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The Role of Arachidonate 5-Lipoxygenase in HIV-associated Pulmonary Hypertension

By

Kristi Michelle Porter
B.S., Clark Atlanta University, 2004

Advisor: Roy L. Sutliff, Ph.D.

An Abstract of
A dissertation submitted to the Faculty of the
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Abstract

The Role of Arachidonate 5-Lipoxygenase in HIV-associated Pulmonary Hypertension
By Kristi Michelle Porter

There are approximately 39 million people infected with human immunodeficiency virus type-1 (HIV-1) worldwide. Since the discovery of HIV-1, one of the hallmark characteristics of the disorder has been enhanced susceptibility to opportunistic infections, such as *Pneumocystis pneumonia* and *Haemophilus influenzae*. The advent of highly active antiretroviral therapy (HAART) has greatly reduced the incidence of infectious disorders and improved survival. However, HIV-infected persons now demonstrate a heightened risk of developing non-infectious lung disorders such as HIV-associated pulmonary arterial hypertension (HIV-PAH). HIV-PAH is a disorder characterized by increased pulmonary vascular tone and remodeling. PAH in HIV-1 patients occurs more frequently and progresses more rapidly than in uninfected individuals. In addition, patients living with HIV-1 have an increased susceptibility to develop severe PAH irrespective of their CD4+ lymphocyte counts. These findings suggest that the interaction of HIV-1 proteins with the pulmonary vascular endothelium may play a critical role in HIV-PAH development by altering pathways that regulate vascular tone and remodeling such as arachidonate 5-lipoxygenase (ALOX5). We hypothesize that the presence of HIV-1 proteins and hypoxia exposure augment the development of pulmonary vascular dysfunction and PAH by altering ALOX5 expression and activity.

This dissertation seeks to determine if ALOX5 contributes to HIV- and hypoxia-induced PH. The central hypothesis of this work is hypoxia exposure and HIV-1 proteins concomitantly promote the development of HIV-PAH by stimulating endothelial cell proliferation and vascular remodeling via increased 5-lipoxygenase expression and activity. *In vitro* results demonstrate that exposure of pulmonary artery endothelial cells to prolonged hypoxia, medium from HIV-infected macrophages and HIV-1 Tat increases ALOX5 expression. Research also reveals that hypoxia exposure induces endothelial proliferation in an ALOX5-dependent manner, and that medium from HIV-infected macrophages potentiates hypoxia-induced cellular proliferation. Furthermore, our findings indicate that excessive reactive oxygen species production and reduced antioxidant expression mediate the hypoxia-induced increases in ALOX5 and cell proliferation. Additionally, i*n vivo* results reveal that HIV-1 transgenic animals develop an exacerbated form of hypoxia-induced PH when compared to wild-type animals. The ALOX5 pathway is implicated in the increased severity of PH in HIV-1 transgenic animals, as they demonstrate elevated levels of ALOX5 and its metabolites. Collectively, these results indicate that the presence of HIV-1 proteins likely impact pulmonary vascular resistance and increase susceptibility to hypoxia-induced PH by stimulating the ALOX5 pathway. These studies identify a novel mechanism whereby HIV-1 proteins contribute to HIV-PAH pathogenesis. This improved understanding of the molecular mechanisms of hypoxia- and HIV-1 Tat-induced cellular proliferation may improve the quality-of-life of HIV-1 patients through the identification of ALOX5 as a biomarker for HIV-PAH and/or the use of agents that target this molecule to treat HIV-PAH.

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LIST OF ABBREVIATIONS

AIDS: Acquired Immunodeficiency Syndrome

AP-1: Activator Protein-1

APC: Antigen-presenting Cells

ARV: Antiretrovirals

BH4: Tetrahydrobiopterin

COPD: Chronic Obstructive Pulmonary Disease

Cys: Cysteine

Cyss: Cystine

d-ROM: Derivatives of Reactive Oxygen Metabolites

DPI: Diphenyleneiodonium

eNOS: endothelial Nitric Oxide Synthase

ET-1: Endothelin-1

GPx: Glutathione Peroxidase

GR: Glutathione Reductase

GSH: Glutathione

GSSG: Glutathione Disulfide

H_2O_2: Hydrogen Peroxide

HAART: Highly Active Antiretroviral Therapies

HPAEC: Human Pulmonary Artery Endothelial Cells

HIV: Human Immunodeficiency Virus type 1

HIV-PH: HIV-associated Pulmonary Hypertension

ICAM: Intracellular Adhesion Molecule

IL-1β: Interleukin-1beta

IL-6: Interleukin-6

iNOS: Inducible Nitric Oxide Synthase

IPAH: Idiopathic Pulmonary Arterial Hypertension

LPS: Lipopolysaccharide

MDA: Malondialdehyde

MDM: Monocyte-derived Macrophages

MMP: Matrix Metalloproteinase

NAC: N-acetylcysteine

NF-kB: Nuclear factor-kappaB

NO: Nitric Oxide

Nox: NADPH Oxidase

NRTI: Nucleoside Reverse Transcriptase Inhibitors

NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitors

ONOO˙: Peroxynitrite

PBMCs: Peripheral blood mononuclear cells

PCNA: Proliferating Cell Nuclear Antigen

PH: Pulmonary Hypertension

PI: Protease Inhibitors

PPAR: Peroxisome Proliferator-activated Receptors

ROS: Reactive Oxygen Species

RV: Right Ventricle

RVSP: Right Ventricular Systolic Pressures

SOD: Superoxide Dismutase

Tg: Transgenic

TNF-α: Tumor Necrosis Factor-alpha

Trx: Thioredoxin

VCAM-1: Vascular Cell Adhesion Molecule-1

CHAPTER 1

INTRODUCTION

Pulmonary Arterial Hypertension: Pulmonary arterial hypertension (PAH) is progressive disorder characterized by a persistent elevation of pulmonary artery pressure and pulmonary vascular resistance. PAH is clinically defined as a sustained elevation of pulmonary arterial pressure greater than 25 mm Hg at rest or greater than 30 mm Hg with exercise (Gaine et al., 1999). The pressure in normal pulmonary arteries is 8-20 mm Hg at rest. Chronic PAH increases the load on the right ventricle (RV) causing RV hypertrophy, right heart failure, the clinical syndrome of cor pulmonale, and ultimately, death (Weir, 1988). The symptoms of PAH are nonspecific and include dyspnea, syncope, fatigue, chest pain, irregular heartbeat, swollen ankles and legs and nonproductive cough.

PAH is a relatively rare disorder. Studies report a prevalence of idiopathic PAH (IPAH), the largest subcategory of PAH, of only 2-15 cases per million annually (Badesch et al., 2010; D'Alonzo GE, 1991; Humbert et al., 2006). Nonetheless, in 2002, pulmonary hypertension led to 15,668 deaths and almost 300,000 hospital visits in the United States (Hyduk et al., 2005). Additionally, the prognosis for PAH is extremely poor. Results from a national registry of IPAH patients indicate that the time from onset of symptoms to death is an average of 2.8 years (Barst, 2008; McLaughlin et al., 2004). Adult females are also much more likely to develop PAH than adult males as established by an analysis of the REVEAL (Registry to Evaluate Early and Long-term PAH Disease Management) registry where a 4.3:1 ratio was noted (Humbert et al., 2010).

Research indicates that PAH is a complex disorder that develops as a result of numerous stimuli and medical conditions. The characteristic vascular pathologies and histological patterns vary widely. As a result, medical professionals and researchers

developed a PH classification system to organize the disease into categories based on common clinical parameters, potential pathogenic mechanisms, and effective therapeutic strategies. There are currently five chronic PH groups (Simonneau et al., 2004; Simonneau et al., 2009). Of this classification system, *Group 1* is termed pulmonary arterial hypertension (PAH). This group, which includes idiopathic PAH, heritable PAH, and acquired PAH, is composed of a group of diverse diseases with similar etiologic and prognostic factors. This group includes PAH induced by drug use or toxin exposure as well as diagnoses attributed to HIV infection. The major histological features of this group include, but are not limited to, medial hypertrophy and intimal proliferation of the pulmonary arteries, and the appearance of muscle in normally non-muscular arteries. Late phase vascular abnormalities include concentric intimal fibrosis and plexiform lesions. *Group 2* is defined as pulmonary hypertension related to left heart disease and/or dysfunction. Pulmonary vascular pathologies such as medial hypertrophy and adventitial thickening as well interstitial fibrosis are associated with this group. *Group 3* is defined as PH that is associated with hypoxia and/or lung disease, such as chronic obstructive pulmonary disease (COPD), interstitial lung disease, and sleep apnea. The main histological features of this group are medial hypertrophy of muscular pulmonary arteries and muscularization of arterioles. *Group 4* PH is composed of only chronic thromboembolic pulmonary hypertension and is associated with eccentric intimal fibrosis, fresh thrombi, and recanalized organized thrombi that form bands and webs. Lastly, *Group 5* pulmonary hypertension is a miscellaneous group and is characterized by unclear multi-factorial mechanisms (Rabinovitch, 2008; Stenmark et al., 2009).

