bullet-}$, it is a major source of endogenous H_2O_2 (Dromparis and Michelakis, 2013; Rehman and Archer, 2010; Richter et al., 1995). In studies where SOD2 is overexpressed, researchers detected decreased lipid peroxidation, increased resistance against Paraquat-induced oxidative stress, and decreased time -related decline in mitochondrial ATP production (Jang et al., 2009). In a hypertension model, overexpression of SOD2 was also found to reduce cellular NADPH oxidase activity, and improved the level of bioavailable NO (Dikalova et al., 2010). Due to the reduction of SOD2 levels in IPAH and studies where application SOD2 has been shown to improve markers of PH, these studies took advantage of a transgenic SOD2 overexpression model (Tg^{hSOD2}) to attenuate $mtO_2^{\bullet-}$ (Dikalova et al., 2010; Jang et al., 2009).

Catalase, glutathione peroxidase (GPx), thioredoxins (Trxs), and peroxiredoxins (Prxs) all catalyze the reduction of H_2O_2 to H_2O and O_2 (Perez-Vizcaino et al., 2010; Schriner and Linford, 2006). Exogenous application of PEG-catalase decreased aberrant cyclinD1 expression in lung tissue in an animal model of PPHN (Wedgwood et al., 2013) and prevented hypoxia-induced Nox4 expression (Lu et al., 2013). Catalase is normally expressed in peroxisomes, whereas the Trx system is endogenous to the mitochondria. Both mitochondrial expression of catalase and overexpression of Trx2 were utilized in the current studies to target H_2O_2 (Schriner et al., 2005). An increased focus on how derangements in ROS can alter mitochondrial metabolism, right ventricular cardiomyocyte hypertrophy, and vascular wall derangements may lead to new and innovative targets for treatment of PH.

Roles and Sources of Oxidative Stress in PH

The modern interpretation of oxidative stress addresses the limitations of defining oxidative stress as solely an imbalance between pro-oxidants and antioxidants (Sies, 1997). This balance concept indicates that distinct biological systems respond equally to decreased pro-oxidants and increased antioxidants. However, since oxidative stress also involves the effects of redox signaling and multiple systems are involved, not all systems will have the same sensitivity to increased oxidants or respond similarly to antioxidants. Therefore, it has been

suggested that oxidative stress be instead defined mechanistically as a disruption of redox signaling and control (Jones, 2006).

ROS are produced as intermediates in redox reactions and are comprised of both free radicals (i.e. $O_2^{\bullet-}$) and non-radical derivatives of O_2 (e.g. H_2O_2) (Sedeek et al., 2009). $O_2^{\bullet-}$ and H_2O_2 play a vital role in vascular cell signaling (Lyle and Griendling, 2006), affecting cellular proliferation, differentiation, and apoptosis (Watson et al., 2003). In PH, ROS are elevated and appear to be mediated through abnormal mitochondrial oxidative phosphorylation and NADPH oxidase (Nox) activity (Datla and Griendling, 2010; Giordano, 2005). There is an incompletely defined link between mitochondria-derived ROS and Noxes (Daiber, 2010; Dikalov, 2011; Perez-Vizcaino et al., 2010). More importantly, these and other studies imply that mitochondrial dysfunction and mtROS may play a heretofore unexplored major role in the regulation of hypoxia-induced PH. It is then vital to explore the interaction between Noxes and mitochondria, two major sources of ROS within the vasculature.

NADPH Oxidases

Background and Isoforms

NADPH oxidases (nicotinamide adenine dinucleotide phosphate-oxidase – Noxes) proteins are multi-subunit, membrane-associated enzymes that catalyze

reduction of molecular oxygen using NADPH as the electron substrate (NADPH + $2O_2 \leftrightarrow NADP^+ + 2O_2^- + H^+$) (Konior et al., 2014). There are seven mammalian isoforms of Noxes, each containing 6 transmembrane domains: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2. Noxes vary by mechanism of activation, localization, and physiological functions (Konior et al., 2014; Lyle and Griendling, 2006; Pendyala and Natarajan, 2010). The various Noxes are by and large ubiquitously expressed however, they can be differentially activated by various agents, including: Ang-II, PDGF, or TGF-β (Lyle and Griendling, 2006). Tissue distribution and intracellular localization of also varies among the Nox isoforms. In the vasculature, Nox1, Nox2, Nox4, and Nox5 are expressed in endothelium, VSMCs, adventitia, fibroblasts, cardiomyocytes and perivascular adipocytes at varying levels (Konior et al., 2014; Lassegue et al., 2012; Paravicini and Touyz, 2008; Pendyala and Natarajan, 2010; Perez-Vizcaino et al., 2010), though cellular distribution of Noxes is less clear.

Noxes are abundantly expressed in lung cells and appear to be upregulated by hypoxia. Noxes can promote cellular dysfunction by activating other enzymes that generate ROS (Landmesser et al., 2003). While Nox2 was initially known to be active as the source of phagocytic respiratory burst, the contribution of Nox2 to ROS generation in all cell types are currently being extensively studied. Chronic activation of Noxes has been linked to many disease states including gastrointestinal inflammation, Alzheimer's disease, and hypertension. Noxes can be inhibited by DPI (diphenylene iodonium) and

apocynin. DPI acts as an inhibitor of the flavin containing subunit. Apocynin inhibits Nox1 and Nox2 by (Konior et al., 2014; Yeligar et al., 2012) inhibiting translocation of p47phox from cytosol to plasma membrane (Lyle and Griendling, 2006). Noxes, important sources of ROS within the vascular wall (Lyle and Griendling, 2006; Sedeek et al., 2009), regulate endothelial function, vascular tone, vascular cell hypertrophy, and apoptosis (Dikalova et al., 2010; Paravicini and Touyz, 2008). ROS derived from Nox isoforms, in particular Nox2 and Nox4, are involved in long-term responses of the pulmonary vasculature to hypoxia (Cutz et al., 2009; Frazziano et al., 2012; Mittal et al., 2007b).

Nox2 and Nox4

The catalytic subunit of the Nox complex, $gp91^{phox}$ (Nox2), has been identified within phagosomes and is present the leading edge of lamellipodia in endothelial cells, and redoxisomes of non-phagocytic cells. Prototype phagocytic Nox2 consists of five subunits. The cytochrome b558 membrane complex consists of gp91phox and p22phox. Catalytic gp91phox binds FAD, p47phox, p67phox, and the G-protein Rac-1 (Konior et al., 2014; Lyle and Griendling, 2006; Perez-Vizcaino et al., 2010). Nox2 activation plays a vital role in signal transduction in endothelial cells, and Nox2-derived ROS regulate p38 MAP-kinase mediated proliferation and vascular endothelial growth factor (VEGF) migration (Konior et al., 2014; Ushio-Fukai et al., 1996). Lack of Nox2 in white blood cells causes the immune disorder, chronic granulomatous disease (Lassegue et al., 2012; Nauseef, 2014). In addition, knockout of $gp91^{phox}$ (Nox2)

prevented hypoxia-induced $O_2^{\bullet-}$ production (Liu et al., 2005). Nox2 is expressed in vascular smooth muscle and endothelial cells (Bedard and Krause, 2007; Brown and Griendling, 2009; Lassegue et al., 2012). Nox2 cellular distribution was initially unknown in VSMC (Lyle and Griendling, 2006) but later found to be localized to the plasma membrane (Lassegue et al., 2012), nucleus, and endoplasmic reticulum (Bayraktutan et al., 2000; Petry et al., 2006).

Nox4, highly expressed in the human vasculature, is detectable in smooth muscle and endothelial vascular wall cells (Geiszt et al., 2000; Gosemann et al., 2013; Schroder et al., 2012). Unlike other Nox isoforms, Nox4 only requires the $p22^{phox}$ subunit to be active and produce ROS and is thought to be constitutively active (Konior et al., 2014). Nox4 has been found compartmentalized in focal adhesions, nuclei, endoplasmic reticulum, and mitochondria, it's intracellular distribution and targeting may vary between cell types (Frazziano et al., 2014; Hilenski et al., 2004; Lyle and Griendling, 2006; Miller et al., 2005). Unlike Nox2, Nox4 is constitutively active and responsible for basal H_2O_2 production in the vasculature (Dikalov et al., 2008; Frazziano et al., 2014; Freund-Michel et al., 2013; Ray et al., 2011; Sedeek et al., 2009). Studies using siNox4 did not reduce DHE detection of $O_2^{\bullet-}$, but did show a decrease in Amplex red detectable H_2O_2. It is possible that Nox4 derived $O_2^{\bullet-}$ is very rapidly converted to H_2O_2, so the $O_2^{\bullet-}$ is not easily detectable (Dikalov et al., 2008; Takac et al., 2011). Nox4-derived ROS promotes vascular remodeling and plays a major role in PH development (Green et al., 2012; Mittal et al., 2007b; Nisbet et al., 2010).

Mitochondrial expression of Nox4 remains controversial. Nox4 expression has been confirmed in the mitochondria of rat kidney cortex (Jernigan et al., 2004) and in cardiac myocytes (Kuroda et al., 2010). Nox4 localization to mitochondria provides a link and possible location for interaction between two major sources of vascular ROS (Noxes and mitochondria). Nox4 localization has been controversial (expression normally expected in focal adhesions (Jones et al., 2000) and nucleus (Marklund, 1976)), because of a lack in the availability of reliable Nox4 antibodies that permit the unequivocal identification of Nox4 in specific cell compartments (Frazziano et al., 2014).

Nox2 and Nox4 in PH

The vascular endothelium predominantly expresses Nox2 and Nox4 (Pendyala et al., 2009; Pendyala and Natarajan, 2010) and many reports (Barman et al., 2014; Fike et al., 2008; Frazziano et al., 2012; Fresquet et al., 2006) have shown that vascular responses to hypoxia may be regulated by both Nox2 and Nox4, so this project focuses on the expression of Nox2 and Nox4 as a vascular derangement in hypoxia-induced PH because both Nox2 and Nox4 function to regulate pulmonary vascular responses to hypoxia (Cutz et al., 2009; Frazziano et al., 2012; Mittal et al., 2007b).

A major pathological derangement in the chronic hypoxia model of PH is smooth muscle cell (SMC) hypertrophy and proliferation. This increased,

dysregulated proliferation of SMCs leads to a decrease in vascular luminal area and increase PVR, culminating in elevated ventricular pressure (Liu et al., 2006; Mittal et al., 2007b). As chronic hypoxia elevates Nox2 and Nox4 expression, increased ROS generation by Noxes may explain the increased ROS signaling that modulates the vascular derangements seen in PH.

Both Nox2 and Nox4 levels were found to be increased in experimental PPHN, with remodeling thought to be through H_2O_2 signaling (Wedgwood et al., 2013). These studies ultimately concluded that H_2O_2 contributes to vasoconstriction and vascular remodeling involving extracellular SOD inactivation, stimulation of vascular remodeling via NFκB activation, and increased cyclinD1 expression (Wedgwood et al., 2013). Frazziano et al. showed that normally quiescent Nox2 can be activated by feed-forward Nox4 induction of ROS in mouse right ventricles caused by pulmonary artery banding (PAB) (Frazziano et al., 2014). In this model, H_2O_2 is rapidly increased in PAB right ventricles, which could be attenuated by administration of catalase (which reduced H_2O_2) or diphenylene iodonium (DPI – a pan-flavin irreversible inhibitor which targets Noxes, XO, and mitochondria complex I) (Konior et al., 2014; Perez-Vizcaino et al., 2010). The successful inhibition of H_2O_2 and Nox activity to prevent PAB-induced right ventricular hypertrophy (RVH) confirms the contribution of both H_2O_2 and Nox to the development of RVH (Frazziano et al., 2014). Though the PAB model only examines the biology of what happens in the

RV in response to pressure/volume overload, these results are consistent with data collected during the subsequent studies.

Nox4 expression is increased in murine models of hypoxia-induced PH, in the pulmonary vasculature of PH patients (Mittal et al., 2007b; Nisbet et al., 2010; Sutliff et al., 2010), and in pulmonary artery endothelial cells isolated from patients with idiopathic pulmonary arterial hypertension (IPAH) (Green et al., 2012). Furthermore, Nox4 co-localizes with hypoxia-induced ROS in adventitia (Barman et al., 2014). Since Nox4 expression is increased during hypoxic oxidative stress, these studies managed to identify a role for mitochondrial-localized Nox4 expression as a contributor to right ventricle failure. Interestingly, mitochondria produce $O_2^{\bullet-}$ (which is converted to H_2O_2 that is diffusible) that participates in normal signaling. So while Nox4 localized to mitochondria could certainly modulate mitochondrial function, H_2O_2 produced by Nox4 elsewhere in the cell could still potentially diffuse into the mitochondrial and modulate mitochondrial function.

Mitochondria

Mitochondrial dysfunction has been implicated in numerous diseases including cardiac disorders, aging, autism, and hypertension. Mitochondria use O_2, adenosine diphosphate (ADP), and NADPH to produce 32 mols of adenosine triphosphate (ATP) for every mole of glucose (Tang et al., 2014; Winslow, 2013).

Compared to hepatocytes (32%) or cardiac myocytes (28%), endothelial cells have a very modest mitochondria composition (2 – 6%) (Dromparis and Michelakis, 2013; Dromparis et al., 2010; Tang et al., 2014). While pulmonary vascular endothelial cells have a low mitochondrial content, mitochondrial dynamics are a key regulator of endothelial cell homeostasis under normal conditions and PAH (Tang et al., 2014). Hypoxia triggers an increase in ROS production after Nox activation in PASMCs, which leads to subsequent mitochondrial ROS production (Rathore et al., 2008). Current evidence suggests that crosstalk between mitochondria and Noxes controls integrated cellular responses to hypoxia (Daiber, 2010; Dikalov, 2011).

Mitochondrial-Derived ROS Production

Under physiological conditions, 2 – 3% of the oxygen consumed by mitochondria for oxidative phosphorylation is incompletely reduced to yield ROS (Dromparis and Michelakis, 2013; Dromparis et al., 2010; Valko et al., 2007). The electron transport chain produces $O_2^{\bullet -}$ at complex I (NADH-ubiquinone oxidoreductase) and complex III (ubiquinol-cytochrome c oxidoreductase) during cellular respiration for the production of ATP (Freund-Michel et al., 2013; Perez-Vizcaino et al., 2010). $O_2^{\bullet -}$ formed at complex I is released into the mitochondrial matrix, whereas complex III releases $O_2^{\bullet -}$ onto either side of the inner mitochondrial membrane depending on the portion of Q cycle (mitochondrial reactions causing oxidation of coenzyme Q10 by ubiquinol and ubiquinone across the inner mitochondrial membrane) (Hansen et al., 2006a; Sommer et al.,

2010; Tang et al., 2014). Mitochondrial ROS generation is dependent on mitochondrial membrane potential, intracellular calcium, and NO levels (Erusalimsky and Moncada, 2007), and mitochondrial ROS production can be modulated by mitochondrial biogenesis, fusion, fission, and the availability of metabolic substrates (Erusalimsky and Moncada, 2007; Perez-Vizcaino et al., 2010).

Mitochondria are O_2 sensing organelles. The terminal cytochrome preferentially binds O_2 making the mitochondria the primary oxygen utilization site in the cell (Weir and Archer, 2010). It has been suggested that though ATP production in the mitochondria does not decrease until O_2 levels are less than 1%, it is possible that the redox status of the electron transport chain (oxidative phosphorylation) can act as the sensor for hypoxic signaling cascades (Schumacker, 2011; Tang et al., 2014). Furthermore, hypoxia is a critical inducer of mitochondrial ROS (mtROS). In pulmonary artery endothelial cells, hypoxia triggers mitochondrial movement to a perinuclear arrangement and mitochondrial ROS release (Al-Mehdi et al., 2012; Tang et al., 2014).

While some studies indicate that ROS production decreases during hypoxia, studies by multiple other labs and our own studies have demonstrated increases in mitochondrial ROS production in response to hypoxia (Chandel et al., 1998; Green et al., 2012; Hoshikawa et al., 2001; Nisbet et al., 2010; Schumacker, 2011), including mitochondrial-derived ROS via protein kinase C

(PKC) and Nox activation (Daiber, 2010; Perez-Vizcaino et al., 2010; Waypa et al., 2010). In PAH, endothelial cell dysfunction is an early event possibly mediated by mtROS-regulated proliferation and apoptosis-resistance (Dromparis and Michelakis, 2013; Dromparis et al., 2010; Sena and Chandel, 2012; Tang et al., 2014). It has been suggested that in PAH, though there is low mitochondria activity and SOD2 levels are reduced, H_2O_2 triggers loss of HIF1-α (Rehman and Archer, 2010; Wang et al., 1995).

Possible Roles of Mitochondrial-Derived ROS in PH

Uncouplers of respiratory chain complexes produce a hypoxia-like environment which induces activation of voltage-gated Ca^{2+}channels (Evans et al., 2011; Wyatt and Buckler, 2004). During hypoxia, mitochondrial oxidative phosphorylation and ATP production is reduced (Sommer et al., 2010), and the cell relies on other metabolic pathways like glycolysis for energy production. 1 – 3% of O_2 becomes mtROS ($mtO_2^{\bullet-}$ and mtH_2O_2) at complex I and complex III of the mitochondria, and Nox2 and Nox4 also produce ROS and regulate proliferation. These two sources of ROS interact and likely influence the others' effect on PH pathophysiology (Dikalov et al., 2008; Dromparis and Michelakis, 2013; Lassegue et al., 2012; Tang et al., 2014). In PAH, mitochondria acquire abnormalities that include epigenetic silencing of SOD2 and disruption of oxygen sensing creating a pseudo-hypoxic environment characterized by normoxic activation of HIF-1α (Perez-Vizcaino et al., 2010; Ryan et al., 2015; Voelkel et al., 2013). Furthermore, hypoxia has been shown to induce acute $O_2^{\bullet-}$ release from

complex III in the pulmonary circulation. These studies provide the first evidence that mtROS differentially contribute to Nox4 upregulation under hypoxic conditions. Since hypoxia differentially regulates vascular responses, it is possible that the dysregulation of mitochondria and subsequent increase in mtROS plays a significant role in the derangements that promote PH.

Summary

PH is defined by an elevation of pulmonary arterial pressures and is characterized by narrowing of the pulmonary vasculature from dysregulated proliferation of pulmonary vascular wall cells, inhibition of apoptosis, and remodeling of the pulmonary vasculature (Rabinovitch, 2012). Current PH therapies target vasoconstriction to promote vasodilation. However, vasodilating agents such as PGI_2, ET-1 receptor antagonists, or agents that promote NO signaling do not address the vascular remodeling associated with long-term PH and indicate the need for therapies that better address these PH derangements.

Hypoxia has been a standard model of PH development for both *in vitro* and *in vivo* studies of PH (Nisbet et al., 2010). ROS production is elevated in cellular models, multiple animal models of PH, and PH patient samples. ROS have been directly linked to pulmonary vascular remodeling, endothelial dysfunction, inflammation, and vasoconstrictive responses through undefined signaling pathways. ROS such as $O_2^{\bullet -}$ and H_2O_2 contribute to signaling cascades and provide likely targets that are integral to pulmonary vasculature responses to hypoxia. In PH, ROS production appears to be related to mitochondrial oxidative phosphorylation and Nox activity (Datla and Griendling, 2010; Giordano, 2005). The current studies aim to further clarify interactions between mtROS and Noxes in PH development.

The compartmentalized regulation of oxidative stress functions as a mechanism for specificity in redox signaling. Increasing evidence indicates that ROS generated from mitochondria, the most reduced organelle in the cell, contributes to endothelial cell dysfunction as seen in PH. Therefore, mitochondrial antioxidant systems may participate in primary defense against ROS and oxidative stress damage to the cell. It is possible that attenuation of mtROS using endogenous antioxidant systems like Trx2, SOD2, or the targeted expression of catalase in mitochondria may provide greater or more effective protection against hypoxia-induced ROS signaling to prevent PH pathophysiology. These considerations led us to the studies proposed below.

Proposed Research

We hypothesize that hypoxia increases mtROS generation to stimulate Nox expression and activity, thereby amplifying and sustaining ROS generation that promotes pulmonary vascular wall cell proliferation, vascular remodeling, and PH. To assess how mtROS generation affects PH development, we used models with targeted mitochondrial antioxidant overexpression to assess if reducing mtROS generation will attenuate hypoxia-induced PH pathobiology.

To test our hypothesis, hypoxia-induced increases in both $O_2^{\bullet-}$ and H_2O_2 generation was confirmed in HPAECs. Expression of antioxidant transgenes were confirmed in our murine models by studies performed to assess both physiological and molecular outcomes of transgene expression (i.e. measurement of RVSP, RVH, vessel muscularization, and H_2O_2 production). To explore the effect of mtROS on molecular markers, we measured levels of Nox2, Nox4, cyclinD1, and PCNA.

To focus more fully on the effects of mitochondrial $O_2^{\bullet-}$, Tg^{hSOD2} mice (obtained by my mentors through a MTA with Dr. Sergey Dikalov, Vanderbilt University), that express human SOD2 driven by the human β-actin promoter (Kowluru et al., 2006), were exposed to 3 weeks of hypoxia, and the effects of

limiting mitochondria-derived superoxide levels on hypoxia-induced Nox expression, pulmonary vascular remodeling, and PH were examined.

To explore targeted attenuation of mitochondrial H_2O_2, a mouse model with a transgene that targets overexpression of catalase to the mitochondria (MCAT) (obtained by my mentors through a material transfer agreement (MTA) with Dr. Peter Rabinovitch, Washington University) (Dai et al., 2009). These mice were exposed to normoxia and hypoxia, and physiological PH markers, Nox expression, and molecular PH derangements were assessed (**Figure 1.1**).

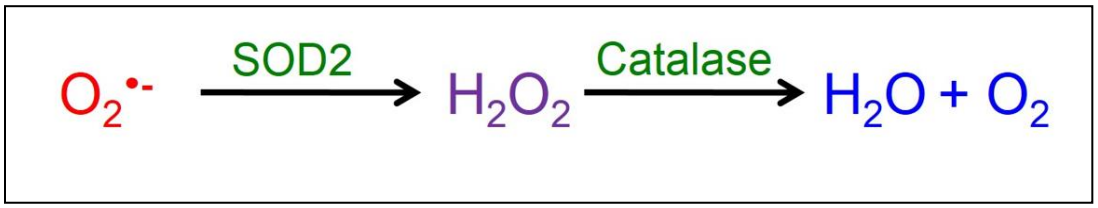


Figure 1.1: Superoxide dismutase reduces superoxide to hydrogen peroxide, and catalase reduces hydrogen peroxide to water and oxygen.

Finally, the effects of compartmentalization of ROS on PH development were examined using Trx2 transgenic mice exposed to hypoxia. These mice express human Trx2 (Tg^{hTrx2}) (obtained by my mentors through a MTA with Dr. Dean Jones, Emory University) localized to the mitochondria. Upon treatment with hypoxia, these mice were assessed for markers of PH. Analysis of the differential effects of antioxidant expression was performed to discern if mitochondrial targeting of antioxidants was beneficial.

Littermate controls, MCAT, Tg^{hSOD2}, and Tg^{hTrx2} mice were subjected to a well-established hypoxia regimen that we and others have reported to increase Nox4 expression and produce PH, RVH, and vascular remodeling in mice (Nisbet et al., 2010). Male mice aged 6 – 9 weeks were exposed to normoxia (21% O_2) or hypoxia (10% O_2) for three weeks (Green et al., 2012; Nisbet et al., 2010). Hypoxia exposure was confirmed by measuring increases in hematocrit in hypoxia-exposed mice. PH progression was characterized by measuring right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH), and in some cases echocardiography as published by our group (Nisbet et al., 2010). Histological examination of the lung to assess pulmonary vascular remodeling in response to hypoxia was also performed by α-smooth muscle actin (α-SMA) staining to assess muscularization of small pulmonary arterioles (Nisbet et al., 2010). SOD2 and catalase levels were also measured in lung tissue to verify overexpression. In addition, Nox2, Nox4, cyclinD1, and PCNA levels were assessed by qRT-PCR and western blot (Jacob et al., 2006; Nisbet et al., 2010; Reed et al., 2011).

These studies will confirm that hypoxia can induce mtROS (both $O_2^{\bullet -}$ and H_2O_2) and assess how this impacts global levels of detectable H_2O_2. Furthermore, these studies will verify if targeted attenuation of mtROS can regulate Nox2 and Nox4 expression. Finally, these studies will also detect the ability of mtROS regulation to modify hypoxia induced molecular (Nox2, Nox4,

cylcinD1, and PCNA protein) and physiological (vessel muscularization, RVSP, and RVH) indicators of PH.

CHAPTER 2: Materials and Methods

Littermate Control and Transgenic Mice

All animal studies were approved by the Atlanta VA IACUC.

Mitochondrial Catalase (MCAT)

A transgenic mouse model with human catalase expression targeted to the mouse mitochondria was employed **(Figure 2.1)**. MCAT mice were provided by Dr. Peter Rabinovitch (University of Washington). Briefly, the MCAT transgene was generated by deleting the carboxy-terminal amino acids, the peroxisomal localization signal, and initiating methionine from the human catalase gene. An ornithine transcarbamylase mitochondria-targeting leader sequence was added to the amino terminus to target catalase expression to mitochondria. The catalase cDNA was driven by the CMV enhancer element and chicken β–actin promoter sequence **(Figure 2.2)**. The MCAT mice lines were generated by microinjection techniques into B6 (B6C3F1) embryos. After 8 backcrosses, line purity was confirmed (Charles River Laboratories, Wilmington, MA) (Schriner et al., 2005). All littermate controls were homozygous for the wild-type genotype. The transgene is ubiquitously expressed, but expression levels vary between different tissues. Both qRT-PCR and WB analysis of mitochondrial fractions confirmed overexpression of catalase in pulmonary tissue. Transgenic mice had similar weights, hematocrits (elevated in all hypoxia samples, confirmed hypoxia exposure), and displayed no phenotypic differences from wild-type littermate

controls. Littermate controls and MCAT mice, ages 6-9 weeks, were utilized for these studies.

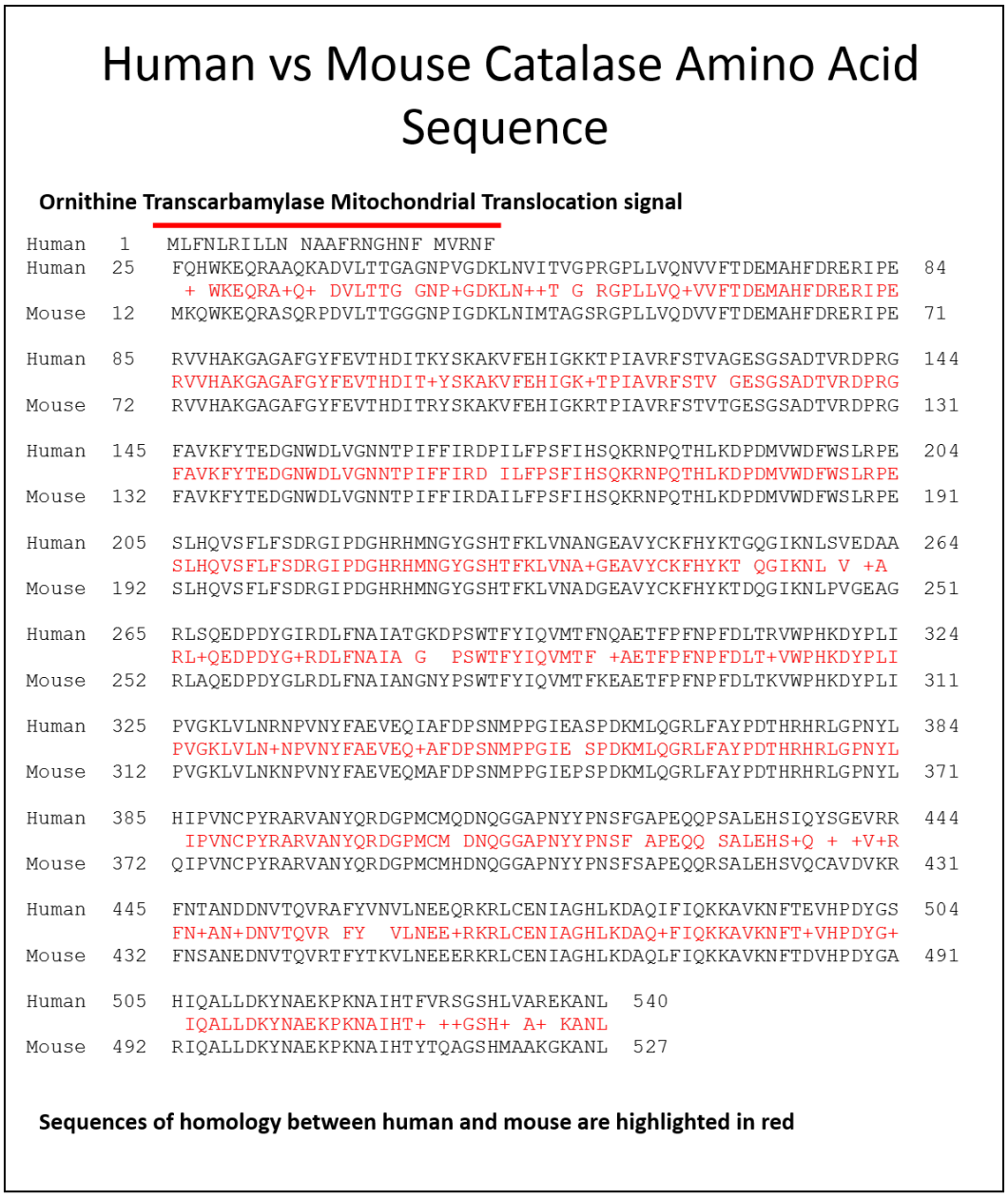


Figure 2.1: Human vs Mouse Catalase Amino Acid sequence.

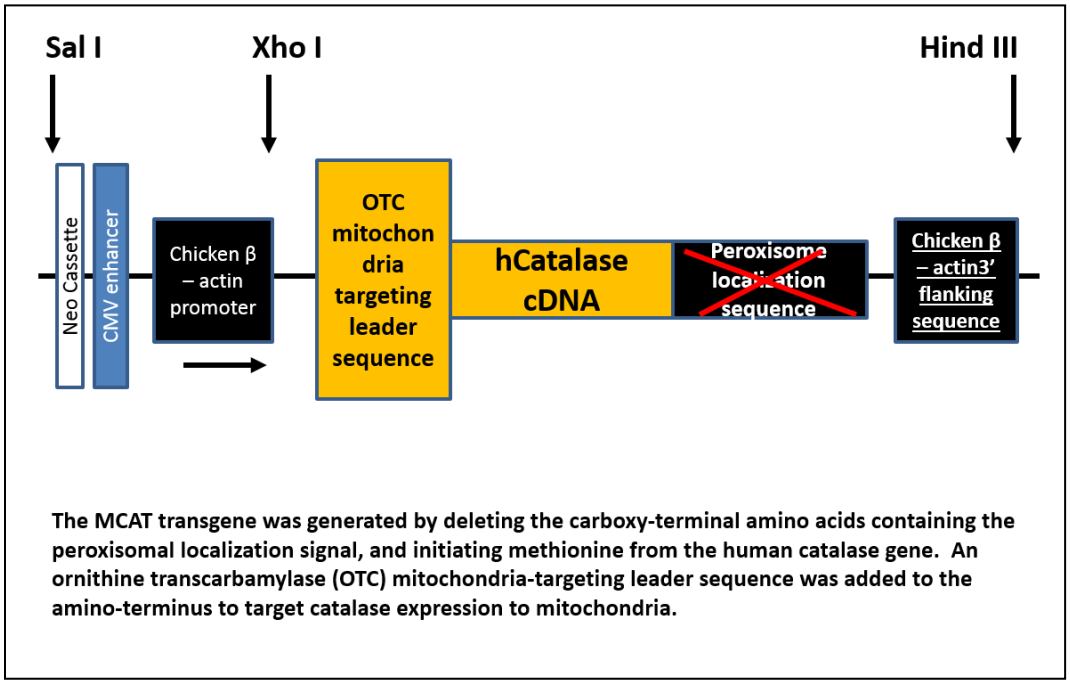


Figure 2.2: MCAT transgene generation.

Transgenic SOD2 Overexpression (Tg^{hSOD2})

A transgenic mouse model overexpressing human mitochondrial superoxide dismutase 2 (SOD2) was also employed **(Figure 2.3)**. Transgenic SOD2 (Tg^{hSOD2}) mice were obtained from Dr. Sergey I. Dikalov and generated by isolating a 13-kb genomic SOD2 clone construct containing the human mitochondrial superoxide dismutase 2 gene from C57BL/6J mice. This SOD2 clone encompassed 2 kb of the native SOD2 promoter. Subsequent generations of Tg^{hSOD2} mice were bred by mating male Tg^{hSOD2} mice to female wild-type (WT) C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, ME) (Jang et al., 2009; Raineri et al., 2001). To allow distinction of the transgene from the endogenous Sod2 gene by PCR, a 344 base pair fragment of the neo gene [nt 754–1086 of pMCIneoPolyA (Stratagene, La Jolla, CA, USA)] was inserted into the unique KpnI site in intron IV **(Figure 2.4)** (Raineri et al., 2001). This fragment did not introduce a start codon or an alternative splice site and served solely as a marker for PCR. pSP72 (Promega, Madison, WI, USA) was used as the cloning vector. Cells of a transgenic embryonic stem cell clone that exhibited a 5-fold increase in SOD2 activity were injected into day 2.5 pc 8-cell to morula stage CD1 embryos. After overnight culture, healthy blastocysts were transferred to day 2 pseudopregnant B6D2F1 females. Tg^{hSOD2} is ubiquitously expressed with expression levels varying in different tissues. Transgenic mice had similar weights, hematocrits (elevated in all hypoxia samples, confirmed hypoxia exposure), and displayed no phenotypic differences from wild-type littermate

controls. Littermate controls and Tg^{hSOD2} mice, ages 6-9 weeks, were utilized for these studies.

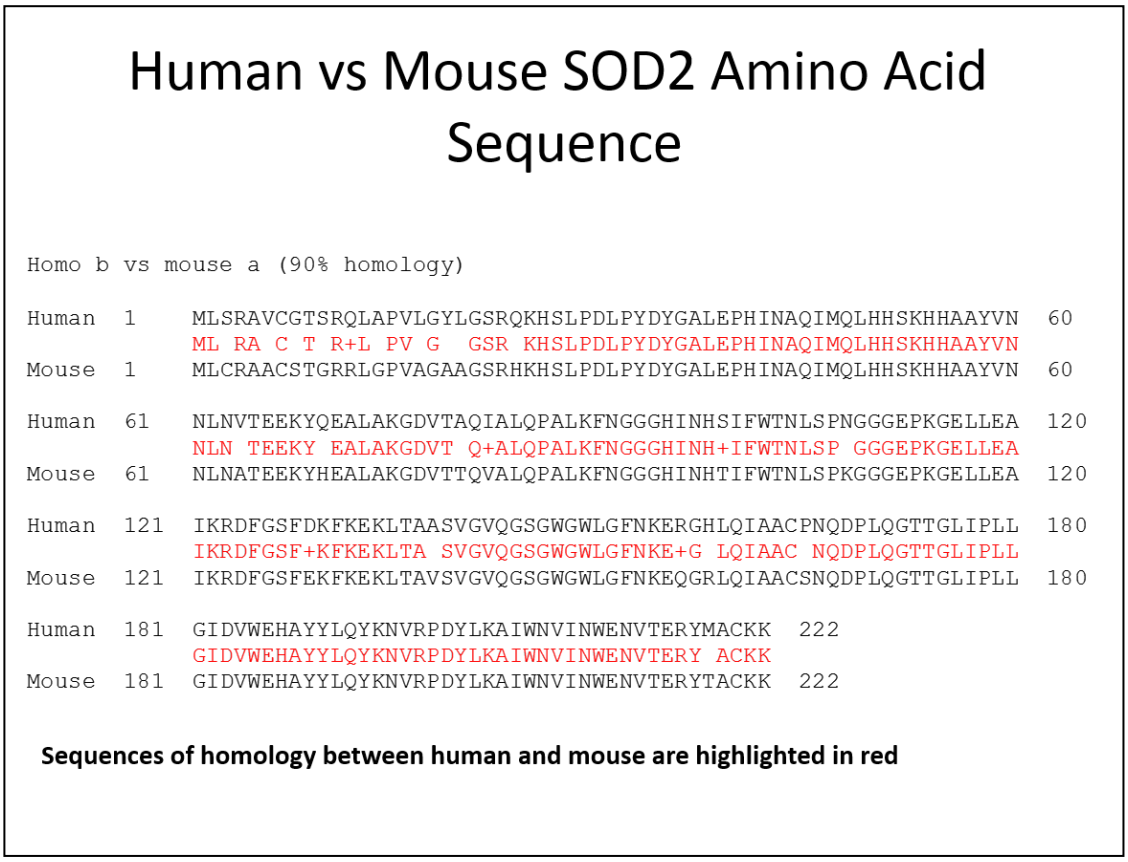
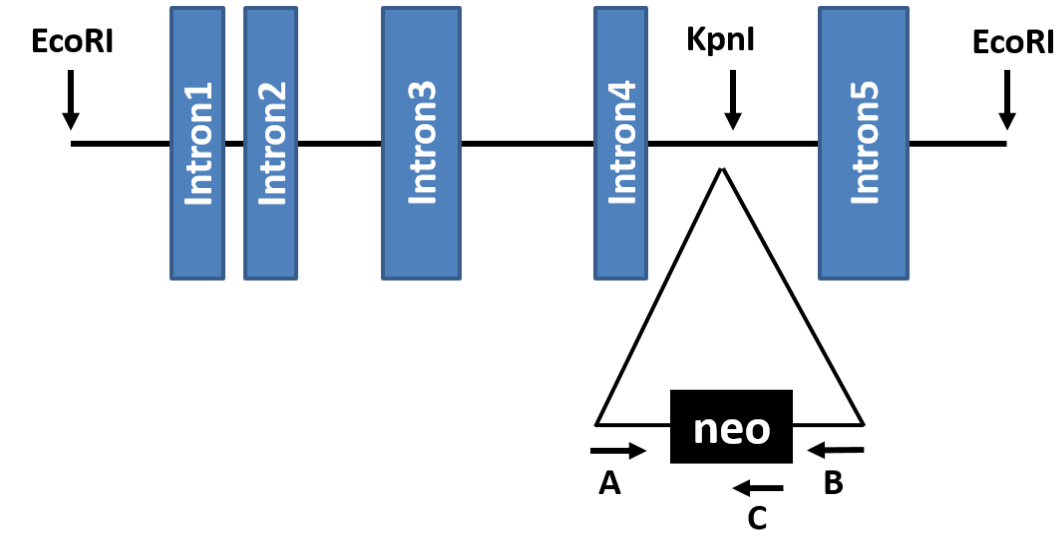


Figure 2.3: Human vs Mouse Manganese Superoxide Dismutase 2 Amino Acid sequence.



13 kb genomic neo DNA fragment, inserted into KpmI site, used for generation of transgenics is shown. Boxes with numbers indicate exons 1 to 5.

Figure Adapted from :Ines Raineri. Free Radical Biology and Medicine, Volume 31, Issue 8, 2001, 1018 - 1030

Figure 2.4: SOD2 transgene generation.