Models of Pulmonary Hypertension: Numerous models are used to investigate the mechanisms underlying pulmonary hypertension. These models of experimental PH are accomplished by prolonged hypoxia exposure, monocrotaline (MCT) injection, Sugen 5416 administration, or left pneumonectomy (surgical lung removal). Of these models, monocrotaline (MCT) treatment and prolonged exposure to hypoxia are the most widely utilized.

The monocrotaline model of PH was first described in 1967 and is routinely used to study both secondary and primary PH (Dorfmuller et al., 2003; Kay et al., 1967). Monocrotaline is a plant-derived toxin that is injected as a single subcutaneous or intraperitoneal dose to induce experimental PH. Activation of monocrotaline by liver oxidases is required to produce a reactive cross-linking compound, monocrotaline pyrrole (MCTP), which induces vascular injury (Lame et al., 2000; Stenmark et al., 2009). As a result of differences in MCTP metabolism, the response to monocrotaline is varied among species and strains. However, studies indicate that monocrotaline-induced PH is most effective in rats (Chesney et al., 1974).

It is believed that monocrotaline injection largely contributes to PH by causing endothelial cell injury and a mononuclear infiltration into the perivascular regions of arterioles and muscular arteries (Jasmin et al., 2001). Although plexiform lesion formation is not typical in this model, monocrotaline-treated rats demonstrate the characteristic pathologies of severe PH including right ventricular hypertrophy leading to right ventricular failure (Buermans et al., 2005) at 2-3 weeks following administration (Meyrick et al., 1980). In addition, high doses of monocrotaline are reported to induce

significant increases in right ventricular systolic pressures (RVSP) (Jasmin et al., 2001; Meyrick et al., 1980).

Hypoxia is another well-established and commonly used model for the study of pulmonary hypertension. Medical disorders that lead to alveolar hypoxia such as COPD, cystic fibrosis, bronchiectasis and asthma are clinical causes of chronic hypoxia and pulmonary hypertension. For example, studies demonstrate that 5-20% of COPD patients develop severe PH without any other major contributing factors (Chaouat et al., 2005; Kessler et al., 2001; Wright et al., 1983). Similarly, children and young adults living in areas of high altitudes experience persistent elevations in PAP (Sime et al., 1963; Vogel et al., 1962). In addition, histological examinations of pulmonary vessels of high-altitude residents who died from causes other than chronic pulmonary sickness reveal typical pathological patterns of pulmonary hypertension (Arias-Stella and Saldana, 1963; Gamboa and Marticorena, 1972).

The second commonly used model to study pulmonary hypertension is hypoxia exposure. This model is most commonly used in mice and rats. Mice or rats are exposed to normobaric (10% O_2) or hypobaric (320 mmHg or 426 kPA) hypoxia for 2-4 weeks to induce experimental PH. Hypoxia-induced PH induces vasoconstriction and remodeling of the pulmonary arteries (Kato and Staub, 1966; Meyrick and Reid, 1979), causing marked increases in pulmonary vascular resistance. These conditions result in a 50% increase in mean PA pressure (PAP) and a doubling of right ventricular weights (Rabinovitch et al., 1979). Phenotypic vascular alterations following hypoxia exposure also includes muscularization of the small, normally non-muscular arteries in the alveolar walls. These changes cause an increase in cells that express α-smooth muscle actin (α-

SMA), which may be attributable to the differentiation of fibroblasts, the migration of smooth muscle cells, and/or the transdifferentiation of endothelial cells into mesenchymal-type cells (Jones et al., 2008; Stenmark et al., 2006). Vasoconstriction is also believed to contribute to hypoxia-induced PH disease development. Hypoxic pulmonary vasoconstriction, which was first described by Von Euler and Liljestrand in 1946, is a physiological response that occurs in most mammals that ensures the preferential distribution of pulmonary blood flow to well ventilated areas of the lung. Reductions in lung NO levels precede the elevations in pulmonary pressure (Weissman et al., 2000) in hypoxic pulmonary vasoconstriction events. As such, hypoxia-induced pulmonary vasoconstriction is thought to also mediate PA remodeling. However, studies suggest that the contribution of vasoconstriction is greatest at early stages of the disease process (Stenmark and McMurtry, 2005; Wagenvoort, 1960).

Of the two more common models, many argue that the hypoxia model is a more physiological model than monocrotaline-induced PH because monocrotaline-induced PH does not occur in nature whereas hypoxia can lead to PH at high altitudes or as a consequence of hypoxic lung diseases. However, disadvantages to the hypoxia model include variability in response between species (Stenmark et al., 2009) and animal age, as developing lungs are more susceptible to decreased oxygen levels (Stenmark et al., 2006). In addition, monocrotaline injection is associated with several unwanted side effects. For example, monocrotaline administration causes significant liver and kidney damage (Roth et al., 1981) produces hepatic veno-occlusive disease in rats (Chen et al., 2008) and promotes myocarditis of the right and left ventricle. These additional pathologies severely complicate the study of RV hypertrophy and failure associated with PH

development (Miyauchi et al., 1993). Furthermore, monocrotaline-induced PH is attenuated and/or reversed by more than 30 agents with limited clinical efficacy (Stenmark et al., 2009). These data question the validity of the monocrotaline model and its value as a predictive tool for future PH therapies. With this evidence in mind, our group utilizes the hypoxia model for the study of pulmonary hypertension pathogenesis.

In addition to the *in vivo* hypoxia model, i*n vitro* hypoxia models are also utilized to study PH. While *in vitro* models vary considerably, most expose pulmonary vascular cells to normobaric hypoxia (0-10% O_2) for 4-24 hours (Pak et al., 2007). *In vitro* hypoxia exposure promotes a similar phenotype to that seen in clinical and experimental PH development (Pak et al., 2007). For example, exposure to 10% or 0% O_2 for 4 hours significantly inhibits endothelial prostacyclin synthesis (Madden et al., 1986) and endothelial nitric oxide synthase (eNOS) expression (McQuillan et al., 1994). Conversely, exposure to 2% oxygen for 24 hours stimulates significant endothelin release from coronary artery endothelial cells (Hieda and Gomez-Sanchez, 1990; Kourembanas et al., 1991). Moreover, hypoxia increases ET-1, endothelin-converting enzyme, and endothelin receptor 1 and B levels in mouse lung and in HPAEC following 72 hours (Kang et al., 2011). Hypoxia exposure also promotes endothelial cytokine (Karakurum et al., 1994; Shreeniwas et al., 1992; Yan et al., 1995) and growth factor release (Namiki et al., 1995; Shweiki et al., 1992). Additionally, proliferation are increased in aortic endothelial cells (Meininger et al., 1988) and human pulmonary artery endothelial cells (Kang et al., 2011) following hypoxia exposure when compared to cells cultured in standard culture conditions. Altogether, these data demonstrate that *in vitro* hypoxia exposure models are useful tools for the study of hypoxia-induced alterations in cellular signaling.

Pulmonary Hypertension Pathogenesis: Although the underlying mechanism of PH remains unknown, extensive research has identified several pathways that likely mediate PH pathogenesis by altering pulmonary vascular tone and/or remodeling. As a result, improved treatments have become available to treat PAH patients for the common pathologies associated with the disease. While not a cure, these treatments including endothelin (ET)-1 receptor antagonists, growth factor inhibitors, and nitric oxide activators, prolong survival and provide clinical improvement (Humbert et al., 2004). In addition, agents that interfere with the actions of 5-lipoxygenase or ROS production have recently been investigated for their potential in PAH treatment (Jones et al., 2004; Van Rheen et al., 2011; Wang et al., 2011). The following section summarizes the evidence implicating each of these pathways in PAH.

Endothelin-1

Endothelin (ET) -1 is a potent vasoconstrictor and smooth muscle cell mitogen. Research indicates that ET-1 contributes to PH pathogenesis as inhibition of ET-1 release and/or signaling attenuates PH development. Endothelin-1 concentrations are elevated in plasma and lung tissue of PH patients (Cacoub et al., 1997). Similarly, distal arteries and lung parenchyma of PH patients exhibit a two-fold increase in ET receptor density when compared with control subjects (Davie et al., 2002). Research employing the fawn-hooded rat (FHR) model of PH demonstrates a threefold increase in preproET-1 mRNA expression and ET-1 levels when compared to Sprague Dawley rats (SDR). These elevations in ET-1 may mediate PH pathogenesis in FHR and contribute to the exacerbated form of PH associated with this strain (Stelzner et al., 1992). Hypoxia

exposure (Elton et al., 1992) and monocrotaline treatment (Frasch et al., 1999) when compared to controls also significantly increase ET-1 levels in lungs and serum.

The hemodynamic effects of ET-1 are regulated by the ET receptors – ET_A and ET_B. The ET_A receptors regulate vasoconstriction and smooth muscle cell proliferation. Conversely, ET_B receptors mediate pulmonary endothelin clearance and vasodilation via the production of nitric oxide and prostacyclin by endothelial cells (Benigni and Remuzzi, 1999). Administration of the ET_A receptor antagonist, BQ123 (Bonvallet et al., 1994) attenuates hypoxia-induced increases in mean pulmonary arterial pressure and vascular remodeling. In addition, the selective inhibition of ET_B receptors for 7 days promotes severe increases in RV hypertrophy and muscularization of small pulmonary arteries (Ivy et al., 2000). Endothelin-1 contributes to hypoxia-induced vasoconstriction of the pulmonary vasculature (McCulloch et al., 1998; Muramatsu et al., 1997), and ET-1 receptor antagonism reverses this effect in newborn lambs (Coe et al., 2000). ET-1 may also contribute to PH pathogenesis by decreasing endothelial NOS expression and activity (Wedgwood and Black, 2005). These data imply that ET-1 plays a major contributing role in PH pathogenesis. As a result, current approaches to the clinical management of PH include the use of ET-1 receptor antagonists such as ambrisentan and bosentan for the treatment of PAH.

Growth Factor Signaling

Platelet-derived growth factor (PDGF) is also involved in PH pathogenesis. PDGF is a potent mitogen and SMC chemoattractant and is known to induce abnormal SMC proliferation and migration (Yu et al., 2003). Hypoxia exposure significantly increases rat PDGF expression (Berg et al., 1998; Katayose et al., 1993). PDGF receptor expression

is also markedly elevated in lungs of IPAH patients (Schermuly et al., 2005). Also, histological analysis of pulmonary arteries from IPAH patients reveals that PDGF expression is localized to PA endothelial and smooth muscle cells whereas PDGF receptors are mainly isolated to PA SMC (Perros et al., 2008). Recent studies indicate that PDGF promotes PA SMC proliferation by enhancing store-operated cytosolic Ca(2+) entry via Akt/mTOR activation (Ogawa et al., 2012). PDGF receptor antagonism reverses hypoxia- and monocrotaline-induced PH and reduces RV hypertrophy, muscularization of small pulmonary arteries, and SMC proliferation (Schermuly et al., 2005). As such, PDGF receptor inhibitors, such as the cancer therapy, imatinib (Gleevec), are under clinical investigation for the treatment of children and adults with PAH.

The growth factor, transforming growth factor-β (TGF-β) is also implicated in PAH pathogenesis. Although the specific contribution of TGF-β to PAH has yet to be identified, some studies suggest that TGF-β is increased in experimental models of PH (Arcot et al., 1993; Perkett et al., 1990) and PAH patients (Botney et al., 1994). Conversely, other studies indicate that TGF-β levels remain low throughout PAH development (Botney et al., 1991; Botney et al., 1992). Although estimations of TGF-β levels in PH remain controversial, alterations in TGF-β signaling are strongly associated with PH pathogenesis. TGF-β receptor blockade attenuates monocrotaline-induced increases in RV systolic pressure, RV hypertrophy and vascular remodeling (Megalou et al., 2010). Similar studies demonstrate that inhibition of TGF-β receptor signaling reduces pulmonary vascular remodeling and MCT-PH development (Zaiman et al., 2008). Additionally, mutations in the type II receptor for bone morphogenetic protein

(BMPR-II), a receptor member of the TGF-β superfamily, contribute to both familial and sporadic forms of PAH (Machado et al., 2005; Machado et al., 2001; Thomson et al., 2000). While the exact role of TGF-β in PH remains unknown, evidence that TGF-β promotes SMC proliferation in a manner partially dependent on Nox4 (Sturrock et al., 2006) suggests that TGF-β contributes to PAH pathogenesis. However, there are currently no PAH therapies targeting TGF-β levels and/or signaling. Future studies may provide a better understanding of TGF-β in PAH and effective TGF-β targeting strategies.

Nitric Oxide (NO) and NO Signaling

NO is a potent endogenous, endothelium-derived vasodilator produced by nitric oxide synthases (NOS). NOS utilizes L-arginine to produce NO and the by-product, L-citrulline. NO directly relaxes vascular smooth muscle by stimulating soluble guanylate cyclase and increasing production of intracellular cyclic guanosine monophosphate (cGMP) (Mehta et al., 2003). PH is associated with reduced nitric oxide (NO) production as well as impaired NO-induced vasodilatation. For example, acute and prolonged exposure to hypoxia causes a 50% decrease in endothelial nitric oxide production in rat pulmonary arteries (Shaul et al., 1993). Also, endothelial NOS expression is significantly decreased and arginase II, the enzyme that decreases the bioavailability of L-arginine for NO synthesis is increased in the pulmonary endothelium of PAH patients (Xu et al., 2004). As a result of these findings and others, studies investigating the effectiveness of pharmacologic therapies targeting the NO and cGMP pathways in experimental and clinical PH have increased dramatically.

Research indicates that activation of guanylyl cyclase by BAY 41-2272 prevents hypoxia-induced RV systolic pressure and RV hypertrophy in rats (Thorsen et al., 2010). Studies also demonstrate the phosphodiesterase type 5 inhibition by sildenafil attenuates PH development by suppressing pulmonary vascular remodeling in MCT-treated rats (Yen et al., 2010). The intravenous administration of sildenafil during right heart catherization dose-dependently reduces pulmonary vascular resistance (Wilkens et al., 2001). Similarly, clinical studies suggest that L-arginine administration reduces pulmonary artery pressure and increases exercise tolerance in patients with PAH (Nagaya et al., 2001). These data and others underscore the importance of appropriate NO levels and NO signaling in vascular health and function. Thusly, several therapies targeting these pathways are approved for the treatment of PAH such sildenafil and tadalafil. Furthermore, additional therapies are undergoing clinical trials for PAH treatment including the soluble guanylate cyclase activators, cinaciguat and riociguat.

Reactive Oxygen Species

Excessive ROS production and altered redox pathways are linked to PH pathogenesis and are currently being evaluated as potential PH therapies. ROS, such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$) as well as the reactive nitrogen species nitric oxide (NO) and peroxynitrite ($ONOO^-$) are biologically active species known to play important roles in vascular biology via redox signaling pathways (Giordano, 2005; Go and Jones, 2011; Kondo et al., 2009). These oxidants are produced by numerous sources such as the NADPH oxidases (Noxes), xanthine oxidase, cytochrome P450, uncoupled endothelial nitric oxide synthase (eNOS) and as byproducts of the mitochondrial respiratory chain (Papaharalambus and Griendling,

2007). To balance ROS levels and combat their toxic effects, cells employ several antioxidant enzymes including the superoxide dismutases (SOD), catalase, glutathione peroxidase (GPx), thioredoxins (Trx), and peroxiredoxins (Prx). Non-enzymatic antioxidant mechanisms also exist, including the vitamins E and C as well as glutathione, which acts as a reducing substrate for glutathione peroxidase (Nordberg and Arnér, 2001). These antioxidant systems are localized throughout the cell and function either in an independent or complementary manner to scavenge ROS. Overall, the balance between ROS generation and antioxidants is essential for normal cell function.

Although cells utilize a variety of mechanisms to regulate ROS generation and inactivation, ROS are essential for normal vascular function and act as key second messengers in numerous signal transduction pathways (Griendling et al., 2000; Irani, 2000; Ushio-Fukai, 2009). Moreover, ROS levels regulate the activity of important transcription factors implicated in vascular function including NF-κB, activator protein-1 (AP-1), and HIF-1α (Canty et al., 1999; Wellman et al., 2003; Wung et al., 1997). Therefore, the excess generation of and/or the reduced ability to remove ROS can lead to detrimental effects such as dysregulated apoptotic or proliferative states, vascular smooth muscle cell migration and endothelial dysfunction. For example, low concentrations of H_2O_2 induce cellular proliferation, whereas high concentrations promote apoptosis and cell cycle arrest (Baas and Berk, 1995). ROS also contribute to TNF-α-induced activation of endothelial apoptosis (Xia et al., 2006) and stimulate vascular smooth muscle cell migration by modulating the matrix metalloproteinases (MMP) -2 and -9 (Luchtefeld et al., 2005). Furthermore, superoxide can cause