Thioredoxin 2 Overexpression (Tg^{hTrx2})

A transgenic mouse model overexpressing mitochondrial thioredoxin 2 (Tg^{hTrx2}) was also employed. Trx2 mice were provided by Dr. Dean Jones (Emory University). Briefly, zygotes expressing human Trx2 DNA were microinjected into pseudopregnant female mice. The chloramphenicol acetyltransferase (CAT) reporter gene was regulated by the CAG promoter (a combination of (C) the cytomegalovirus (CMV) early enhancer element (A), the promoter, the first exon, the first intron of chicken beta-actin gene, and (G) the splice acceptor of the rabbit beta-globin gene) **(Figure 2.5)**. Transgenic offspring were genotyped to confirm the insertion of the construct into the genome. Mice were then crossed with mice where Cre recombinase expression is regulated by the Protamine promoter. The Protamine promoter expresses specifically in the male germ line and the flox-stop-Trx2 mouse was created with the option of tissue-specific expression (He; Kleinhenz, 2015; O'Gorman et al., 1997). The product of Cre gene recombination (Cre recombinase) excises the DNA sequences that are between the two LoxP sites. This allows free transcription of the transgene, hTrx2 with V5 epitope on the carboxyl terminus, under the CAG promoter. The initial mice that were produced from the pseudopregnant mice (founder lines) were mated with Protamine-Cre recombination mice. The DNA containing the transgene was microinjected into fertilized eggs isolated from C57/BL mice and transplanted into CD-1 female pseudopregnant recipient mice. The offspring of this crossing were bred with 129SVev wildtype mice (Taconic) to generate

transgenic mice used for the study (He). The Trx2 is ubiquitously expressed with expression levels varying in different tissues. Transgenic mice had similar weights, hematocrits (elevated in all hypoxia samples, confirmed hypoxia exposure), and displayed no phenotypic differences from wild-type littermate controls. Littermate controls and Tg^{hTrx2} mice, ages 6-9 weeks, were utilized for these studies.

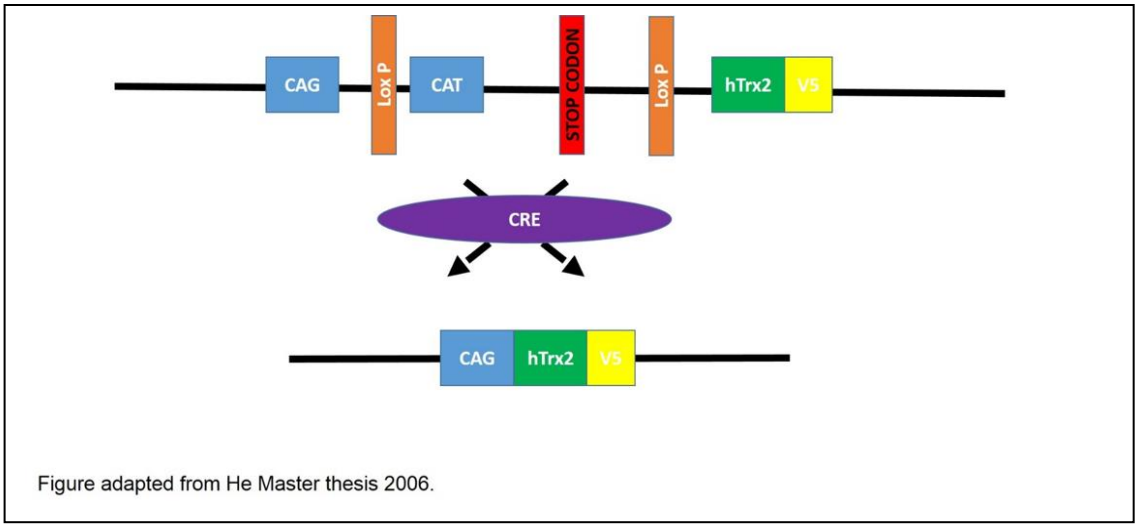


Figure 2.5: Trx2 Transgene generation.

Hypoxia Exposure

To assess the effects of transgene overexpression on pulmonary hypertension development, mice were either housed in ambient air (normoxia, 21% O_2) or hypoxic conditions (10% O_2) for three weeks (Green et al., 2012; Nisbet et al., 2010). A ProOx C-Chamber hypoxia chamber run by the ProOx model 110 (BioSpherix Lacona, NY) was used to maintain hypoxic conditions.

Right Ventricular Systolic Pressure Assessment

Right ventricular systolic pressures (RVSP) were assessed using a 0.8 F micro-tip pressure transducer. Mice were anesthetized with isoflurane, and depilatory cream was applied to the neck to remove hair. A ventral midline incision was made from the lower mandible to the sternum. The submaxillary glands were gently separated by blunt dissection. The jugular vein was isolated using fine forceps. Two #6-0 surgical silk sutures were passed under the jugular vein and used for both ligation and retraction. The superior suture was tied off and the inferior suture was placed as far caudal as possible. Using a 25 g needle with a bend at the beveled tip, a small hole was made in the jugular vein. The needle tip was used to hold open the jugular vein while the SciSense pressure catheter was inserted. The catheter was advanced into the right ventricle, and right ventricular pressure was monitored for 20 minutes. Data were analyzed using the Powerlab system (AD Instruments, Denver, CO) (Nisbet

et al., 2010). Waveforms were initially assessed at 500:1 visual ratio to identify any irregularities in waveforms. Using the Windows tab within the Powerlab program, we used the search feature to identify comments. All comments provided a measurement of blood pressure and heart rate assigned to identify every mouse. Datapad feature was used to identify the values for the average height of each waveform – the systolic pressure within the right ventricle. Three to six 10-second pressure replicates per mouse were averaged for each treatment group. Following completion of data collection, the catheter was removed, and the neck of the mouse was sutured. The mouse was euthanized using CO_2 within 30 minutes of the procedure.

Right Ventricular Hypertrophy Measurement

Right ventricular hypertrophy was assessed by determining right ventricle/left ventricle + septum weight ratios (Fulton Index). Mouse hearts were removed and the right ventricle was dissected from the left ventricle and septum. The weight of the right ventricle was measured and recorded in milligrams. The weight of the left ventricle plus septum was measured and recorded in milligrams. The Fulton Index was calculated as the weight of right ventricle divided by the weight of the left ventricle plus septum. The weight ratio of the right ventricle to the left ventricle and septum (Fulton Index) were averaged for each treatment group.

Echocardiography

Transthoracic echocardiograms (TTEs) were performed on mice using a Vevo 770 High-Resolution In Vivo Imaging System (VisualSonics, Toronto, ON, Canada) equipped with a RMV 707B High-Frame-Rate Scanhead (frequency band 15– 45 MHz) and a SONOS 5500 ultrasound unit (Philips Medical Systems, Bothell, WA) equipped with a 15-MHz linear-array transducer and a 12-MHz phase-array transducer (as described previously (Nisbet et al., 2009). Echocardiograms were acquired before hypoxia exposure (baseline), then again at the end of 3-weeks of hypoxia exposure. During echocardiography, the animals were lightly anesthetized with 1% isoflurane, and the body temperature was continuously monitored using a rectal thermometer probe to maintain body temperature at 36-37°C. Under these conditions, the animal's heart rate could be maintained between 400 and 550 beats per minute. Two-dimensional and M-mode echocardiography was used to assess wall motion, chamber dimensions, and wall thickness and to calculate the fractional shortening. Color flow Doppler was used to assess valve functions and the area (cm^2) of all heart chambers.

Assessment of Pulmonary Arteriolar Muscularization

Immunohistochemical and morphometric analysis was performed to assess pulmonary vascular remodeling. Lungs were perfused blood-free at

atmospheric pressure with Calcium-free PBS and placed in formalin overnight. Lung tissue was then paraffin embedded and sliced into 5 μm sections using a Microtome Microm HM340E. These lung sections were fixed in 4% formaldehyde, washed three times (5 min each) in PBS, and endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS. Sections were then permeabilized with 0.05% Tween-20 (PBS-T), blocked with 5% donkey serum and incubated overnight at 4°C with rabbit anti-α-smooth muscle actin (α-SMA) antibody (LabVision Corporation, Fremont, CA) or PCNA antibody (Santa Cruz) . Sections were incubated with biotinylated donkey anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) followed by horseradish peroxidase–streptavidin (Vectastain kit, Vector Laboratories, Burlingame, CA) as previously reported by our laboratory (Green et al., 2012; Nisbet et al., 2010). Primary antibody was removed, and lung sections were washed 3x for 5 minutes with PBS-Tris. 100 μL of ImmPRESS (ImmPRESS HRP Anti-Rabbit Ig (Peroxidase) Polymer Detection Kit, Vector Laboratories, Burlingame, CA) anti-rabbit secondary HRP solution was dripped onto each section then incubated for 1 hour at room temperature. ImmPRESS solution was removed and each section washed again with PBS-Tris. ImmPACT DAB was applied to each slide for 2-5 minutes. After color change, the slides were washed in water and incubated for 5 minutes. After a 30-second hematoxylin bath, and then a PBS bath, slides were exposed to dehydration series: 2 minutes 95% ethanol, 2 minutes 100% ethanol, 1 minute 50% ethanol and 50% xylene, and finally 10 minutes in 100% xylene. After drying in a fume hood, slides were

covered in 6 drops of Permount and a coverslip. The following formula was used to assess vessel thickness: lumen cross-sectional (subtract inner radius from outer radius to assess vessel thickness – r = P/2π (r, radius, P, perimeter, π, pi 3.14)). Five μm thick lung sections were stained with PCNA. Brown staining indicated by arrows represents PCNA positive staining in the media of small pulmonary arterioles (≤ 100 μm). PCNA staining was quantified as PCNA positive cells per 100 μm^2 area.

Mitochondria Isolation

Cardiac and pulmonary tissues were perfused with 0.5% EDTA-PBS and processed for mitochondria isolation using a mitochondria isolation kit (Thermo Scientific, Waltham, MA). Four lungs per condition were pooled to increase mitochondrial concentration. Immediately before use, protease inhibitors were added to BSA/Reagent A solution and Reagent C. Briefly, the tissues were lysed by manual cutting and further glass – on – glass dounce homogenization and added to 800 μL of BSA/Reagent A. 800 μL of Mitochondria Isolation Reagent C was added to Eppendorf tubes containing sample and then inversion mixed. Samples were then subjected to a centrifugation protocol: 700 x g for 10 minutes at 4°C, transfer supernatant, 3,000 x g for 15 minutes at 4°C, remove the supernatant (cytosolic fraction). 500 μL of wash buffer was added to the mitochondrial pellet, and then centrifuged at 12,000 x g for 5 minutes. The supernatant was discarded and the mitochondrial fraction resuspended in

mitochondria lysis buffer (2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) in Tris Buffered Saline).

Amplex Red Detection of H_2O_2 in Pulmonary Tissue

H_2O_2 was measured by horseradish peroxidase-catalyzed oxidation of the non-fluorescent molecule N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; Invitrogen) into the highly fluorescent molecule resorufin. Lung tissue sections were pre-incubated with Krebs Ringer's Phosphate Glucose (KRPG) buffer for 1 hour. KRPG was composed of: 145 mM NaCl, 5.7 mM KH_2PO_4, 4.86 mM KCl, 0.54mM $CaCl_2$, 1.22 mM $MgSO_4$, and 5.5 mM glucose. Samples were then incubated in KRPG buffer containing 100 µl/ml Amplex Red and 0.2 U/ml HRP for 1 hour at 37˚C. Menadione (0.5 M) was used as positive control or 1000 Units PEG-Catalase was used as negative control to assess H_2O_2 production. Lung tissue sections are cut into sections and incubated in Amplex Red reaction mix for 1 hour. Resorufin fluorescence was measured with a Wallac fluorimeter (PerkinElmer**,** Waltham, MA) at excitation and emission wavelengths of 540 nm and 590 nm, respectively. Sample fluorescence was compared to that generated by a H_2O_2 standard curve to calculate the concentrations of H_2O_2 released from tissue. H_2O_2 concentrations were normalized to wet tissue weight as described (Green et al., 2012; Williams et al., 2012).

Real-time qRT-PCR mRNA Analysis

Quantitative real-time 2 – step PCR was employed to measure mRNA levels of Trx2, catalase, Nox2, Nox4, cyclinD1, proliferating cell nuclear antigen (PCNA), SOD2, Prx3, and Trx2R and GAPDH *in vitro* and *in vivo* (HPAECs, HPASMCs, MCAT, Tg^{hTrx2}, and Tg^{hSOD2}) (Table 2.1). Total RNA was isolated from HPAECs and mouse lung tissue using Trizol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Briefly, homogenized samples were resuspended in 1 mL Trizol. 200 µL of chloroform was added to each sample and inverted vigorously before being centrifuged at 12,000 rpm for 10 minutes at 4°C to separate phases. The top aqueous layer containing RNA was placed into a new tube with 500 µL isopropanol which was inverted gently, then placed on ice for 10 minutes. Samples were again centrifuged at 12,000 rpm for 30 minutes at 4°C to pellet RNA. The liquid was aspirated, and the remaining pellet was washed with 70% ethanol. The samples were again centrifuged at 12,000 rpm for 30 minutes at 4°C, then placed in -80°C for 30 minutes to better visualize the RNA pellet. Ethanol was aspirated, and pellets were allowed to air dry. The RNA pellet was finally resuspended in 20 µL nuclease-free water. RNA concentration was estimated by spectrophotometer.

Total RNA was reverse transcribed to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). 10 µL of 1 µg RNA was mixed with

4 µL 5X iScript Reaction Mix, 5 µL DEPC water, and 1 µL iScript Reverse Transcriptase Mix for a final volume of 20 µL. Samples were cycled at 25°C for 5 minutes, 42°C for 45 minutes, 85°C for 5 minutes, and held at 4°C until cDNA samples could be diluted 1:10 with deionized water.

Target cDNA was amplified using SYBR Green master mix (Applied Biosystems, Hercules, CA). Briefly, samples were held at 95°C for 10 minutes. Sequences were denatured at 95°C for 15 seconds, then annealed and extended at 60°C for 1 minute for 40 cycles. Master mix contains: dimethyl sulfoxide, glycerine, Tris-HCl, and sodium azide. All data were normalized to the content of endogenous GAPDH in the same sample as previously described (Green et al., 2012).

Sample	Genotype	Sequence (5' → 3')
Catalase	Human	For: AAT TTT CGG TGT GGA CAA CCA Rev: CTC CTT CCA GTG CTG CAT CTG
Nox2	Mouse	For: TGA GAG GTT GGT TCG GTT TT Rev: GTT TTG AAA GGG TGG GTG AC
Nox4	Mouse	For: CAG TCC TGG CTT ATC TTC GAG Rev: GAG TCT TGC TTT TAT CCA ACA ATC T
GAPDH	Mouse	For: AGC TTG TCA TCA ACG GGA AG Rev: TTT GAT GTT AGT GGG GTC TCG
GAPDH	Human	For: GCC CAA TAC GAC CAA ATC C Rev: AGC CAC ATC GCT CAG ACA C
PCNA	Mouse	For: CTA GCC ATG GGC GTG AAC Rev: GAA TAC TAG TGC TAA GGT GTC TGC AT
Trx2	Human	For: AGG GAG GGC TAG GCT GTG REV: ACT GAC CCT GAG AGG GCT TC
Trx2	Mouse	For: CAC ACA GAC CTT GCC ATT GA Rev: CAC GTC CCC GTT CTT GAT
SOD2	Mouse	For: TGC TCT AAT CAG GAC CCA TTG Rev: GTA GTA AGC GTG CTC CCA CAC
SOD2	Human	For: AAG TAC CAG GAG GCG TTG G Rev: TGA ACT TCA GTG CAG GCT GA
CyclinD1	Mouse	For: TTT CTT TCC AGA GTC ATC AAG TGT Rev: TGA CTC CAG AAG GGC TTC AA
CyclinD1	Human	For: GCT GTG CAT CTA CAC CGA CA Rev: TTG AGC TTG TTC ACC AGG AG

Table 2.1: Sequence of all primers for qRT-PCR.

Western Blot Analysis

Levels of selected proteins including catalase, Nox2, Nox4, CyclinD1, PCNA, cytochrome c, and β-actin were assessed in various tissues by Western blotting. After washing in PBS, tissues or cells were manually homogenized in buffer (20 mM Tris pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4). Samples were sonicated 10x for 2-seconds. Next, homogenates were centrifuged for 5 minutes at 4°C and supernatants transferred. The protein concentrations were measured by BCA (BioRad) by comparing colorimetric intensity of samples to bovine serum albumin (BSA) standards. 30 µg of protein was loaded in 10% Bis-Tris gels (Invitrogen, Carlsbad, CA), then transferred onto a nitrocellulose membrane (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat dry milk (NFDM). Nitrocellulose membranes were probed with rabbit anti-catalase (Athens Research & Technology), mouse anti-$gp91^{phox}$/Nox2 (BD Transduction Laboratories), rabbit anti-Nox4 (Abcam), rabbit anti-cyclinD1 (Santa Cruz), mouse anti-PCNA (BD Transduction Laboratories), mouse anti-Cytochrome C (BD Pharmingen), with β-Actin (Santa Cruz) used as protein loading control in NFDM overnight at 4°C (Table 2.2). After washing 3x for 10 minutes with PBS-Tris, membranes were incubated with UV-conjugated secondary antibody (LiCor Biosciences, Lincoln, Nebraska) for 1 hour at room temperature. Immunodetection was performed using a UV method (LiCor). Bands were quantified by comparing the densitometric intensity of bands of interest

normalized to β-actin densitometry in same sample. Nitrocellulose membranes were probed with rabbit anti - Trx2 (synthesized by Young-Mi Go PhD, Emory University), rabbit anti-Nox4 (Abcam), with β-Actin (Santa Cruz) used as protein loading control in NFDM overnight at 4°C. Nitrocellulose membranes were probed with SOD2 and β-Actin (Santa Cruz) was used as protein loading control in NFDM overnight at 4°C. After washing 3x for 10 minutes with PBS-Tris, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour at room temperature. Immunodetection was performed using an enhanced chemiluminescence (ECL) (emission at 428 nm) method (ChemiDoc).

To assess oxidized or reduced levels of Trx2, native gels were manually prepared. Samples were treated with 10% trichloroacetic acid (TCA) then transferred to microcentrifuge tubes. Samples were centrifuged for 5 minutes at 12,000 x g at 4°C, and the supernatants were removed. 100% acetone was added to each pellet, vortexed, and incubated for 30 minutes on ice. These preparations were then centrifuged at 12,000 x g at 4°C, then resuspended in 100 µL of lysis/derivatization buffer (0.05 mL 1M Tris-HCL, pH8, 0.01 mL 10% SDS, 0.94 mL deionized water, and 8.04 mg 4-acetamido-4'-maleimidylstilbene-2,2'- disulfonic acid (AMS)) for 3 hours in the dark. Separation and stacking gel preparation quantities and protocol for native conditions are detailed in Table 2.3. Native running buffer was prepared at 10x concentration (120 g Tris base and

576 g glycine in 4 liters distilled water). The loading buffer for non-reducing samples was prepared at 5x (Table 2.3).

Antibody	**Secondary**	**Concentration**	**Item Number**
Catalase	Rabbit	1:100	BD Pharmingen 554532
hCatalase	Mouse	1:100	Santa Cruz sc-365738
Nox2	Mouse	1:200	BD Transduction Laboratories 611415
Nox4	Rabbit	1:200	Abcam ab109225
CyclinD1	Rabbit	1:250	Santa Cruz sc-753
PCNA	Rabbit	1:200	Santa Cruz sc-7907
PCNA	Mouse	1:200	BD Transduction Laboratories
Cytochrome C	Mouse	1:100	BD Pharmingen 554532
β – Actin	Goat	1:2000	Santa Cruz sc-1616
Trx2	Rabbit	1:100	Young-Mi Go (Go et al., 2010)
SOD2	Rabbit	1:1000	Cell Signaling 13141

Table 2.2: Antibody Information.

15% Polyacrylamide Separation gel	15 mL Native (mL)
30% polyacrylamide/0.8% Bis	8
1.5M Tris-HCL, pH8.8	3.2
10% APS	0.06
TEMED	0.01
Distilled H_2O	3.8
6% Polyacrylamide Stacking gel	8 mL Native (mL)
30% polyacrylamide/0.8% Bis	2
0.5M Tris-HCL, pH6.8	2
10% APS	0.04
TEMED	0.008
Distilled H_2O	3.9
Non-Reducing Sample Loading Buffer 5x	
1.5M Tris-HCL, pH6.8	20.8 mL
Glycerol	50 mL
SDS	10 g
Bromophenol Blue	0.05 g
Distilled H_2O	100 mL

Table 2.3: Native Gel Preparation Protocol.

Confocal Microscopy of Mitochondrial ROS

MitoPy1

Human pulmonary arterial endothelial cells (HPAECs) (Clonetics, San Diego, CA) were cultured at 37˚C in 5% CO_2 in endothelial cell growth medium (EGM, Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS), 10 ng/ml human epidermal growth factor, 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 ng/ml amphotericin-B and 12 µg/ml bovine brain extract as we have described previously (Green et al., 2012; Nisbet et al., 2010; Williams et al., 2012). HPAECs were cultured on glass bottom microscope slide plates for 72 hours in normoxia (21% O_2) or hypoxia (1% O_2). A ProOx C hypoxia chamber run by a ProOx model C21 controller (BioSpherix Lacona, NY) was employed for all hypoxia exposures. A subset of the HPAECs were treated with PEG-Catalase (1000 U/mL) for the last 24 hours of exposure to normoxic or hypoxic conditions. To determine if hypoxia induces mitochondrial H_2O_2, cells were incubated with DAPI (1.5 µM) to visualize nuclei, MitoTracker red (1 µM) to identify mitochondria, and MitoPy1 (10 µM) to assess mitochondrial H_2O_2. These probes were added in KRPG. Cells were washed 3x with KRPG buffer and fixed with 1% paraformaldehyde for 30 minutes. Cells were then kept in PBS in the dark until confocal microscopy was performed. Using an Olympus BX51 60x water immersion lens, HPAECs were examined and photographed at 1.5x magnification. All microscope and camera settings were kept consistent across

all conditions and images. Using Fluoview analysis program, individual cells were outlined, and the intensity of MitoPY1 fluorescence was averaged as relative fluorescence units (RFU) per cell. 50 – 100 cells per condition were counted in at least 4 different HPAEC cell lines.

MitoSOX

To detect mitochondrial $O_2^{\cdot-}$, HPAECs were incubated with DAPI (1.5 μM) to identify the nucleus, MitoTracker green (1 μM) to identify mitochondria, and MitoSOX (7.5 μM) to probe for mitochondrial $O_2^{\cdot-}$ in KRPG for 30 minutes in the dark. Cells were washed 3x with KRPG buffer and fixed in 1% paraformaldehyde for 30 minutes. Cells were then kept in PBS in the dark until confocal microscopy was performed. Using an Olympus BX51 60x lens, HPAECs were examined and photographed at 1.5x magnification. All microscope and camera settings were kept consistent across all conditions and images. Using Fluoview analysis program, individual cells were outlined and the intensity of MitoSOX fluorescence was averaged as relative fluorescence units (RFU) per cell. 50 – 100 cells per condition were counted in 4 different HPAEC cell lines.

Statistical Analysis

For all experiments comparing only two groups, statistical analysis was performed by Student's t-test. When more than two groups were analyzed, two-

way ANOVA (samples varied by genotype and oxygen exposure) followed by post-hoc analysis with the Tukey HSD (honest significant difference) test to detect differences between experimental groups (Littermate Control Normoxia, Transgenic Normoxia, Littermate Control Hypoxia, and Transgenic Hypoxia) was employed. A value of $p < 0.05$ was considered statistically significant (Bijli et al., 2015; Green et al., 2012; Kang et al., 2011; Lu et al., 2013; Nisbet et al., 2010; Williams et al., 2012; Yeligar et al., 2012).

CHAPTER 3:
Mitochondrial Catalase Expression Prevents Hypoxia – induced PH

Figures contained in this section have been published:

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INTRODUCTION

PH is a disease of low incidence but high mortality and morbidity (Rabinovitch, 2012). PH is characterized by vasoconstriction and proliferation of pulmonary endothelial and smooth muscle cells that cause pulmonary vascular remodeling, increased pulmonary vascular resistance, and right ventricular hypertrophy that can progress to right heart failure and death (Rabinovitch, 2012). Late stage PAH is characterized pathologically by obliterative plexiform lesions comprised of proliferative, apoptosis-resistant, vascular wall cells (Rabinovitch, 2012; Ryan et al., 2013; Tuder et al., 1994). These vascular changes are caused by multiple stimuli that promote pulmonary endothelial injury (Sakao et al., 2009). Current data suggest that ROS such as $O_2^{\bullet-}$ and H_2O_2 contribute to PH pathogenesis by altering vascular cell proliferation and apoptosis (Fike et al., 2008; Liu and Folz, 2004; Mittal et al., 2007a; Weir and Archer, 2010).

Hypoxia causes pulmonary vasoconstriction through complex mechanisms that involve increased intracellular ROS generation and increased expression of Noxes (Wang and Zheng, 2010). Noxes are important sources of ROS within the vascular wall (Lyle and Griendling, 2006; Sedeek et al., 2009), that can regulate endothelial function, vascular tone, vascular cell hypertrophy, proliferation, and apoptosis (Dikalova et al., 2010; Paravicini and Touyz, 2008). Accumulating evidence implicates Noxes, namely Nox2 and Nox4, act as sources of ROS that can impact long-term responses of the pulmonary

vasculature to hypoxia (Frazziano et al., 2012). Nox2 was the first NADPH oxidase isoform described (Babior et al., 1973; Brown and Griendling, 2009). Nox2 is expressed in cells of the vascular wall including vascular smooth cells and endothelial cells (Bedard and Krause, 2007; Brown and Griendling, 2009). Knockout of $gp91^{phox}$ (Nox2) prevented hypoxia-induced $O_2^{\bullet -}$ production (Liu et al., 2005). Unlike Nox2, Nox4 is a constitutively active isoform responsible for basal H_2O_2 production in the vasculature (Dikalov et al., 2008; Frazziano et al., 2014; Ray et al., 2011; Sedeek et al., 2009). The expression of Nox4 is increased in murine models of hypoxia-induced PH (Green et al., 2012; Nisbet et al., 2010), in the pulmonary vasculature of PH patients (Mittal et al., 2007b; Nisbet et al., 2010; Sutliff et al., 2010), and in pulmonary artery endothelial cells isolated from patients with idiopathic pulmonary arterial hypertension (IPAH) (Green et al., 2012). In chronic hypoxia models, Nox4-derived ROS directly regulate voltage-gated potassium channels (Mittal et al., 2007a), and Nox4 has been suggested to function as an oxygen sensor that produces H_2O_2 and is localized at multiple sub-cellular compartments, including the mitochondria (Dikalov et al., 2008; Frazziano et al., 2014; Sedeek et al., 2009). Taken together, these studies emphasize that hypoxia can increase Nox expression and activity to generate ROS that likely contribute to PH pathogenesis.

Mitochondria are viewed as both targets of Nox-derived ROS and as sources of ROS. The ROS generated by the mitochondria can stimulate Nox activity (Dikalov, 2011; Dikalova et al., 2010). mtROS levels depend on the rate of

O_2 reduction to $O_2^{\bullet -}$ and on the activity of mitochondrial antioxidant mechanisms (Aon et al., 2010). Increasing evidence indicates that mtROS contribute to endothelial cell dysfunction (Min W et al., 2010; Widder et al., 2009) and alter redox-signaling pathways that modulate vascular tone (Fuchs et al., 2010; Shimoda and Undem, 2010; Waypa et al., 2010). In the Ang-II model of hypertension, mtROS activate redox-sensitive transcription factors that stimulate Nox expression (Dikalova et al., 2010). Studies have confirmed that Noxes stimulate mtROS in response to Ang-II, which ultimately results in mitochondrial dysfunction. Apocynin, a Nox inhibitor, blocked the activation of Noxes, and also prevented mtROS generation (Doughan et al., 2008). **Therefore, since mitochondrial dysfunction is associated with increased Nox expression (Frazziano et al., 2014), and mtROS have been shown to stimulate the activity of Noxes (Dikalova et al., 2010), we hypothesized that mtROS may stimulate increases in Nox expression and activity in the lung during PH pathogenesis.**

Because Nox-mediated and mitochondrial-derived ROS may amplify each other, we sought to further explore this mitochondria-NADPH oxidase axis in PH. To assess this, we analyzed the impact of targeted overexpression of catalase (normally expressed in the peroxisome) in the mitochondria (Frazziano et al., 2012; Sies, 1997). This transgenic mouse model with ubiquitously expressed human catalase targeted to the mitochondria (MCAT) has an extended lifespan (Schriner et al., 2005). Evidence that mtROS alter endothelial function (Widder et

al., 2009) and interact with Noxes (Dikalov, 2011) suggests that dysregulation of mitochondrial H_2O_2 (mtH_2O_2) production may participate in PH pathogenesis and that targeted attenuation of mtH_2O_2 generation may prevent PH development. Therefore, this study took advantage of the mitochondrial catalase expression (MCAT) transgenic mouse model to determine the impact of mitochondria derived H_2O_2 on PH progression.

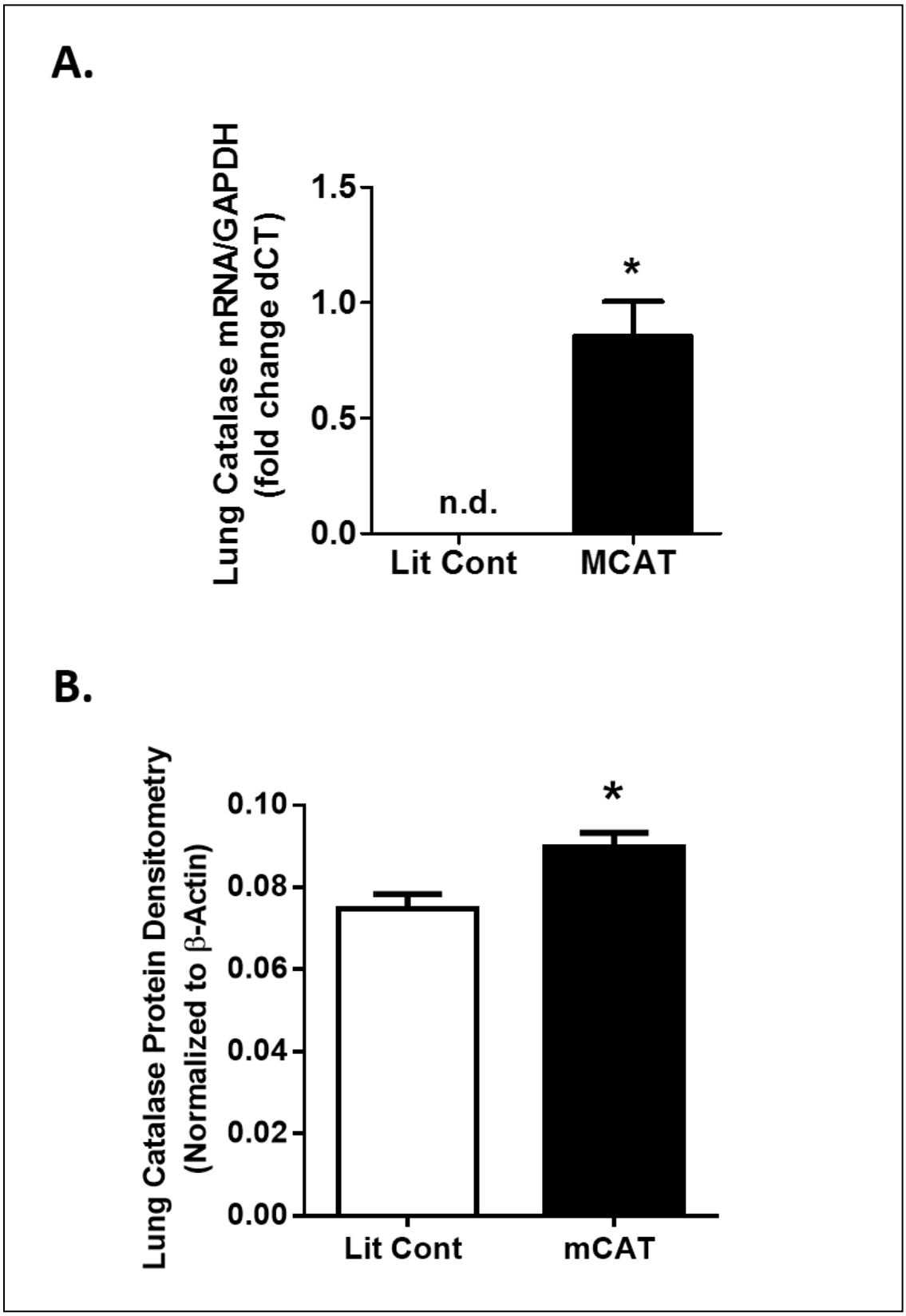
RESULTS

Confirmation of human catalase expression in the MCAT mouse model

Before studies assessing the benefit of mitochondrial catalase could be performed, we established that the transgenic model expressed our target gene of interest, human catalase. Human catalase mRNA and elevated catalase protein were detected in MCAT lung tissue, but not in littermate control (Lit Cont) samples (**Figure 3.1A and 3.1B)**. Since mitochondria content isn't robust in pulmonary tissue, the modest increase in total catalase in MCAT samples is likely due to an added contribution of human catalase from MCAT mitochondria. Human catalase protein was only detected in MCAT samples (**Figure 3.1C)**. Furthermore, in isolated mitochondria samples, exogenous catalase was only present in MCAT samples (**Figure 3.1D)**.

Figure 3.1: Confirmation of human catalase overexpression in MCAT mouse model. Whole lung homogenates were collected from Lit Cont and MCAT mice. qRT-PCR and Western blots were employed to detect expression of human catalase. **(A)** Human catalase mRNA levels were detected only in lungs from mice that carried the MCAT transgene. Each bar represents mean ± SEM of catalase mRNA levels relative to GAPDH expressed as fold-change vs. Lit Cont ($n = 3$), $*p < 0.05$ compared to Lit Cont. **(B)** Total catalase protein expression was increased in MCAT samples. Each bar represents mean ± SEM catalase protein relative to β-actin ($n = 3$), $*p < 0.05$ compared to Lit Cont. **(C)** Human catalase

protein expression was detectable in MCAT samples only. **(D)** Mitochondria were isolated from hearts of Lit Cont and MCAT mice, and western blotting followed by densitometry was employed to measure human catalase levels. Human catalase was exclusively expressed in mitochondria isolated from mice expressing the MCAT transgene. Each bar represents mean ± SEM human catalase protein relative to Cytochrome C expressed as fold-change vs. Lit Cont (n = 3), *$p < 0.05$ compared to Lit Cont.


A.
Lung Catalase mRNA/GAPDH
(fold change dCT)
1.5
1.0
0.5
0.0
*
n.d.
Lit Cont
MCAT
B.
Lung Catalase Protein Densitometry
(Normalized to β-Actin)
0.10
0.08
0.06
0.04
0.02
0.00
*
Lit Cont
mCAT

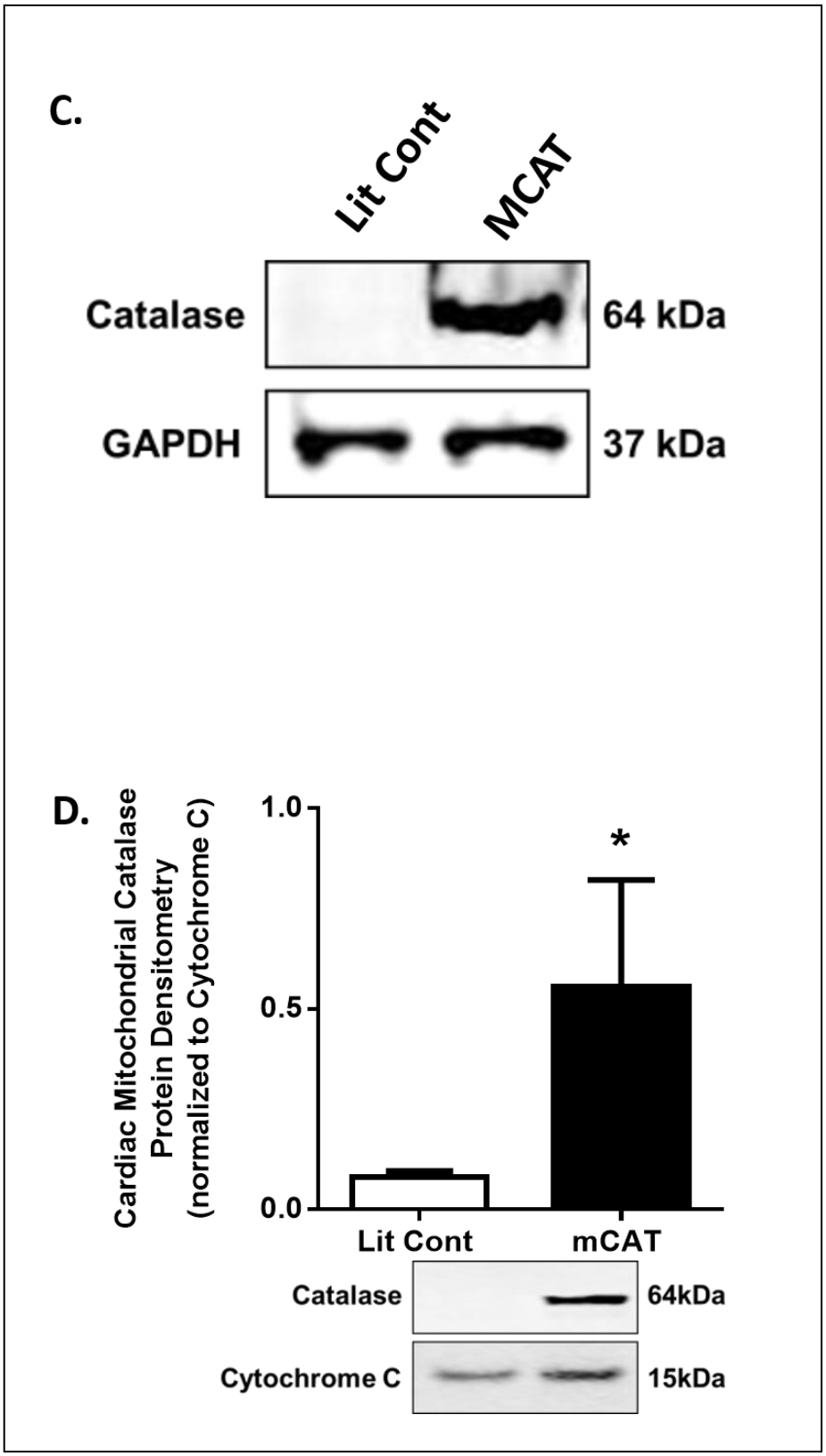


Figure 3.1: Confirmation of MCAT model.

Hypoxia exposure increases mitochondrial H_2O_2 generation in HPAECs.

To examine the effect of hypoxia on mtH_2O_2 levels, HPAECs were cultured under either normoxic or hypoxic conditions for 72 hours and incubated in KRPG buffer containing DAPI, MitoPy1, and MitoTracker Red. MitoPy1 is a mitochondrial-targeted H_2O_2 probe. HPAECs were treated with 1000 U/mL PEG-catalase for the last 24 hours to confirm H_2O_2 specificity. Conjugation of catalase with polyethylene glycol facilitates intracellular delivery (Beckman et al., 1988). Exposure of HPAECs to chronic hypoxia increased MitoPY1 signals in a PEG-catalase inhibitable manner **(Figure 3.2A and 3.2B).** These results indicate that hypoxia increases mitochondria-derived H_2O_2.

Figure 3.2: Hypoxia exposure increases mitochondrial H_2O_2 generation in HPAECs. HPAECs were exposed to normoxia (21% O_2) or hypoxia (1% O_2) for 72 hours. Following exposure, cells were labeled with MitoPy1, MitoTracker Red, and DAPI and assessed for mitochondrial H_2O_2 production by confocal microscopy. **(A)** HPAECs were treated with 1000 U/mL PEG-catalase or DMSO vehicle during the last 24 hours of exposure. Representative images at 90x magnification are presented. Scale bar = 10 microns. **(B)** The fluorescence intensity in 50 – 100 cells from each treatment group is presented as mean ± SEM MitoPy1 relative fluorescence units (RFU)/cell. PEG-catalase treatment eliminates the hypoxia-induced mtH_2O_2 signal (n = 3), *$p < 0.05$ compared to all other groups.

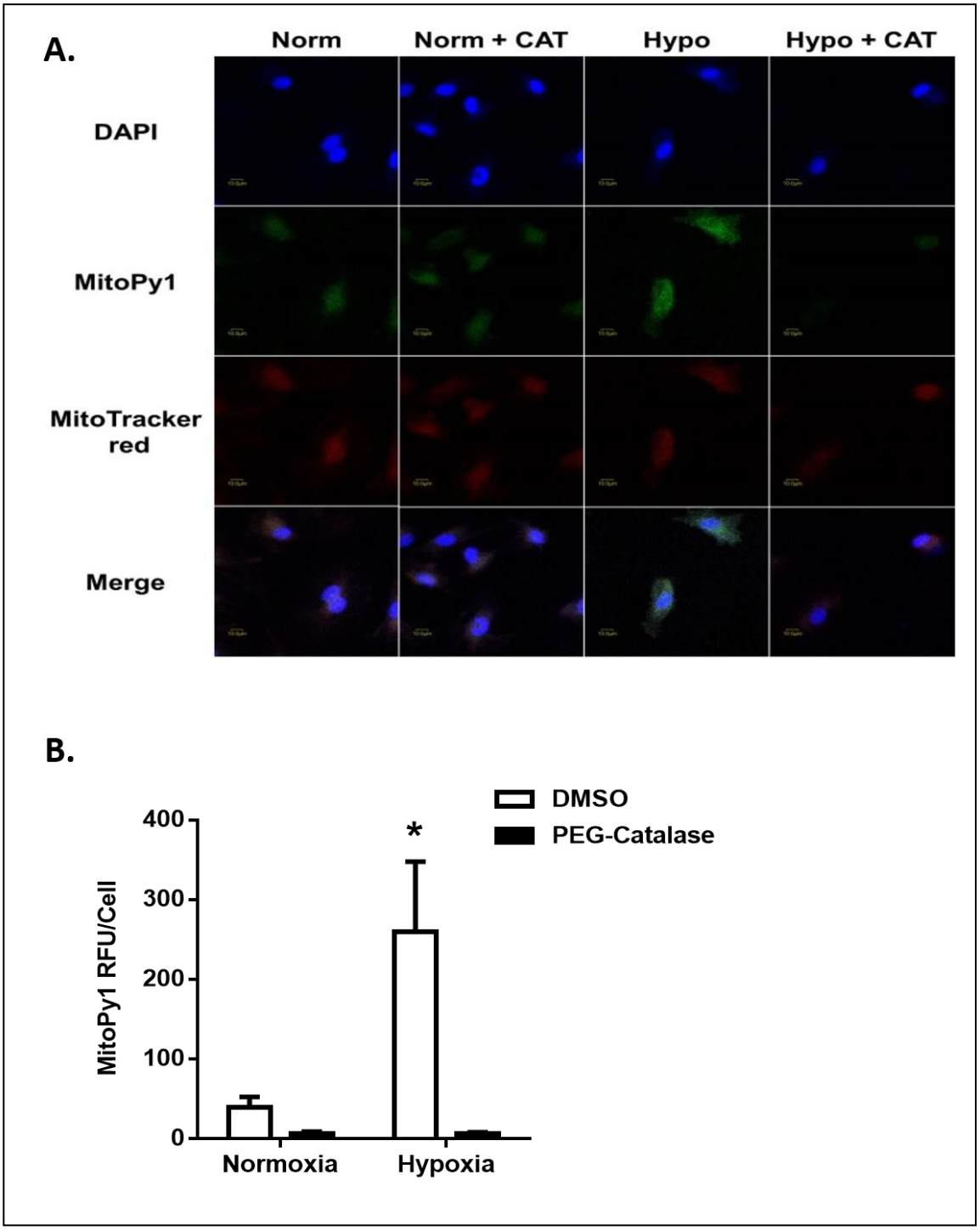


Figure 3.2: Hypoxia increases mitochondrial H_2O_2 generation in HPAECs.

Hypoxia exposure increases NADPH Oxidase and Proliferation Markers in HPAECs.

To determine if hypoxia modulates Nox expression under conditions of increased mtROS, we assessed the effects of hypoxia-on Nox2 and Nox4 mRNA and protein levels. Hypoxia increased mRNA levels of Nox2 **(Figure 3.3A)** and Nox4 **(Figure 3.3B)**. An increase in Nox2 and Nox4 protein was also detected. Because increases in Nox2 and Nox4 have been shown to stimulate HPAEC growth and proliferation, we measured levels of the proliferation markers, cyclinD1 and PCNA (Frazziano et al., 2014; Wedgwood et al., 2013). Hypoxia increased cyclinD1 **(Figure 3.3C)** and PCNA **(Figure 3.3D)**, consistent with hypoxia-induced HPAEC proliferation.

Figure 3.3: Hypoxia increases Nox2, Nox4, CyclinD1, and PCNA levels in HPAECs. HPAECs were exposed to normoxia (21% O_2) or hypoxia (1% O_2) for 72 hours. Following exposure, Nox levels and markers of cell proliferation were assessed by qRT-PCR and Western blotting. **(A – D)** Nox and proliferation marker levels are expressed relative to GAPDH as fold-change vs. Normoxia. Hypoxia elevates levels of Noxes and proliferation markers in HPAECs. Each bar represents mean ± SEM mRNA **(A)** Nox2, **(B)** Nox4, **(C)** PCNA, **(D)** and cyclinD1 ($n = 3$), $^*p < 0.05$ compared to Normoxia.

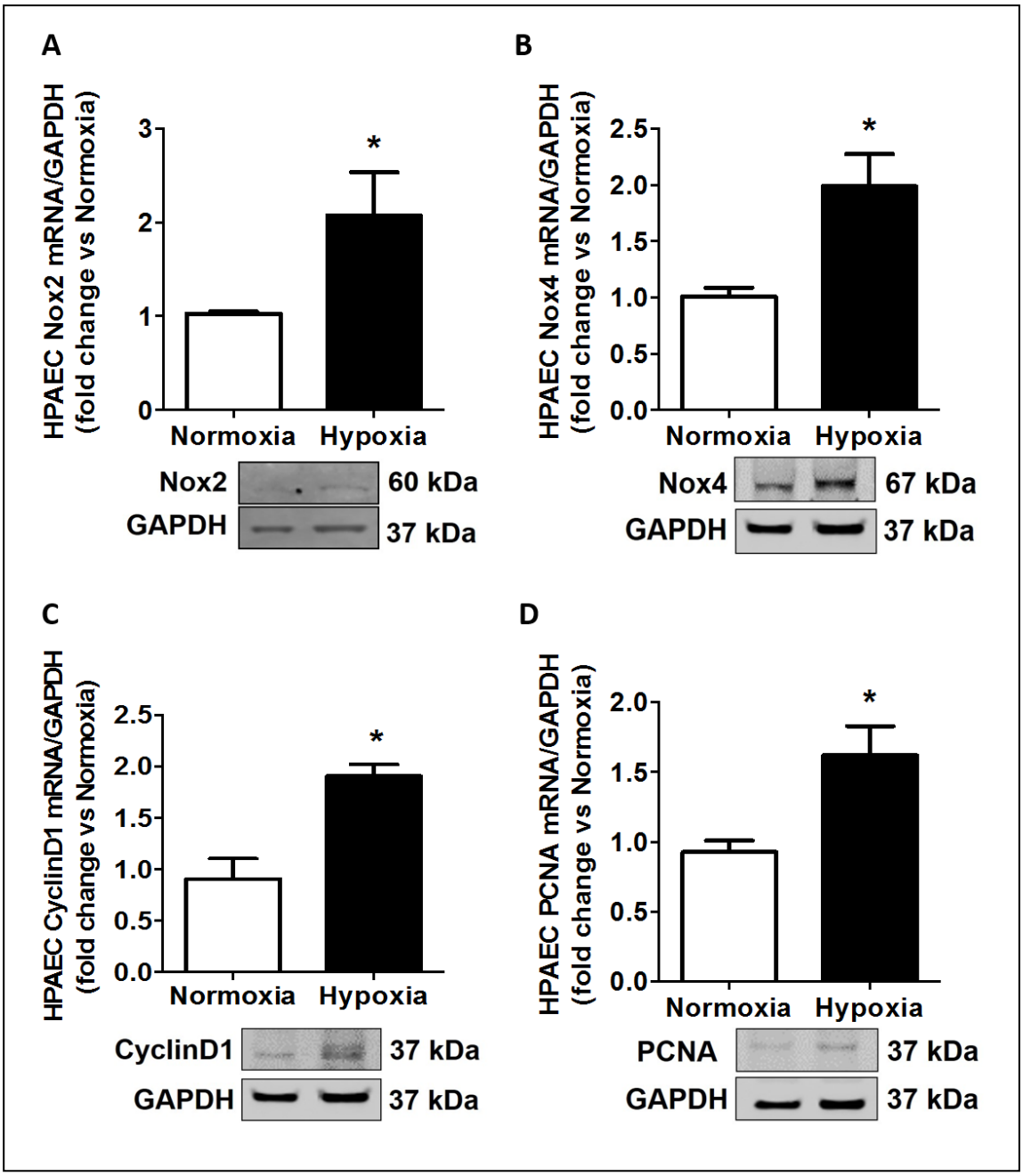


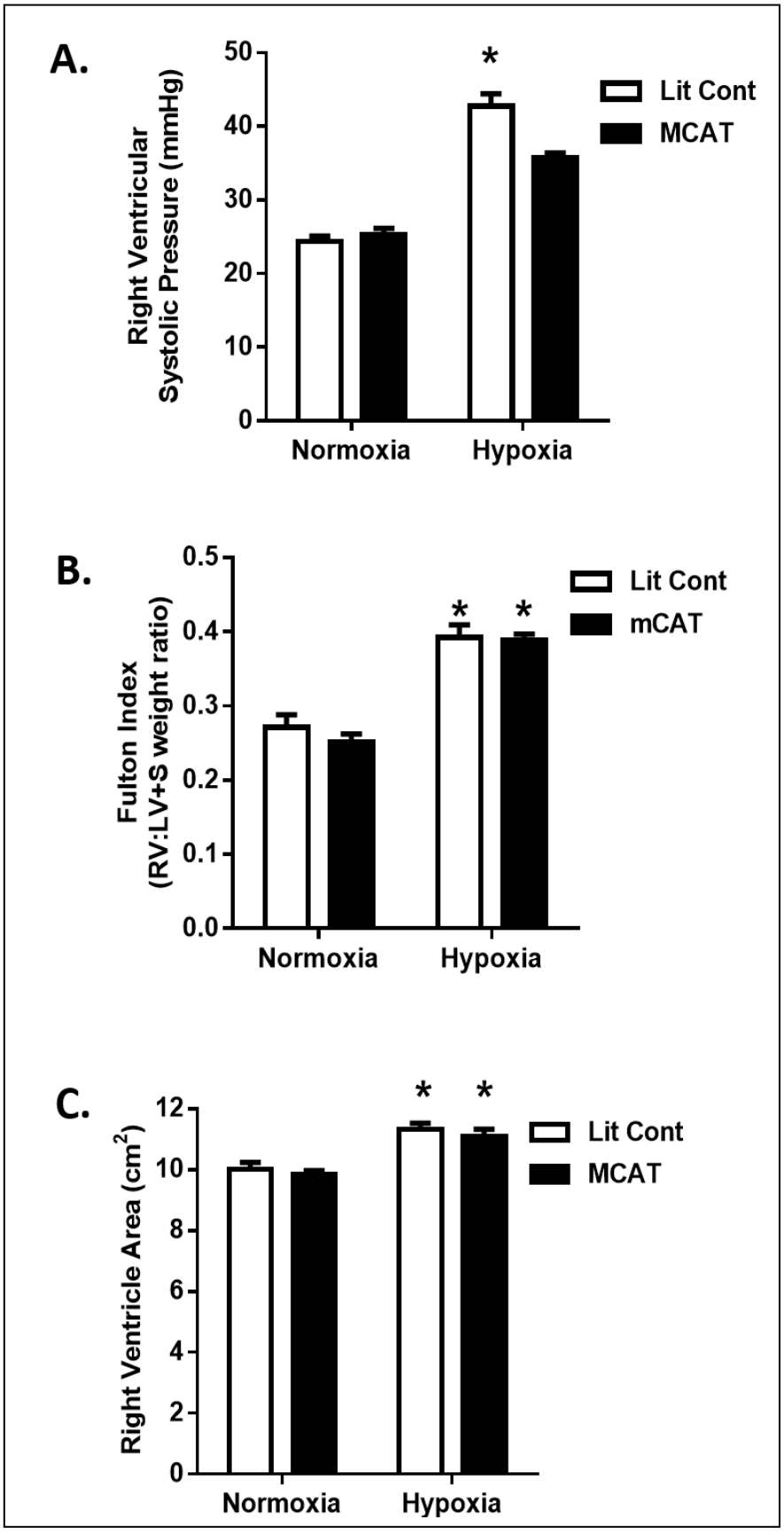
Figure 3.3: Hypoxia increases Nox2, Nox4, CyclinD1, and PCNA levels in HPAECs.

Hypoxia-induced PH is attenuated in the MCAT model

To assess the role of mtH_2O_2 in hypoxia-induced PH *in vivo*, littermate control and MCAT mice (Schriner et al., 2005) were studied following 3 weeks of hypoxia exposure. Hypoxia exposure significantly increased RVSP in littermate controls; however, the increase in RVSP was attenuated in MCAT mice **(Figure 3.4A)**. In addition, compared to hypoxia-exposed Lit Cont, MCAT mice also demonstrated significantly less vascular remodeling, detected as α-SMA staining of small pulmonary arterioles, a critical derangement in PH pathogenesis (Freund-Michel et al., 2013; Morrell et al., 2009; Rabinovitch, 2012). In contrast to the protective effects on RVSP and vessel muscularization, MCAT expression failed to attenuate hypoxia-induced RVH measured by either the Fulton Index, **(Figure 3.4B)** or echocardiographic measurements of the 2 dimensional area of the right ventricular cavity **(Figure 3.4C)**. These results suggest that MCAT expression attenuates hypoxia-induced increases in RVSP and muscularization of small pulmonary vessels, without attenuating RVH.

Figure 3.4: Hypoxia-induced PH is attenuated in the MCAT model. Mitochondrial-targeted catalase expression attenuates hypoxia-induced PH and muscularization of small pulmonary arteries. Lit Cont and MCAT mice were exposed to normoxia (21% O_2) or hypoxia (10% O_2) for 3-weeks. **(A)** Right ventricular systolic pressure (RVSP) was determined with a pressure transducer. MCAT expression attenuated hypoxia-induced elevations in RVSP. Each bar

represents mean ± SEM RVSP in mm Hg (n = 10), *p < 0.05 compared to all other groups. **(B)** Right ventricular hypertrophy was assessed by dissecting and weighing the right ventricle (RV) and the left ventricle + septum (LV + S) and calculating the RV: LV+S weight ratio. Hypoxia induces elevations in RVH. Each bar represents the mean ± SEM RV: LV+S weight ratio (n = 7 – 8), *p < 0.05 compared to both normoxia groups. **(C)** Right ventricular hypertrophy was also assessed by cardiac echocardiography and measurement of the right ventricular area. Each bar represents mean ± SEM RV area in cm^2 (Portnoy and Rudski, 2015). Hypoxia increases right ventricular area (n = 7 - 8), *p < 0.05 compared to both normoxia groups. **(D)** 10 – 20 lung sections (5 μm thick) were stained with α-SMA. Representative images are displayed as indicated. Brown staining indicated by arrows represents α-SMA positive staining in the media of small pulmonary arterioles. Magnification = 40x. **(E)** The wall thickness calculated by dividing total thickness of vessel by inner vessel radius. MCAT expression prevents hypoxia-induced elevations in α-SMA staining of small arterioles (n = 3 - 4), *p < 0.05 compared to all other groups.


A.
Right Ventricular Systolic Pressure (mmHg)
50
40
30
20
10
0
*
Lit Cont
MCAT
Normoxia
Hypoxia
B.
Fulton Index (RV:LV+S weight ratio)
0.5
0.4
0.3
0.2
0.1
0.0
* *
Lit Cont
mCAT
Normoxia
Hypoxia
C.
Right Ventricle Area (cm2)
12
10
8
6
4
2
0
* *
Lit Cont
MCAT
Normoxia
Hypoxia

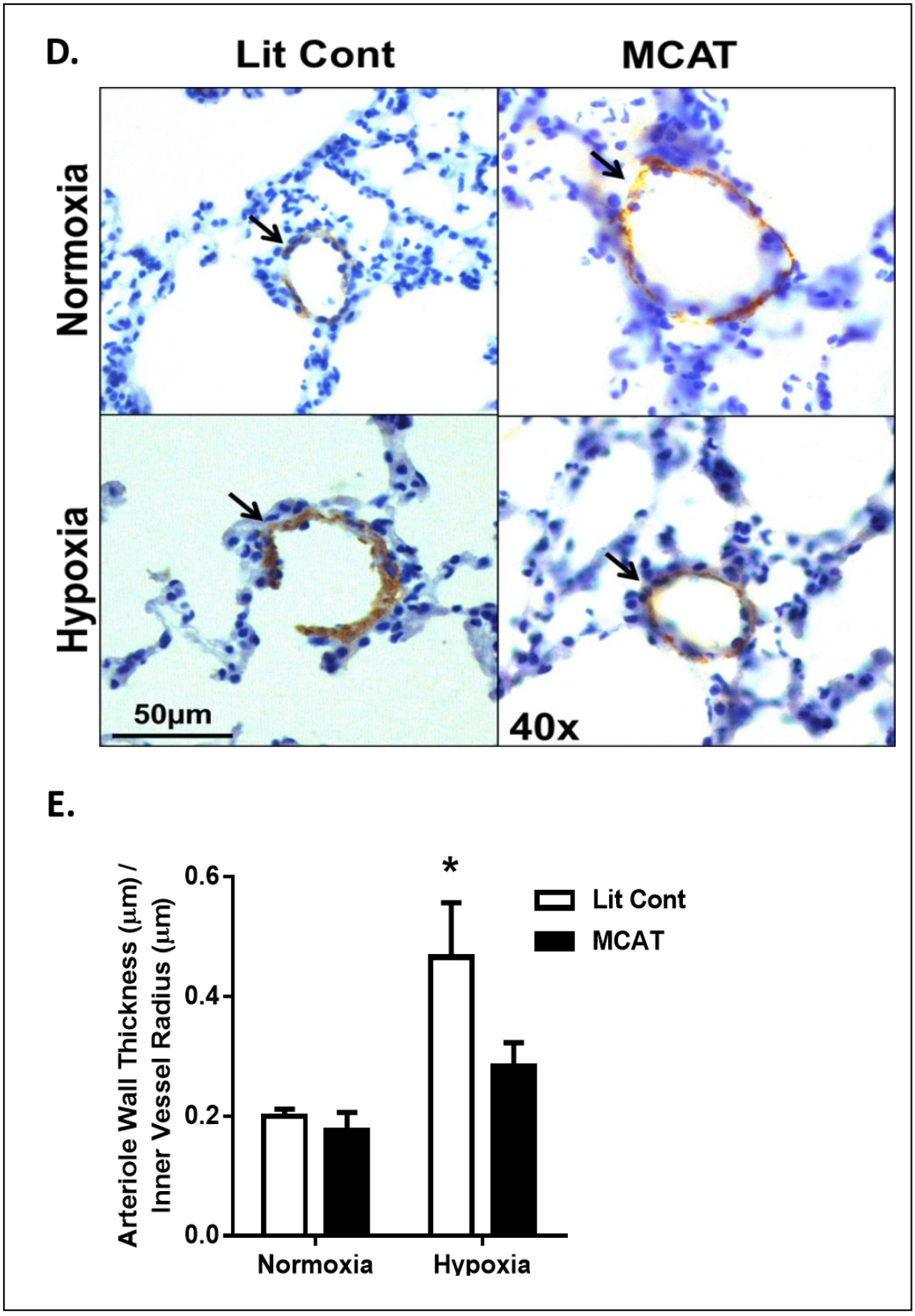


Figure 3.4: Hypoxia-induced PH is attenuated in the MCAT model.

MCAT expression prevents hypoxia-induced increases in Nox expression.

The targets of mtH_2O_2 in these models have not been established. Noxes have been shown to contribute to the physiological derangements seen in hypoxia-induced PH. Nox2 and Nox4 are suggested to contribute to pulmonary vascular responses to hypoxia (Nisbet et al., 2009). To determine the effect of mtH_2O_2 on Nox expression, we assessed the effect of MCAT expression on Nox2 and Nox4 levels in hypoxia-induced PH. Nox2 mRNA and protein levels were attenuated in hypoxia-exposed MCAT pulmonary tissue **(Figure 3.5A and 3.5B)**. MCAT expression also prevented hypoxia-induced elevations in Nox4 mRNA and protein **(Figure 3.5C and 3.5D)**. These data indicate that mtH_2O_2 signaling regulates Nox expression. Furthermore, since Noxes produce ROS as well, hypoxia-induced attenuation of Nox2 and Nox4 expression in the MCAT model may contribute to a global decrease in H_2O_2 levels.

Figure 3.5: Attenuation of mtH_2O_2 in the MCAT model prevents hypoxia-induced increases in Nox 2 and 4 mRNA and protein levels. Lit Cont and MCAT mice were exposed to normoxic or hypoxic conditions for 3 weeks. Whole lung homogenates were collected from Lit Cont and MCAT mice. Levels of Nox 2 and 4 mRNA are calculated relative to GAPDH and expressed as fold-change vs. Normoxia. Protein samples are normalized to β-Actin. **(A)** MCAT expression attenuated hypoxia-induced Nox2 mRNA. Each bar represents mean ± SEM lung

Nox2 mRNA (n = 9), *p < 0.05 compared to all other groups. **(B)** MCAT expression inhibited hypoxia-induced Nox2 protein levels. Each bar represents mean ± SEM lung Nox2 protein (n = 3), *p < 0.05 compared to all other groups. **(C)** MCAT expression prevented elevation of hypoxia-induced Nox4 mRNA levels. Each bar represents mean ± SEM lung Nox4 mRNA (n = 7 - 10), *p < 0.05 compared to all other groups. **(D)** Nox4 hypoxia-induced protein expression was attenuated in MCAT mice. Each bar represents mean ± SEM lung Nox4 protein (n = 3), *p < 0.05 compared to all other groups.

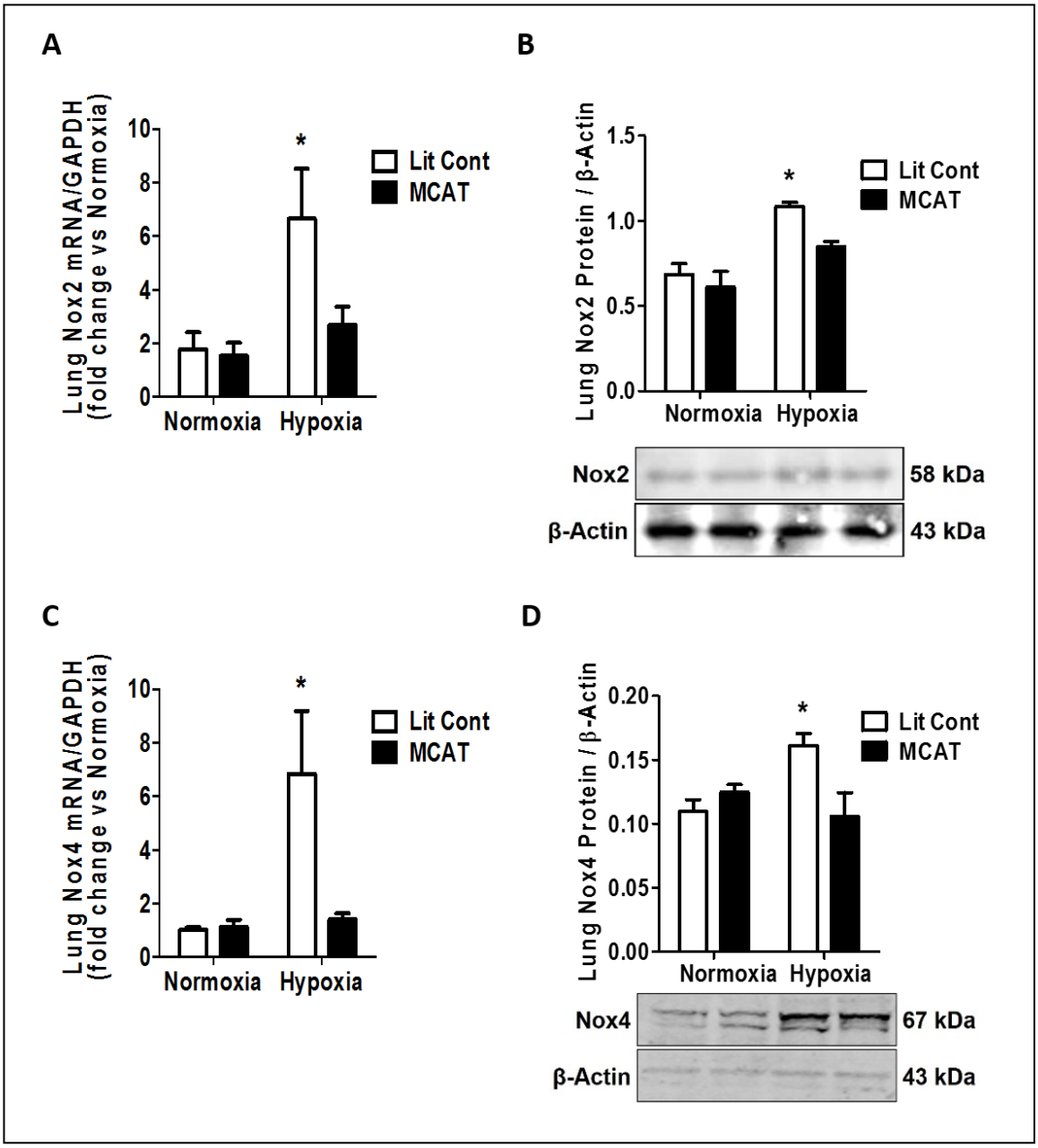


Figure 3.5: MCAT prevents hypoxia-induced increases in Nox levels.

MCAT expression attenuates hypoxia-induced increases in H_2O_2 levels

ROS generation is elevated in PH and our previous results verified elevation of Nox2 and Nox4, which was significantly attenuated in MCAT model. Consistent with reductions in Nox2 and Nox4, MCAT expression significantly attenuated hypoxia-induced elevations in H_2O_2 production **(Figure 3.6)**. These data indicates that targeted attenuation of mtH_2O_2 can prevent hypoxia-induced elevations in total H_2O_2.

Figure 3.6: MCAT expression attenuates hypoxia-induced H_2O_2 production. Amplex Red assay was utilized to assess lung extracellular H_2O_2 levels in lung tissue. (A) MCAT expression significantly decreased hypoxia-induced H_2O_2 production. Each bar represents mean ± SEM H_2O_2 concentration relative to lung tissue wet weight (n = 5 - 6), *$p < 0.05$ compared to all other groups.

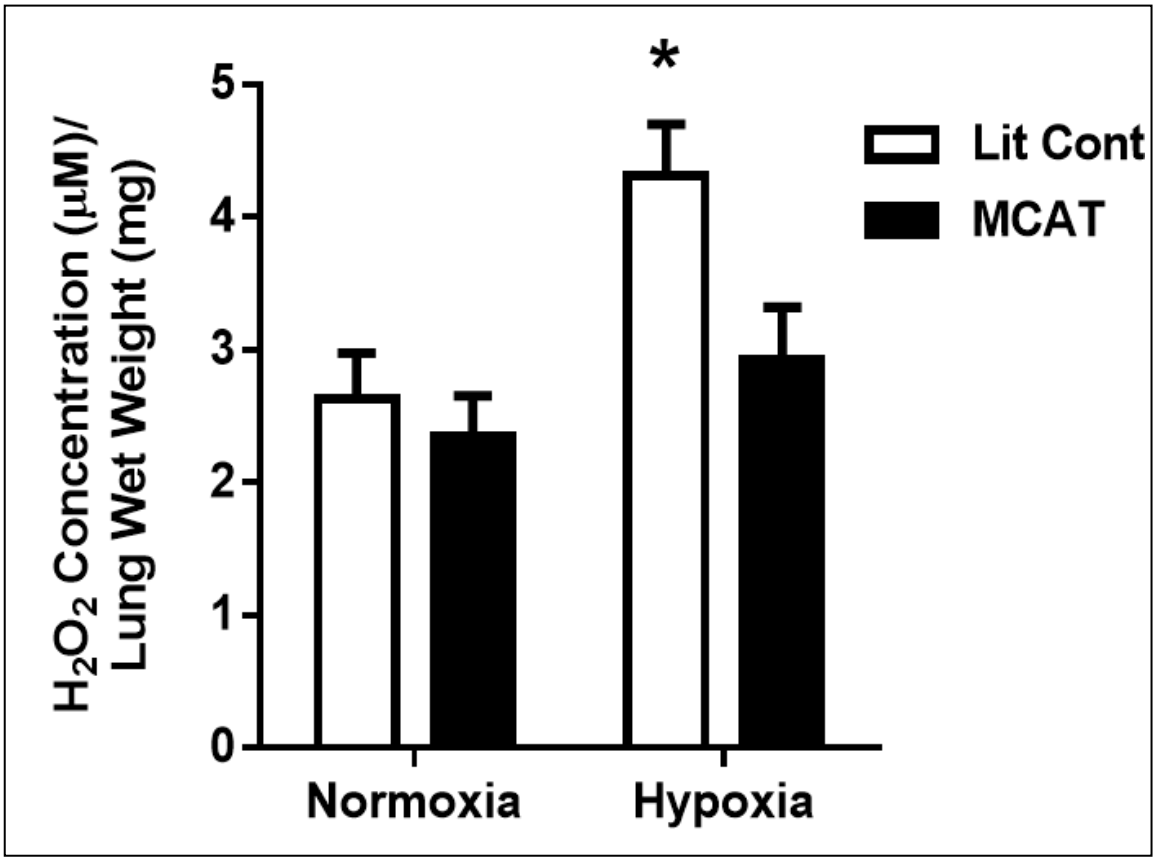
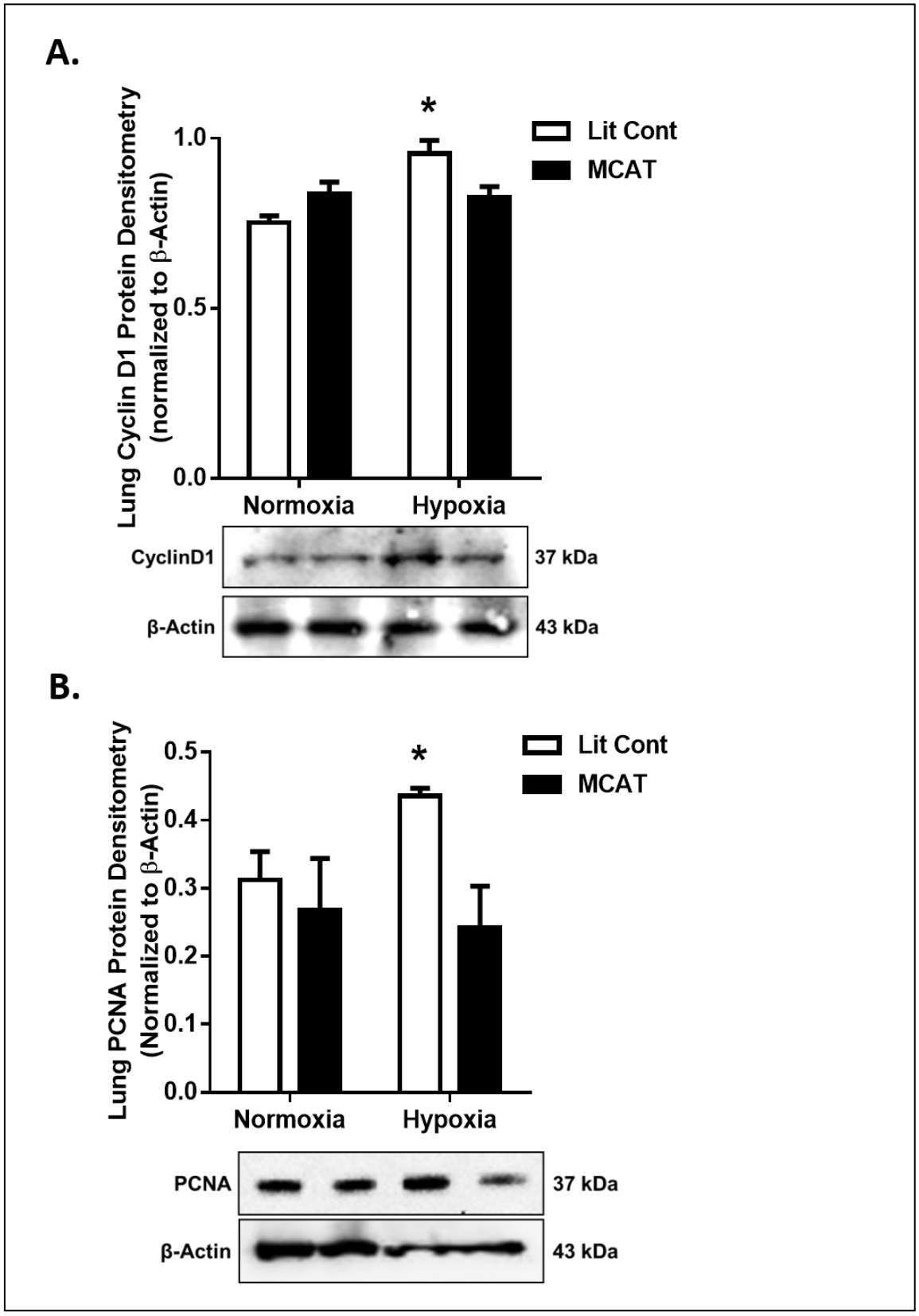


Figure 3.6: MCAT expression attenuates hypoxia-induced H_2O_2 production.

MCAT expression attenuates hypoxia-induced increases in cyclinD1 and PCNA expression

To determine if MCAT expression is protective against hypoxia-induced proliferation, the effects of MCAT on expression of cell cycle regulating proteins and proliferation markers were examined. CyclinD1 regulates cell cycle progression and hypertrophy and can promote vascular cell proliferation and activation of PCNA (Jing et al., 2014; Samaga et al., 2014; Wedgwood et al., 2013). MCAT expression prevented hypoxia-induced elevation of CyclinD1 when compared to normoxia controls **(Figure 3.7A)**. Furthermore, lungs from MCAT mice displayed less downstream hypoxia-induced proliferation as measured by PCNA protein expression in pulmonary tissue **(Figure 3.7B)**. These results suggest that targeting hypoxia-induced mitochondrial H_2O_2 generation can prevent induction of cell cycle and vascular proliferation markers. To further confirm pulmonary vascular smooth muscle cell proliferation, immunohistochemistry was performed to assess nuclear localization of PCNA (brown staining) within endothelial and smooth muscle cell nuclei (blue staining). Lung sections (5-μm thick) were stained with PCNA. Brown staining indicated by arrows represents PCNA positive staining in the media of small pulmonary arterioles (≤ 100 μm). PCNA staining was quantified as PCNA positive cells per 100 μm^2 area. MCAT – representative image of PCNA IHC detection **(Fig. 3.7C)** MCAT expression attenuates hypoxia-induced PCNA IHC detection **(Fig. 3.7D)**

Figure 3.7: Targeting mtH_2O_2 attenuates markers of hypoxia-induced proliferation. Lit Cont and MCAT mice were exposed to normoxic or hypoxic conditions for 3 weeks. Whole lung homogenates were collected from Lit Cont and MCAT mice. MCAT expression prevented hypoxia-induced induction of cyclinD1 and PCNA protein expression. CyclinD1 and PCNA values are normalized to β-Actin or CDK4. **(A)** MCAT expression inhibited hypoxia-induced elevation of cyclinD1 protein. Each bar represents mean ± SEM lung cyclinD1 protein (n = 5 - 6), *p < 0.05 compared to all other groups. **(B)** MCAT expression inhibited hypoxia-induced elevation of PCNA protein. Each bar represents mean ± SEM lung PCNA protein (n =3 - 5), *p < 0.05 compared to all other groups. **(C)** Hypoxia-exposed MCAT mice demonstrated significantly less vascular PCNA staining co-localizing with nuclei – representative image. Nuclear localization of PCNA (brown staining) with endothelial and smooth muscle cell nuclei (blue staining) were analyzed by a reviewer blinded to treatment group. Arrows indicate overlap between brown PCNA staining and blue nuclei. Scale bar = 50 μm; magnification = 40x. **(D)** MCAT expression attenuates hypoxia-induced PCNA IHC detection. Each bar represents mean ± SEM, (n = 3), *p < 0.05 compared to Lit Cont normoxia.


A.
Lung Cyclin D1 Protein Densitometry (normalized to β-Actin)
1.0
0.5
0.0
*
Lit Cont
MCAT
Normoxia
Hypoxia
CyclinD1
37 kDa
β-Actin
43 kDa
B.
Lung PCNA Protein Densitometry (Normalized to β-Actin)
0.5
0.4
0.3
0.2
0.1
0.0
*
Lit Cont
MCAT
Normoxia
Hypoxia
PCNA
37 kDa
β-Actin
43 kDa

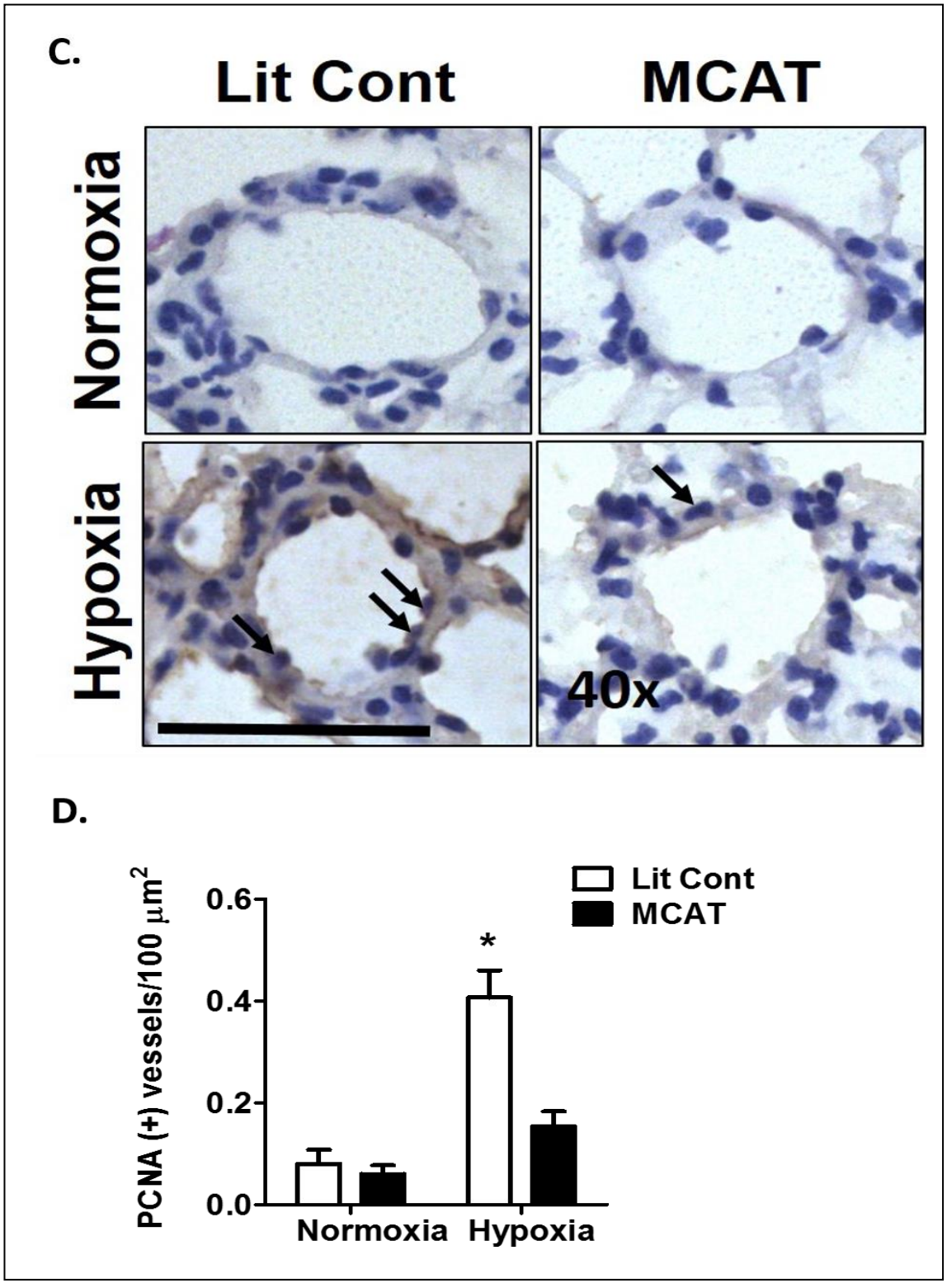


Figure 3.7: Hypoxia-induced cyclinD1 and PCNA expression is attenuated in the MCAT model.