endothelial dysfunction by combining with nitric oxide to produce the highly reactive radical, peroxynitrite ($ONOO^-$) and decrease NO levels (Bauersachs et al., 1996). Peroxynitrite is then able to oxidize the essential eNOS cofactor, tetrahydrobiopterin (BH4), stimulating eNOS uncoupling and further contributing to endothelial dysfunction by increasing superoxide and reducing NO availability (Kuzkaya et al., 2003). ROS-induced endothelial dysfunction is pivotal to vascular injury and the inflammatory response, and endothelial dysfunction is known to be an early predictor of cardiovascular events in patients without (Suwaidi et al., 2000) and with known vascular disease (Gokce et al., 2003; Schachinger and Zeiher, 2000). The extensive effects of ROS on the vessel wall support a role for ROS in the development of numerous vascular disorders including PH. For example, increased superoxide production has been observed in experimental models of PH (Brennan et al., 2003), and biomarkers of oxidative stress are elevated in PH patients (Bowers R, 2004). PH patients exhibit low NO levels in their exhaled breath (Kaneko et al., 1998; Machado RF, 2004), which suggests that NO scavenging by superoxide radicals likely results in reductions in bioavailable NO (Cai and Harrison, 2000). Moreover, superoxide regulates characteristic PH pathologies such as modulating pulmonary vasoconstriction (Liu et al., 2004) and stimulating pulmonary smooth muscle cell proliferation (Wedgwood and Black, 2003). Superoxide and other oxygen radicals also promote other cardiovascular pathologies such as atherosclerosis by altering NO and activating redox-sensitive pathways that mediate vessel remodeling and plaque stability (Szocs et al., 2002). In addition, coronary arteries from CAD patients express greater levels of the NADPH oxidase subunits, p22phox, p67phox, and p47phox (Guzik et al., 2006) and produce significantly larger amounts of superoxide (Sorescu et al., 2002).

Oxidative stress also enhances vessel inflammation by stimulating the release of endothelin-1 (ET-1) and pro-inflammatory cytokines interleukin-6 (IL-6). Patients with atherosclerosis demonstrate elevations in plasma ET-1 levels (Lerman et al., 1991) and enhanced immunoreactive staining for ET-1 in the vasculature (Zeiher et al., 1995). Similarly, increases in pulmonary vascular ET-1 and IL-6 (Golembeski et al., 2005b; Sadaie et al., 1988; Savale et al., 2009; Steiner et al., 2009) are implicated in PH development. ROS mediate the secretion of ET-1 from endothelial cells (Michael et al., 1997; Sethi et al., 2006) and polymorphonuclear leukocytes (Syeda et al., 2008). Antioxidant administration, however, inhibits the IL-6 and ET-1 release in healthy volunteers (Böhm et al., 2007), as well as, ET-1-induced release of IL-6 from vascular smooth muscle cells (Browatzki et al., 2000). Altogether, ROS and redox-sensitive pathways greatly contribute to vessel injury and vascular disease pathogenesis which further support a role for ROS in PAH development.

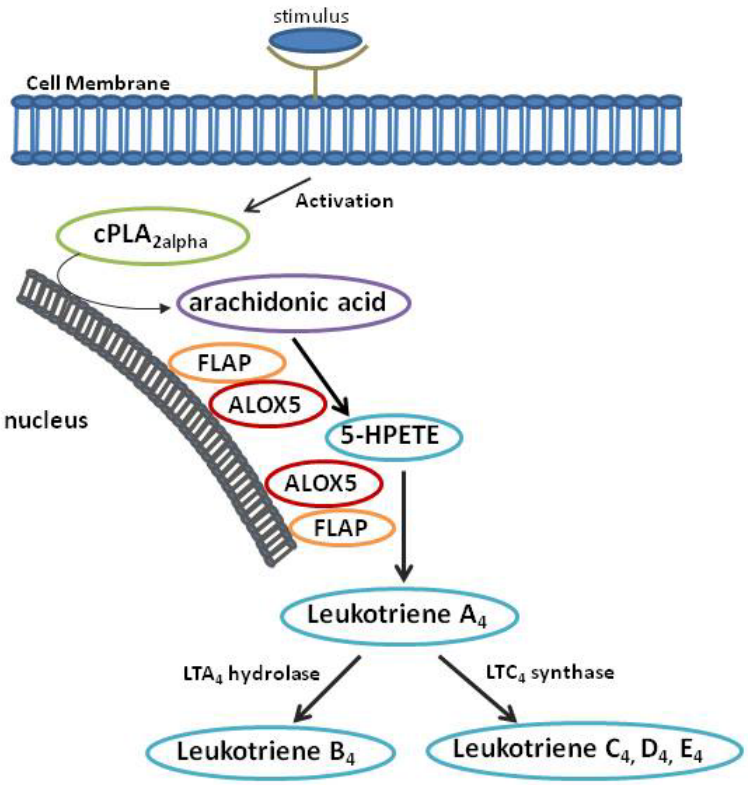
Arachidonate 5-Lipoxygenase

The correlation between PH and arachidonate 5-lipoxygenase (ALOX5) is based on data showing that agents that block ALOX5 attenuate the development of PH. ALOX5 is the enzyme that produces leukotrienes (LTs) by catalyzing the metabolism of arachidonic acid, released following cellular activation. Traditionally, ALOX5 expression was thought to be limited to cells of myeloid origin, such as monocytes, macrophages, eosinophils, neutrophils, and inflammatory cells. However, studies have shown ALOX5 is expressed in pulmonary artery endothelial cells (Zhang et al., 2002) as well as in the small artery endothelium of hypoxic rat lungs (Voelkel et al., 1996).

To produce the intermediate leukotriene LTA_4 from arachidonic acid, ALOX5 requires both calcium and the 5-lipoxygenase activating protein (FLAP) for its activation and translocation to the nuclear membrane. LTA_4 is highly unstable and is rapidly converted into two metabolites, LTB_4 or LTC_4. LTB_4 is a potent chemotactic agent formed by the action of LTA_4 hydrolase on LTA_4. Alternatively, in eosinophils, monocytes, and mast cells, LTA_4 can be conjugated with glutathione to form LTC_4, D_4 and E_4, which are collectively known as cysteinyl leukotrienes (Peters-Golden and Brock, 2000). LTC_4 synthase catalyzes the production of intracellular LTC_4, however, LTD_4 and LTE_4 are generated from extracellular LTC_4 following its export by the multidrug-resistance protein 1(MRP1) (Nguyen and Gupta, 1997) (Figure 1.1).

ALOX5 activity and leukotriene production are regulated by numerous signaling pathways. 5-lipoxygenase activating protein (FLAP) acts as a primary ALOX5 regulator. In intact cells, ALOX5 requires the presence of FLAP for leukotriene synthesis (Dixon et al., 1990; Miller et al., 1990) FLAP serves as a membrane anchor for ALOX5 and binds arachidonic acid for ALOX5 activity. Calcium serves as an important regulator of ALOX5 activity, and ALOX5 exhibits minimal activity in the absence of calcium (Aharony and Stein, 1986; Rouzer and Samuelsson, 1985). Conversely, the addition of calcium stimulates ALOX5. Kinase-mediated phosphorylation also stimulates ALOX5. ALOX5 is activated by p38 kinases as stimuli-induced ALOX5 is ablated by the p38 kinase inhibitor, SB203580 (Werz et al., 2000a). Additionally, ALOX5 phosphorylation at Ser271 and Ser663 by MAP kinase-activated protein kinase 2 and by ERK1/2, respectively induces ALOX5 activation (Werz et al., 2000a).

Figure 1.1. 5-Lipoxygenase (ALOX5) activation and leukotriene biosynthesis. 5-Lipoxygenase is expressed in macrophages, inflammatory cells, and endothelial cells. Upon activation, intracellular calcium concentrations rise, activating cytosolic phospholipase A_2 which produces arachidonic acid from phospholipids found in the nuclear membrane. ALOX5, along with its required co-factor, 5-lipoxygenase activating protein (FLAP), catalyze two consecutive reactions thereby converting arachidonic acid into leukotriene A_4. Leukotriene A_4 is then converted into either leukotriene B4 via LTA_4 hydrolase or leukotriene C_4 by LTC_4 synthase.



Yet, cAMP-dependent PKA-induced phosphorylation at Ser523 inhibits ALOX5 activation and translocation (Luo et al., 2004).

In addition, excessive ROS production is believed to affect ALOX5 activity (Riendeau et al., 1989; Werz et al., 2000b). *In vitro* studies demonstrate that ALOX5 is activated in conditions that promote lipid peroxidation (Riendeau et al., 1989) particularly following glutathione depletion (Hatzelmann et al., 1989; Hatzelmann and Ullrich, 1987). Similarly, stimulation of endogenous ROS release by antimycin A causes an almost 4-fold increase in leukotriene formation in transformed B lymphocytes (Werz et al., 2000b). These effects may in part be due to ROS-induced arachidonic acid release. Studies indicate that ROS enhance arachidonic acid release (Sporn et al., 1988) and subsequent arachidonic acid metabolism (Martinez and Moreno, 2001). Oxidative stress also activates p38 MAPK, which may also stimulate ALOX5 activity by promoting ALOX5 phosphorylation (Werz et al., 2001). However, nitric oxide (NO) is a potent inhibitor of LT synthesis (Brunn et al., 1997; Coffey et al., 2000). Taken together, these studies emphasize the reactive oxygen and nitrogen species production can play important roles in regulating ALOX5 activity.