DISCUSSION

The current studies provide novel evidence supporting an important role for mtH_2O_2 in the development of hypoxia-induced PH. As summarized in **Figure 3.8**, hypoxia increases mtH_2O_2 generation which stimulates the expression of Nox 2 and 4 and the physiological and molecular derangements associated with PH pathogenesis. These derangements were targeted using our transgenic MCAT mouse model. Hypoxia-induced H_2O_2 detection and Nox expression, in addition to proliferation, muscularization, and elevation of RVSP were attenuated in the MCAT model. Ultimately, these studies suggest that targeted attenuation of mtH_2O_2 may be sufficient to prevent PH pathogenesis.

Figure 3.8: Role of mtH_2O_2 in the development of PH. Targeted attenuation of mtH_2O_2 with MCAT model (left side of schema) prevents hypoxia-induced PH molecular and physiological derangements.

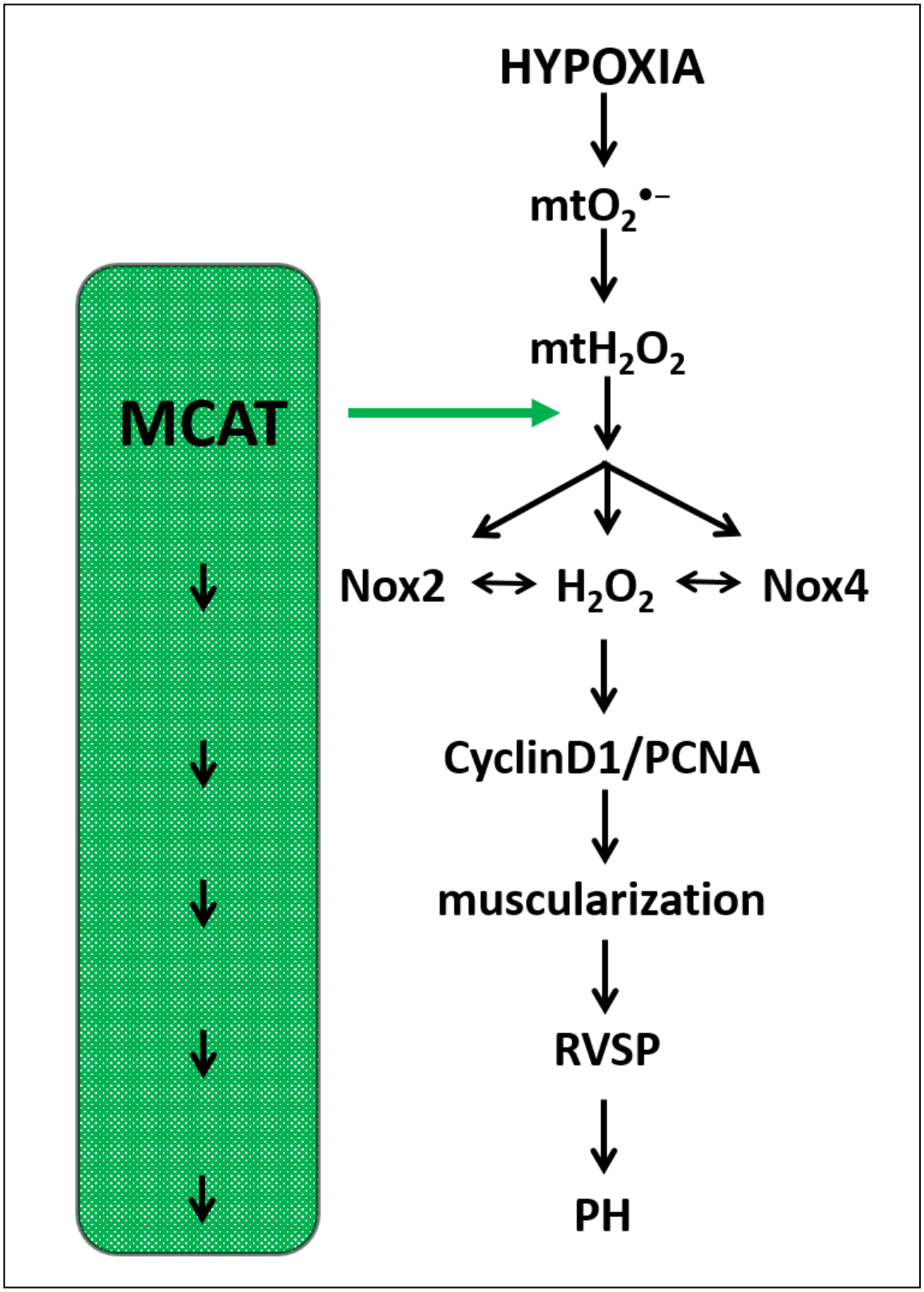


Figure 3.8: Role of mtH_2O_2 in the development of PH.

Murine models of PH have a number of important limitations that merit consideration. As models of human disease, mouse PH models are limited by potential species-dependent differences in vascular responses to hypoxia. In addition, chronic hypoxia serves as a model for Group 3 PH (Simonneau et al., 2013), and hypoxia alone fails to induce the proliferative, plexiform arteriopathy seen in patients with more severe PH (Stenmark et al., 2009). Similar limitations pertain to other rodent models of PH, including Monocrotaline, fawn hooded rats (a genetic model of PH), and hypoxia + SU5416, all of which induce a more severe vasculopathy than hypoxia alone and PH of varying severity (Barman et al., 2014; Gomez-Arroyo et al., 2012; Ryan et al., 2013). Despite these limitations, murine models provide distinct advantages including being highly reproducible, affordable, and amenable to strategies that permit knockout or overexpression of targeted genes, as illustrated by the current studies (Bauer et al., 2007; Granger et al., 1986; Maarman et al., 2013; Stenmark et al., 2009).

Our previous studies show that hypoxia increases RVSP and RVH in the mouse (Green et al., 2012; Nisbet et al., 2010; Nisbet et al., 2009). The current study demonstrates that hypoxia-induced elevations in RVSP (but not RVH) are attenuated by MCAT expression. Many studies find that exposure to chronic hypoxia stimulates vasoconstriction and pulmonary vascular remodeling which result in increases in RVSP, which if sustained, cause RVH in response to this increased afterload (Campen et al., 2014). However, previous reports from our

lab and others have provided evidence where interventions that attenuate hypoxia-induced RVSP fail to attenuate RVH (Nisbet et al., 2010) (Green et al., 2012). The current study provides additional evidence that MCAT mice had reduced RVSP when compared to hypoxia-exposed littermate controls without accompanying reductions in RVH. Although the precise mechanisms for these findings remain to be defined, we considered several potential possibilities. For example, differential expression of mitochondrial catalase in pulmonary and cardiac tissue could account for relative lack of response in the RV and reductions in RVH. Alternatively, these and other findings may suggest that RVH, rather than being mediated only by responses to increased afterload in the pulmonary circulation, may be caused by systemic factors promoting RVH and that RV mtH_2O_2 plays little or no role in this process. These interesting considerations remain an active area of investigation in our laboratories.

Increasing evidence indicates that ROS generated from mitochondrial respiration contribute to endothelial cell dysfunction (Min W et al., 2010; Widder et al., 2009) and subsequent vascular derangements. While the role of ROS in hypoxic pulmonary vasoconstriction has been controversial (Fuchs et al., 2010; Lyle and Griendling, 2006; Shimoda and Undem, 2010; Wolin et al., 2011), growing support demonstrates that hypoxia stimulates mtROS generation (Chandel et al., 1998; Waypa et al., 2013). Similar to several previous studies (Liu et al., 2005; Wedgwood S et al., 2011; Wedgwood et al., 2013), our confocal microscopy studies confirmed that hypoxia increases mtH_2O_2. Similar to our

studies, others have confirmed that MCAT expression reduces H_2O_2 release from pulmonary tissue (Song et al., 2014). Mitochondria adjust intracellular redox-signaling pathways to modulate vascular tone and regulate hypoxia-induced redox signaling by ROS (Fuchs et al., 2010; Guzy et al., 2005; Shimoda and Undem, 2010; Waypa et al., 2010). Enhanced ROS production also contributes to vascular cell proliferation which can lead to PH (Blanquicett et al., 2010). $O_2^{\bullet -}$ and H_2O_2 play a vital role in vascular cell signaling (Lyle and Griendling, 2006), regulating cellular proliferation, differentiation, and apoptosis (Watson et al., 2003). In HPASMC, PEG-catalase prevented HPASMC proliferation, ERK 1/2 and NF-κB activation, and Nox4 expression, indicating that H_2O_2 participates in feed-forward activation of the signaling events (Bijli et al., 2015). mtROS have been implicated in the pathophysiological and molecular proliferative and apoptotic derangements seen in vascular wall cells (Chandel et al., 1998; Gillespie et al., 2013; Waypa et al., 2013). We observed that, targeted attenuation of mtH_2O_2 with the MCAT model was beneficial. These findings support our conclusions that H_2O_2, mtH_2O_2 in particular, appears to drive PH vascular derangements.

Noxes, another major source of ROS within the vasculature (Lyle and Griendling, 2006; Sedeek et al., 2009), regulate endothelial function, vascular tone, vascular cell hypertrophy, and apoptosis (Dikalova et al., 2010; Paravicini and Touyz, 2008). Our study not only confirmed previous observations that hypoxia induced Nox4 expression (Fig. 4) (21,40-42,61), but our data also

demonstrated that hypoxia increased Nox2 (Fig. 4). While the regulation of mtROS and its effects on PH remain incompletely defined, studies are beginning to highlight that mitochondrial ROS may stimulate Noxes (Dikalov, 2011; Dikalov and Ungvari, 2013). Our study is the first to demonstrate that mtH_2O_2 directly contributes to induction of Nox2 and Nox4 expression. ROS generated by Noxes contributes to the aberrant pulmonary arterial responses. Accumulating evidence indicates that ROS derived from Nox2 and Nox4 are involved in long-term responses of the pulmonary vasculature to hypoxia. Although Nox4 mRNA upregulation in PH has been well demonstrated (Barman et al., 2014; Frazziano et al., 2014; Lu et al., 2013; Wedgwood et al., 2013), there remains debate whether Nox 4 is localized to the mitochondria, which may be related to cell type (Ago et al., 2010; Frazziano et al., 2014). Furthermore, Noxes have been implicated in upregulation of cyclinD1 (Veit et al., 2013), possibly explaining the increased vascular proliferation seen in hypoxia-induced PH. Taken together these studies indicate the mtROS drives Nox expression which may in turn promote a proliferative pulmonary vascular cell phenotype (Dikalova et al., 2010; Frazziano et al., 2014).

CyclinD1 promotes transition from G1 phase to S phase of the cell cycle thereby stimulating proliferation, and PCNA is a marker of vascular cell proliferation (Green et al., 2012; Wedgwood et al., 2013). We found that hypoxia exposure increased both cyclinD1 and PCNA protein levels and that these biochemical changes were attenuated in the MCAT model. Our studies, and

others (Wedgwood et al., 2013), implicate a role for ROS-induced cellular proliferation in the development of PH by demonstrating the elevation of Nox2, Nox4, cyclinD1, and PCNA in hypoxia exposed samples. Our findings suggest that increases in mtH_2O_2 play a critical role in mediating these hypoxia-induced alterations. This study and evidence that hypoxia-induced PH is attenuated in Nox2 KO mice (Liu et al., 2006) and by treatment with a Nox4 inhibitor, GKT137831 (Green et al., 2012), emphasize the important connections between mtROS, Noxes and the vascular proliferation seen in PH (Liu and Folz, 2004; Wedgwood and Black, 2003).

Our results confirm that hypoxia increases mtH_2O_2 and that targeted expression of catalase in the mitochondria and reductions in mtH_2O_2 prevented many physiological derangements associated with PH including increases in RVSP and vessel muscularization. Furthermore, MCAT also prevented hypoxic induction of molecular aberrations in Nox2, Nox4, cyclinD1, and PCNA protein expression. Consistent with our data showing the mitochondrial catalase expression can improve many markers of PH, MCAT expression also extends murine lifespan (Schriner et al., 2005). This established efficacy of targeted expression of catalase in the mitochondria supports a critical role for mtH_2O_2 in pulmonary vascular responses to hypoxia. These studies suggest that novel interventions targeting mitochondrial redox balance may provide improved efficacy in PH treatment. To our knowledge, our study is the first to demonstrate that targeting mtH_2O_2 prevents both physiological and molecular derangments

associated with hypoxia-induced PH. These results provide novel evidence for the involvement of mtH_2O_2 in Nox induction and maintenance of a proliferative pulmonary vascular cell phenotype.

CHAPTER 4: Overexpression of SOD2 Exacerbates Hypoxia-induced PH

Figures contained in this section have been published:

Adesina SE, Kang BY, Bijli KM, Ma J, Cheng J, Murphy T, Michael Hart C and Sutliff RL (2015) Targeting mitochondrial reactive oxygen species to modulate hypoxia-induced pulmonary hypertension. Free radical biology & medicine.

INTRODUCTION

Current evidence suggests that reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) generated by mitochondria and NADPH oxidases (Noxes) contribute to PH pathogenesis by altering vascular cell proliferation (Fike et al., 2008; Liu and Folz, 2004; Mittal et al., 2007a; Weir and Archer, 2010) and apoptotic signaling pathways (Bedard and Krause, 2007; Fike et al., 2008; Weir and Archer, 2010). The exact contribution of mitochondria-derived $O_2^{\bullet-}$ in the development of PH remains poorly defined, and the role of mitochondria-derived ROS in dysregulation of pulmonary vascular wall cells remains controversial.

Hypoxia causes pulmonary vasoconstriction, and increasing evidence indicates that hypoxic increases in intracellular ROS and Nox expression contribute to pulmonary vascular alterations (Wang and Zheng, 2010). Noxes are a major source of ROS within the vessel (Lyle and Griendling, 2006; Sedeek et al., 2009) and regulate endothelial function, vascular tone, vascular cell hypertrophy, and apoptosis (Dikalova et al., 2010; Paravicini and Touyz, 2008). Two Nox isoforms that are broadly implicated in long-term responses of the pulmonary vasculature to hypoxia are Nox2 and Nox4. (Frazziano et al., 2012). Nox4 has been found to be increased in murine models of hypoxia-induced PH, in the pulmonary vasculature of PH patients (Nisbet et al., 2010; Sutliff et al., 2010), and in pulmonary artery endothelial cells isolated from patients with IPAH (Green et al., 2012).

In PH, ROS production in pulmonary vascular cells is increased, and there appears to be crosstalk between Noxes and mitochondria (Dikalov, 2011; Paravicini and Touyz, 2008). Mitochondria, implicated as cellular O_2 sensors, adjust redox-signaling pathways to modulate vascular tone and regulate hypoxia-induced redox signaling by ROS (Fuchs et al., 2010; Hansen et al., 2006b). Mitochondria can be viewed as both a target of NADPH oxidase-derived ROS and as a source of ROS that may stimulate Nox expression and activity (Dikalov, 2011; Dikalova et al., 2010). While the role of ROS in hypoxic pulmonary vasoconstriction may be controversial (Fuchs et al., 2010; Lyle and Griendling, 2006; Shimoda and Undem, 2010; Wolin et al., 2011), it can be postulated that mitochondria-derived ROS (mtROS) are stimulated by hypoxia and promote increased expression of Noxes in the lung to contribute to the pathogenesis of PH.

Previous reports have demonstrated increased $O_2^{\bullet-}$ levels in the lungs of experimental models of PH (Brennan et al., 2003; Fresquet et al., 2006; Liu and Folz, 2004; Nisbet et al., 2010). Consistent with these experimental observations, PH patients exhale lower levels of nitric oxide (NO) suggesting that increased levels of $O_2^{\bullet-}$ are scavenging NO to mediate reductions in nitric oxide bioavailability (Kaneko et al., 1998). $O_2^{\bullet-}$, which is converted to H_2O_2 by SOD2 in the mitochondria, regulates pulmonary vasoconstriction and stimulates proliferation of smooth muscle cells (Liu and Folz, 2004; Wedgwood and Black, 2003). Lung tissue from PH patients displays decreased SOD2 activity (Bowers

et al., 2004) suggesting that increased mitochondrial-derived $O_2^{\bullet-}$ may contribute to PH pathogenesis. Nox 2 constitutes another source of $O_2^{\bullet-}$ generation in pulmonary vascular cells emphasizing that to effectively address increased ROS generation in PH may require strategies that target both enzymatic and mitochondrial sources of ROS as well as the interaction of those sources (Dikalov, 2011; Dikalova et al., 2010).

To further examine the role of mitochondria-derived ROS in PH pathogenesis, we hypothesized that mitochondria-targeted overexpression of SOD2 will reduce mitochondria-derived ROS and hypoxia-induced PH pathogenesis. The current study establishes that chronic hypoxia increases HPAEC mitochondria-derived $O_2^{\bullet-}$ generation. To experimentally manipulate $mtO_2^{\bullet-}$**,** an SOD2 overexpression (Tg^{hSOD2}) transgenic mouse model was used to determine if targeting $mtO_2^{\bullet-}$ generation alters hypoxia-induced PH, Nox expression, and proliferative markers *in vivo*.

RESULTS

Confirmation of SOD2 overexpression in the Tg^{hSOD2} model

In order to establish that the mitochondrial SOD2 transgenic model overexpresses the target gene of interest, human SOD2 mRNA levels in lung tissue (**Figure 4.1A)** and total (human and mouse) SOD2 protein expression were measured. An increase in SOD2 was confirmed in the Tg^{hSOD2} model compared to Lit Cont (**Figure 4.1B)**.

Figure 4.1: Confirmation of SOD2 overexpression in the Tg^{hSOD2} model. Whole lung homogenates were collected from littermate control (Lit Cont) and Tg^{hSOD2} mice. qRT-PCR and Western blot were employed to detect human SOD2 mRNA and protein. **(A)** Human SOD2 mRNA levels were detected in lungs from Tg^{hSOD2} mice but not in littermate controls. Each bar represents mean ± SEM. hSOD2 mRNA levels are expressed as fold-change relative to GAPDH expression (n = 3), *p < 0.05 compared to Lit Cont. **(B)** Total (human and mouse) SOD2 protein expression was increased in Tg^{hSOD2} samples. Each bar represents mean ± SEM SOD2 protein relative to β-actin (n = 3), *p < 0.05 compared to Lit Cont.

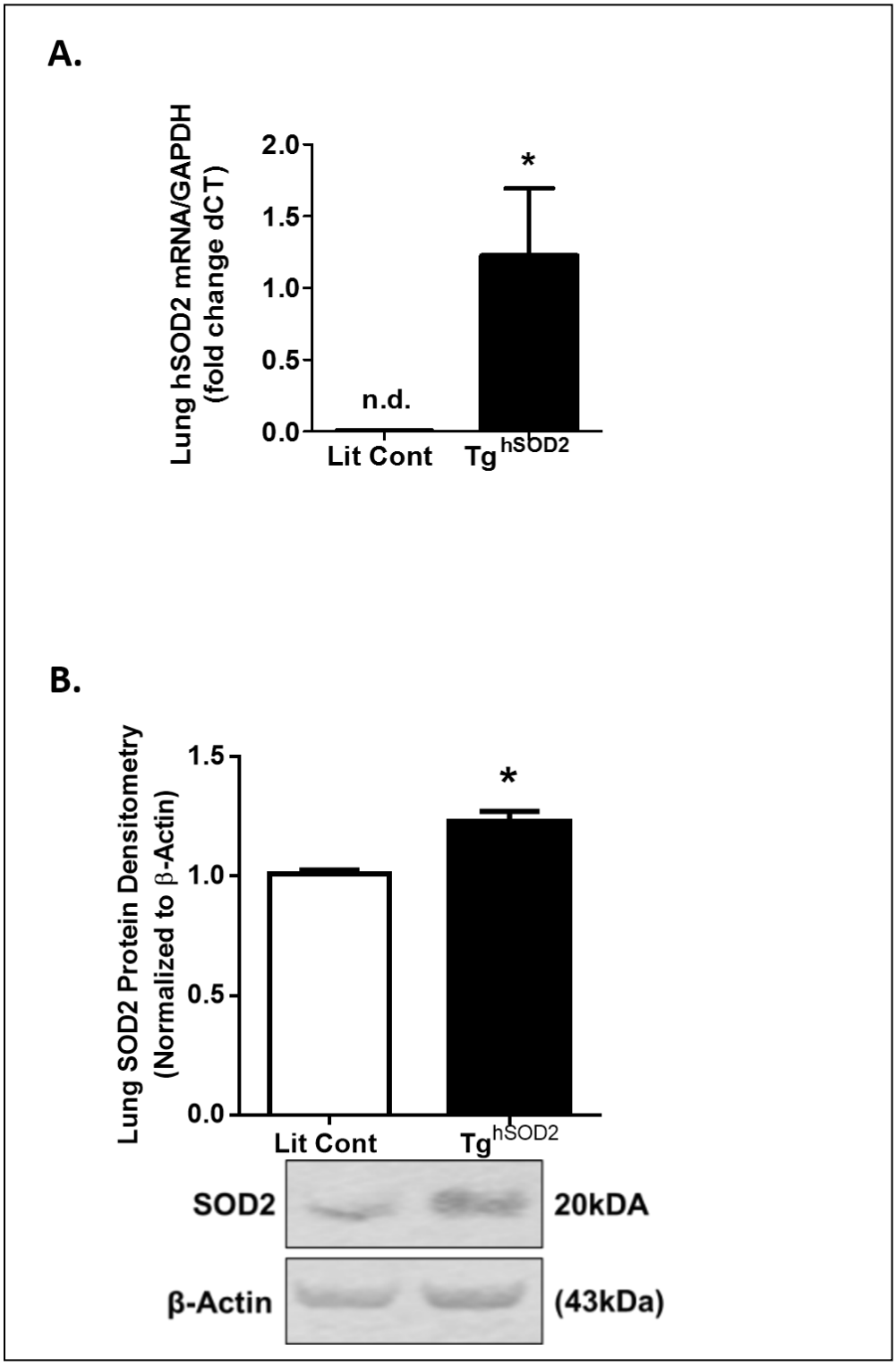


Figure 4.1: Confirmation of SOD2 overexpression in the Tg^{hSOD2} model.

Hypoxia exposure increases mitochondrial $O_2^{\bullet-}$ generation in HPAECs.

To examine the effect of hypoxia on mt$O_2^{\bullet-}$ levels, HPAECs were cultured under either normoxic or hypoxic conditions for 72 hours and incubated in KRPG buffer containing DAPI, MitoSOX, and MitoTracker Green. HPAECs were treated with MitoTEMPO (100 nM, a mitochondria-targeted SOD mimetic) daily to confirm the specificity of the measured $O_2^{\bullet-}$ signal. Exposure of HPAECs to chronic hypoxia increased the intensity of MitoSOX fluorescence which was decreased by treatment with MitoTEMPO **(Figure 4.2A)**. These results indicate that hypoxia increases mitochondria-derived $O_2^{\bullet-}$ **(Figure 4.2B).**

Figure 4.2: Hypoxia exposure increases mitochondrial $O_2^{\bullet-}$ generation in HPAECs. HPAECs were exposed to normoxia (Norm) (21% O_2) or hypoxia (Hypo) (1% O_2) for 72 hours. Following exposure, cells were examined for mitochondrial $O_2^{\bullet-}$ by confocal microscopy. **(A)** HPAECs were treated with 100 nM MitoTEMPO (MT) or DMSO vehicle daily and then treated with MitoSOX, MitoTracker green, and DAPI. Representative images at 90x magnification are presented. **(B)** The fluorescence intensity in each treatment group was measured in 50-100 cells and is presented as mean ± SEM MitoSOX RFU/cell. MitoTEMPO treatment catalyzes the degradation of hypoxia-induced $O_2^{\bullet-}$ (n = 3), *$p < 0.05$ compared to all other groups.

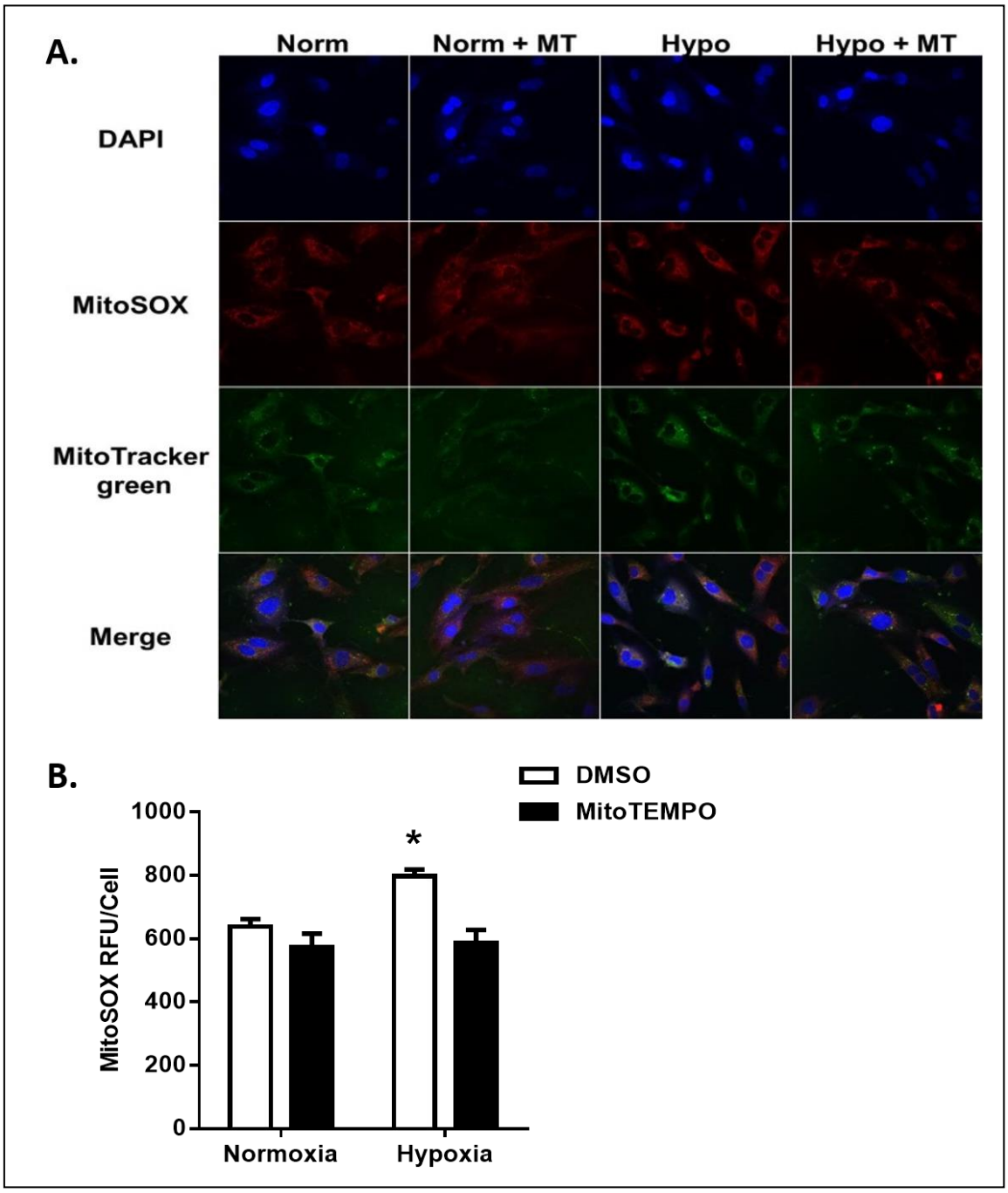


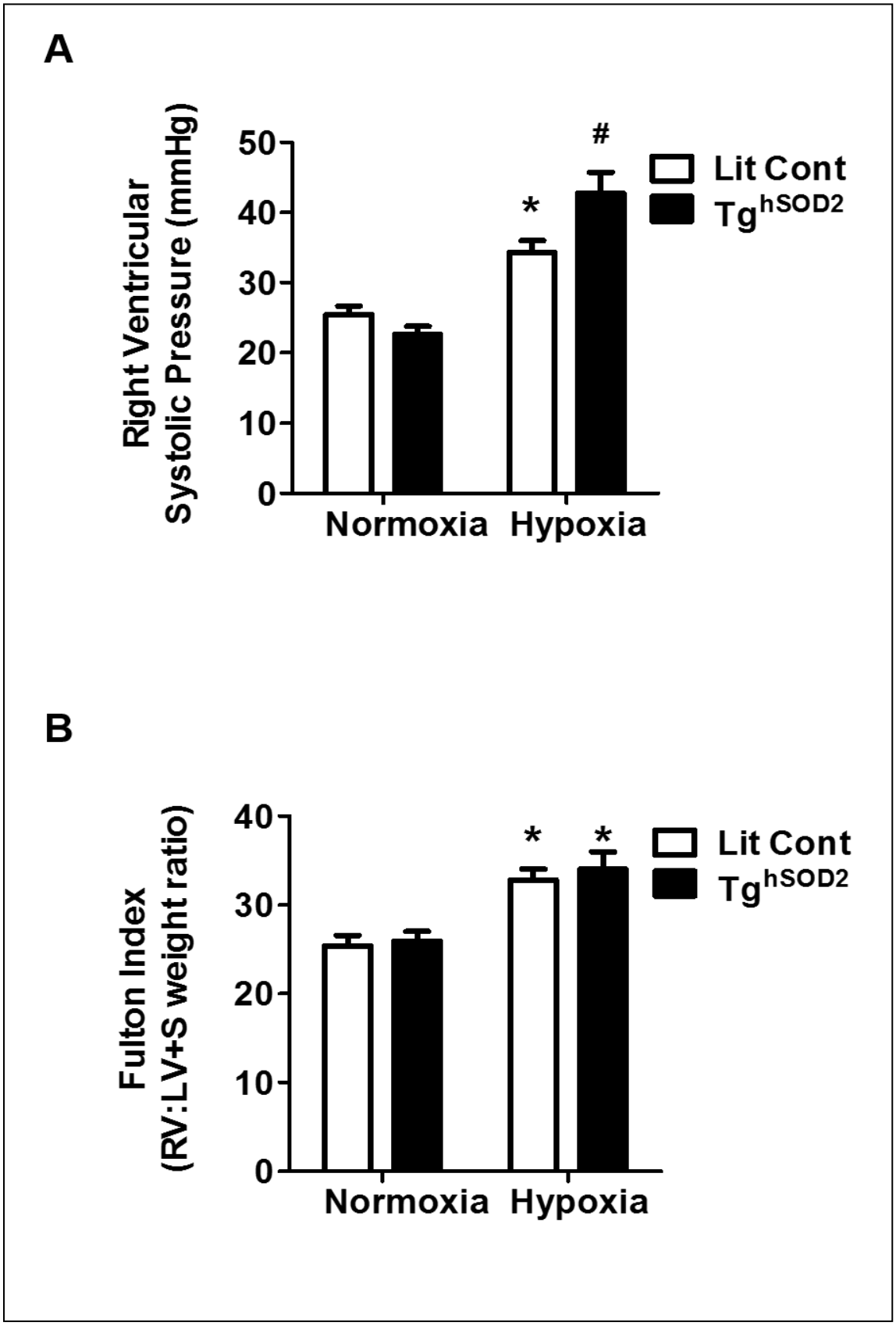
Figure 4.2: Hypoxia exposure increases mitochondrial $O_2^{\bullet -}$ generation in HPAECs.

Effects of SOD2 overexpression on hypoxia-induced PH.

To elucidate the role of mitochondrial $O_2^{\bullet-}$ in hypoxia-induced PH pathophysiological outcomes were assessed in littermate control and Tg^{hSOD2} mice (Kowluru et al., 2006) exposed to 3 weeks of either normoxia or hypoxia. After 3 weeks of hypoxia exposure, Tg^{hSOD2} mice had significantly higher RVSP when compared to normoxia-exposed groups and hypoxia-exposed littermate controls **(Figure 4.3A)**. Furthermore, hypoxia-induced muscularization of small pulmonary vessels was exacerbated in Tg^{hSOD2} mice **(Figure 4.3B and 4.3C)**, whereas Tg^{hSOD2} expression had no significant effect on hypoxia-induced RVH **(Figure 4.3D)**.

Figure 4.3: Effects of SOD2 overexpression on hypoxia-induced PH. SOD2 overexpression exacerbates hypoxia-induced RVSP and muscularization of small pulmonary arteries whereas Tg^{hSOD2} had no effect on hypoxia-induced RVH. Lit Cont and Tg^{hSOD2} mice were exposed to normoxia (21% O_2) or hypoxia (10% O_2) for 3-weeks in 2 independent studies. **(A)** RVSPs were recorded with a pressure transducer. Hypoxia-induced increases in RVSP were exacerbated in Tg^{hSOD2} mice. Each bar represents mean ± SEM RVSP in mm Hg ($n = 4 – 5$), *$p < 0.05$ compared to Lit Cont Normoxia and $^{\#}p < 0.05$ compared to Lit Cont Hypoxia. **(B)** Tg^{hSOD2} expression had no significant effect on hypoxia-induced RVH. Each bar represents the mean ± SEM RV: LV+S weight ratio ($n = 8 - 12$), *$p < 0.05$ compared to both Normoxia groups.

(C) 10 – 20 lung sections (5 μm thick) were stained with α-SMA antibodies. Representative images are displayed as indicated. Brown staining indicated by arrows represents α-SMA positive staining in the media of small pulmonary arterioles. Magnification = 40x. **(D)** The thickness of the pulmonary vascular wall was calculated by dividing total thickness of the vessel by the inner vessel radius. Hypoxia-induced vascular remodeling and muscularization as detected by α-SMA staining was increased in Tg^{hSOD2} mice (n = 3), *$p < 0.05$ compared to Lit Cont Normoxia and $^{\#}p < 0.05$ compared to Lit Cont Hypoxia.


A
Right Ventricular Systolic Pressure (mmHg)
50
40
30
20
10
0
*
#
Lit Cont
Tg hSOD2
Normoxia
Hypoxia
B
Fulton Index (RV:LV+S weight ratio)
40
30
20
10
0
*
*
Lit Cont
Tg hSOD2
Normoxia
Hypoxia

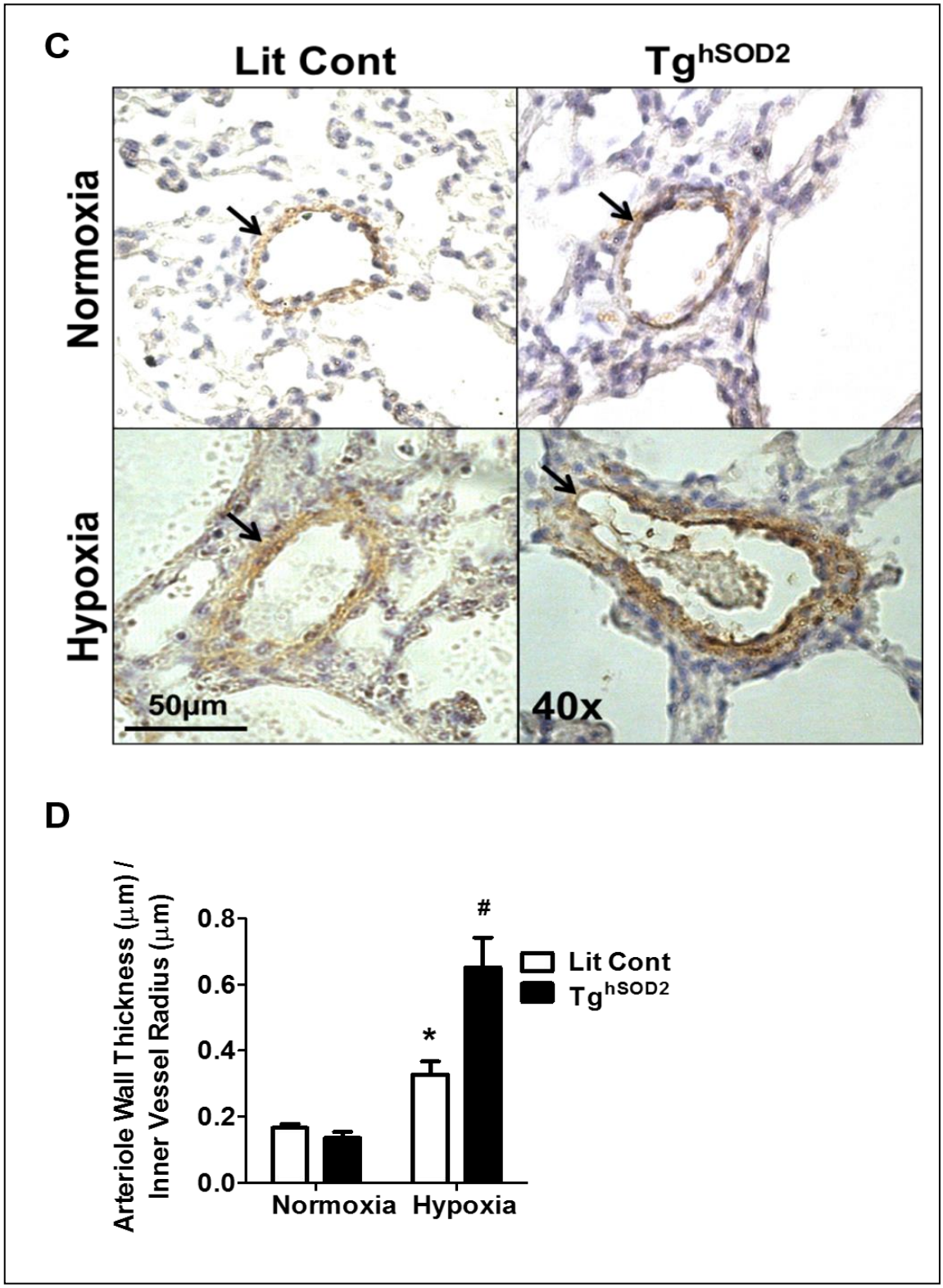


Figure 4.3: Pathophysiological Effects of SOD2 Overexpression in Hypoxia-induced PH.

Effects of SOD2 on hypoxia-induced Nox expression.

Noxes have been shown to contribute to the physiological derangements seen in hypoxia-induced PH. Nox2 and Nox4 are suggested to contribute to the long-term response of the pulmonary vasculature to hypoxia (Nisbet et al., 2009). Our group previously determined that targeted reduction of mtH_2O_2 attenuates hypoxia-induced PH. To determine the effect of targeted reduction of mt$O_2^{\bullet -}$ (by increased conversion to H_2O_2) via increased SOD2 on Nox expression, we assessed Nox2 and Nox4 levels in lungs from littermate controls and Tg^{hSOD2} following normoxia or hypoxia exposure. SOD2 overexpression in the Tg^{hSOD2} model failed to prevent hypoxia-induced increases in Nox2 mRNA and protein levels **(Figure 4.4A and 4.4B)**. In addition, Nox4 mRNA and protein levels are significantly increased in hypoxia Tg^{hSOD2} compared to hypoxia-exposed Lit Cont **(Figure 4.4C and 4.4D)**. These data indicate that SOD2 overexpression exacerbates Nox expression.

Figure 4.4: Effects of SOD2 overexpression on hypoxia-induced Nox levels. Lit Cont and Tg^{hSOD2} mice were exposed to normoxic or hypoxic conditions for 3 weeks. Whole lung homogenates were collected from Lit Cont and Tg^{hSOD2} mice. Nox mRNA values are normalized to 9S and expressed as fold-change vs. Normoxia. Protein samples are normalized to β-Actin. **(A)** Hypoxia-induced lung Nox2 mRNA levels were exacerbated in Tg^{hSOD2}. Each bar represents mean ± SEM lung Nox2 mRNA (n = 6 - 11), *$p < 0.05$ compared to Lit Cont Normoxia and

$^{\#}p < 0.05$ compared to Lit Cont Hypoxia. **(B)** Hypoxia elevates lung Nox2 protein in both Lit Cont and Tg^{hSOD2}. Each bar represents mean ± SEM lung Nox2 protein (n = 3), *p < 0.05 compared to all other groups. **(C)** Hypoxia-induced Nox4 mRNA levels are exacerbated in hypoxia-exposed Tg^{hSOD2}. Each bar represents mean ± SEM lung Nox4 mRNA (n = 6), *p < 0.05 compared to Lit Cont Normoxia and $^{\#}p < 0.05$ compared to Lit Cont Hypoxia. **(D)** Hypoxia-induced Nox4 protein expression is exacerbated in hypoxia-exposed Tg^{hSOD2}. Each bar represents mean ± SEM lung Nox4 mRNA (n = 3 – 6), *p < 0.05 compared to Lit Cont Normoxia and $^{\#}p < 0.05$ compared to Lit Cont Hypoxia.