Increased ALOX5 expression has been found in the lung tissue of patients with primary pulmonary hypertension, within infiltrating perivascular alveolar macrophages and in small pulmonary artery endothelial cells (Wright et al., 1998). MK-886 and zileuton, inhibitors of FLAP (5-Lipoxygenase Activating Protein) and ALOX5 respectively, have been effectively used in the prevention of PAH in experimental models (Jones et al., 2004). Studies utilizing mice exposed to hypoxia and monocrotaline (MCT)-treated rat models have provided valuable evidence that ALOX5 contributes to

PH (Stenmark et al., 1985). These experimental PH models mimic human PH cases by stimulating either hypoxic vasoconstriction of the pulmonary vasculature or endothelial dysfunction, which are directly involved in the pathogenesis of PH. ALOX5 inhibition has been shown to significantly prevent and improve this aspect of PH pathophysiology. Furthermore, overexpression of ALOX5 in MCT-treated rats markedly increases right ventricular systolic pressures (RVSP) when compared to rats treated with MCT without ALOX5 overexpression (Jones et al., 2004). On the other hand, daily inhibition of ALOX5 with either MK-886 or zileuton during a 5-week monocrotaline (MCT) study in rats attenuates elevations in RVSP by more than 50% (Jones et al., 2004). These results suggest that ALOX5 critically contributes to MCT-induced pulmonary hypertension progression and that increased ALOX5 expression may exacerbate PH pathologies.

The ALOX5 metabolites, cysteinyl leukotriene (CysLTs) are also associated with PH. CysLT levels are increased in lung lavage fluid of neonates with persistent pulmonary hypertension (Stenmark et al., 1983), patients with chronic obstructive pulmonary disease (COPD) (Piperno et al., 1993) as well as animals exposed to hypoxia (Morganroth et al., 1984b). Additionally, CysLTs are known to increase vascular permeability and vasoconstriction (Friedman et al., 1984) of pulmonary arteries. CysLTs also induce a variety of proinflammatory activities in cardiovascular tissues. For example, the administration of CysLT receptor antagonists attenuate increases in intracellular calcium concentrations and prevent vessel contraction in a dose dependent manner (Morganroth et al., 1984a). In addition, cysteinyl leukotrienes stimulate vasoconstriction in distal segments of pulmonary arteries (Friedman et al., 1984). *In vivo* injections of cysteinyl leukotrienes increase pulmonary arterial pressures in monkeys

(Smedegard et al., 1982) and pigs (Ohwada et al., 1990). Studies using isolated human pulmonary arteries also show that LTC_4 and LTD_4 induce contraction (Back et al., 2000a; Back et al., 2000b; Schellenberg and Foster, 1984). Therefore, due to their pronounced effects on the vasculature, the cysteinyl leukotrienes are implicated in recent studies as contributing mediators in PH development.

Furthermore, ALOX5 expression and activity are linked to cellular proliferation (Fischer et al., 2010; Ishii et al., 2009; Svensson Holm et al., 2008; Walker et al., 2002). Inhibitors of cysteinyl leukotriene (CysLT) production attenuate the basal proliferation of pulmonary artery endothelial cells (Walker et al., 2002). Also ALOX5 blockade with the specific inhibitors AA861 and MK886 significantly ablate the proliferation of three different lung tumor cell lines (Avis et al., 1996). Moreover, zileuton treatment induced a 54% reduction in abnormal proliferation of rat mammary tissue following challenge with the chemical carcinogen, DMBA (Chatterjee et al., 2011). Altogether, these data suggest that ALOX5 may play a major contributing role in PH pathogenesis and progression by altering pulmonary vascular tone and promoting pulmonary vascular remodeling.

PAH development is strongly associated with human immunodeficiency virus-1 (HIV-1) infection. Knowledge of HIV-PAH and its underlying etiology is minimal. However, many believe that alterations of the aforementioned pathways caused by HIV-1 or HIV-secreted mediators greatly contribute to HIV-PAH pathogenesis. The following section will summarize what is known about PAH in the context of HIV-1 and highlight the evidence implicating these potential factors.

HIV-1 structure and replication cycle: HIV-1 is a retrovirus belonging to the lentivirus (or slow virus) category. The HIV-1 virion is surrounded by a glycoprotein-rich envelope

and contains the double-stranded RNA genome within a nucleocapsid core (Krogstad, 2003). The HIV-1 genome contains 9 genes: *gag*, *pol*, *env*, *tat*, *rev*, *vpu*, *vpr*, *vif*, and *nef*. These genes encode for approximately 15 viral proteins (Turner and Summers, 1999) that are essential for HIV-1 structure, function, and infection.

HIV-1 enters target cells through high-affinity interaction between the viral envelope protein gp120 and the CD4 cell surface molecule, which is expressed by multiple cell types (Bour et al 1995, Dalgleish et al 1984, Klatzmann et al 1984, McDougal et al 1986). Following the attachment of the envelope complex to the CD4 protein receptor, the chemokine binding domains of gp120 are exposed and able to bind the target chemokine receptor. Once HIV has bound to the target cell, the virus content including HIV RNA and the reverse transcriptase, integrase, and protease enzymes are released into the cell. Upon entry into target cells, the viral genome is converted into double-stranded DNA by the virally encoded reverse transcriptase enzyme. HIV proteins then facilitate the transport of the newly synthesized viral DNA to the host cell nucleus, integrase then covalently incorporates the HIV DNA into the host genome (Turner and Summers, 1999), forcing the host cell machinery to synthesize numerous viral proteins. The resulting proteins are essential for HIV-1 replication and infection of target cells. For example, the cleavage of the Env protein produces the virion envelope glycoproteins gp120 and gp41. The regulatory proteins Tat and Rev, as well as the accessory proteins Vif, Vpu, Vpr, and Nef are also synthesized. New HIV-1 virions are then assembled along with a copy of the double stranded RNA genome and released into the bloodstream to continue replication in other cells.

Continued infection and replication of HIV-1 depletes CD4+ lymphocytes, the most commonly infected cells of the bloodstream. These cells regulate immune cell communication via lymphokine release and cell surface signaling (Gottlieb et al., 1981). With significant reductions in CD4+ lymphocytes, the HIV-1-infected subjects become immune-compromised and are unable to fight against foreign pathogens. HIV-1 infection is also associated with Kaposi's sarcoma (Boyle et al., 1993; Mitsuyasu, 1988), dementia (Merrill and Chen, 1991; Price et al., 1988), non-Hodgkin's lymphoma (Karp and Broder, 1991), nephropathy (Bourgoignie and Pardo, 1991; Cohen and Nast, 1988), and AIDS wasting syndrome (Melchior, 1997).

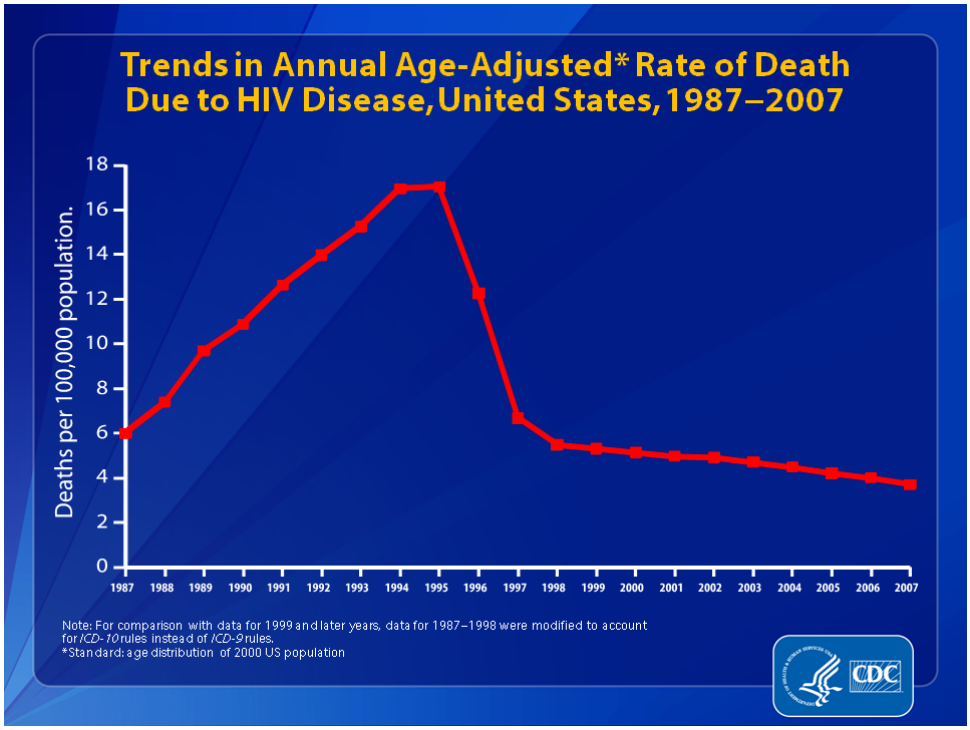
HIV- 1 and AIDS Statistics: An estimated 1.2 million United States residents are infected with HIV-1 (Source: CDC, 2008). According to the 2006 World Health Organization report, HIV-1 is the causative agent of Acquired Immune Deficiency Syndrome (AIDS). Since the HIV-1/AIDS epidemic began, over 600,000 people have succumbed to HIV-1/AIDS in the United States and over 30,000 new cases are diagnosed each year (Source: CDC, 2009). The Centers for Disease Control and Prevention estimates that the number of yearly HIV-1 diagnoses remained stable between 2007 and 2010 with 48,079 adults and adolescents being newly diagnosed in 2010. Of these new diagnoses, 79% were male and 21% were female. The distribution of HIV diagnoses has also remained stable with the largest percentage (61%) of HIV-1 diagnoses resulting from male-to-male sexual contact; 28% attributed to heterosexual sexual contact, injection drug use (8%), and receipt of blood or blood products or perinatal exposure (3%) constitute the remaining HIV diagnoses. Before the advent of highly active antiretroviral therapies (HAART), HIV infection was the leading cause death of among persons 25-44

years old. However, with the introduction of highly active antiretroviral therapies (HAART) in 1996, deaths of persons with AIDS have declined substantially (See Figure 1.2). Nonetheless, HIV-1 is still ranked as the 5th ranking cause of death for individuals between 25 and 44 years of age.

HIV/AIDS Antiretroviral Therapies (ARVs): ARVs are divided into 5 major classes: the nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors, and entry inhibitors (Kline and Sutliff, 2008). Although HAART fails to cure HIV-1 patients, these therapies reduce HIV replication, immune activation, and chronic inflammation as well as improve CD4+ lymphocyte counts. As a result, many HIV-infected patients demonstrate a considerable improvement in health. Additionally, the morbidity and mortality associated with HIV disease has decreased dramatically (Source: CDC, 2008).

HIV-1 infection of endothelial cells: It remains controversial as to whether HIV-1 can infect vascular endothelial cells *in vivo.* Evidence of productive infection of brain microvascular endothelial cells (BMVECs) has been reported (Moses et al., 1993) and refuted (Ades et al., 1993; Ades et al., 1992; Poland et al., 1995). In addition, research reveals that HIV binds but fails to infect non-replicating human umbilical vein endothelial cells (HUVECs) *in vitro* (Conaldi et al., 1995). Conversely, HIV-1 may be able to infect replicating HUVEC. In addition, exposure to IL-1β and tumor necrosis factor (TNF)-α enhanced this effect (Conaldi et al., 1995). Though interesting, the clinical relevance of these studies is unclear as vascular endothelial cells are believed to be non-replicative *in vivo*. Other studies demonstrate that while HIV-1 can enter endothelial cells via macropinocytosis (Liu et al., 2002) or through cytoplasmic vacuoles

Figure 1.2. Improved Survival of HIV-infected Patients in the United States. Trends in annual age-adjusted rate of death due to HIV disease between 1987 and 2007. The decrease in the rate in 1996 and 1997 is attributed to the introduction of antiretroviral therapy. Prophylactic medications for opportunistic infections and the prevention of HIV infection may have also contributed to this decrease.



(Gujuluva C, 2001), no infection occurs. Therefore, the majority of data suggest that HIV-1 is incapable to infecting endothelial cells under physiological conditions. However, additional research is needed for confirmation.

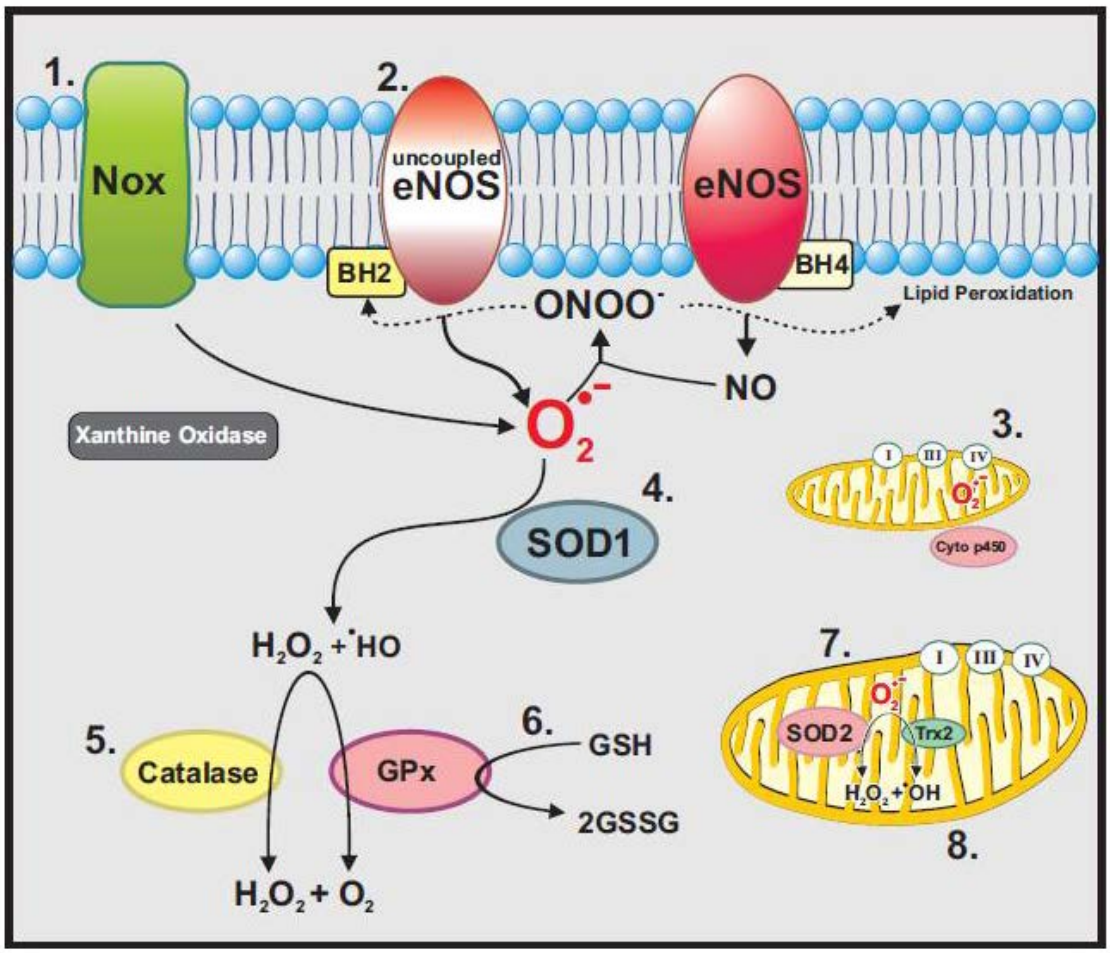
HIV-1 and endothelial dysfunction: Although the ability of HIV-1 to infect endothelial cells remains controversial, the deleterious effects of HIV-1 on endothelial cell function is well documented (Bussolino et al., 2001). For example, HIV-1 patients consistently demonstrate marked increases in ROS production as well as significant reductions in antioxidant availability and activity (Buhl et al., 1989; Eck et al., 1989; Mandas et al., 2009) In a study with treated and untreated HIV-infected subjects, the oxidative stress marker, d-ROM (derivatives of reactive oxygen metabolites) was shown to be greater in serum of HIV-1 patients than that of healthy controls (Mandas et al., 2009). Malondialdehyde (MDA), an index of lipid peroxidation, was also significantly elevated in the serum from both symptomatic and asymptomatic HIV-1 patients (Revillard JP, 1992; Sonnerborg A, 1988; Suresh et al., 2009). The marked increases in ROS biomarkers, d-ROM and MDA in HIV-1 patients demonstrate a HIV-induced imbalance between oxidant generation and antioxidant activity. These alterations are likely attributable to numerous mediators. However, several studies suggest that the increased ROS production documented in HIV-1 patients results from diminished antioxidant expression and activity. For example, a dramatic attenuation in the total antioxidant capacity including vitamin A and C serum concentrations has been noted in HIV-1 seropositive patients (Lacey et al., 1996; Suresh et al., 2009; Wang and Watson, 1994). Glutathione (GSH), the predominant antioxidant in the lung, is also significantly altered in HIV-1 patients. Studies demonstrate that total and reduced GSH in the epithelial

lining fluid of symptom-free HIV-seropositive individuals was 60% less than those in normal subjects (Buhl et al., 1989). GSH levels are also reduced in the blood of HIV-1 patients (Eck et al., 1989; Staal et al., 1992a; Staal et al., 1992b). Plasma of HIV-infected patients displays a 30% reduction in glutathione when compared to healthy controls. There was no difference in glutathione levels in the untreated HIV-infected group when compared to subjects on ART regimens (Wanchu A, 2009). Interestingly, however, HIV-1 produces a contradictory effect in the antioxidant, thioredoxin (Trx) which is significantly elevated in the plasma of HIV-infected healthy volunteers (Nakamura et al., 1996). Trx functions as an antioxidant in both the cytosol and mitochondria and contributes to cell growth, DNA repair and transcription factor regulation (Watson et al., 2004). The HIV-induced increase in Trx may, therefore, function as a cellular compensatory mechanism or an attempt to normalize antioxidant capacity.