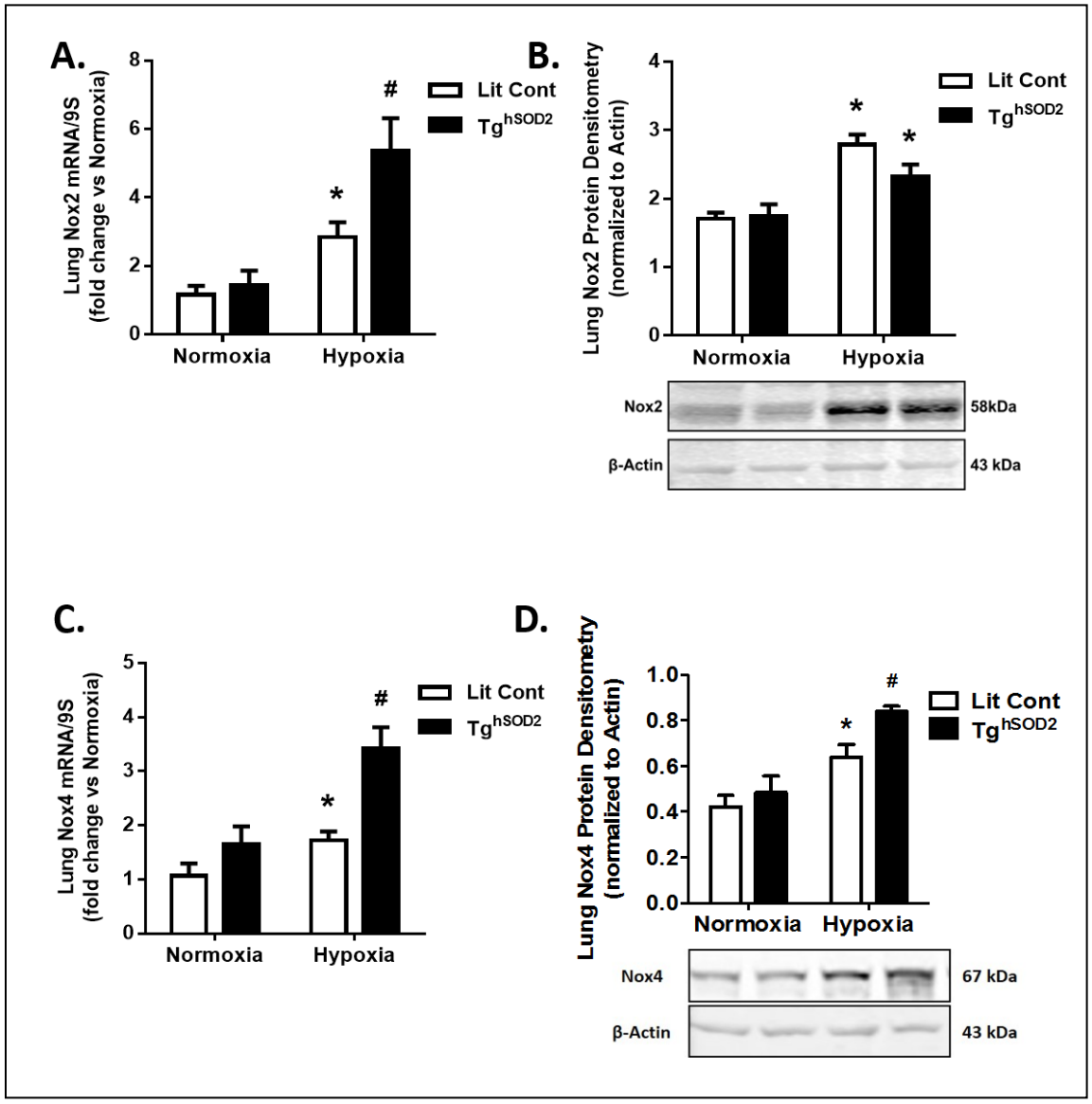


Figure 4.4: Effects of SOD2 overexpression on hypoxia-induced Nox2 and 4 levels.

Tg^{hSOD2} expression exacerbated hypoxia-induced H_2O_2 production

Since $mtO_2^{\bullet-}$ is converted to H_2O_2 via SOD2 and our studies demonstrated exacerbated hypoxia-induced PH in Tg^{hSOD2}, it is expected that increased SOD2 expression may lead to an elevation of global H_2O_2 levels (Connor et al., 2005; Dasgupta et al., 2006). Hypoxia-induced H_2O_2 production is exacerbated in lungs from Tg^{hSOD2} **(Fig. 5.7A)**. These data indicate that enhanced upstream conversion of $O_2^{\bullet-}$ to H_2O_2 in Tg^{hSOD2} mice increases pulmonary H_2O_2 levels and suggest that elevated SOD2 levels may exacerbate hypoxia-induced PH pathophysiology by increasing H_2O_2 levels.

Figure 4.5: Tg^{hSOD2} expression exacerbates hypoxia-induced H_2O_2 production. Lit Cont and Tg^{hSOD2} mice were exposed normoxia (21% O_2) or hypoxia (10% O_2) for 3-weeks. **(A)** Tg^{hSOD2} expression significantly increased hypoxia- induced H_2O_2 levels. Each bar represents mean ± SEM H_2O_2 concentration relative to lung tissue wet weight (n = 5 - 9), *$p < 0.05$ compared to Lit Cont Normoxia and $^{\#}p < 0.05$ compared to Lit Cont Hypoxia.

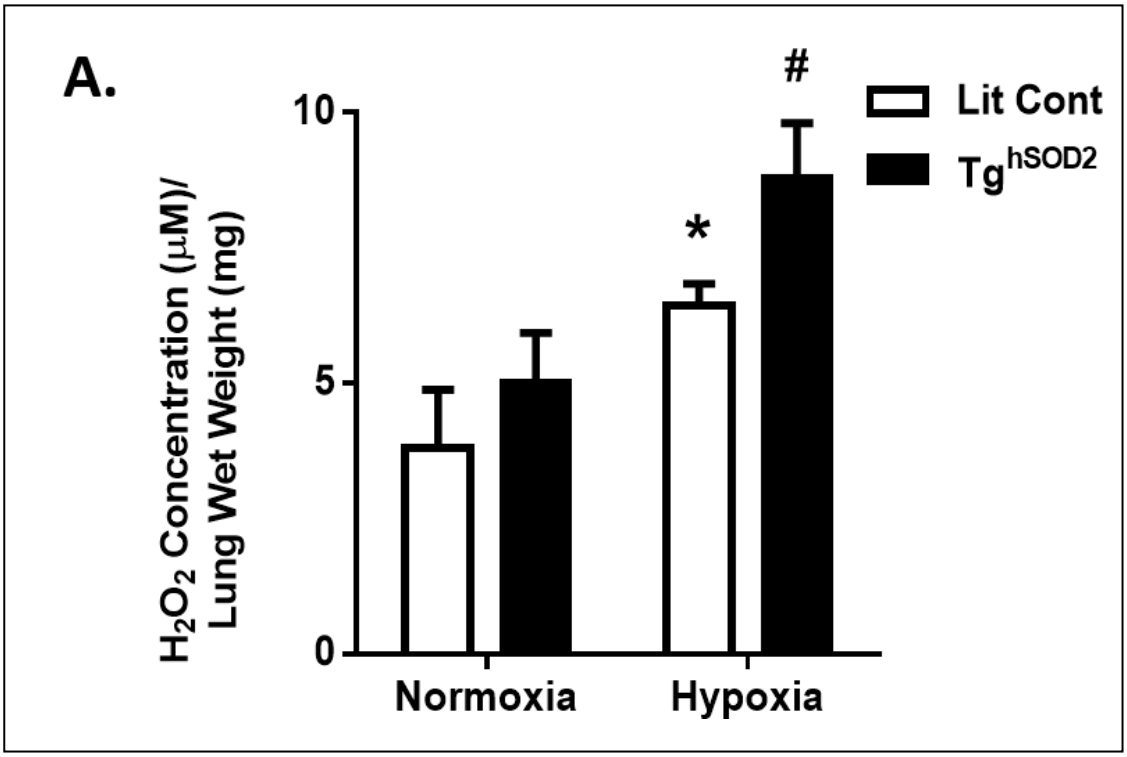


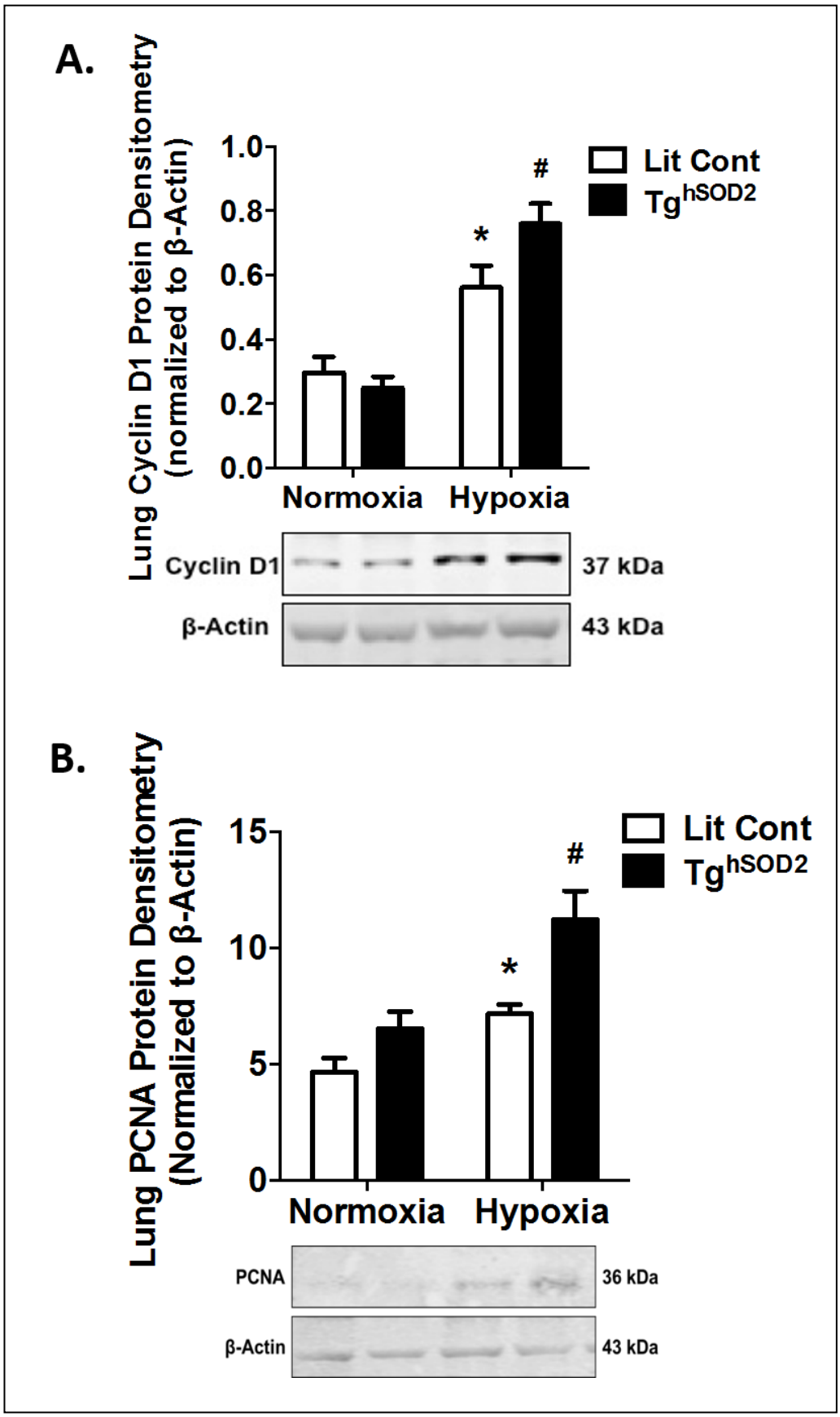
Figure 4.5: Tg^{hSOD2} expression exacerbated hypoxia-induced H_2O_2 production.

Hypoxia-induced CyclinD1 and PCNA protein expression is exacerbated in lungs from the Tg^{hSOD2} model.

To identify mechanistic modulators of hypoxia-induced PH, the expression of mediators and markers of cell proliferation was evaluated. CyclinD1 is a cell cycle regulator that can promote expression of vascular cell proliferation markers and activate PCNA (Jing et al., 2014; Samaga et al., 2014; Wedgwood et al., 2013). SOD2 overexpression exacerbated hypoxia-induced CyclinD1 **(Figure 4.6A)** and PCNA protein expression **(Figure 4.6B)**. These results suggest that overexpression of SOD2 enhances hypoxia-induced vascular cell proliferation in the lung. To further confirm proliferation of smooth muscle vascular cells, immunohistochemistry was performed to assess nuclear localization of PCNA (brown staining) with endothelial and smooth muscle cell nuclei (blue staining). 5 μm thick lung sections were stained with PCNA. Brown staining, indicated by arrows, indicates PCNA positive staining in the media of small pulmonary arterioles (≤ 100 μm; **Fig. 4.6C**). Quantification of PCNA positive cells per 100 μm^2 revealed that Tg^{hSOD2} expression exacerbates hypoxia-induced PCNA IHC **(Fig. 4.6D)**.

Figure 4.6: Hypoxia-induced increases in proliferation markers are exacerbated in the Tg^{hSOD2} model. Lit Cont and transgenic mice were exposed to normoxic or hypoxic conditions for 3 weeks. Whole lung homogenates were collected from Lit

Cont and Tg^{hSOD2} mice. **(A)** Hypoxia-induced cyclinD1 protein expression was exacerbated in Tg^{hSOD2} mice. Each bar represents mean ± SEM lung cyclinD1 protein (n =3 - 6), *p < 0.05 compared to Lit Cont Normoxia and $^{\#}$ p < 0.05 compared to Lit Cont Hypoxia. **(B)** SOD2 overexpression exacerbated hypoxia-induced PCNA protein expression. Each bar represents mean ± SEM lung PCNA protein (n =3 - 6), *p < 0.05 compared to Lit Cont Normoxia and $^{\#}$p < 0.05 compared to Lit Cont Hypoxia. **(C)** Nuclear localization of PCNA (brown staining) with endothelial and smooth muscle cell nuclei (blue staining) were analyzed by a reviewer blinded to treatment group. Arrows indicate overlap between brown PCNA staining and blue nuclei. Scale bar = 50 μm; magnification = 40x. PCNA staining was increased in hypoxia exposed Tg^{hSOD2} sections – representative image. **(D)** Tg^{hSOD2} expression exacerbates hypoxia-induced PCNA IHC. Each bar represents mean ± SEM, (n = 3), *p < 0.05 compared to Lit Cont Normoxia and #p < 0.05 compared to Lit Cont Hypoxia.


A.
Lung Cyclin D1 Protein Densitometry (normalized to β-Actin)
1.0
0.8
0.6
0.4
0.2
0.0
Lit Cont
Tg hSOD2
*
#
Normoxia
Hypoxia
Cyclin D1
37 kDa
β-Actin
43 kDa
B.
Lung PCNA Protein Densitometry (Normalized to β-Actin)
15
10
5
0
Lit Cont
Tg hSOD2
*
#
Normoxia
Hypoxia
PCNA
36 kDa
β-Actin
43 kDa

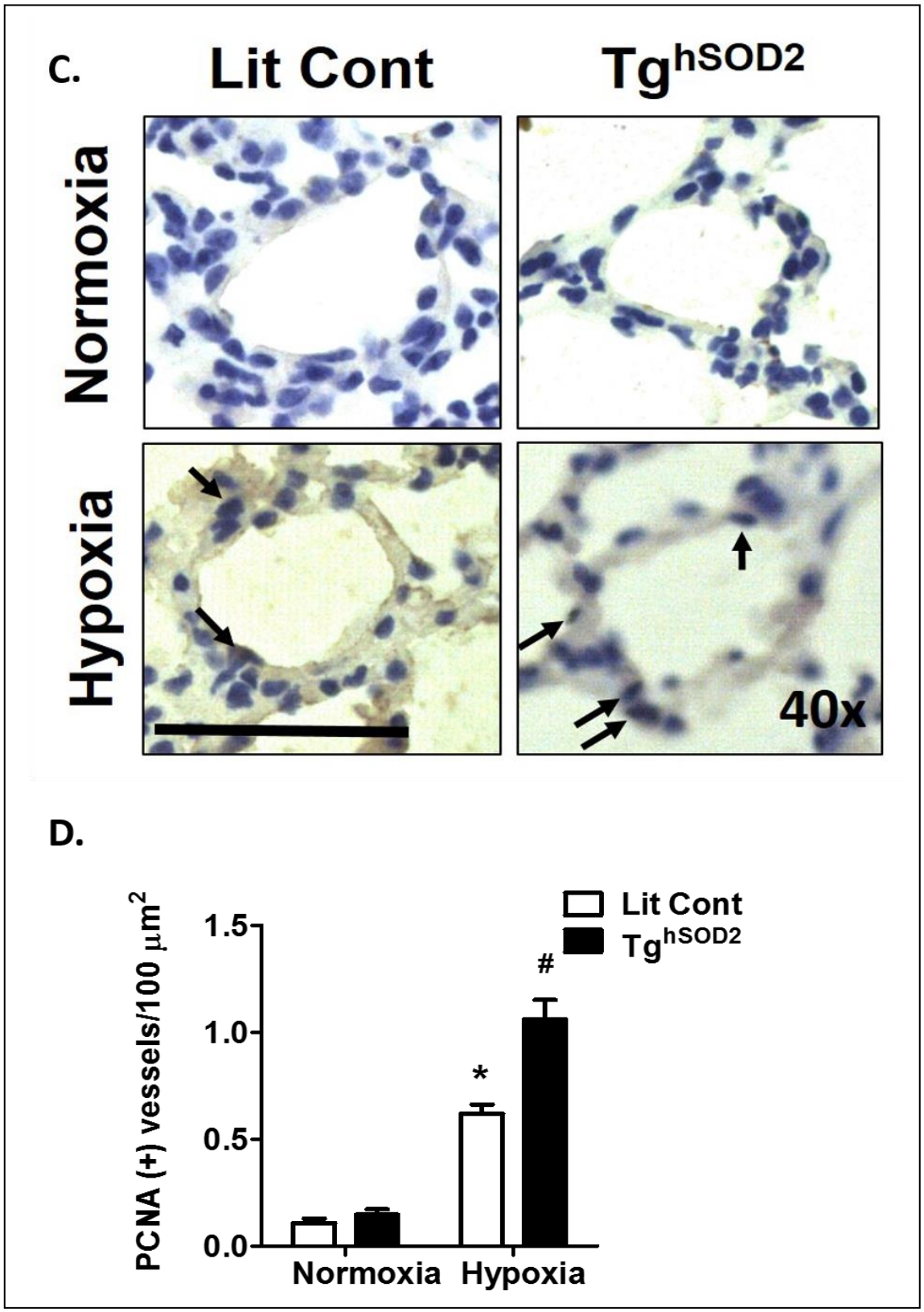


Figure 4.6: Hypoxia-induced CyclinD1 and PCNA protein expression exacerbated in Tg^{hSOD2} model.

DISCUSSION

ROS are implicated in the pathogenesis of PH. Enhanced ROS production contributes to endothelial dysfunction and hypertension (Blanquicett et al., 2010). It is our belief that mtROS in particular, play a critical role in the pro-proliferative and apoptosis resistant phenotype of pulmonary vascular wall cells in PH. The current studies employ established animal models with targeted mitochondrial antioxidant overexpression. Our studies demonstrate that overexpression of SOD2 exacerbates hypoxia-induced increases in RVSP (Fig. 4.3), α-SMA staining (Fig. 4.3), H_2O_2 production (Fig. 4.5), Nox expression (Fig. 4.4), and pulmonary vascular cell proliferation (Fig. 4.6) (Wedgwood et al., 2013). Collectively, these results support a signaling cascade wherein hypoxia increases mtROS that activate downstream pathways causing pulmonary vascular remodeling and PH **(Figure 4.7)**.

Figure 4.7: Schema depicting effects of SOD2 overexpression on PH pathogenesis. Targeted conversion of $mtO_2^{\bullet-}$ to H_2O_2 (left side of schema) exacerbates hypoxia-induced derangements that contribute to PH pathogenesis.

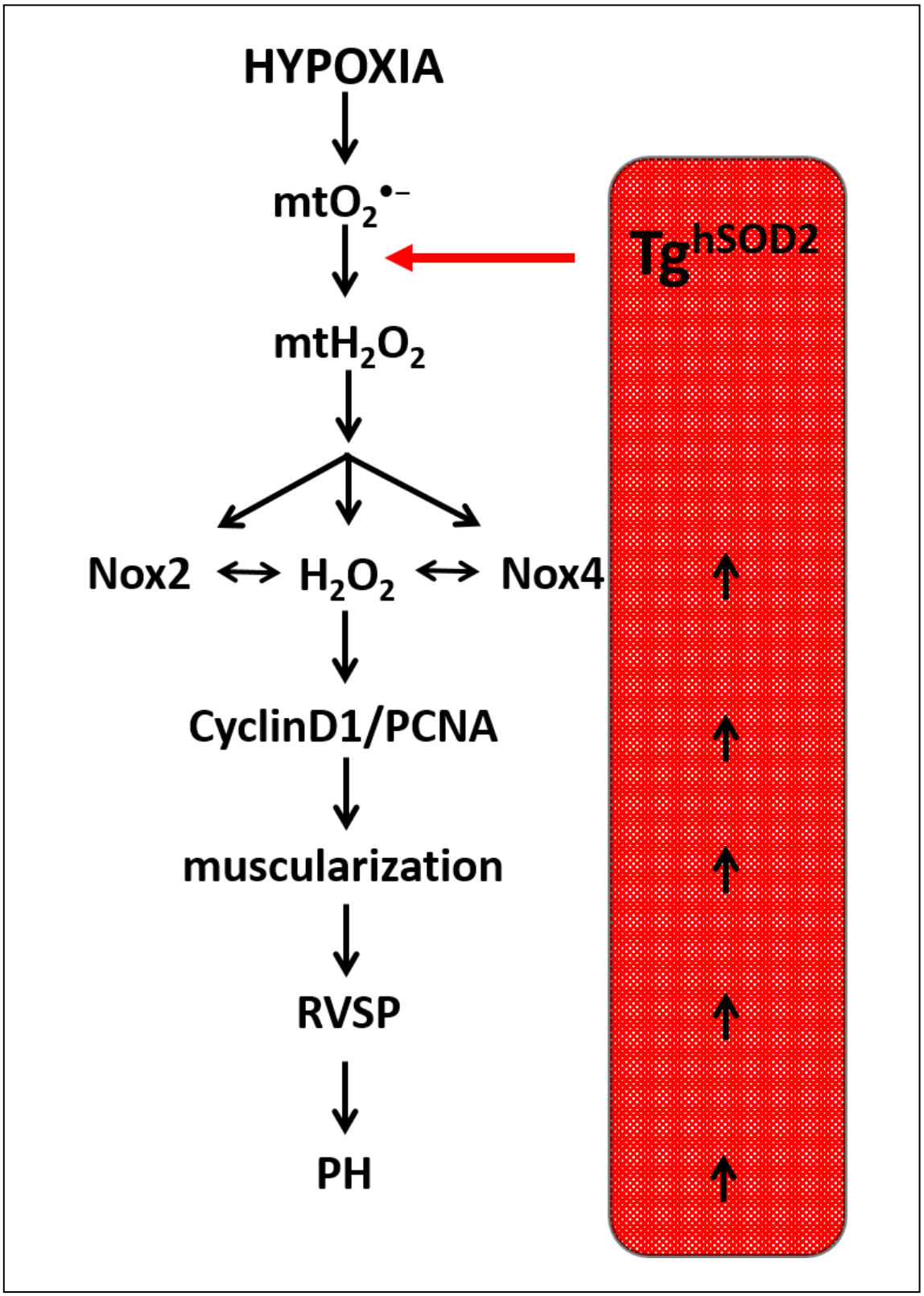


Figure 4.7: Schema depicting effects of SOD2 overexpression to PH pathogenesis.

$O_2^{\bullet-}$ plays a vital role in vascular cell signaling, proliferation, differentiation, and apoptosis (Lyle and Griendling, 2006; Watson et al., 2003). In PH, ROS production is increased (Paravicini and Touyz, 2008) and disrupted mitochondrial oxidative phosphorylation or NADPH oxidase (Nox) activity appear to be the predominant sources of these ROS (Datla and Griendling, 2010; Giordano, 2005). We confirmed that hypoxia increases HPAEC $O_2^{\bullet-}$ levels as detected by MitoSOX. Our studies also established that hypoxia-induced H_2O_2 production in the mouse lung was exacerbated by SOD2 overexpression. These results suggest that enhanced conversion of hypoxia-induced $O_2^{\bullet-}$ production to H_2O_2 by SOD2 overexpression leads to global increases in mitochondria-derived H_2O_2 (Connor et al., 2005).

Our findings are consistent with other studies that demonstrated hypoxia-induced increases in Nox4 (Nisbet et al., 2010; Sutliff et al., 2010) and Nox2 (Mittal et al., 2007b; Nisbet et al., 2010). Nox4 mRNA, protein, and H_2O_2 measurement were exacerbated in hypoxic Tg^{hSOD2} samples which we believe corresponds to increased production of H_2O_2 via enhanced SOD2 activity (Connor et al., 2005; Dasgupta et al., 2006). Based on earlier data confirming that the attenuated mtH_2O_2 via the MCAT model was able to prevent many markers of PH, these data suggest that mitochondria-derived H_2O_2 plays a role in the pathogenesis of PH. Hypoxia-induced PH and the expression of PH markers

was exacerbated in Tg^{hSOD2} mice but attenuated in the MCAT model. These results support the postulate that the Tg^{hSOD2} model, through SOD2 overexpression, converts more $O_2^{\bullet-}$ to H_2O_2 whereas the MCAT model reduces H_2O_2. These findings emphasize that regulation of mtH_2O_2 may be a viable target for PH therapy.

In contrast to our study, overexpression of EC-SOD attenuated hypoxia-induced PH (Nozik-Grayck et al., 2014; Voelkel et al., 2013). Current evidence has shown that SOD2 overexpression in fawn-hooded rat PASMCs reverses hyperproliferative PAH (Archer et al., 2010), SOD2 overexpression exacerbated muscularization and ROS detection in these studies. This discrepancy is likely due to model differences, as our studies modeled group 3 PH, not PA. Furthermore, fawn-hooded rats PASMCs have higher proliferation and lower apoptosis rates than Sprague-Dawley rat PASMCs, hyperpolarized mitochondria, low H_2O_2 production, and reduced cytoplasmic and mitochondrial redox state. ROS generated by Noxes contribute to the aberrant pulmonary arterial responses, further supported by data suggesting that in intrapulmonary arteries, hypoxia-induced endothelial dysfunction depends on gp91phox/Nox2 (Fike et al., 2008; Fresquet et al., 2006; Griffith et al., 2009). SOD2 overexpression significantly exacerbated hypoxia-induced PCNA protein expression which corresponds with the exacerbated α-SMA detected vascular remodeling and muscularization of small pulmonary arterioles. Both intermittent and chronic hypoxia exposure causes muscularization of small peripheral vessels in lung

tissue (Green et al., 2012; Nisbet et al., 2010; Nisbet et al., 2009). Consistent with our findings, elevation of Nox2, Nox4, cyclinD1, and PCNA correlated with H_2O_2 signaling (Wedgwood S et al., 2011). With molecular signaling for proliferation increased and physiological assessment of muscularization of small peripheral vessels increased in hypoxia-exposed mice, we have shown that targeted attenuation of mitochondrial $O_2^{\bullet-}$ levels through conversion to H_2O_2 enhanced molecular and physiological derangements contributing to PH pathogenesis.

Similar to previous studies from our labs, hypoxia exposure increased RVSP (Green et al., 2012; Nisbet et al., 2010; Nisbet et al., 2009). Our studies show consistent elevations in hypoxia-induced right ventricular systolic pressures, that SOD2 overexpression exacerbated. Though we saw the expected increase in hypoxia-induced RVH, overexpression of SOD2 with the Tg^{hSOD2} model did not to attenuate RVH to baseline normoxic values.

In summary, these studies demonstrate that mitochondrial H_2O_2 contributes to the progression of PH. Increased H_2O_2 (by virtue of increased SOD2 activity) significantly exacerbated molecular and physiological PH markers. While these studies attempted to assess the beneficial effects of targeting $mtO_2^{\bullet-}$ production using Tg^{hSOD2}, strategies to attenuate $mtO_2^{\bullet-}$ by converting it to H_2O_2 in fact exacerbated RVSP, muscularization and remodeling of pulmonary arterioles, and markers of PASMC proliferation. In addition, total

H_2O_2 production was increased by SOD2 overexpression resulting in increases in both hypoxia-induced Nox 2 and Nox 4 mRNA and protein levels. Tg^{hSOD2} mice had significantly increased induction of PCNA, suggesting enhanced proliferation and remodeling. Ultimately, these studies suggest that targeted attenuation of mitochondria $O_2^{\bullet -}$ is insufficient to prevent PH pathogenesis. To detect any beneficial effect of SOD2 overexpression, it may be necessary to also increase reduction of H_2O_2. This data suggests that mtH_2O_2 is the initial signal that needs to be targeted to attenuate hypoxia-induced PH. Even though some studies have suggested that SOD expression is reduced in PH, these findings indicate that increasing that activity with SOD2 overexpression worsens hypoxia-induced PH pathobiology. These findings do not exclude the possibility that strategies that reduce $mtO_2^{\bullet -}$ generation might also be protective if mtH_2O_2 is also reduced.

CHAPTER 5:
Overexpression of Thioredoxin2 in Hypoxia-induced PH

INTRODUCTION

Thioredoxins (Trx) are a family of multifunctional antioxidants first described in1964 by Laurent et al (Powis and Montfort, 2001). There are three thioredoxin (Trx) isozymes: cytosolic Trx1, mitochondrial Trx2, and spermatozoa spTrx. All mammalian Trxs are able to reduce disulfides to dithiols as a result of 2 conserved cysteine residues in the active site (Tyr -Cys32 – Gly – Pro – Cys35) (Babior et al., 1973; Beckman et al., 1988; Hansen et al., 2006a). Trxs act with Peroxiredoxins (Prxs) to exert cytoprotective effects against oxidative stress and influence cell function via modulation of redox status. Prxs are non-heme peroxidases that are responsible for the reduction of endogenously produced H_2O_2. Trxs reduce oxidized Prxs, becoming oxidized themselves (Ebrahimian and Touyz, 2008; Lowes and Galley, 2011). Thioredoxin reductase (TrxR) reduces oxidized Trxs to restore antioxidant capacity (Ebrahimian and Touyz, 2008). Trx proteins are present in all eukaryotic and prokaryotic organisms and are essential for cell viability. Homozygous knockout of either Trx1 or Trx2 is embryonic lethal in mice (Dunn et al., 2010). Furthermore, Trx2 is oxidized at cysteine 32 and 35, and this active site is present in the transgenic human Trx2 (Tg^{hTrx2}) model used in subsequent studies **(Figure 5.1)**. Prx I, II, and VI are localized to the cytosol, Prx III and V are in the mitochondria, and Prx IV is localized to the extracellular space (Dunn et al., 2010). Prxs, not catalase, are responsible for the reduction of endogenously generated H_2O_2 within the mitochondria. Using NADPH as a source of electrons, Trx2 works with Prx family

members and TrxR to reduce exposed protein disulfides (a S—S bond) to dithiols (two –SH groups) (Watson et al., 2003). The critical role of H_2O_2 in hypoxia-induced pulmonary vascular cell proliferation and PH provide the rationale for exploring the role of Trxs in these studies (Ebrahimian and Touyz, 2008). Though Trx2 does not directly interact with H_2O_2, Trx2 modulation is a novel strategy of modulating mitochondrial redox.

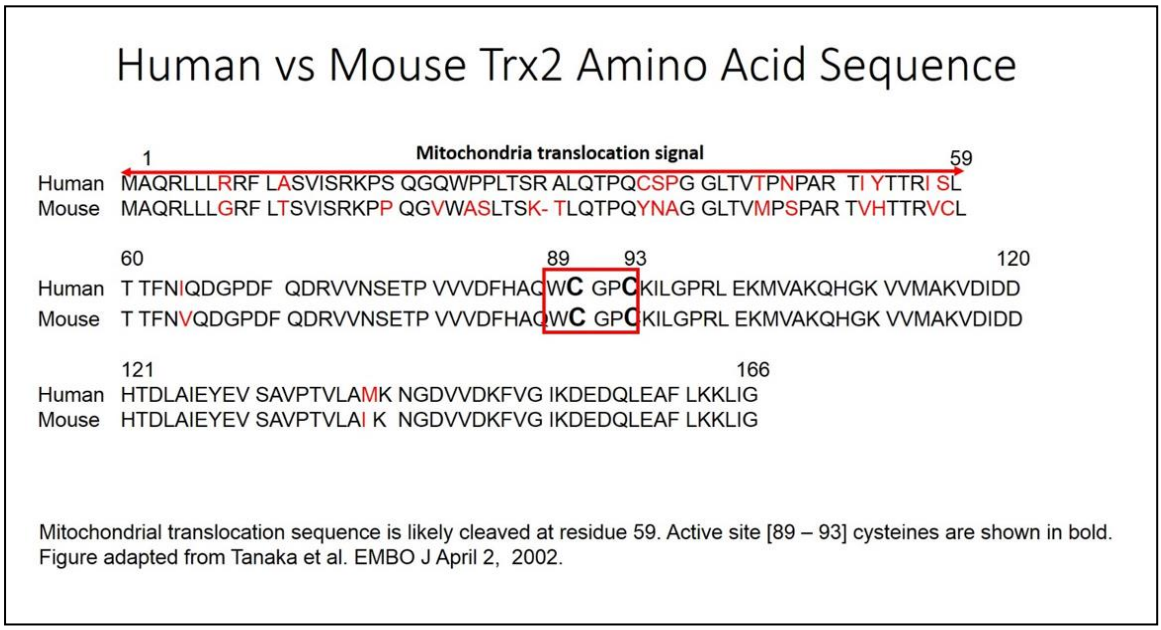
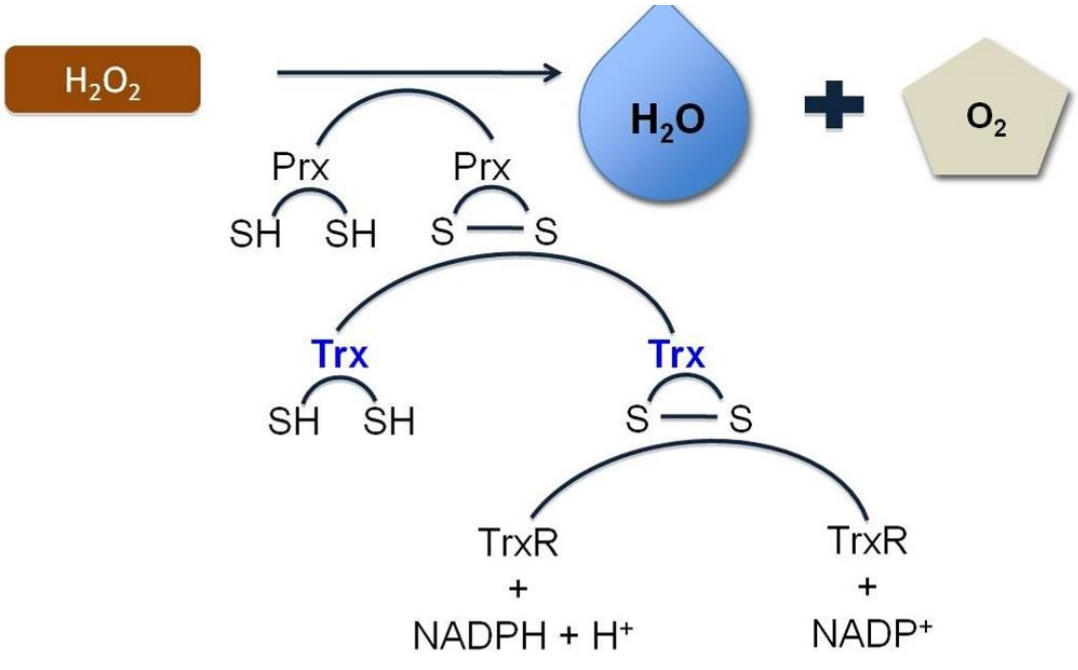


Figure 5.1: Human vs Mouse Trx2 Amino Acid Sequence. Modified cysteines are detailed in red box.

ROS from both the cellular respiration of the mitochondria and the activity of NADPH oxidases likely play a vital role in PH pathogenesis (Datla and Griendling, 2010). Therefore attenuation of mitochondrial pools of antioxidants, such as Trx2, may differentially regulate hypoxia-induced vascular remodeling and PH via divergent effects on the expression of Noxes, proliferation genes, and apoptotic signaling in pulmonary vascular wall cells. The present study examines the effects of overexpression of Trx2, a mitochondrial ROS scavenger, on the development of hypoxia-induced PH *in vitro* and *in vivo*.

In the case of hypoxia-induced PH, the production of ROS is in excess of the endogenous antioxidant capacity leading to an environment of oxidative stress (Jones, 2006; Sies, 1997). Mitochondria act as cellular O_2 sensors that adjust redox-signaling pathways to modulate vascular tone and regulate hypoxia-induced redox signaling by ROS (Fuchs et al., 2010; Hansen et al., 2006b). Noxes, in particular Nox2 and Nox4, also produce ROS within the vasculature (Cutz et al., 2009; Frazziano et al., 2012; Mittal et al., 2007b) which may impact endothelial function, vascular tone, vascular cell proliferation, and apoptosis (Dikalova et al., 2010; Paravicini and Touyz, 2008). **We hypothesize that Trx2 differentially regulates hypoxia-induced vascular remodeling and PH via attenuation of mitochondrial H_2O_2 and modifying the effects of H_2O_2 on the expression of Noxes.** Overexpression of Trx2 would increase the availability of reduced Prx, allowing for more H_2O_2 to be converted to H_2O and O_2, and thereby prevent elevations in hypoxia-induced PH markers **(Figure 5.2)**.



Abbreviation	Molecule
H_2O_2	Hydrogen peroxide
H_2O	Water
O_2	Oxygen
Prx	Peroxiredoxin
SH	Dithiol
S – S	Disulfide bond
Trx	Thioredoxin
TrxR	Thioredoxin reductase
NADPH	Nicotinamide adenine dinucleotide phosphate

Figure 5.2: Role of Trx2 in H_2O_2 reduction.

RESULTS

Chronic hypoxia exposure decreases Trx2 expression and activity in HPAECs

Mitochondrial ROS plays a critical role in the development of multiple vascular complications (Min W et al., 2010; Widder et al., 2009). To further explore how mitochondrial antioxidants might be altered during PH, we examined the effects of hypoxia on Trx2 in human pulmonary arterial endothelial cells (HPAECs) exposed to normoxic (21% O_2) or hypoxic (1% O_2) conditions for 72 hours. Hypoxia exposure decreased Trx2 mRNA and Trx2 protein levels compared to HPAECs exposed to Normoxia, as measured by qRT-PCR (**Figure 5.3A)** and western blotting (**Figure 5.3B)**, respectively.

Figure 5.3: Hypoxia decreased Trx2 expression *in vitro*. HPAECs were exposed to 72 hours of hypoxia (1% O_2) or Normoxia (21% O_2), and Trx2 mRNA and protein levels were measured. **(A)** Trx2 mRNA levels were decreased in HPAECs exposed to 72 hours of hypoxia (mean ± SEM, n = 3 *p < 0.05.). **(B)** Trx2 protein levels were decreased in HPAECs exposed to 72 hours of hypoxia (mean ± SEM, n = 5 *p< 0.05). A representative western blot for Trx2 in HPAECs exposed to 72 hours of hypoxia. β-Actin was used as loading control.

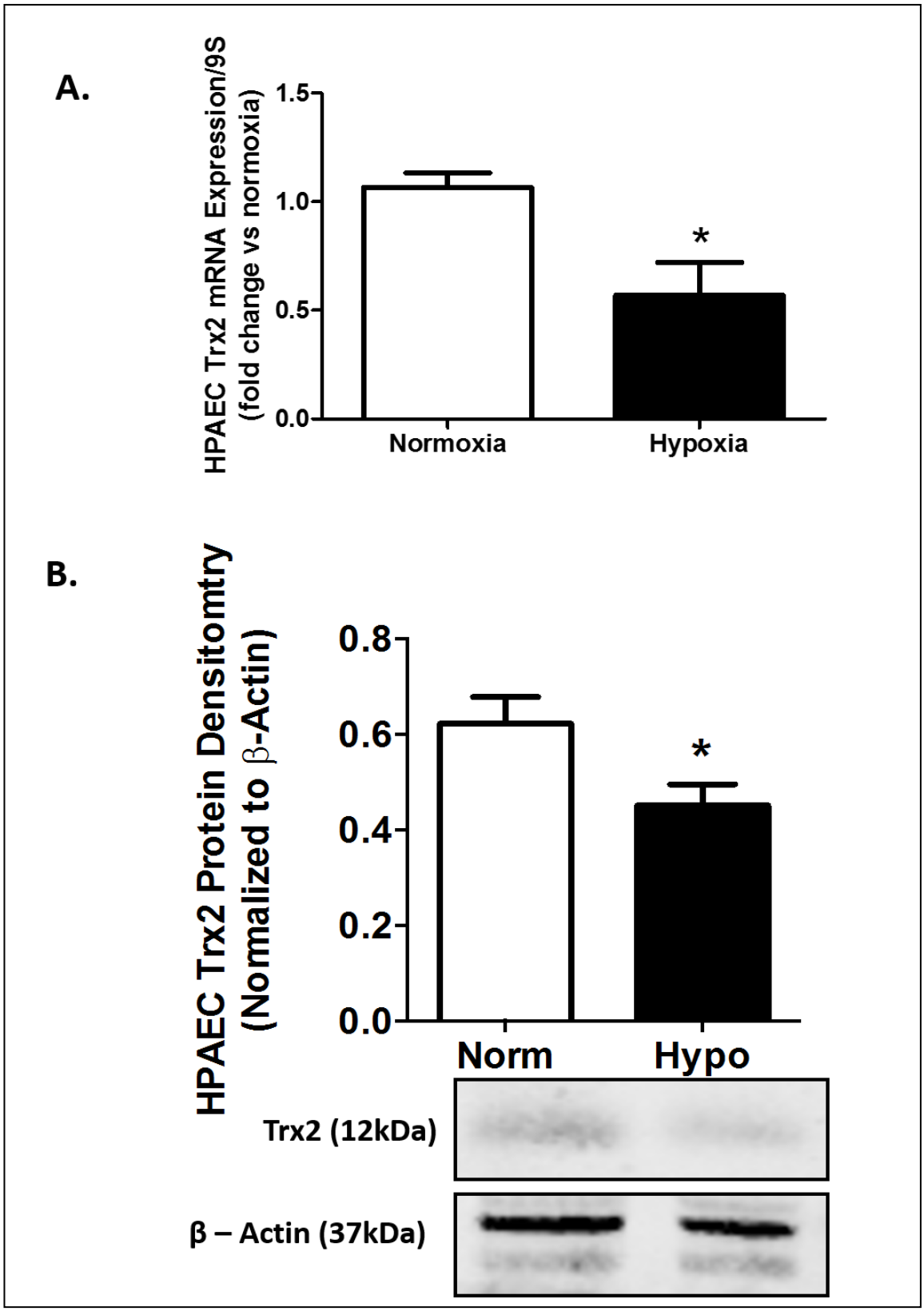


Figure 5.3: Chronic hypoxia exposure decreased Trx2 expression in HPAECs.

Chronic hypoxia exposure decreased Trx2 RNA levels in C57Bl/6 mice in vivo

Because hypoxia reduced Trx2 levels *in vitro* and because increases in mitochondrial ROS generation may contribute to endothelial dysfunction in PH (Min W et al., 2010; Widder et al., 2009), the levels of Trx2 in mice following exposure to hypoxia for 3 weeks was examined. Hypoxia decreased Trx2 mRNA levels compared to mice exposed to normoxic conditions as measured by qRT-PCR **(Figure 5.4)**.

Figure 5.4: Chronic hypoxia exposure decreased Trx2 expression *in vivo*. C57Bl/6 mice were exposed to hypoxia for 3 weeks, and Trx2 mRNA levels were measured in whole lung homogenates. Hypoxia reduced Trx2 mRNA levels in C57Bl/6 mice (mean ± SEM, n = 5 *p < 0.05).

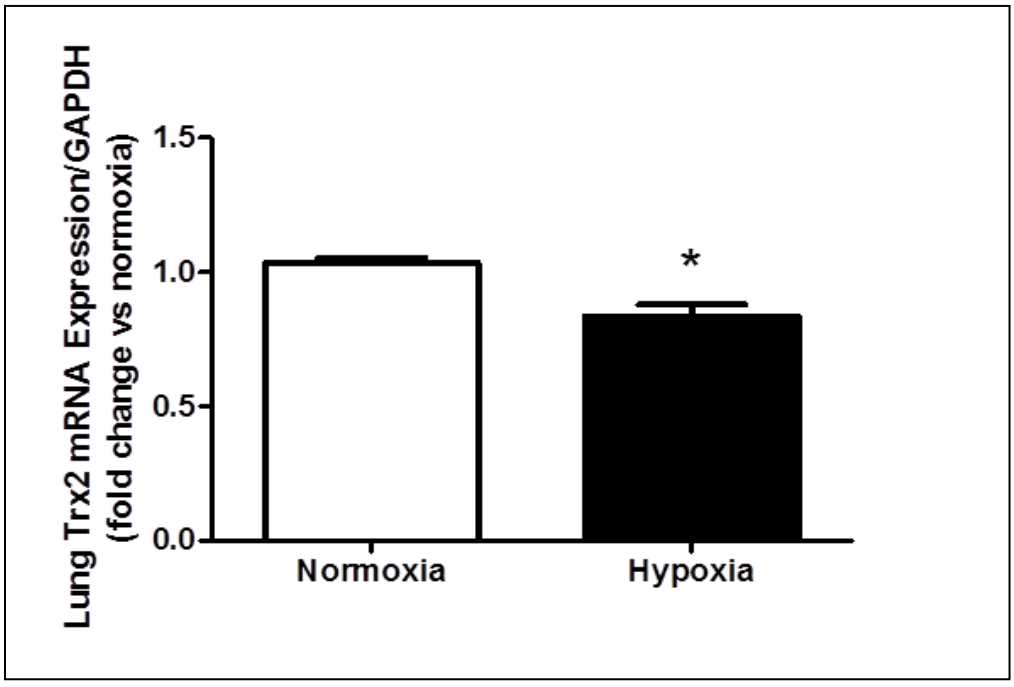


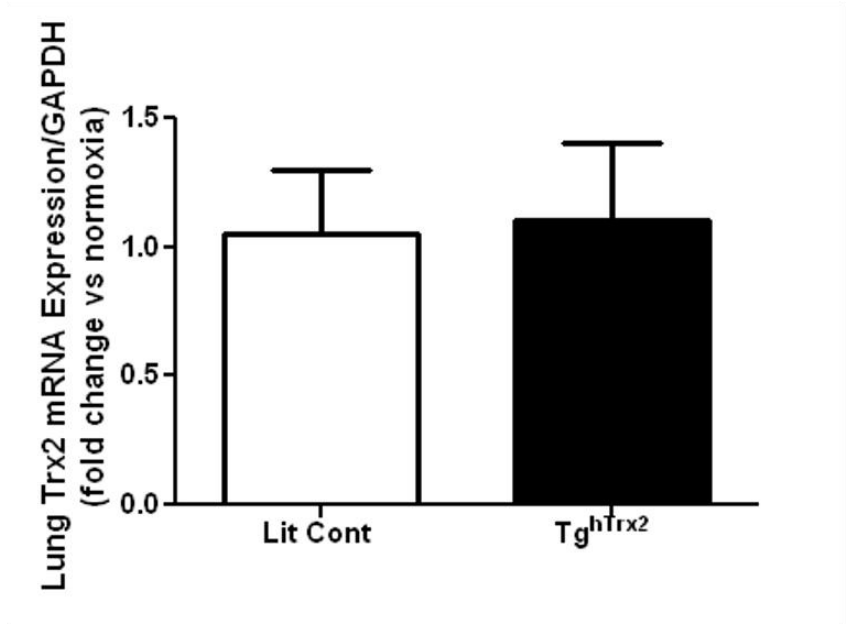
Figure 5.4: Chronic hypoxia exposure decreased Trx2 mRNA levels in mouse lung *in vivo*.

Confirmation of Trx2 overexpression in the Tg^{hTrx2} model

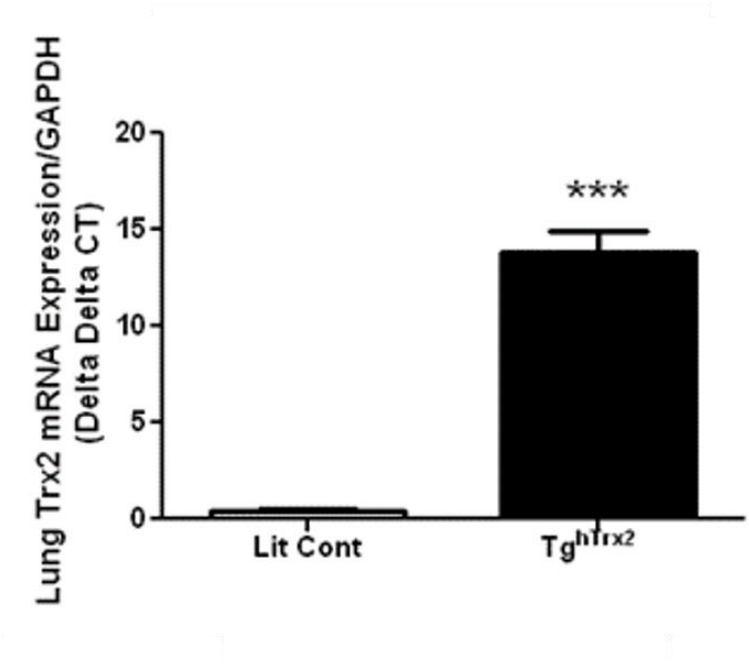
Having established that endogenous Trx2 protein expression was decreased by hypoxia, studies were completed to confirm that our transgenic model appropriately overexpressed Trx2. Murine (**Figure 5.5A)** and human (**Figure 5.5B)** Trx2 mRNA levels and protein levels were examined. Western blots made use of the V5 epitope present in the Tg^{hTrx2} model (**Figure 5.5C)**.

Figure 5.5: Confirmation of Trx2 overexpression in the Tg^{hTrx2} model. **(A)** Mouse Trx2 mRNA levels were equal when comparing littermate controls (Lit Cont) to Tg^{hTrx2} (mean ± SEM, n = 3 *p < 0.5). **(B)** Human Trx2 mRNA was only detected in the Tg^{hTrx2} model compared to Lit Cont animals (Lit Cont mice expressed only endogenous mouse Trx2 and not the human transgene) (mean ± SEM, n = 3 ***p < 0.05). **(C)** Following exposure to normoxia (21% O_2) or hypoxia (10% O_2) for 3 weeks, levels of human Trx2 protein, as detected by probing for the V5 epitope, remained significantly elevated in the Tg^{hTrx2} mice (mean ± SEM, n = 6, ***p < 0.0001).

A.


Lung Trx2 mRNA Expression/GAPDH
(fold change vs normoxia)
1.5
1.0
0.5
0.0
Lit Cont
Tg^hTrx2

B.


Lung Trx2 mRNA Expression/GAPDH
(Delta Delta CT)
20
15
10
5
0
***
Lit Cont
Tg^hTrx2

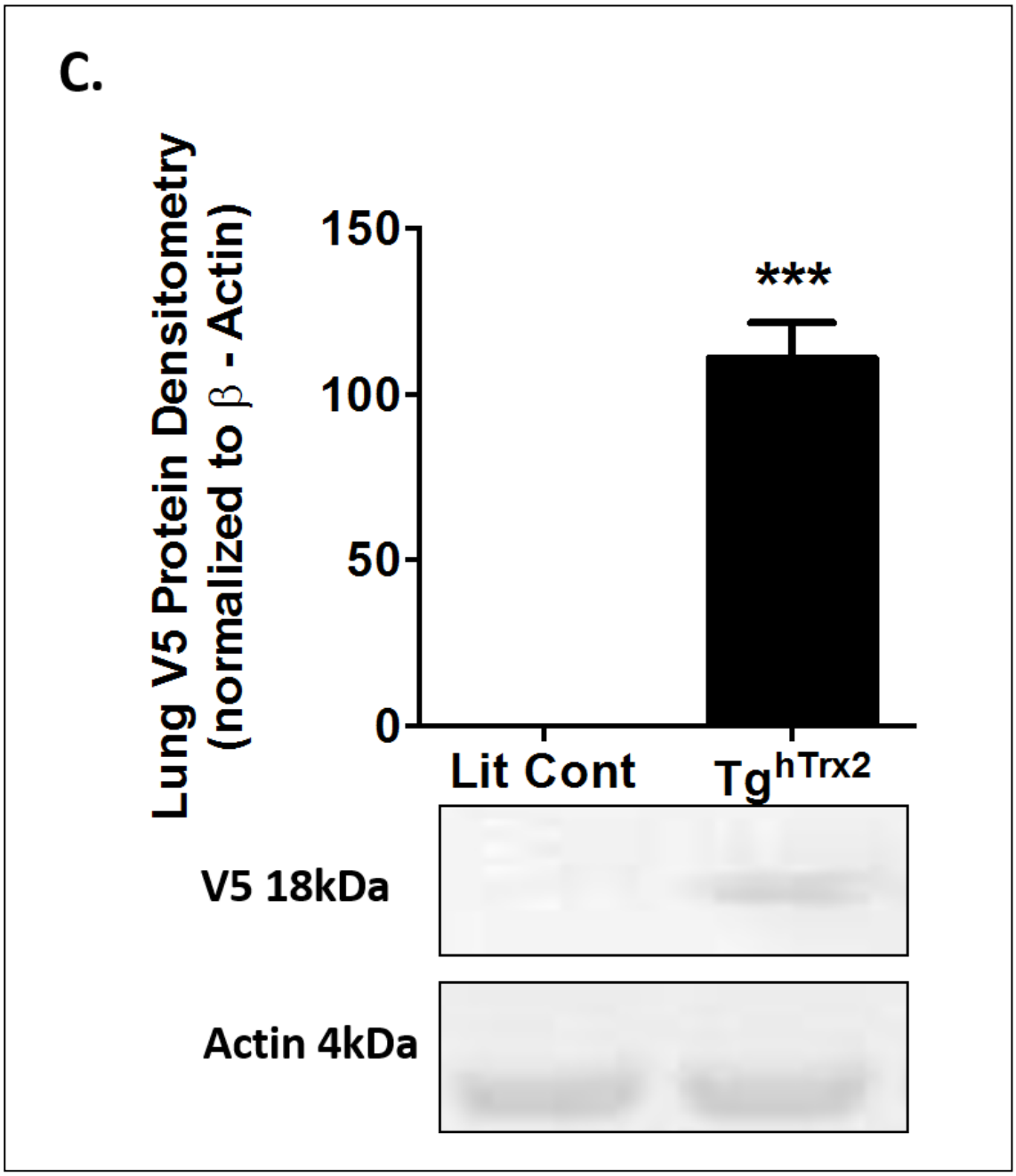
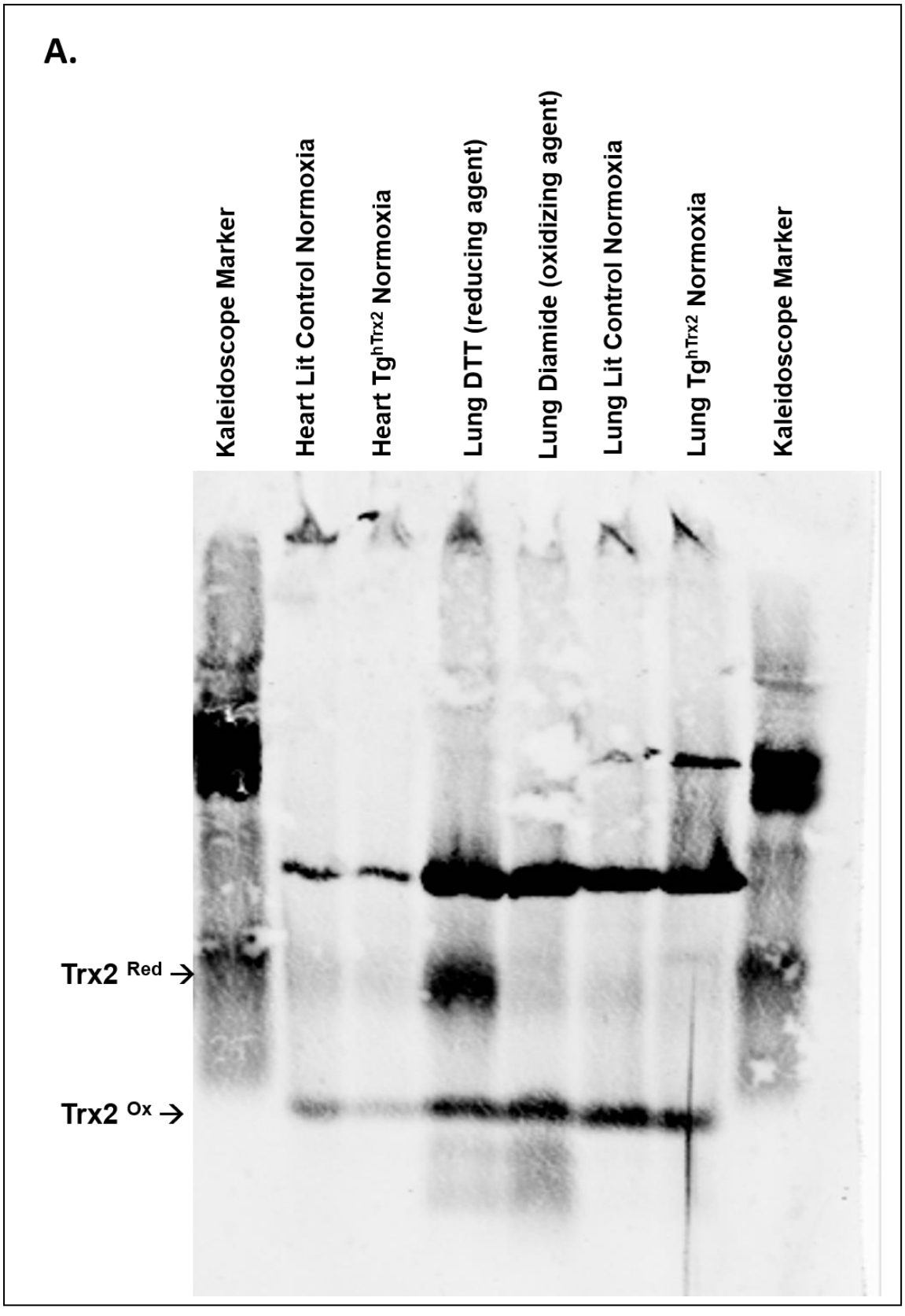


Figure 5.5: Confirmation of Trx2 overexpression in Tg^{hTrx2} mice.

Hypoxia increases oxidized Trx2 levels

To further assess the activity of the transgene, we utilized redox western blot techniques. This technique allowed us to compare reduced Trx2 (active) to oxidized Trx2 (inactive) protein levels in cardiac and pulmonary tissue **(Figure 5.6 A)**. The control agent dithiothreitol (DTT) was used to reduce samples and diamide was used as an oxidizing agent. Densitometric intensity of Trx2 bands were averaged to permit quantitative comparisons of inactive (oxidized) hypoxic samples **(Figure 5.6 B)**.

Figure 5.6: Hypoxia increased oxidized Trx2 protein levels. **(A)** A representative immunoblot is presented showing levels of reduced (upper band Trx2 ^{Red}) and oxidized (lower band – Trx2 ^{Ox}) Trx2 in cardiac and pulmonary tissue. **(B)** Preliminary densitometry of band intensity indicated an increase in oxidized Trx2 in hypoxic samples, including Tg^{hTrx2} samples, possibly due to a decrease in reduced levels, as opposed to an increase in oxidized form (mean ± SEM, n ≥ 3), warranting follow-up. Lit Cont N (Littermate Control Normoxia), Lit Cont H (Littermate Control Hypoxia), Trx2 N (Tg^{hTrx2} Normoxia), Trx2 H (Tg^{hTrx2} Hypoxia).


A.
Kaleidoscope Marker
Heart Lit Control Normoxia
Heart Tg^hTrx2 Normoxia
Lung DTT (reducing agent)
Lung Diamide (oxidizing agent)
Lung Lit Control Normoxia
Lung Tg^hTrx2 Normoxia
Kaleidoscope Marker
Trx2 Red →
Trx2 Ox →

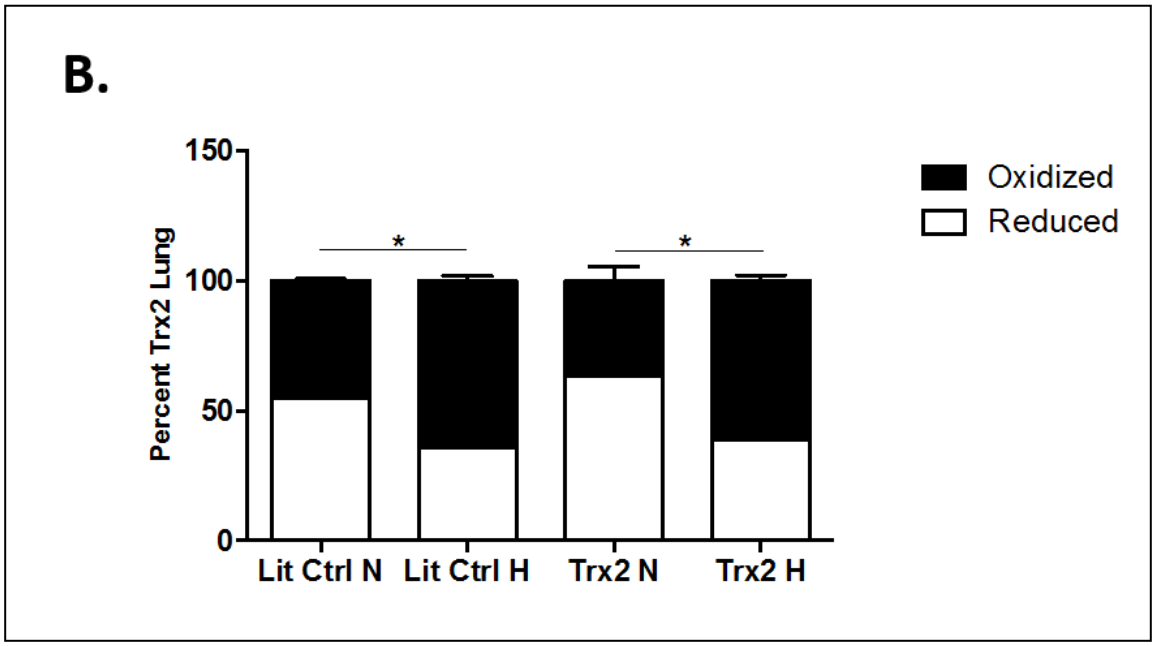


Figure 5.6: Hypoxia increases oxidized Trx2 protein expression.

Hypoxia decreases Prx3 and Trx2R mRNA levels

Since our studies confirmed that hypoxia decreases Trx2 levels and increases oxidized (inactive) Trx2 levels, qRT-PCR was used to assess upstream Prx3 levels (reduces H_2O_2) and downstream Trx2R levels (reduces Trx2) (Figure 5.2). Using both HPAECS and HPASMCs exposed to 72 hours of normoxia or hypoxia, we detected that hypoxia significantly decreases mRNA levels of Prx3, Trx2, and Trx2R. Prx3 and Trx2 mRNA levels were significantly reduced in HPAECs **(Figure 5.7A and 5.7B)** and HPASMCs **(Figure 5.7C and 5.7D)**.

Figure 5.7: Hypoxia decreases Prx3 and Trx2R mRNA levels in HPAECs and HPASMCs. Hypoxia decreases HPAEC **(A)** Prx3 mRNA (mean ± SEM, n ≥ 5, *p < 0.05 compared to normoxic group) and **(B)** Trx2R mRNA levels (mean ± SEM, n ≥ 3, *p < 0.05 compared to normoxic group). This hypoxia-induced decrease in **(C)** Prx3 mRNA (mean ± SEM, n ≥ 5, *p < 0.05 compared to normoxic group) and **(D)** Trx2R mRNA levels (mean ± SEM, n ≥ 5, *p < 0.05 compared to normoxic group) was also present in HPASMCs.

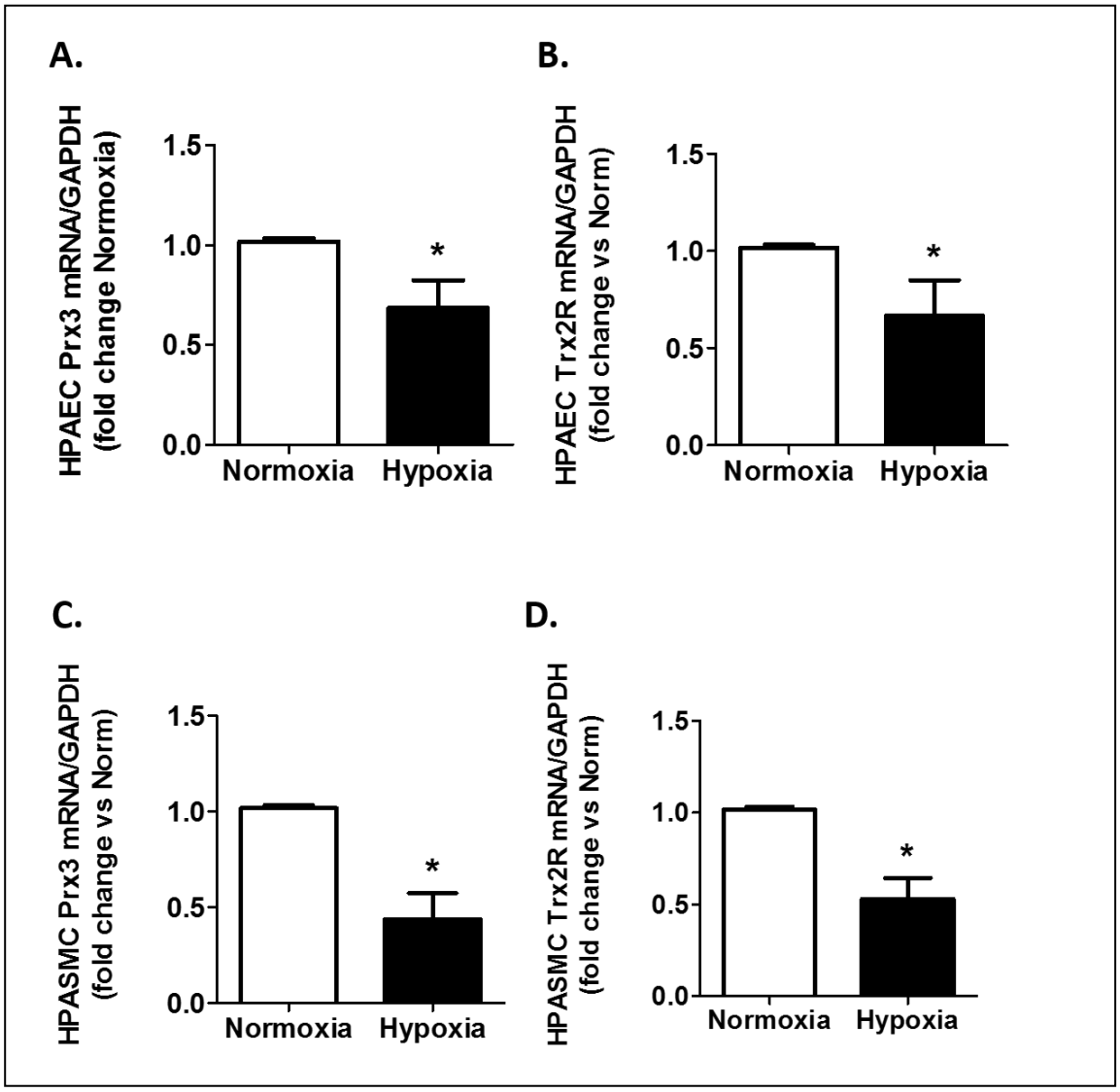


Figure 5.7: Hypoxia decreases Prx3 and Trx2R mRNA levels in HPAECs and HPASMCs.

Overexpression of Trx2 does not prevent hypoxia-induced PH

Because redox systems are not in equilibrium, the concept of a simple balance between pro-oxidant and antioxidant systems may represent an oversimplification of oxidative stress. Rather, from a mechanistic perspective, oxidative stress would be better defined as disruption of redox signaling and control in various cellular compartments (Jones, 2006). In order to determine if overexpression of Trx2 affects the development of hypoxia-induced PH in normoxia or hypoxia, Lit Cont and Tg^{hTrx2} mice were exposed to 3 weeks normoxia or hypoxia. There were no weight differences between Lit Cont or Tg^{hTrx2} mice (data not shown). Hypoxia-exposed Lit Cont and Tg^{hTrx2} mice showed similar increases in hematocrit (data not shown) confirming hypoxia exposure by indicating increase in red blood cell content. Hypoxia-induced PH **(Figure 5.8A)** and RVH **(Figure 5.8B)**, when compared to Lit Cont, were either unchanged or exacerbated in Tg^{hTrx2} mice, respectively.

Figure 5.8: Overexpression of Trx2 does not prevent hypoxia-induced PH. Tg^{hTrx2} mice demonstrated no decrease in physiological markers of PH. **(A)** Overexpression of Trx2 did not attenuate hypoxia-induced elevations in RVSP (mean ± SEM, n = 6, *p < 0.05 compared to normoxia groups). **(B)** Trx2 overexpression significantly increased hypoxia-induced RVH (mean ± SEM, n = 6, *p < 0.05 compared to normoxic groups, **p < 0.01 compared to Lit Cont Hypoxia).

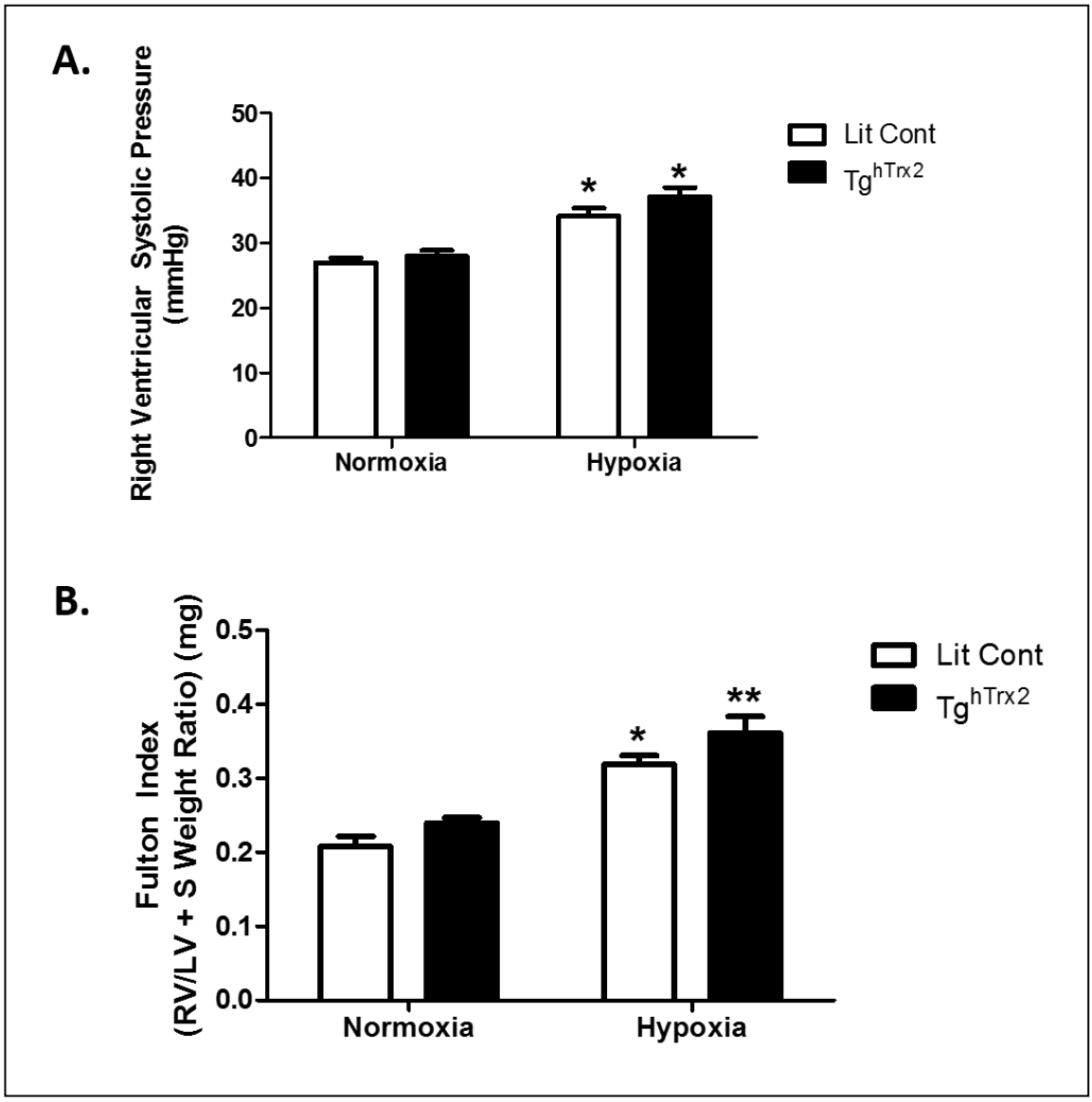


Figure 5.8: Overexpression of Trx2 failed to prevent hypoxia-induced PH and RVH.

Overexpression of Trx2 does not prevent hypoxia-induced increases in ROS

Imbalances in redox signaling and increases in ROS promote the development of PH (Nisbet et al., 2010; Watson et al., 2004). Increases in H_2O_2 levels are observed in various models of PH (Green et al., 2012; Nisbet et al., 2010). To determine if Trx2 overexpression modified H_2O_2 levels, Amplex red was used to assess extracellular H_2O_2 levels in lung tissue from Lit Cont and Tg^{hTrx2} mice exposed to normoxia or hypoxia for 3 weeks. Trx2 overexpression had no significant effect on hypoxia-induced increases in lung H_2O_2 levels **(Figure 5.9A)**. Consistent with a lack of effect of Tg^{hTrx2}, hypoxia-induced increases in lung Nox4 mRNA levels were not altered by Trx2 overexpression **(Figure 5.9B)**.

Figure 5.9: Trx2 overexpression failed to alter hypoxia-induced increases in lung H_2O_2 and Nox4 levels. Amplex red assays were used to measure lung H_2O_2 levels *ex vivo*. **(A)** Hypoxia-induced increases in lung H_2O_2 levels were not altered by Trx2 overexpression in Tg^{hTrx2} mice (mean ± SEM, n = 6, *p < 0.05 compared to normoxic groups). **(B)** Hypoxia-induced increases in lung Nox4 mRNA levels were not altered by Trx2 overexpression in Tg^{hTrx2} mice (mean ± SEM, n = 3, *p<0.05 compared to normoxia groups).

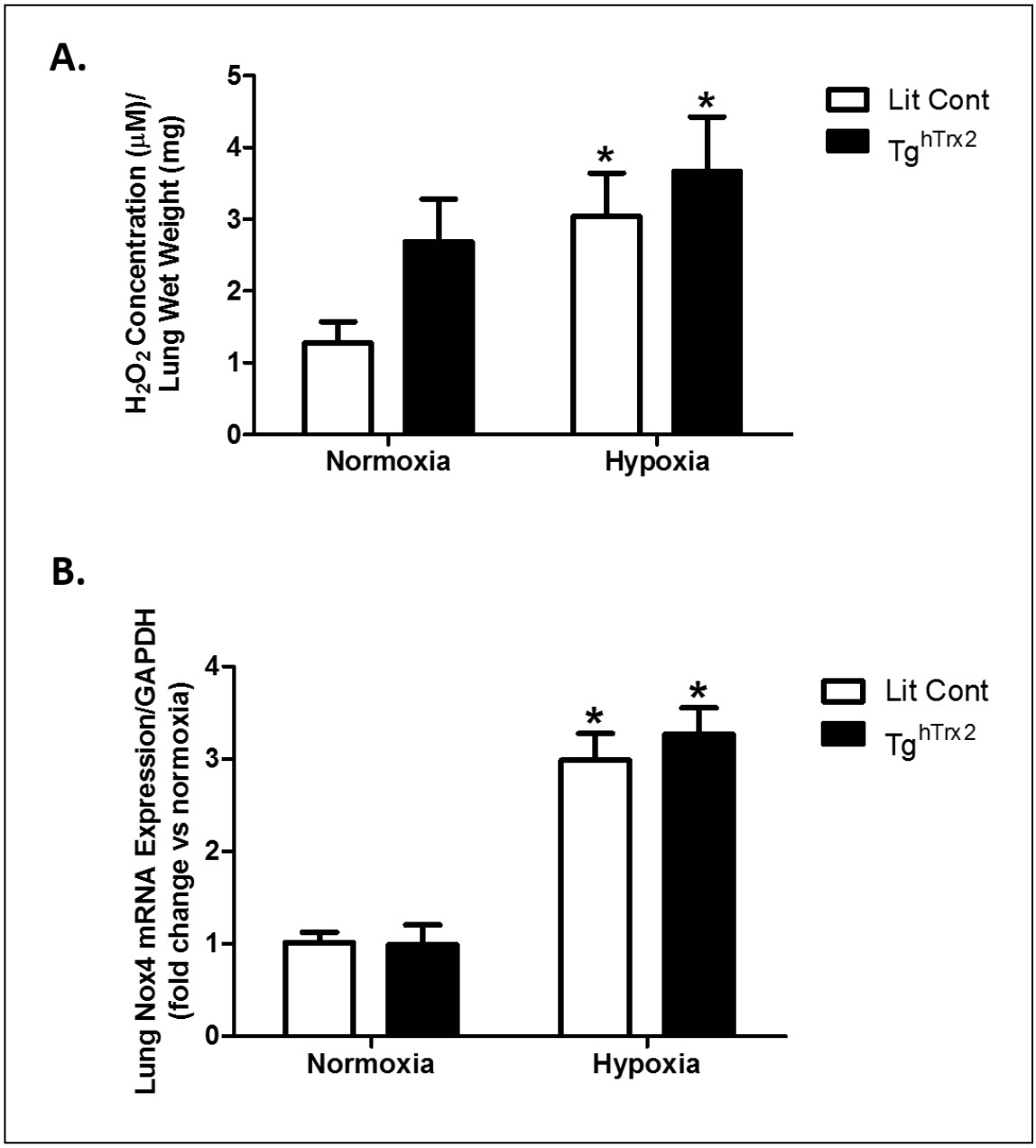


Figure 5.9: Trx2 overexpression failed to modulate hypoxia-induced increases in H_2O_2 or Nox4 levels in mouse lung.

DISCUSSION

The studies performed establish that regulation of mitochondrial ROS through modulation of Trx2 has little ability to alter mitochondrial ROS generation and PH development **(Figure. 5.10)**. These studies confirm that hypoxia decreases Trx2 expression *in vitro* (Fig. 5.3) and *in vivo* (Fig. 5.4). The present study confirms expression of human Trx2 in the Tg^{hTrx2} mouse lung by qRT-PCR and WB (Fig. 5.5). However, overexpression of Trx2 did not prevent hypoxia-induced increases in: RVSP, RVH, H_2O_2 generation, and Nox4 mRNA (Figs. 5.7 and Fig. 5.8).