In vitro models of HIV-1 infection mirror clinical studies demonstrating both increases in ROS and declines in antioxidant activity. HIV-1 infection of human primary macrophages produces a 6-fold increase in malondialdehyde (MDA) (Aquaro et al., 2007). This finding implicates HIV-1 infection as the principal cause for elevated macrophage ROS levels. However, it remains controversial whether HIV-1 infection or HIV-1-induced mediators contribute to the increased ROS production and antioxidant depletion seen in infected patients. Data suggest that HIV-induced mediators, independent of HIV-1 infection, are sufficient to increase cellular ROS levels. In an *in vitro* model of HIV-induced oxidative stress, podocytes expressing the NL4-3 HIV-1 construct with a deleted *gag/pol* region exhibit a marked increase in ROS generation over a 3-hour interval of HIV exposure. The NL4-3-induced increase in podocyte ROS

Figure 1.3. Effects of HIV-1 and HAART on ROS Sources and Scavengers

1. NADPH Oxidases (Noxes), the primary producer of ROS in vascular cells, are dramatically up-regulated by HIV-1 (Husain et al., 2009; Salmen et al., 2010; Wu et al., 2010) and ART (Papparella et al., 2007). 2. Uncoupled eNOS produces superoxide, instead of nitric oxide. Superoxide may then couple with NO to generate the highly reactive radical peroxynitrite ($ONOO^-$) (Bauersachs et al., 1996), which oxidizes tetrahydrobiopterin and causes lipid peroxidation (Kuzkaya et al., 2003). HIV-1 (Aquaro et al., 2007; Suresh et al., 2009) and ART (Manda et al., 2011) stimulate elevations in lipid peroxidation markers such as MDA and nitrotyrosine. 3. HIV-1 (Flores et al., 1993; Westendorp et al., 1995) and ART (Caron M, 2008; Gao RY, 2011; Kline et al., 2009; Opii et al., 2007) promote ROS release by inducing mitochondrial dysfunction. 4. HIV (Kline et al., 2008) and ART (Chandra et al., 2009; Prakash et al., 1997) reduce SOD expression and activity. 5. HIV-1 negatively modulates catalase expression and activity. 6. GSH levels are significantly decreased in HIV-1 patients (Sundaram et al., 2008; Wanchu A, 2009). In addition, HIV-1 proteins alter GSH release (Opalenik et al., 1998) and regulation (Fan et al., 2011; Richard et al., 2001). ART also deplete cellular GSH (Kline et al., 2009; Manda et al., 2011). 7. Mitochondrial antioxidant, SOD2 is decreased in HIV-1 (Flores et al., 1993; Prakash et al., 1997; Westendorp, 1995).


1.
Nox
2.
uncoupled
eNOS
eNOS
BH2
BH4
ONOO-
Lipid Peroxidation
NO
Xanthine Oxidase
O2•-
3.
I
III
IV
O2•-
Cyto p450
4.
SOD1
H2O2 + •HO
7.
I
III
IV
O2•-
SOD2
Trx2
H2O2 + •OH
8.
5.
Catalase
GPx
6.
GSH
2GSSG
H2O2 + O2

generation was attenuated by diphenyleneiodonium (DPI) administration, implicating flavin-containing enzymes such as NADPH oxidase as the primary source for ROS increases (Husain et al., 2009). This study suggests that virus infectivity due to *gag* and *pol* function is not necessary for HIV-induced ROS release.

In vivo models studying the effect of HIV-1 proteins on oxidative stress reach similar conclusions. Mice expressing the HIV-1 Tat protein (HIV-1 Tat+) exhibit a significant reduction in total intracellular GSH content in both the liver and erythrocytes. Additionally, glutathione synthetase activity in HIV-1 Tat+ mouse liver was decreased to 73% of control levels (Choi et al., 2000). Similarly, studies from our group employing an HIV-1 transgenic rat model indicate that the expression of HIV-1 proteins is sufficient to augment ROS production and alter antioxidant expression. These animals express a HIV-1 provirus that encodes for the viral genes *env*, *tat*, *nef*, *rev*, *vif*, *vpu*, and *vpr*. However, due to the deletion of the *gag* and *pol* regions, the HIV-1 transgene is both nonreplicative and noninfectious. Studies using this model show that HIV-1 transgenic (Tg) rat aortas display significant increases in superoxide and 3-nitrotyrosine levels compared to wild-type controls. HIV-1 Tg rats also exhibit marked decreases in circulating nitric oxide (NO) and total GSH as well as reductions in aortic SOD1 expression and activity (Kline et al., 2008). HIV-1 transgene expression also induces marked elevations in rat lung superoxide, hydrogen peroxide (H_2O_2), and NO metabolite levels as well as concomitant decreases in lung lavage fluid GSH when compared to wild-type rats (Jacob et al., 2006; Lassiter et al., 2009).

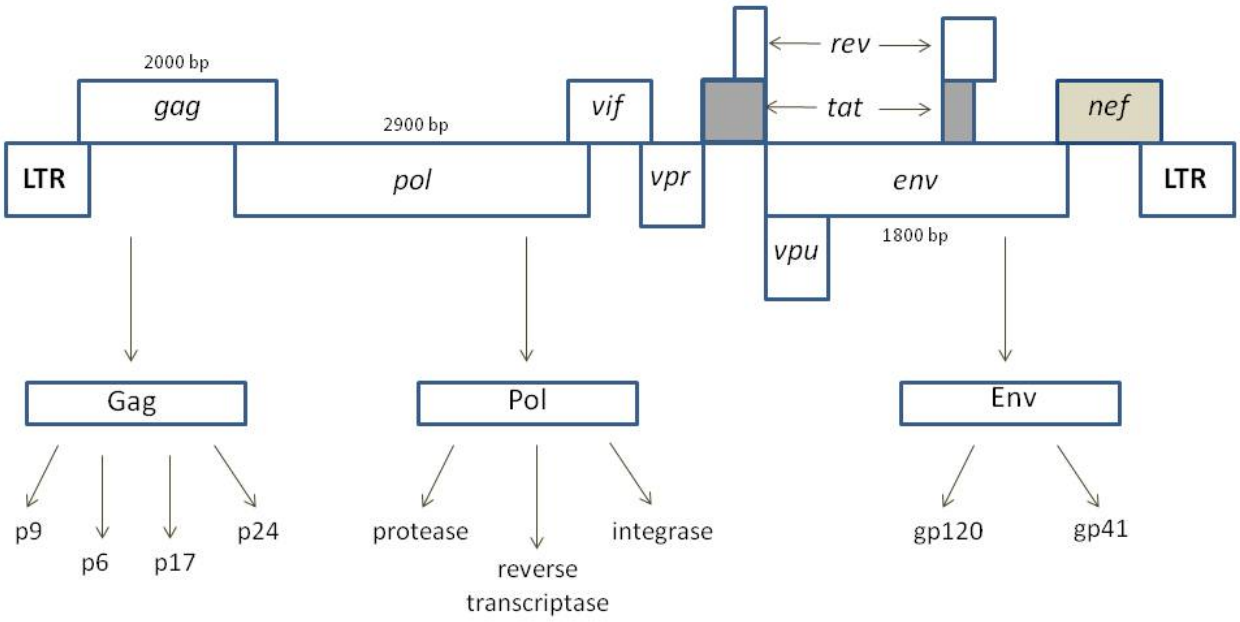
Altogether, these studies highlight the ability of HIV-1 proteins to independently alter endothelial function and induce vascular injury *in vivo.* Indeed, more details

regarding whether HIV-1 protein concentrations in these models are physiologically relevant are needed. Nonetheless, research demonstrates marked alterations in ROS and antioxidant levels in HIV-1 models. These data suggest a potential correlation between HIV-1 and oxidative stress, and raise the question of whether the oxidant/antioxidant imbalance found in HIV-1 patients contributes to the characteristic pathologies associated with this population.