Figure 5.10: Effect of Tg^{hTrx2} in the development of PH. The effect of targeted attenuation of mtH_2O_2 with Tg^{hTrx2} model (right side of schema) remains undefined in hypoxia-induced PH.

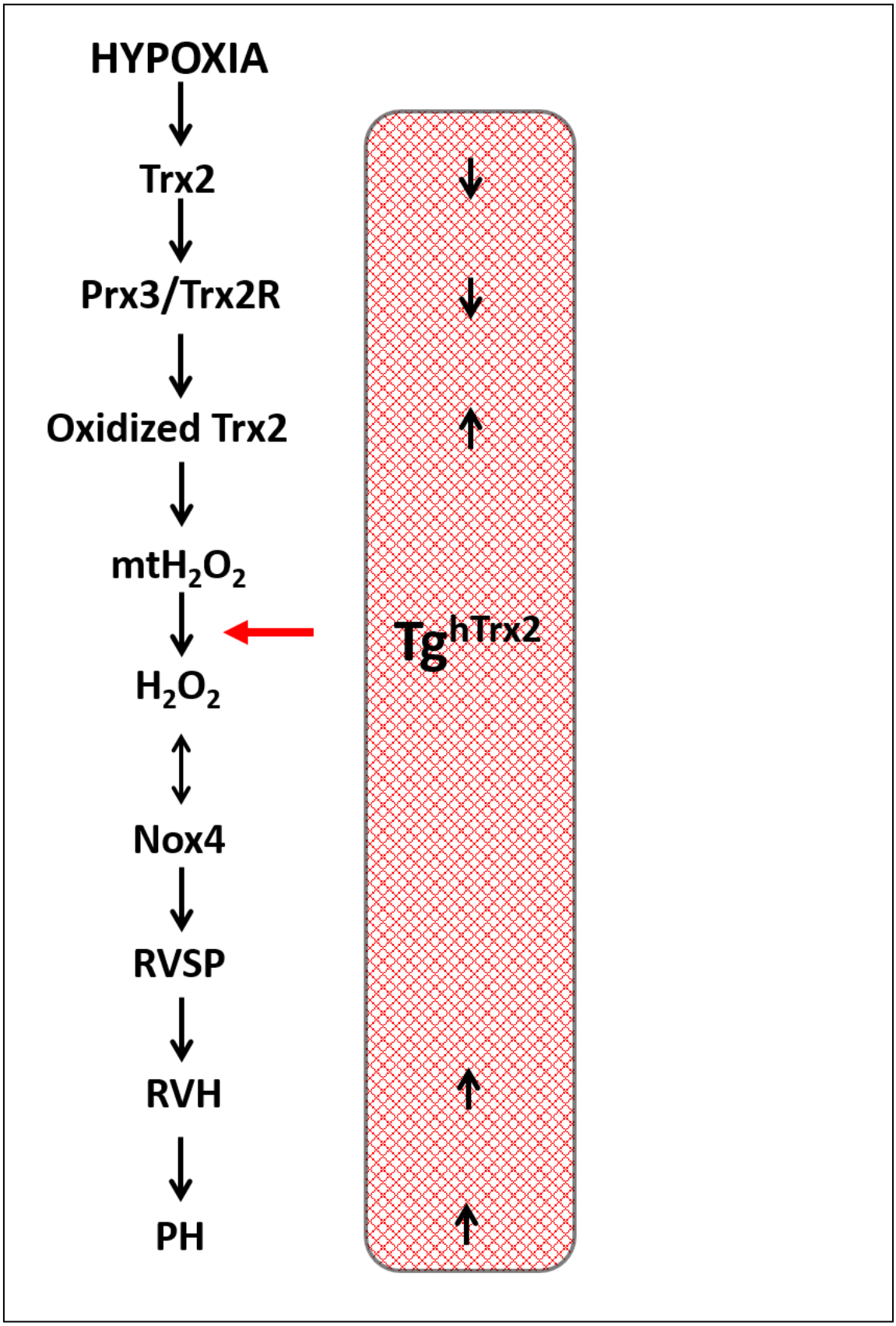


Figure 5.10: Schema depicting effect of Tg^{hTrx2} in a mouse model of PH.

Accumulating data suggests that ROS generated from the mitochondria in the endothelial cells of the vasculature significantly contributes to pulmonary disease (Min W et al., 2010). Hypoxia exposure has previously been shown to enhance ROS and Nox expression (Nisbet et al., 2009). Since the thioredoxin system is key in regulating mitochondria redox state (Min W et al., 2010), it follows that increased antioxidant expression within the mitochondria may be able to prevent hypoxia-induced PH. Reduction of oxidized proteins by Trx2 is crucial for maintaining the mitochondria in a reduced state. The reduced state of the mitochondria is crucial for maintaining the electrochemical gradient, ATP generation, and ROS elimination (Min W et al., 2010). Similar to these results, Trx2 mRNA levels are decreased in the lungs of hypoxia-exposed animals (Zhou et al., 2012).

These studies allow us to conclude that modulation of Trx2 alone is not sufficient to regulate mitochondrial H_2O_2. Admittedly, the study was limited by the murine model, which cannot replicate all of the derangements observed in human PH (Bauer et al., 2007; Maarman et al., 2013). Though we predicted that Trx2 overexpression would lower mtH_2O_2 production (by rapidly reducing Prx3 to constantly reduce hypoxia-induced H_2O_2), Trx2 overexpression had no effect on Amplex® Red detected H_2O_2 levels. Furthermore, we detected that the Trx2 was in the inactive oxidized form. Additional studies are needed to confirm that there is an increase in the reduced (active) Trx2 compared to oxidized (inactive) Trx2

since the reduced form of Trx2 is required to reduce Prx for a protective effect. Increasing levels of TrxR to increase Trx2 reduction may also be beneficial. Also, *in vitro* studies overexpressing Trx2 can be used to assess the effect of Trx2 overexpression in HPAECs. It should also be considered that though our other studies established a vital role for mtH_2O_2 in PH development, H_2O_2 is freely diffusible out of the mitochondria and elevated H_2O_2 may exert detrimental effects without ever interacting with Trx2, Prx3, or other mitochondrial antioxidant proteins.

Since these studies incompletely assess the benefit of Trx2 overexpression, additional studies are needed to assess if Trx2 overexpression is a useful target for PH therapy. Studies involving *in vitro* overexpression to assess downstream effects on Nox4, H_2O_2 generation, and proliferation would provide additional evidence for the role of Trx2 in PH development. Furthermore, additional studies are necessary to confirm Trx2 activity in the Tg^{hTrx2} model. We can conclude that though there may be a significant role for mitochondrial ROS in PH regulation, attenuation of mitochondria-ROS via Trx2 overexpression is insufficient to address the dilemma. Since Trx2 actually functions to reduce Prx3, not H_2O_2, it is possible that the effects of Trx2 overexpression are too far removed from the H_2O_2 to show any global level of PH benefit. A model that has elevated Trx2 and Prx3 may better serve to assess the benefit of targeted attenuation of mitochondrial H_2O_2.

CHAPTER 6: Discussion

Mitochondrial ROS Regulation of Noxes

The present studies demonstrate that mtROS generation is important in the development of PH. To our knowledge, these studies are the first PH studies to focus on the regulation of NADPH oxidase expression by mtROS. While previous studies established a feedback loop between mtROS and Nox activity (Dikalov, 2011), these studies established that hypoxia-induced mtROS generation increased Nox mRNA levels and Nox protein expression. Further, the present studies determined that mt-derived H_2O_2 generation was the ROS critical for Nox induction and PH pathogenesis (Dikalov et al., 2008).

In the current studies, targeted attenuation of mtH_2O_2 with mitochondrial-targeted catalase overexpression prevented hypoxia induced elevation of both Nox2 and Nox4. These two Noxes are of particular importance because previous studies have established their contribution to increased ROS levels and to vascular responses to hypoxia (Barman et al., 2014; Fike et al., 2008; Frazziano et al., 2014; Fresquet et al., 2006; Griffith et al., 2009). Accumulating evidence indicates that ROS derived from Nox2 and Nox4 are involved in long-term responses of the pulmonary vasculature to hypoxia (Frazziano et al., 2012; Griffith et al., 2009). Our study is the first to demonstrate that mtH_2O_2 directly contributes to induction of Nox2 and Nox4 expression. This increase in H_2O_2 may sustain the effects of hypoxia by activating Noxes which further produce ROS that contribute to dysregulated pulmonary artery responses. Nox4 mRNA

upregulation in PH has been demonstrated previously (Barman et al., 2014; Frazziano et al., 2014; Lu et al., 2013; Wedgwood et al., 2013) and Nox4 expression in focal adhesions, endoplasmic reticulum, and nucleus in vascular smooth muscle cells and endothelial cells has been established (Clempus et al., 2007; Desouki et al., 2005; Nauseef, 2008). Nevertheless, the localization of Nox4 remains unclear. Nox4 mitochondrial localization remains uncertain (Ago et al., 2010; Frazziano et al., 2014) but appears to be cell type specific. Mitochondrial Nox4 was first detected in WB from cultured mesangial cells and kidney cortex that revealed the presence of Nox4 in crude mitochondria, mitochondria-enriched heavy fractions, and in purified mitochondria. These studies also detected that Nox4 localizes with the mitochondrial marker MitoTracker and that the mitochondrial localization prediction program MitoProt indicated that the probability score for Nox4 was equal to known mitochondrial protein cytochrome c oxidase subunit IV. Ultimately this study provided initial evidence that a functional Nox4 is present in and can be regulated by mitochondria (Block et al., 2009).

In a mouse catecholaminergic neuronal cell model (CATH.a), Nox4 was shown to be present in neuron mitochondria by co-localization with SOD2 and MitoTracker Red. This study also found Nox4 expression significantly increased in enriched mitochondrial fractions compared to whole cell lysates (Case et al., 2013). Another study using pulmonary artery banding (PAB) confirmed the role of mitochondria-localized Nox4 in early ROS generation possibly contributing to

progression of RV dysfunction and failure (Frazziano et al., 2014). Finally, Nox4 has also been shown to be overexpressed in the majority of breast cancer cell lines, primary breast tumors, and in ovarian tumors. In these cell types, Nox4 protein contained a 73 amino acid mitochondrial localization signal at the N-terminus that is capable of transporting a passenger protein GFP into the mitochondria (Graham et al., 2010). While attempts to detect Nox4 in isolated mitochondria from pulmonary tissue were unsuccessful (data not shown), the possibility that Nox4 (and the H_2O_2 it produces) can be localized to the mitochondria further raise the possibility that Nox4 direct production of mtH_2O_2 may contribute to PH pathogenesis.

Treatment of Nox4 overexpressing cells with catalase resulted in decreased tumorigenic characteristics (i.e. dysregulated proliferation) (Graham et al., 2010). Hypoxia plays a major role in tumorigenicity (prior to angiogenesis, tumor interiors are hypoxic) and since our studies were performed using a hypoxia model of PH, these Nox4 cancer studies further our belief that mtROS does interact with Nox activity. In addition, since Nox-derived ROS can upregulate cyclinD1 (Veit et al., 2013) and promote vascular proliferation, and MCAT prevents cyclinD1 induction it is reasonable to conclude that preventing hypoxia-induced increases in Nox prevents hypoxia-induced proliferative phenotype. Taken together these studies indicate the mtROS drives Nox expression which may in turn promote a proliferative pulmonary vascular cell phenotype (Dikalova et al., 2010; Frazziano et al., 2014). Our studies align well

with previous studies that found that Nox2 KO protected against hypoxia-induced PH (Liu et al., 2006). Additional studies have also found that hypoxia/reoxygenation-induced vasoconstriction was mediated by intracellular superoxide overproduction via endothelial Nox2 and that increasing endogenous levels of CuZn-SOD in coronary arteries may be cardioprotective (Liu et al., 2004). In endothelial cells, Nox2-derived ROS regulate p38 MAP-kinase mediated proliferation and vascular endothelial growth factor (VEGF) migration (103, 198). These studies integrate and support a major role for Noxes in proliferation, which a hallmark of PH.

In contrast to targeting mtH_2O_2 by overexpressing catalase in mitochondria, the Tg^{hSOD2} model targeted mt$O_2^{\bullet-}$. Using human SOD2 to attenuate mitochondrial $O_2^{\bullet-}$ did not prevent hypoxia-induced elevation of Nox2 or Nox4. In fact, Nox2 and Nox4 mRNA levels and protein expression were exacerbated in the Tg^{hSOD2} model. Since Nox2 and Nox4 can regulate vascular responses to hypoxia (Barman et al., 2014; Fike et al., 2008; Fresquet et al., 2006), this increase in Nox2 and Nox4 expression likely contributed to exacerbation of PH in the model. Studies using a shunt lamb model of PH also found that the expression and activity of Rac1, $p47^{phox}$, and $p67^{phox}$ were increased and $O_2^{\bullet-}$ production was enhanced in PH (Wedgwood et al., 2013). In addition, uncoupled eNOS activity related to oxidation of tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2) contributed to enhanced $O_2^{\bullet-}$ production in PH animals (Grobe et al., 2006). This data solidifies a connection between elevation

in Nox expression and activity leading to elevations in $O_2^{\bullet -}$, and our novel findings imply that increased conversion of $O_2^{\bullet -}$ to H_2O_2 can increase Nox expression.

In addition, the Tg^{hTrx2} model failed to inhibit hypoxia-induced PH development. It is possible that because Trx2 does not directly interact with H_2O_2, its effects on PH development were more difficult to detect. In the present studies, Trx2 overexpression had no beneficial effect on RVSP, RVH, Nox4 mRNA levels, or H_2O_2 production. Unsurprisingly, since Nox4 has been shown to preferentially produce H_2O_2 (Dikalov et al., 2008), the lack of hypoxia-induced Nox4 inhibition in this model correlates with a lack of reduction in Amplex® red detected H_2O_2. Furthermore, it may remain difficult to detect any beneficial effect of Trx2 overexpression unless we are able to also increase levels of other proteins in this pathway (Prx and/or TrxR) or increase Trx2 activity.

Mitochondrial H_2O_2 Regulation of PH

The findings with the MCAT model indicate that mtH_2O_2 plays a major role in regulating pulmonary vascular responses to hypoxia. Our studies indicate that H_2O_2 is the functional reactive oxygen species because overexpression of SOD2 exacerbated PH pathogenesis. Though not conclusive in our studies Tg^{hTrx2} has been shown to attenuate vascular superoxide, H_2O_2, and Nox subunit activation in Ang-II model of hypertension (Widder et al., 2009). Additionally, Tg^{hTrx2} expression has improved endothelial cell function and decrease atherosclerosis development by decreasing oxidative stress (Zhang et al., 2007b). In the MCAT model, attenuated mtH_2O_2 reduced hypoxia-induced increases in RVSP, and vessel muscularization as well as, decreased hypoxia-induced H_2O_2 generation, increases in Nox2 and Nox4 expression, and expression of the proliferation markers cyclinD1 and PCNA. Conversely, the Tg^{hSOD2} model resulted in increased mtH_2O_2. This elevated mtH_2O_2 likely contributed to the elevation of RVSPs, increased vessel muscularization, hypoxia-induced H_2O_2 generation, exacerbated Nox2 and Nox4 expression, and increased PCNA levels indicative of a more proliferative PH phenotype. Since the Tg^{hTrx2} model does not directly target mtH_2O_2, results of the model cannot be used to accurately define the role of mtH_2O_2 in hypoxia-induced PH development. Ongoing studies are assessing the activity/function of the two antioxidant systems regulated by Trx2.

Increased production of mtH_2O_2, within the Tg^{hSOD2} model indicates that elevation of mtH_2O_2 can induce exacerbations in various markers of PH

pathophysiology. Though beyond the scope of these studies, the results indicate that increased expression of SOD2 resulted in elevated production of mtH_2O_2. Studies have found that the anti-apoptotic effects of SOD2 in HT-1080 cells are attributed to its ability to generate H_2O_2 (Dasgupta et al., 2006). SOD2 overexpression in HT-1080 cells resulted in production of mtH_2O_2 and led to PTEN oxidation which promoted an angiogenic switch. SOD2 can also serve as an alternative physiological source of H_2O_2 as a signaling molecule (Connor et al., 2005). A global increase in the intracellular steady-state production of H_2O_2 by SOD2 overexpression is likely responsible for the exacerbated PH phenotype observed in the Tg^{hSOD2} model. In fact, the same exacerbations (H_2O_2, Nox2/Nox4, cyclinD1/PCNA, RVSP, and vessel muscularization) in the Tg^{hSOD2} were subsequently attenuated in the MCAT model.

With the MCAT model, we directly attenuated mtH_2O_2 using catalase. This prevented many markers of PH pathobiology leading us to conclude that mtH_2O_2 plays a major role in the dysregulated signaling observed in PH. To better discern the efficacy of targeted mtH_2O_2, a model that overexpresses SOD2 and simultaneously targets mtH_2O_2 (possibly by overexpression of endogenous Prx3), could be used to confirm the importance of mtH_2O_2.

Several lines of evidence support our findings that increased ROS production plays a critical role in PH pathogenesis. The antioxidant, N-acetyl cysteine (NAC), prevented increases in phosphatidylcholine hydroperoxide

(PCOOH – an oxidative stress marker) in rats and reduced hypoxia-induced cardiopulmonary alterations (Hoshikawa et al., 2001; Voelkel et al., 2013). Unlike our study, SOD2 augmentation has been able to regress experimental PAH (Archer et al., 2010). This disparity in our results is likely due to different models, tissues, and cells used for each study. Furthermore, that study assessed Group 1 PAH (Archer et al., 2010), whereas the hypoxia model used in these studies is more applicable to Group 3 PH. Pharmacological NO synthase inhibition with NG-nitro-l-arginine methyl ester (L-NAME) treatment prevented adverse lung remodeling in caveolin KO mice (Wunderlich et al., 2008). Activation of peroxisome proliferator-activated receptor gamma with the synthetic agonist, rosiglitazone, attenuated hypoxia-induced activation of HIF-1alpha, NF-κB activation, and hypoxia-induced ET-1 signaling in PH pathogenesis. This same study also found that Caffeic acid phenethyl ester (CAPE – a 5-lipoxygenase and ROS inhibitor (Song et al., 2015)) prevented hypoxia-induced increases in HPAEC ET-1 mRNA and protein levels (Kang et al., 2011). These studies all concluded that increased ROS was involved with PH pathology.

Future Studies

While these studies are sufficient to conclude that targeted attenuation of aberrant mtH_2O_2 levels can prevent PH pathogenesis, additional studies are needed to further extend this work.

There are many studies necessary to further explore the effects of overexpression of Trx2. Though V5 epitope expression was only identified in the Tg^{hTrx2} model, mitochondrial isolation and probe for Trx2 in mitochondrial fraction could further confirm exclusive expression of human Trx2 in transgenic mice in only the mitochondria. Since results of the Tg^{hTrx2} model are currently inconclusive, confirming that Trx2 expression in the correct compartment in the model would be beneficial. Additionally, *in vitro* overexpression studies can be performed to detect how overexpression of Trx2 affects proliferation (as measured by cell counting), mitochondrial function (as assessed by MTT assay), mtH_2O_2 generation (as qualified by confocal microscopy), mitochondrial redox levels (Prx3 levels), and PH markers (Nox4). Comparing HPAECs with and without vector-based overexpression of Trx2 exposed to 72 hours of Normoxia (21% O_2) or Hypoxia (1% O_2) and measuring the above targets will elucidate if there is a beneficial role of Trx2 overexpression. Furthermore, elucidating the activity of Trx2 in the overexpression model will be beneficial as well. Quantifying the levels of reduced (active) to oxidized (inactive) Trx2, will elucidate the role of Trx2 oxidation in PH progression. In addition, overexpressing a non-oxidizable

Trx2 would establish if Trx2 oxidation is important in mtROS levels (Hansen et al., 2006b; Imhoff and Hansen, 2010; Zhang et al., 2007a).

To further confirm that hypoxia elevates mtH_2O_2, it is necessary to confirm that increased SOD2 expression allows for increased mtH_2O_2 production. Studies by other groups have confirmed that overexpression of SOD2 results in an increase in mtH_2O_2, as detected by H_2O_2-sensitive dye, dichlorodihydrofluorescein diacetate (DCFDA) (Connor et al., 2005; Dasgupta et al., 2006). It is our belief that overexpression of SOD2 increases the rate of dismutation of $mtO_2^{\bullet-}$ to mtH_2O_2, leading to elevated levels of mtH_2O_2. Furthermore, we posit that the increase in mtH_2O_2 in the Tg^{hSOD2} model causes an exacerbated PH phenotype. Studies using confocal microscopy can assess if targeted conversion of $mtO_2^{\bullet-}$ increases mtH_2O_2. Using HPAECs exposed to 72 hours of hypoxia, treated with MitoTEMPO (SOD2 mimetic), and probed with mtH_2O_2-specific MitoPy1, will allow us to measure if reducing $mtO_2^{\bullet-}$ leads to a subsequent increase in mtH_2O_2 detection.

Additional studies focusing on the effect of mtROS on hypoxia-induced activation of transcription factors like HIF1-α will further confirm that mtROS can activate transcription factors that promote PH (Chandel et al., 1998). Studies have confirmed HIF1-α-dependent transcriptional gene regulation of critical mitochondrial metabolism proteins (Voelkel et al., 2013). Additional, future studies to contribute to our findings could utilize a Nox4 knockout murine model

to confirm our work establishing mtH_2O_2 activation of Nox4 production to be crucial to PH vascular dysregulation.

Other *in vivo* studies that may allow for clarity on the efficacy and specificity of the model include using a pulmonary tissue specific MCAT expression model. Using a model developed by Dr. Peter Rabinovitch that previously targeted cardiac tissue, we would redirect the high catalase (hiCAT) or low catalase (lowCAT) expression to pulmonary tissue (pulmonary smooth muscle directed – Tamoxifen-induced SM myosin heavy chain Cre, and endothelial cell directed – Tamoxifen-induced vascular endothelial cadherin Cre) transgenic mice (Dai et al., 2011; Song et al., 2014). This model would be exposed to the standard 3 weeks of normoxia/hypoxia regimen and assessed for molecular and physiological markers of PH. This model could confirm if increased catalase expression in pulmonary tissue alone is sufficient to induce the beneficial effects seen with the MCAT model in the studies discussed within. In addition, studies assessing the effect of targeted mitochondrial H_2O_2 reduction on the reversal of the PH phenotype may give more insight as to the role of mtH_2O_2. Our studies confirmed that targeted attenuation of mtH_2O_2 is capable of preventing many molecular and physiological markers of PH. If reduction of mtH_2O_2 is capable of reversing PH once it has already occurred, this goes to further to establish the vital role of mtH_2O_2.

Conclusions

These studies used several novel and mitochondrial specific transgenes to assess the benefits of targeting mtROS to prevent hypoxia-induced PH. The studies focused on attenuating mitochondrial sources of ROS as there remains significant controversy in regards to the ability of a single cellular compartment to generate enough ROS to produce global molecular and physiological effects. The studies clearly establish that targeting mtROS, specifically mtH_2O_2, is sufficient to prevent PH pathogenesis.

Several limitations of the current studies merit additional consideration. For protein western blot detection studies, whole lung homogenates were used to detect protein expression. Since the lung is composed of over 40 unique cell types, analysis of specific protein expression patterns in whole lung homogenates runs the risk of diluting alterations in vascular endothelial and smooth muscle cells. Strategies to overcome this limitation might include analysis of isolated pulmonary endothelial or smooth muscle cells or the use of laser capture microscopy. In addition, and as reviewed earlier, the murine models employed fail to mimic many of the pathological aspects of severe human pulmonary arterial hypertension (Morrell et al., 2009; Stenmark et al., 2009). Studies using hypoxia plus a single dose of VEGFR2 antagonist (SUGEN, SU5416) have been shown to lead to a progressive form of pulmonary hypertension in the rat, similar to human PAH, causing proliferative, occlusive vascular lesions and right ventricular failure. Adaptation of that protocol to mouse

models produces more severe PH in mice (Voelkel et al., 2013; West and Hemnes, 2011). These considerations suggest that existing and alternative rodent models of PH might be useful to confirm the importance of mtH_2O_2 in Nox induction and PH pathogenesis. Finally to confirm the utility of mtH_2O_2 as a therapeutic target in PH (Lu and McLoughlin, 2014), intervention studies are needed wherein mtH_2O_2 is reduced only after PH pathogenesis has begun. For example, wild-type mice could be exposed to Hypoxia + SU5416 to induce severe PH. After the 3 week exposure, interventions with mitochondrial-targeted catalase or Prx3 via tail vein injection or via nebulization directly into the lung could be employed. These studies could thereby determine if interventions to reduce mtH_2O_2 effectively modulate disease outcomes if applied only after the disease is present.

The novel findings of these studies are two-fold: mitochondrial ROS signaling is vital and aberrant mtH_2O_2 signaling can regulate Noxes and PH pathology. Initially, the ability of mitochondria-generated ROS to alter physiological responses (tissue H_2O_2, RVSP, vessel muscularization, and remodeling) was controversial. Though the importance of mitochondria in physiology is not under debate, the ability of mtROS dysregulation to cause the molecular and physiological derangements found in PH was contested. The MCAT and Tg^{hSOD2} studies independently confirm that changes in mtROS levels can affect both molecular and physiological markers of PH. These studies emphasize: a) the importance of mtROS in hypoxia-induced PH pathogenesis, b)

the critical importance of understanding the role of specific ROS and their intracellular locus of generation, and c) these results suggest that targeted therapies directed at lowering mtH_2O_2 may be uniquely effective in reducing pulmonary vascular cell proliferation, remodeling, and PH.

Our studies support mtH_2O_2 as a critical mediator in the molecular and physiological derangements causing hypoxia-induced PH. Regulation of mtH_2O_2 in the MCAT model decreased cell cycle initiation (cyclinD1) and proliferation (PCNA) markers, which were both found to be exacerbated in the hypoxia Tg^{hSOD2} model. Our data suggest that increased conversion of superoxide to H_2O_2 is a possible source of physiological and molecular exacerbations in the Tg^{hSOD2} model. It is likely that the Tg^{hSOD2} model provides evidence that increased conversion of upstream mt$O_2^{\bullet -}$ to mtH_2O_2, requires direct attenuation of downstream mtH_2O_2. The relationship between mtROS and Nox expression is poorly defined in relationship to PH. The present studies tested the hypothesis that mitochondria-derived $O_2^{\bullet -}$ and H_2O_2 regulate Nox expression promoting PH pathogenesis. Targeting hypoxia-induced mtROS could prevent PH by attenuating mtROS-induced Nox expression and downstream signaling.

In summary, the current study demonstrates that exposing HPAEC to chronic hypoxia increases mitochondria-derived $O_2^{\bullet -}$ and H_2O_2. Additionally, transgenic mouse models were employed to demonstrate that selective mtROS

targeting attenuates hypoxia-induced PH, Nox expression, and proliferative markers *in vivo*. Our results establish that mitochondria-derived H_2O_2 is likely necessary for hypoxia-induced elevations of Nox 2 and 4 in PH. These findings contribute to the current field of PH by identifying mtROS as a novel target to attenuate hypoxia-induced PH. Our results suggest that selective targeting of specific ROS and subcellular compartments has potential to enhance the effectiveness of future PH therapies.

References

Ago T, Kuroda J, Pain J, Fu C, Li H and Sadoshima J (2010) Upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes. *Circulation research* **106**(7): 1253-1264.

Al-Mehdi AB, Pastukh VM, Swiger BM, Reed DJ, Patel MR, Bardwell GC, Pastukh VV, Alexeyev MF and Gillespie MN (2012) Perinuclear mitochondrial clustering creates an oxidant-rich nuclear domain required for hypoxia-induced transcription. *Science signaling* **5**(231): ra47.

Aon MA, Cortassa S and O'Rourke B (2010) Redox-optimized ROS balance: A unifying hypothesis. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1797**(6,Äì7): 865-877.

Archer SL, Marsboom G, Kim GH, Zhang HJ, Toth PT, Svensson EC, Dyck JR, Gomberg-Maitland M, Thebaud B, Husain AN, Cipriani N and Rehman J (2010) Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: a basis for excessive cell proliferation and a new therapeutic target. *Circulation* **121**(24): 2661-2671.

Ardanaz N and Pagano PJ (2006) Hydrogen peroxide as a paracrine vascular mediator: regulation and signaling leading to dysfunction. *Experimental biology and medicine* **231**(3): 237-251.

Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC and Morrell NW (2002) Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation* **105**(14): 1672-1678.

Babior BM, Kipnes RS and Curnutte JT (1973) Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *The Journal of clinical investigation* **52**(3): 741-744.

Badesch DB, Raskob GE, Elliott CG, Krichman AM, Farber HW, Frost AE, Barst RJ, Benza RL, Liou TG, Turner M, Giles S, Feldkircher K, Miller DP and McGoon MD (2010) Pulmonary arterial hypertension: baseline characteristics from the REVEAL Registry. *Chest* **137**(2): 376-387.

Barbera JA and Blanco I (2009) Pulmonary hypertension in patients with chronic obstructive pulmonary disease: advances in pathophysiology and management. *Drugs* **69**(9): 1153-1171.

Barman SA, Chen F, Su Y, Dimitropoulou C, Wang Y, Catravas JD, Han W, Orfi L, Szantai-Kis C, Keri G, Szabadkai I, Barabutis N, Rafikova O, Rafikov R, Black SM, Jonigk D, Giannis A, Asmis R, Stepp DW, Ramesh G and Fulton DJ (2014) NADPH oxidase 4 is expressed in pulmonary artery adventitia and contributes to hypertensive vascular remodeling. *Arteriosclerosis, thrombosis, and vascular biology* **34**(8): 1704-1715.

Barst RJ (2008) Pulmonary hypertension: past, present and future. *Annals of thoracic medicine* **3**(1): 1-4.

Bauer NR, Moore TM and McMurtry IF (2007) Rodent models of PAH: are we there yet? *American journal of physiology Lung cellular and molecular physiology* **293**(3): L580-582.

Bayraktutan U, Blayney L and Shah AM (2000) Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology* **20**(8): 1903-1911.

Beckman JS and Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* **271**(5 Pt 1): C1424-1437.

Beckman JS, Minor RL, Jr., White CW, Repine JE, Rosen GM and Freeman BA (1988) Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. *The Journal of biological chemistry* **263**(14): 6884-6892.

Bedard K and Krause KH (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews* **87**(1): 245-313.

Benza RL, Gomberg-Maitland M, Miller DP, Frost A, Frantz RP, Foreman AJ, Badesch DB and McGoon MD (2012) The REVEAL Registry risk score calculator in patients newly diagnosed with pulmonary arterial hypertension. *Chest* **141**(2): 354-362.

Bijli KM, Kleinhenz JM, Murphy TC, Kang BY, Adesina SE, Sutliff RL and Hart CM (2015) Peroxisome proliferator-activated receptor gamma depletion stimulates Nox4 expression and human pulmonary artery smooth muscle cell proliferation. *Free radical biology & medicine* **80**: 111-120.

Blanquicett C, Kang BY, Ritzenthaler JD, Jones DP and Hart CM (2010) Oxidative stress modulates PPAR gamma in vascular endothelial cells. *Free radical biology & medicine* **48**(12): 1618-1625.

Block K, Gorin Y and Abboud HE (2009) Subcellular localization of Nox4 and regulation in diabetes. *Proceedings of the National Academy of Sciences of the United States of America* **106**(34): 14385-14390.

Bowers R, Cool C, Murphy RC, Tuder RM, Hopken MW, Flores SC and Voelkel NF (2004) Oxidative Stress in Severe Pulmonary Hypertension. *Am J Respir Crit Care Med* **169**(6): 764-769.

Brennan LA, Steinhorn RH, Wedgwood S, Mata-Greenwood E, Roark EA, Russell JA and Black SM (2003) Increased superoxide generation is associated with pulmonary hypertension in fetal lambs: a role for NADPH oxidase. *Circulation research* **92**(6): 683-691.

Brown DI and Griendling KK (2009) Nox proteins in signal transduction. *Free radical biology & medicine* **47**(9): 1239-1253.

Campen MJ, Paffett ML, Colombo ES, Lucas SN, Anderson T, Nysus M, Norenberg JP, Gershman B, Hesterman J, Hoppin J and Willis M (2014) Muscle RING finger-1 promotes a maladaptive phenotype in chronic hypoxia-induced right ventricular remodeling. *PloS one* **9**(5): e97084.

Cantin AM (2004) Potential for antioxidant therapy of cystic fibrosis. *Current opinion in pulmonary medicine* **10**(6): 531-536.

Case AJ, Li S, Basu U, Tian J and Zimmerman MC (2013) Mitochondrial-localized NADPH oxidase 4 is a source of superoxide in angiotensin II-stimulated neurons. *American journal of physiology Heart and circulatory physiology* **305**(1): H19-28.

Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC and Schumacker PT (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences of the United States of America* **95**(20): 11715-11720.

Chang SW, Stelzner TJ, Weil JV and Voelkel NF (1989) Hypoxia increases plasma glutathione disulfide in rats. *Lung* **167**(5): 269-276.

Chen X, Touyz RM, Park JB and Schiffrin EL (2001) Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. *Hypertension* **38**(3 Pt 2): 606-611.

Chettimada S, Gupte R, Rawat D, Gebb SA, McMurtry IF and Gupte SA (2015) Hypoxia-induced glucose-6-phosphate dehydrogenase overexpression and -activation in pulmonary artery

smooth muscle cells: implication in pulmonary hypertension. *American journal of physiology Lung cellular and molecular physiology* **308**(3): L287-300.

Ciuclan L, Bonneau O, Hussey M, Duggan N, Holmes AM, Good R, Stringer R, Jones P, Morrell NW, Jarai G, Walker C, Westwick J and Thomas M (2011) A novel murine model of severe pulmonary arterial hypertension. *American journal of respiratory and critical care medicine* **184**(10): 1171-1182.

Clempus RE, Sorescu D, Dikalova AE, Pounkova L, Jo P, Sorescu GP, Schmidt HH, Lassegue B and Griendling KK (2007) Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. *Arteriosclerosis, thrombosis, and vascular biology* **27**(1): 42-48.

Connor KM, Subbaram S, Regan KJ, Nelson KK, Mazurkiewicz JE, Bartholomew PJ, Aplin AE, Tai YT, Aguirre-Ghiso J, Flores SC and Melendez JA (2005) Mitochondrial H2O2 regulates the angiogenic phenotype via PTEN oxidation. *The Journal of biological chemistry* **280**(17): 16916-16924.

Cutz E, Pan J and Yeger H (2009) The role of NOX2 and "novel oxidases" in airway chemoreceptor O(2) sensing. *Advances in experimental medicine and biology* **648**: 427-438.

D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Kernis JT and et al. (1991) Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Annals of internal medicine* **115**(5): 343-349.

Dai D-F, Santana LF, Vermulst M, Tomazela DM, Emond MJ, MacCoss MJ, Gollahon K, Martin GM, Loeb LA, Ladiges WC and Rabinovitch PS (2009) Overexpression of Catalase Targeted to Mitochondria Attenuates Murine Cardiac Aging. *Circulation* **119**(21): 2789-2797.

Dai DF, Johnson SC, Villarin JJ, Chin MT, Nieves-Cintron M, Chen T, Marcinek DJ, Dorn GW, 2nd, Kang YJ, Prolla TA, Santana LF and Rabinovitch PS (2011) Mitochondrial oxidative stress mediates angiotensin II-induced cardiac hypertrophy and Galphaq overexpression-induced heart failure. *Circulation research* **108**(7): 837-846.

Daiber A (2010) Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species. *Biochimica et biophysica acta* **1797**(6-7): 897-906.

Dasgupta J, Subbaram S, Connor KM, Rodriguez AM, Tirosh O, Beckman JS, Jourd'Heuil D and Melendez JA (2006) Manganese superoxide dismutase protects from TNF-alpha-induced apoptosis by increasing the steady-state production of H2O2. *Antioxidants & redox signaling* **8**(7-8): 1295-1305.

Datla SR and Griendling KK (2010) Reactive Oxygen Species, NADPH Oxidases, and Hypertension. *Hypertension* **56**(3): 325-330.

Demiryurek AT and Wadsworth RM (1999) Superoxide in the pulmonary circulation. *Pharmacology & therapeutics* **84**(3): 355-365.

Desouki MM, Kulawiec M, Bansal S, Das GM and Singh KK (2005) Cross talk between mitochondria and superoxide generating NADPH oxidase in breast and ovarian tumors. *Cancer biology & therapy* **4**(12): 1367-1373.

Dikalov S (2011) Cross talk between mitochondria and NADPH oxidases. *Free radical biology & medicine* **51**(7): 1289-1301.

Dikalov SI, Dikalova AE, Bikineyeva AT, Schmidt HH, Harrison DG and Griendling KK (2008) Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. *Free radical biology & medicine* **45**(9): 1340-1351.

Dikalov SI and Harrison DG (2014) Methods for detection of mitochondrial and cellular reactive oxygen species. *Antioxidants & redox signaling* **20**(2): 372-382.

Dikalov SI and Ungvari Z (2013) Role of mitochondrial oxidative stress in hypertension. *American journal of physiology Heart and circulatory physiology* **305**(10): H1417-1427.

Dikalova AE, Bikineyeva AT, Budzyn K, Nazarewicz RR, McCann L, Lewis W, Harrison DG and Dikalov SI (2010) Therapeutic targeting of mitochondrial superoxide in hypertension. *Circulation research* **107**(1): 106-116.

Doughan AK, Harrison DG and Dikalov SI (2008) Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circulation research* **102**(4): 488-496.

Dromparis P and Michelakis ED (2013) Mitochondria in vascular health and disease. *Annual review of physiology* **75**: 95-126.

Dromparis P, Sutendra G and Michelakis ED (2010) The role of mitochondria in pulmonary vascular remodeling. *J Mol Med (Berl)* **88**(10): 1003-1010.

Dunn LL, Buckle AM, Cooke JP and Ng MK (2010) The emerging role of the thioredoxin system in angiogenesis. *Arteriosclerosis, thrombosis, and vascular biology* **30**(11): 2089-2098.

Ebrahimian T and Touyz RM (2008) Thioredoxin in vascular biology: role in hypertension. *Antioxidants & redox signaling* **10**(6): 1127-1136.

Erusalimsky JD and Moncada S (2007) Nitric oxide and mitochondrial signaling: from physiology to pathophysiology. *Arteriosclerosis, thrombosis, and vascular biology* **27**(12): 2524-2531.

Evans AM, Hardie DG, Peers C and Mahmoud A (2011) Hypoxic pulmonary vasoconstriction: mechanisms of oxygen-sensing. *Curr Opin Anaesthesiol* **24**(1): 13-20.

Fike CD, Slaughter JC, Kaplowitz MR, Zhang Y and Aschner JL (2008) Reactive oxygen species from NADPH oxidase contribute to altered pulmonary vascular responses in piglets with chronic hypoxia-induced pulmonary hypertension. *American journal of physiology Lung cellular and molecular physiology* **295**(5): L881-888.

Frazziano G, Al Ghouleh I, Baust J, Shiva S, Champion HC and Pagano PJ (2014) Nox-derived ROS are acutely activated in pressure overload pulmonary hypertension: indications for a seminal role for mitochondrial Nox4. *American journal of physiology Heart and circulatory physiology* **306**(2): H197-205.

Frazziano G, Champion HC and Pagano PJ (2012) NADPH oxidase-derived ROS and the regulation of pulmonary vessel tone. *American journal of physiology Heart and circulatory physiology* **302**(11): H2166-2177.

Fresquet F, Pourageaud F, Leblais V, Brandes RP, Savineau JP, Marthan R and Muller B (2006) Role of reactive oxygen species and gp91phox in endothelial dysfunction of pulmonary arteries induced by chronic hypoxia. *British journal of pharmacology* **148**(5): 714-723.

Freund-Michel V, Guibert C, Dubois M, Courtois A, Marthan R, Savineau JP and Muller B (2013) Reactive oxygen species as therapeutic targets in pulmonary hypertension. *Therapeutic advances in respiratory disease* **7**(3): 175-200.

Fuchs B, Sommer N, Dietrich A, Schermuly RT, Ghofrani HA, Grimminger F, Seeger W, Gudermann T and Weissmann N (2010) Redox signaling and reactive oxygen species in hypoxic pulmonary vasoconstriction. *Respiratory Physiology & Neurobiology* **174**(3): 282-291.

Fukai T, Siegfried MR, Ushio-Fukai M, Griendling KK and Harrison DG (1999) Modulation of extracellular superoxide dismutase expression by angiotensin II and hypertension. *Circulation research* **85**(1): 23-28.

Geiszt M, Kopp JB, Varnai P and Leto TL (2000) Identification of renox, an NAD(P)H oxidase in kidney. *Proceedings of the National Academy of Sciences of the United States of America* **97**(14): 8010-8014.

George MG, Schieb LJ, Ayala C, Talwalkar A and Levant S (2014) Pulmonary hypertension surveillance: United States, 2001 to 2010. *Chest* **146**(2): 476-495.

Gillespie MN, Al-Mehdi AB and McMurtry IF (2013) Mitochondria in hypoxic pulmonary vasoconstriction: potential importance of compartmentalized reactive oxygen species signaling. *American journal of respiratory and critical care medicine* **187**(4): 338-340.

Giordano FJ (2005) Oxygen, oxidative stress, hypoxia, and heart failure. *The Journal of clinical investigation* **115**(3): 500-508.

Go YM, Gipp JJ, Mulcahy RT and Jones DP (2004) H2O2-dependent activation of GCLC-ARE4 reporter occurs by mitogen-activated protein kinase pathways without oxidation of cellular glutathione or thioredoxin-1. *The Journal of biological chemistry* **279**(7): 5837-5845.

Go YM, Park H, Koval M, Orr M, Reed M, Liang Y, Smith D, Pohl J and Jones DP (2010) A key role for mitochondria in endothelial signaling by plasma cysteine/cystine redox potential. *Free radical biology & medicine* **48**(2): 275-283.

Goeptar AR, Scheerens H and Vermeulen NP (1995) Oxygen and xenobiotic reductase activities of cytochrome P450. *Critical reviews in toxicology* **25**(1): 25-65.

Gologanu D, Stanescu C and Bogdan MA (2012) Pulmonary hypertension secondary to chronic obstructive pulmonary disease. *Rom J Intern Med* **50**(4): 259-268.

Gomez-Arroyo JG, Farkas L, Alhussaini AA, Farkas D, Kraskauskas D, Voelkel NF and Bogaard HJ (2012) The monocrotaline model of pulmonary hypertension in perspective. *American journal of physiology Lung cellular and molecular physiology* **302**(4): L363-369.

Gosemann JH, Friedmacher F, Hunziker M, Alvarez L, Corcionivoschi N and Puri P (2013) Increased activation of NADPH oxidase 4 in the pulmonary vasculature in experimental diaphragmatic hernia. *Pediatric surgery international* **29**(1): 3-8.

Graham KA, Kulawiec M, Owens KM, Li X, Desouki MM, Chandra D and Singh KK (2010) NADPH oxidase 4 is an oncoprotein localized to mitochondria. *Cancer biology & therapy* **10**(3): 223-231.

Granger DN, Hollwarth ME and Parks DA (1986) Ischemia-reperfusion injury: role of oxygen-derived free radicals. *Acta physiologica Scandinavica Supplementum* **548**: 47-63.

Green DE, Murphy TC, Kang BY, Kleinhenz JM, Szyndralewiez C, Page P, Sutliff RL and Hart CM (2012) The Nox4 inhibitor GKT137831 attenuates hypoxia-induced pulmonary vascular cell proliferation. *American journal of respiratory cell and molecular biology* **47**(5): 718-726.

Griendling KK, Minieri CA, Ollerenshaw JD and Alexander RW (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circulation research* **74**(6): 1141-1148.

Griendling KK, Sorescu D and Ushio-Fukai M (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. *Circulation research* **86**(5): 494-501.

Griffith B, Pendyala S, Hecker L, Lee PJ, Natarajan V and Thannickal VJ (2009) NOX enzymes and pulmonary disease. *Antioxidants & redox signaling* **11**(10): 2505-2516.

Grobe AC, Wells SM, Benavidez E, Oishi P, Azakie A, Fineman JR and Black SM (2006) Increased oxidative stress in lambs with increased pulmonary blood flow and pulmonary hypertension: role of NADPH oxidase and endothelial NO synthase. *American journal of physiology Lung cellular and molecular physiology* **290**(6): L1069-1077.

Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC, Hammerling U and Schumacker PT (2005) Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell metabolism* **1**(6): 401-408.

Hansen JM, Go YM and Jones DP (2006a) Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling. *Annu Rev Pharmacol Toxicol* **46**: 215-234.

Hansen JM, Zhang H and Jones DP (2006b) Mitochondrial thioredoxin-2 has a key role in determining tumor necrosis factor-alpha-induced reactive oxygen species generation, NF-kappaB activation, and apoptosis. *Toxicol Sci* **91**(2): 643-650.

He J, DP He Masters Thesis.

Hilenski LL, Clempus RE, Quinn MT, Lambeth JD and Griendling KK (2004) Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arteriosclerosis, thrombosis, and vascular biology* **24**(4): 677-683.

Hoeper MM, Mayer E, Simonneau G and Rubin LJ (2006) Chronic thromboembolic pulmonary hypertension. *Circulation* **113**(16): 2011-2020.

Hoshikawa Y, Ono S, Suzuki S, Tanita T, Chida M, Song C, Noda M, Tabata T, Voelkel NF and Fujimura S (2001) Generation of oxidative stress contributes to the development of pulmonary hypertension induced by hypoxia. *Journal of applied physiology* **90**(4): 1299-1306.

Huie RE and Padmaja S (1993) The reaction of no with superoxide. *Free Radic Res Commun* **18**(4): 195-199.

Humbert M, Lau EM, Montani D, Jais X, Sitbon O and Simonneau G (2014) Advances in therapeutic interventions for patients with pulmonary arterial hypertension. *Circulation* **130**(24): 2189-2208.

Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, Yaici A, Weitzenblum E, Cordier JF, Chabot F, Dromer C, Pison C, Reynaud-Gaubert M, Haloun A, Laurent M, Hachulla E and Simonneau G (2006) Pulmonary arterial hypertension in France: results from a national registry. *American journal of respiratory and critical care medicine* **173**(9): 1023-1030.

Humbert M, Sitbon O and Simonneau G (2004) Treatment of pulmonary arterial hypertension. *The New England journal of medicine* **351**(14): 1425-1436.

Hyduk A, Croft JB, Ayala C, Zheng K, Zheng ZJ and Mensah GA (2005) Pulmonary hypertension surveillance--United States, 1980-2002. *Morbidity and mortality weekly report Surveillance summaries* **54**(5): 1-28.

Imhoff BR and Hansen JM (2010) Tert-butylhydroquinone induces mitochondrial oxidative stress causing Nrf2 activation. *Cell Biol Toxicol* **26**(6): 541-551.

Imlay JA and Fridovich I (1991) Assay of metabolic superoxide production in Escherichia coli. *The Journal of biological chemistry* **266**(11): 6957-6965.

Ismail S, Sturrock A, Wu P, Cahill B, Norman K, Huecksteadt T, Sanders K, Kennedy T and Hoidal J (2009) NOX4 mediates hypoxia-induced proliferation of human pulmonary artery smooth muscle cells: the role of autocrine production of transforming growth factor-{beta}1 and insulin-like growth factor binding protein-3. *American journal of physiology Lung cellular and molecular physiology* **296**(3): L489-499.

Jacob BA, Porter KM, Elms SC, Cheng PY, Jones DP and Sutliff RL (2006) HIV-1-induced pulmonary oxidative and nitrosative stress: exacerbated response to endotoxin administration in HIV-1 transgenic mouse model. *American journal of physiology Lung cellular and molecular physiology* **291**(4): L811-819.

Jang YC, Perez VI, Song W, Lustgarten MS, Salmon AB, Mele J, Qi W, Liu Y, Liang H, Chaudhuri A, Ikeno Y, Epstein CJ, Van Remmen H and Richardson A (2009) Overexpression of Mn superoxide dismutase does not increase life span in mice. *The journals of gerontology Series A, Biological sciences and medical sciences* **64**(11): 1114-1125.

Jernigan NL, Walker BR and Resta TC (2004) Endothelium-derived reactive oxygen species and endothelin-1 attenuate NO-dependent pulmonary vasodilation following chronic hypoxia. *American journal of physiology Lung cellular and molecular physiology* **287**(4): L801-808.

Jing L, Peng X, Xie MJ, Yu ZY and Wang W (2014) Different responses of cell cycle between rat vascular smooth muscle cells and vascular endothelial cells to paclitaxel. *Journal of Huazhong University of Science and Technology Medical sciences = Hua zhong ke ji da xue xue bao Yi xue Ying De wen ban = Huazhong keji daxue xuebao Yixue Yingdewen ban* **34**(3): 370-375.

Jones DP (2006) Redefining oxidative stress. *Antioxidants & redox signaling* **8**(9-10): 1865-1879.

Jones DP, Carlson JL, Mody VC, Cai J, Lynn MJ and Sternberg P (2000) Redox state of glutathione in human plasma. *Free radical biology & medicine* **28**(4): 625-635.

Jung HJ, Shim JS, Lee J, Song YM, Park KC, Choi SH, Kim ND, Yoon JH, Mungai PT, Schumacker PT and Kwon HJ (2010) Terpestacin inhibits tumor angiogenesis by targeting UQCRB of mitochondrial complex III and suppressing hypoxia-induced reactive oxygen species production and cellular oxygen sensing. *The Journal of biological chemistry* **285**(15): 11584-11595.

Kaneko FT, Arroliga AC, Dweik RA, Comhair SA, Laskowski D, Oppedisano R, Thomassen MJ and Erzurum SC (1998) Biochemical Reaction Products of Nitric Oxide as Quantitative Markers of Primary Pulmonary Hypertension. *Am J Respir Crit Care Med* **158**(3): 917-923.

Kang BY, Kleinhenz JM, Murphy TC and Hart CM (2011) The PPARgamma ligand rosiglitazone attenuates hypoxia-induced endothelin signaling in vitro and in vivo. *American journal of physiology Lung cellular and molecular physiology* **301**(6): L881-891.

Kleinhenz JM (2015) Trx2 Transgene, (Bello SA ed).

Klinke A, Moller A, Pekarova M, Ravekes T, Friedrichs K, Berlin M, Scheu KM, Kubala L, Kolarova H, Ambrozova G, Schermuly RT, Woodcock SR, Freeman BA, Rosenkranz S, Baldus S, Rudolph V and Rudolph TK (2014) Protective Effects of 10-Nitro-Oleic Acid in a Hypoxia-Induced Murine Model of Pulmonary Hypertension. *American journal of respiratory cell and molecular biology*.

Konior A, Schramm A, Czesnikiewicz-Guzik M and Guzik TJ (2014) NADPH oxidases in vascular pathology. *Antioxidants & redox signaling* **20**(17): 2794-2814.

Koskenkorva-Frank TS, Weiss G, Koppenol WH and Burckhardt S (2013) The complex interplay of iron metabolism, reactive oxygen species, and reactive nitrogen species: insights into the potential of various iron therapies to induce oxidative and nitrosative stress. *Free radical biology & medicine* **65**: 1174-1194.

Kowluru RA, Kowluru V, Xiong Y and Ho Y-S (2006) Overexpression of mitochondrial superoxide dismutase in mice protects the retina from diabetes-induced oxidative stress. *Free Radical Biology and Medicine* **41**(8): 1191-1196.

Kuroda J, Ago T, Matsushima S, Zhai P, Schneider MD and Sadoshima J (2010) NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart. *Proceedings of the National Academy of Sciences of the United States of America* **107**(35): 15565-15570.

Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE and Harrison DG (2003) Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *The Journal of clinical investigation* **111**(8): 1201-1209.

Lassegue B and Clempus RE (2003) Vascular NAD(P)H oxidases: specific features, expression, and regulation. *American journal of physiology Regulatory, integrative and comparative physiology* **285**(2): R277-297.

Lassegue B, San Martin A and Griendling KK (2012) Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circulation research* **110**(10): 1364-1390.

Libby P, Ridker PM and Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. *Nature* **473**(7347): 317-325.

Liu JQ, Erbynn EM and Folz RJ (2005) Chronic hypoxia-enhanced murine pulmonary vasoconstriction: role of superoxide and gp91phox. *Chest* **128**(6 Suppl): 594S-596S.

Liu JQ and Folz RJ (2004) Extracellular superoxide enhances 5-HT-induced murine pulmonary artery vasoconstriction. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **287**(1): L111-L118.

Liu JQ, Zelko IN, Erbynn EM, Sham JS and Folz RJ (2006) Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase (gp91phox). *American journal of physiology Lung cellular and molecular physiology* **290**(1): L2-10.

Liu JQ, Zelko IN and Folz RJ (2004) Reoxygenation-induced constriction in murine coronary arteries: the role of endothelial NADPH oxidase (gp91phox) and intracellular superoxide. *The Journal of biological chemistry* **279**(23): 24493-24497.

Lowes DA and Galley HF (2011) Mitochondrial protection by the thioredoxin-2 and glutathione systems in an in vitro endothelial model of sepsis. *Biochem J* **436**(1): 123-132.

Lu L and McLoughlin P (2014) Choice of Vehicle in the Sugen/Hypoxia Model of Pulmonary Hypertension: Carboxymethylcellulose or Simethyl Sulfide, in *American Thorasic Society 2014*, San Diego.

Lu X, Bijli KM, Ramirez A, Murphy TC, Kleinhenz J and Hart CM (2013) Hypoxia downregulates PPARgamma via an ERK1/2-NF-kappaB-Nox4-dependent mechanism in human pulmonary artery smooth muscle cells. *Free radical biology & medicine* **63**: 151-160.

Lyle AN and Griendling KK (2006) Modulation of vascular smooth muscle signaling by reactive oxygen species. *Physiology (Bethesda)* **21**: 269-280.

Maarman G, Lecour S, Butrous G, Thienemann F and Sliwa K (2013) A comprehensive review: the evolution of animal models in pulmonary hypertension research; are we there yet? *Pulmonary circulation* **3**(4): 739-756.

Machado RD, Eickelberg O, Elliott CG, Geraci MW, Hanaoka M, Loyd JE, Newman JH, Phillips JA, 3rd, Soubrier F, Trembath RC and Chung WK (2009) Genetics and genomics of pulmonary arterial hypertension. *Journal of the American College of Cardiology* **54**(1 Suppl): S32-42.

Mailloux RJ (2015) Teaching the fundamentals of electron transfer reactions in mitochondria and the production and detection of reactive oxygen species. *Redox biology* **4**: 381-398.

Marklund S (1976) Spectrophotometric study of spontaneous disproportionation of superoxide anion radical and sensitive direct assay for superoxide dismutase. *The Journal of biological chemistry* **251**(23): 7504-7507.

McKeown SR (2014) Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. *The British journal of radiology* **87**(1035): 20130676.

McLaughlin VV (2006) 'Raising the bar' for the treatment of pulmonary arterial hypertension. *Eur Heart J* **27**(5): 510-511.

McLaughlin VV, Presberg KW, Doyle RL, Abman SH, McCrory DC, Fortin T, Ahearn G and American College of Chest P (2004) Prognosis of pulmonary arterial hypertension: ACCP evidence-based clinical practice guidelines. *Chest* **126**(1 Suppl): 78S-92S.

Mielniczuk LM, Swiston JR and Mehta S (2014) Riociguat: a novel therapeutic option for pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension. *The Canadian journal of cardiology* **30**(10): 1233-1240.

Mik EG (2013) Special article: measuring mitochondrial oxygen tension: from basic principles to application in humans. *Anesthesia and analgesia* **117**(4): 834-846.

Miller AA, Drummond GR, Schmidt HH and Sobey CG (2005) NADPH oxidase activity and function are profoundly greater in cerebral versus systemic arteries. *Circulation research* **97**(10): 1055-1062.

Min W, Xu LK, Zhou HJ, Huang Q, Zhang H, He Y, Zhe X and Y L (2010) Thioredoxin and redox signaling in vasculature-studies using Trx2 endothelium-specific transgenic mice. *Methods Enzymology* **474**: 315 - 324.

Mittal M, Gu XQ, Pak O, Pamenter ME, Haag D, Fuchs DB, Schermuly RT, Ghofrani HA, Brandes RP, Seeger W, Grimminger F, Haddad GG and Weissmann N (2007a) Hypoxia induces Kv channel current inhibition by increased NADPH oxidase-derived reactive oxygen species. *Free Radical Biology and Medicine* **52**(6): 1033-1042.

Mittal M, Roth M, Konig P, Hofmann S, Dony E, Goyal P, Selbitz AC, Schermuly RT, Ghofrani HA, Kwapiszewska G, Kummer W, Klepetko W, Hoda MA, Fink L, Hanze J, Seeger W, Grimminger F, Schmidt HH and Weissmann N (2007b) Hypoxia-dependent regulation of nonphagocytic NADPH oxidase subunit NOX4 in the pulmonary vasculature. *Circulation research* **101**(3): 258-267.

Morrell NW, Adnot S, Archer SL, Dupuis J, Jones PL, MacLean MR, McMurtry IF, Stenmark KR, Thistlethwaite PA, Weissmann N, Yuan JX and Weir EK (2009) Cellular and molecular basis of pulmonary arterial hypertension. *Journal of the American College of Cardiology* **54**(1 Suppl): S20-31.

Morrell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK and Trembath RC (2001) Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation* **104**(7): 790-795.

Mouthon L, Guillevin L and Humbert M (2005) Pulmonary arterial hypertension: an autoimmune disease? *The European respiratory journal* **26**(6): 986-988.

Nauseef WM (2008) Nox enzymes in immune cells. *Seminars in immunopathology* **30**(3): 195-208.

Nauseef WM (2014) Detection of superoxide anion and hydrogen peroxide production by cellular NADPH oxidases. *Biochimica et biophysica acta* **1840**(2): 757-767.

Nisbet RE, Bland JM, Kleinhenz DJ, Mitchell PO, Walp ER, Sutliff RL and Hart CM (2010) Rosiglitazone attenuates chronic hypoxia-induced pulmonary hypertension in a mouse model. *American journal of respiratory cell and molecular biology* **42**(4): 482-490.

Nisbet RE, Graves AS, Kleinhenz DJ, Rupnow HL, Reed AL, Fan TH, Mitchell PO, Sutliff RL and Hart CM (2009) The role of NADPH oxidase in chronic intermittent hypoxia-induced pulmonary hypertension in mice. *American journal of respiratory cell and molecular biology* **40**(5): 601-609.

Norboo T, Stobdan T, Tsering N, Angchuk N, Tsering P, Ahmed I, Chorol T, Kumar Sharma V, Reddy P, Singh SB, Kimura Y, Sakamoto R, Fukutomi E, Ishikawa M, Suwa K, Kosaka Y, Nose M, Yamaguchi T, Tsukihara T, Matsubayashi K, Otsuka K and Okumiya K (2015) Prevalence of hypertension at high altitude: cross-sectional survey in Ladakh, Northern India 2007-2011. *BMJ Open* **5**(4): e007026.

Nozik-Grayck E, Woods C, Taylor JM, Benninger RK, Johnson RD, Villegas LR, Stenmark KR, Harrison DG, Majka SM, Irwin D and Farrow KN (2014) Selective depletion of vascular EC-SOD augments chronic hypoxic pulmonary hypertension. *American journal of physiology Lung cellular and molecular physiology* **307**(11): L868-876.

O'Gorman S, Dagenais NA, Qian M and Marchuk Y (1997) Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **94**(26): 14602-14607.

Ono S and Voelkel NF (1991) PAF antagonists inhibit monocrotaline-induced lung injury and pulmonary hypertension. *Journal of applied physiology* **71**(6): 2483-2492.

Paravicini TM and Touyz RM (2008) NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* **31 Suppl 2**: S170-180.

Partovian C, Adnot S, Raffestin B, Louzier V, Levame M, Mavier IM, Lemarchand P and Eddahibi S (2000) Adenovirus-mediated lung vascular endothelial growth factor overexpression protects against hypoxic pulmonary hypertension in rats. *American journal of respiratory cell and molecular biology* **23**(6): 762-771.

Pauvert O, Lugnier C, Keravis T, Marthan R, Rousseau E and Savineau JP (2003) Effect of sildenafil on cyclic nucleotide phosphodiesterase activity, vascular tone and calcium signaling in rat pulmonary artery. *British journal of pharmacology* **139**(3): 513-522.

Peacock AJ, Murphy NF, McMurray JJ, Caballero L and Stewart S (2007) An epidemiological study of pulmonary arterial hypertension. *The European respiratory journal* **30**(1): 104-109.

Pendyala S, Gorshkova IA, Usatyuk PV, He D, Pennathur A, Lambeth JD, Thannickal VJ and Natarajan V (2009) Role of Nox4 and Nox2 in hyperoxia-induced reactive oxygen species generation and migration of human lung endothelial cells. *Antioxidants & redox signaling* **11**(4): 747-764.

Pendyala S and Natarajan V (2010) Redox regulation of Nox proteins. *Respir Physiol Neurobiol* **174**(3): 265-271.

Perez-Vizcaino F, Cogolludo A and Moreno L (2010) Reactive oxygen species signaling in pulmonary vascular smooth muscle. *Respir Physiol Neurobiol* **174**(3): 212-220.

Peskin AV, Low FM, Paton LN, Maghzal GJ, Hampton MB and Winterbourn CC (2007) The high reactivity of peroxiredoxin 2 with H(2)O(2) is not reflected in its reaction with other oxidants and thiol reagents. *The Journal of biological chemistry* **282**(16): 11885-11892.

Petry A, Djordjevic T, Weitnauer M, Kietzmann T, Hess J and Gorlach A (2006) NOX2 and NOX4 mediate proliferative response in endothelial cells. *Antioxidants & redox signaling* **8**(9-10): 1473-1484.

Porter KM, Kang BY, Adesina SE, Murphy TC, Hart CM and Sutliff RL (2014) Chronic hypoxia promotes pulmonary artery endothelial cell proliferation through H2O2-induced 5-lipoxygenase. *PloS one* **9**(6): e98532.

Portnoy SG and Rudski LG (2015) Echocardiographic evaluation of the right ventricle: a 2014 perspective. *Current cardiology reports* **17**(4): 578.

Powis G and Montfort WR (2001) Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol* **41**: 261-295.

Rabinovitch M (2012) Molecular pathogenesis of pulmonary arterial hypertension. *The Journal of clinical investigation* **122**(12): 4306-4313.

Raineri I, Carlson EJ, Gacayan R, Carra S, Oberley TD, Huang TT and Epstein CJ (2001) Strain-dependent high-level expression of a transgene for manganese superoxide dismutase is associated with growth retardation and decreased fertility. *Free radical biology & medicine* **31**(8): 1018-1030.

Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK and Harrison DG (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide

production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *The Journal of clinical investigation* **97**(8): 1916-1923.

Rathore R, Zheng YM, Niu CF, Liu QH, Korde A, Ho YS and Wang YX (2008) Hypoxia activates NADPH oxidase to increase [ROS]i and [Ca2+]i through the mitochondrial ROS-PKCepsilon signaling axis in pulmonary artery smooth muscle cells. *Free radical biology & medicine* **45**(9): 1223-1231.

Ray R, Murdoch CE, Wang M, Santos CX, Zhang M, Alom-Ruiz S, Anilkumar N, Ouattara A, Cave AC, Walker SJ, Grieve DJ, Charles RL, Eaton P, Brewer AC and Shah AM (2011) Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure in vivo. *Arteriosclerosis, thrombosis, and vascular biology* **31**(6): 1368-1376.

Reed AL, Tanaka A, Sorescu D, Liu H, Jeong EM, Sturdy M, Walp ER, Dudley SC, Jr. and Sutliff RL (2011) Diastolic dysfunction is associated with cardiac fibrosis in the senescence-accelerated mouse. *American journal of physiology Heart and circulatory physiology* **301**(3): H824-831.

Rehman J and Archer SL (2010) A proposed mitochondrial-metabolic mechanism for initiation and maintenance of pulmonary arterial hypertension in fawn-hooded rats: the Warburg model of pulmonary arterial hypertension. *Advances in experimental medicine and biology* **661**: 171-185.

Rhee SG, Kang SW, Netto LE, Seo MS and Stadtman ER (1999) A family of novel peroxidases, peroxiredoxins. *BioFactors* **10**(2-3): 207-209.

Rich JD and Rich S (2014) Clinical diagnosis of pulmonary hypertension. *Circulation* **130**(20): 1820-1830.

Rich S, Rubin L, Walker AM, Schneeweiss S and Abenhaim L (2000) Anorexigens and pulmonary hypertension in the United States: results from the surveillance of North American pulmonary hypertension. *Chest* **117**(3): 870-874.

Richter C, Gogvadze V, Laffranchi R, Schlapbach R, Schweizer M, Suter M, Walter P and Yaffee M (1995) Oxidants in mitochondria: from physiology to diseases. *Biochimica et biophysica acta* **1271**(1): 67-74.

Ryan J, Bloch K and Archer SL (2011) Rodent models of pulmonary hypertension: harmonisation with the world health organisation's categorisation of human PH. *International journal of clinical practice Supplement*(172): 15-34.

Ryan J, Dasgupta A, Huston J, Chen KH and Archer SL (2015) Mitochondrial dynamics in pulmonary arterial hypertension. *J Mol Med (Berl)* **93**(3): 229-242.

Ryan JJ, Marsboom G and Archer SL (2013) Rodent models of group 1 pulmonary hypertension. *Handbook of experimental pharmacology* **218**: 105-149.

Sakao S, Tatsumi K and Voelkel NF (2009) Endothelial cells and pulmonary arterial hypertension: apoptosis, proliferation, interaction and transdifferentiation. *Respir Res* **10**: 95.

Samaga KK, Rao GV, Chandrashekara Reddy G, Kush AK and Diwakar L (2014) Synthetic racemates of abyssinone I and II induces apoptosis through mitochondrial pathway in human cervix carcinoma cells. *Bioorganic chemistry* **56C**: 54-61.

Sato K, Morio Y, Morris KG, Rodman DM and McMurtry IF (2000) Mechanism of hypoxic pulmonary vasoconstriction involves ET(A) receptor-mediated inhibition of K(ATP) channel. *American journal of physiology Lung cellular and molecular physiology* **278**(3): L434-442.

Satoh K, Godo S, Saito H, Enkhjargal B and Shimokawa H (2014) Dual roles of vascular-derived reactive oxygen species-With a special reference to hydrogen peroxide and cyclophilin A. *J Mol Cell Cardiol* **73C**: 50-56.

Savale L, Chaumais MC, Cottin V, Bergot E, Frachon I, Prevot G, Pison C, Dromer C, Poubeau P, Lamblin N, Habib G, Reynaud-Gaubert M, Bourdin A, Sanchez O, Tubert-Bitter P, Jais X, Montani D, Sitbon O, Simonneau G and Humbert M (2012) Pulmonary hypertension associated with benfluorex exposure. *The European respiratory journal* **40**(5): 1164-1172.

Schmitt FJ, Renger G, Friedrich T, Kreslavski VD, Zharmukhamedov SK, Los DA, Kuznetsov VV and Allakhverdiev SI (2014) Reactive oxygen species: re-evaluation of generation, monitoring and role in stress-signaling in phototrophic organisms. *Biochimica et biophysica acta* **1837**(6): 835-848.

Schriner SE and Linford NJ (2006) Extension of mouse lifespan by overexpression of catalase. *Age* **28**(2): 209-218.

Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC and Rabinovitch PS (2005) Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* **308**(5730): 1909-1911.

Schroder E and Eaton P (2008) Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations. *Current opinion in pharmacology* **8**(2): 153-159.

Schroder K, Zhang M, Benkhoff S, Mieth A, Pliquett R, Kosowski J, Kruse C, Luedike P, Michaelis UR, Weissmann N, Dimmeler S, Shah AM and Brandes RP (2012) Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase. *Circulation research* **110**(9): 1217-1225.

Schumacker PT (2011) Lung cell hypoxia: role of mitochondrial reactive oxygen species signaling in triggering responses. *Proc Am Thorac Soc* **8**(6): 477-484.

Sedeek M, Hebert RL, Kennedy CR, Burns KD and Touyz RM (2009) Molecular mechanisms of hypertension: role of Nox family NADPH oxidases. *Curr Opin Nephrol Hypertens* **18**(2): 122-127.

Sena LA and Chandel NS (2012) Physiological roles of mitochondrial reactive oxygen species. *Molecular cell* **48**(2): 158-167.

Shaffer JB, Treanor CP and Del Vecchio PJ (1990) Expression of bovine and mouse endothelial cell antioxidant enzymes following TNF-alpha exposure. *Free radical biology & medicine* **8**(5): 497-502.

Shanmugam E, Jena A and George M (2015) Riociguat: Something new in pulmonary hypertension therapeutics? *Journal of pharmacology & pharmacotherapeutics* **6**(1): 3-6.

Shimoda LA and Undem C (2010) Interactions between calcium and reactive oxygen species in pulmonary arterial smooth muscle responses to hypoxia. *Respiratory Physiology & Neurobiology* **174**(3): 221-229.

Sies H (1997) Oxidative stress: oxidants and antioxidants. *Experimental physiology* **82**(2): 291-295.

Simonneau G, Galie N, Rubin LJ, Langleben D, Seeger W, Domenighetti G, Gibbs S, Lebrec D, Speich R, Beghetti M, Rich S and Fishman A (2004) Clinical classification of pulmonary hypertension. *Journal of the American College of Cardiology* **43**(12 Suppl S): 5S-12S.

Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, Gomez Sanchez MA, Krishna Kumar R, Landzberg M, Machado RF, Olschewski H, Robbins IM and Souza R (2013) Updated clinical classification of pulmonary hypertension. *Journal of the American College of Cardiology* **62**(25 Suppl): D34-41.

Sommer N, Pak O, Schorner S, Derfuss T, Krug A, Gnaiger E, Ghofrani HA, Schermuly RT, Huckstorf C, Seeger W, Grimminger F and Weissmann N (2010) Mitochondrial cytochrome redox

states and respiration in acute pulmonary oxygen sensing. *The European respiratory journal* **36**(5): 1056-1066.

Song H, Han IY, Kim Y, Kim YH, Choi IW, Seo SK, Jung SY, Park S and Kang MS (2015) The NADPH oxidase inhibitor DPI can abolish hypoxia-induced apoptosis of human kidney proximal tubular epithelial cells through Bcl2 up-regulation via ERK activation without ROS reduction. *Life Sci* **126**: 69-75.

Song M, Chen Y, Gong G, Murphy E, Rabinovitch PS and Dorn GW, 2nd (2014) Super-suppression of mitochondrial reactive oxygen species signaling impairs compensatory autophagy in primary mitophagic cardiomyopathy. *Circulation research* **115**(3): 348-353.

Stenmark KR, Meyrick B, Galie N, Mooi WJ and McMurtry IF (2009) Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *American Journal of Physiology - Lung Cellular and Molecular Physiology* **297**(6): L1013-L1032.

Stenmark KR, Nozik-Grayck E, Gerasimovskaya E, Anwar A, Li M, Riddle S and Frid M (2011) The adventitia: Essential role in pulmonary vascular remodeling. *Comprehensive Physiology* **1**(1): 141-161.

Sun X, Kumar S, Sharma S, Aggarwal S, Lu Q, Gross C, Rafikova O, Lee SG, Dasarathy S, Hou Y, Meadows ML, Han W, Su Y, Fineman JR and Black SM (2014) Endothelin-1 Induces a Glycolytic Switch in Pulmonary Arterial Endothelial Cells via the Mitochondrial Translocation of Endothelial Nitric Oxide Synthase. *American journal of respiratory cell and molecular biology* **50**(6): 1084-1095.

Sutliff RL, Kang BY and Hart CM (2010) PPARgamma as a potential therapeutic target in pulmonary hypertension. *Therapeutic advances in respiratory disease* **4**(3): 143-160.

Taichman DB and Mandel J (2013) Epidemiology of pulmonary arterial hypertension. *Clinics in chest medicine* **34**(4): 619-637.

Takac I, Schroder K, Zhang L, Lardy B, Anilkumar N, Lambeth JD, Shah AM, Morel F and Brandes RP (2011) The E-loop is involved in hydrogen peroxide formation by the NADPH oxidase Nox4. *The Journal of biological chemistry* **286**(15): 13304-13313.

Tang X, Luo YX, Chen HZ and Liu DP (2014) Mitochondria, endothelial cell function, and vascular diseases. *Frontiers in physiology* **5**: 175.

Tuder RM, Archer SL, Dorfmuller P, Erzurum SC, Guignabert C, Michelakis E, Rabinovitch M, Schermuly R, Stenmark KR and Morrell NW (2013a) Relevant issues in the pathology and pathobiology of pulmonary hypertension. *Journal of the American College of Cardiology* **62**(25 Suppl): D4-12.

Tuder RM, Groves B, Badesch DB and Voelkel NF (1994) Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *The American journal of pathology* **144**(2): 275-285.

Tuder RM, Stacher E, Robinson J, Kumar R and Graham BB (2013b) Pathology of pulmonary hypertension. *Clinics in chest medicine* **34**(4): 639-650.

Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N and Griendling KK (1996) p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. *The Journal of biological chemistry* **271**(38): 23317-23321.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M and Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology* **39**(1): 44-84.

Veit F, Pak O, Egemnazarov B, Roth M, Kosanovic D, Seimetz M, Sommer N, Ghofrani HA, Seeger W, Grimminger F, Brandes RP, Schermuly RT and Weissmann N (2013) Function of NADPH Oxidase 1 in Pulmonary Arterial Smooth Muscle Cells After Monocrotaline-Induced Pulmonary Vascular Remodeling. *Antioxidants & redox signaling*.

Visner GA, Chesrown SE, Monnier J, Ryan US and Nick HS (1992) Regulation of manganese superoxide dismutase: IL-1 and TNF induction in pulmonary artery and microvascular endothelial cells. *Biochemical and biophysical research communications* **188**(1): 453-462.

Voelkel NF, Bogaard HJ, Al Husseini A, Farkas L, Gomez-Arroyo J and Natarajan R (2013) Antioxidants for the treatment of patients with severe angioproliferative pulmonary hypertension? *Antioxidants & redox signaling* **18**(14): 1810-1817.

Voelkel NF and Tuder RM (2000) Hypoxia-induced pulmonary vascular remodeling: a model for what human disease? *The Journal of clinical investigation* **106**(6): 733-738.

Wang GL, Jiang BH and Semenza GL (1995) Effect of altered redox states on expression and DNA-binding activity of hypoxia-inducible factor 1. *Biochemical and biophysical research communications* **212**(2): 550-556.

Wang YX and Zheng YM (2010) ROS-dependent signaling mechanisms for hypoxic Ca(2+) responses in pulmonary artery myocytes. *Antioxidants & redox signaling* **12**(5): 611-623.

Watson WH, Chen Y and Jones DP (2003) Redox state of glutathione and thioredoxin in differentiation and apoptosis. *BioFactors* **17**(1-4): 307-314.

Watson WH, Yang X, Choi YE, Jones DP and Kehrer JP (2004) Thioredoxin and Its Role in Toxicology. *Toxicological Sciences* **78**(1): 3-14.

Waypa GB, Marks JD, Guzy R, Mungai PT, Schriewer J, Dokic D and Schumacker PT (2010) Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. *Circulation research* **106**(3): 526-535.

Waypa GB, Marks JD, Guzy RD, Mungai PT, Schriewer JM, Dokic D, Ball MK and Schumacker PT (2013) Superoxide generated at mitochondrial complex III triggers acute responses to hypoxia in the pulmonary circulation. *American journal of respiratory and critical care medicine* **187**(4): 424-432.

Wedgwood S, Lakshminrusimha S, Fukai T, Russell JA, Schumacker PT and RH. S (2011) Hydrogen peroxide regulates extracellular superoxide dismutase activity and expression in neonatal pulmonary hypertension. *Antioxidant Redox Signal* **15**(6): 1497-1506.

Wedgwood S and Black SM (2003) Molecular mechanisms of nitric oxide-induced growth arrest and apoptosis in fetal pulmonary arterial smooth muscle cells. *Nitric Oxide* **9**(4): 201-210.

Wedgwood S, Dettman RW and Black SM (2001) ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. *American journal of physiology Lung cellular and molecular physiology* **281**(5): L1058-1067.

Wedgwood S, Lakshminrusimha S, Czech L, Schumacker PT and Steinhorn RH (2013) Increased p22(phox)/Nox4 expression is involved in remodeling through hydrogen peroxide signaling in experimental persistent pulmonary hypertension of the newborn. *Antioxidants & redox signaling* **18**(14): 1765-1776.

Weir EK and Archer SL (2010) The role of redox changes in oxygen sensing. *Respir Physiol Neurobiol* **174**(3): 182-191.

West J and Hemnes A (2011) Experimental and transgenic models of pulmonary hypertension. *Comprehensive Physiology* **1**(2): 769-782.

Widder JD, Fraccarollo D, Galuppo P, Hansen JM, Jones DP, Ertl G and Bauersachs J (2009) Attenuation of angiotensin II-induced vascular dysfunction and hypertension by overexpression of Thioredoxin 2. *Hypertension* **54**(2): 338-344.

Williams C, Lu X, Sutliff R and Hart C (2012) Rosiglitazone attenuates NF-κB-mediated Nox4 upregulation in hyperglycemia-activated endothelial cells. *Am J Physiol Cell Physiol* **303**(2): C213-223.

Wind S, Beuerlein K, Armitage ME, Taye A, Kumar AH, Janowitz D, Neff C, Shah AM, Wingler K and Schmidt HH (2010) Oxidative stress and endothelial dysfunction in aortas of aged spontaneously hypertensive rats by NOX1/2 is reversed by NADPH oxidase inhibition. *Hypertension* **56**(3): 490-497.

Winslow RM (2013) Oxygen: the poison is in the dose. *Transfusion* **53**(2): 424-437.

Wolin MS, Ahmad M and Gupte SA (2011) Role of Oxygen-Derived Species in the Regulation of Pulmonary Vascular Tone, in *Textbook of Pulmonary Vascular Disease* (Yuan JX-J and al e eds) pp 301 -311, Springer, New York.

Woolley JF, Stanicka J and Cotter TG (2013) Recent advances in reactive oxygen species measurement in biological systems. *Trends in biochemical sciences* **38**(11): 556-565.

Wunderlich C, Schmeisser A, Heerwagen C, Ebner B, Schober K, Braun-Dullaeus RC, Schwencke C, Kasper M, Morawietz H and Strasser RH (2008) Chronic NOS inhibition prevents adverse lung remodeling and pulmonary arterial hypertension in caveolin-1 knockout mice. *Pulmonary pharmacology & therapeutics* **21**(3): 507-515.

Wyatt CN and Buckler KJ (2004) The effect of mitochondrial inhibitors on membrane currents in isolated neonatal rat carotid body type I cells. *The Journal of physiology* **556**(Pt 1): 175-191.

Yeligar SM, Harris FL, Hart CM and Brown LA (2012) Ethanol induces oxidative stress in alveolar macrophages via upregulation of NADPH oxidases. *Journal of immunology* **188**(8): 3648-3657.

Zhang H, Go YM and Jones DP (2007a) Mitochondrial thioredoxin-2/peroxiredoxin-3 system functions in parallel with mitochondrial GSH system in protection against oxidative stress. *Arch Biochem Biophys* **465**(1): 119-126.

Zhang H, Luo Y, Zhang W, He Y, Dai S, Zhang R, Huang Y, Bernatchez P, Giordano FJ, Shadel G, Sessa WC and Min W (2007b) Endothelial-specific expression of mitochondrial thioredoxin improves endothelial cell function and reduces atherosclerotic lesions. *The American journal of pathology* **170**(3): 1108-1120.

Zhou W, Li S, Wan N, Zhang Z, Guo R and Chen B (2012) Effects of various degrees of oxidative stress induced by intermittent hypoxia in rat myocardial tissues. *Respirology* **17**(5): 821-829.

Zuo L, Rose BA, Roberts WJ, He F and Banes-Berceli AK (2014) Molecular characterization of reactive oxygen species in systemic and pulmonary hypertension. *American journal of hypertension* **27**(5): 643-650.