HIV-1 Proteins: Considerable research indicates that HIV-1 significantly alters vascular cell function. However, research utilizing HIV- and Tat-Tg animal models argues that virus-induced mediators such as HIV-1 proteins may serve as sufficient inducers of vessel injury via increased oxidative stress. As a result, the investigation of HIV-1 proteins and their effects on ROS release and regulation has increased substantially. This research sheds light on the imbalance between oxidants and antioxidants, and strongly suggests that HIV-1 proteins contribute to both increased production of ROS and diminished antioxidant activity.

HIV-1 proteins are encoded by 9 genes located within the virion capsid (See Figure 1.4). Three of these genes, *gag*, *pol*, and *env*, are found in all retroviruses and are vital to the structure of HIV-1. For example, the *gag* and *pol* regions encode for the reverse transcriptase and integrase enzymes necessary for efficient HIV-infection and replication. The *env* gene encodes for gp160, the precursor for the envelope proteins gp120 and gp41, which are necessary for virus entry into cells. The 6 remaining "accessory" genes are unique to HIV-1. Two of these, *tat* and *rev*, perform a regulatory function and are essential for viral replication (Sadaie et al., 1988). However, the roles of HIV-1 genes, *vpr*, *vpu*, *vif*, and *nef* are less fully understood (Frankel and Young, 1998).

Figure 1.4. HIV-1 genome and mature HIV-1 protein cleavage products. The HIV-1 genome is 9.75 kB in length and encodes for nine translation products. Three of these genes, *gag*, *pol*, and *env*, are vital to the structure of HIV-1. The 6 remaining genes are unique to HIV-1 and directly encode for viral accessory and regulatory factors. The HIV-1 genome is regulated by a single promoter located in the 5'-LTR. This promoter contains binding sites for multiple cellular transcription factors such as Sp1 and NF-κB. Adapted from (Bruggeman et al., 1994)



The following section will focus on the role of HIV-1 proteins thought to play a contributing role in HIV-PH pathogenesis – Tat, Nef, and gp120.

Tat

Of all the HIV-1 proteins, the early viral protein, Tat is the most widely studied. Composed of 86-101 amino acids, Tat serves as a transcriptional transactivator of viral gene expression by binding to a transactivation-responsive region in the HIV long terminal repeat (LTR). The expression of Tat is critical for productive HIV infection, as Tat-deficient viruses are non-infectious. In HIV-1 patients, Tat can be secreted from infected T cells and monocytes (Ensoli et al., 1993) and following its release, circulates in the bloodstream. In 1995, Westendorp et al reported plasma Tat levels between 1-3 ng/mL in HIV-infected patients (Westendorp, 1995). More recently, however, Tat serum levels in HIV-1-infected patients were estimated to fall between 2 and 40 ng/mL (Xiao et al., 2000). It is also suggested that Tat concentrations are higher around HIV-infected perivascular cells and in the proximity of endothelial cells (András et al., 2003). This effect is thought to occur because macrophages and monocytes act as viral reservoirs and secrete Tat as well as cytokines and oxidants near endothelial cells.

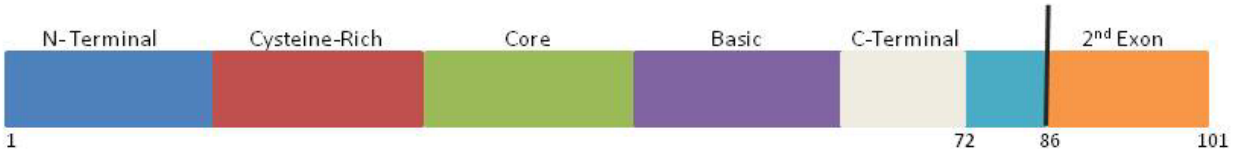
From the circulation, Tat enters uninfected cells (Ensoli et al., 1993; Helland et al., 1991; Marcuzzi et al., 1992; Westendorp et al., 1995). Five distinct functional domains have been identified in the Tat protein. These domains include the N-terminal, cysteine-rich, core, basic, and C-terminal (See Figure 1.5). The Tat C-terminal domain is thought to serve as the principal cell attachment and internalization moiety via the arginine-glycine-aspartic (RGD) sequence (Brake et al., 1990). This sequence is recognized by

integrin receptors and thereby enables Tat to bind to integrins α5β1 and αvβ3 with high affinity (Barillari et al., 1993; Urbinati et al., 2005b).

Additionally, conditions promoting the expression of these integrin receptors stimulate Tat-induced effects (Albini et al., 1995). Tat internalization may also be mediated by cell surface heparin sulfate proteoglycans. Research demonstrates that soluble heparin and treatment with glycosaminoglycan lysases specific for heparin sulfate chains inhibit Tat internalization (Tyagi et al., 2001).

Following internalization, Tat alters cellular physiology by positively or negatively affecting gene expression. Tat activates transcription of numerous genes including tumor necrosis factor β (Buonaguro et al., 1994) and interleukin-6 (Ambrosino et al., 1997) by binding to the RNA stem-loop structures generated by the 5' end of target transcripts, such as the HIV-1 transctivation-responsive element (TAR) (Berkhout et al., 1989). Studies also indicate that HIV-1 Tat stimulates the expression of the adhesion molecule E-selectin in endothelial cells (Cota-Gomez et al., 2002; Hofman et al., 1993). Similarly, Tat induces pulmonary artery endothelial cell VCAM-1 (Liu et al., 2005b) as well as astrocyte VCAM-1 and ICAM-1 expression in a dose- and time-dependent manner (Woodman et al., 1999). Twenty fours of hour exposure to Tat decreases the expression of the tight junction proteins claudin-1, claudin-5, and zonula occludens (ZO)-2 in brain microvascular endothelial cells (Andras et al., 2003). The Tat arginine-rich, or basic, and cysteine rich domains are essential for NF-kB induction as modification of these Tat sequences completely block HIV replication and NF-κB activity in monocytes (Devadas et al., 2006). The arginine- and cysteine-rich domains of

Figure 1.5. HIV-1 Tat Functional Domains.



Tat are required for IκB-α and p65 association, respectively, and for sustaining NF-κB activation (Fiume et al., 2011).

Extensive research also demonstrates that HIV-1 Tat increases ROS levels and decreases antioxidant levels. For example, Tat causes a dose-dependent increase of ROS in cultured brain microvascular cells (Toborek M, 2003) and significantly induces ROS production and lipid peroxidation in rat brain endothelial cells (Price TO, 2005). In the HIV indicator (HeLa-CD4-LTR-B-gal), or MAGI cells, transfection with a Tat-expressing plasmid for 48 hours significantly increases ROS levels and reduces intracellular GSH levels by 50%. This study also showed that the Tat-induced alterations are reversed by pretreatment with the antioxidant, N-acetylcysteine (NAC) (Zhang et al., 2009). Murine fibroblasts expressing the full-length HIV-1 Tat protein exhibit similar reductions in cellular GSH concentrations (Opalenik et al., 1998). Additionally, *in vivo* studies demonstrate that the intravenous injection of Tat protein decreases mouse brain GSH levels by 85% (Banerjee et al., 2010). Tat over-expression in HeLa cells results in a 3-fold reduction in the glutathione peroxidase (GPx) mRNA ratio as well as a 2.5 fold decrease in GPx activity (Richard et al., 2001). Moreover, HeLa cells stably producing the Tat protein express 48% less SOD2 compared to control cells (Flores et al., 1993; Westendorp, 1995), which may be caused by Tat-induced disruption of Sp1 and Sp3 binding in the SOD2 basal promoter (Marecki JC, 2004).

These studies demonstrate that Tat alters cellular ROS and antioxidant regulation. Yet, the exact mechanism and source of Tat-induced oxidative stress remain unclear. Recent studies, however, have demonstrated Tat-induced activation of several ROS-producing enzymes. For example, Gu et al showed that Tat acutely increases

intracellular oxidant levels in ECV-304 cells. This Tat-induced oxidant activity is decreased by pretreatment with two NADPH oxidase inhibitors, DPI and apocynin (Gu et al., 2001). Co-culture of human umbilical vein endothelial cells with HeLa-Tat cells also significantly induces endothelial H_2O_2 production via Nox 4 activation (Wu et al., 2010). These studies implicate NADPH oxidases as potential mediators of Tat-induced ROS. However, other oxidases may contribute to HIV-induced ROS release as DPI and apocynin are somewhat nonspecific inhibitors.

Conversely, cellular redox state may negatively affect HIV-1 gene expression and transcription by altering HIV-1 Tat internalization and function. Previous studies demonstrate that the oxidation state of the cysteine-rich region of Tat strongly influences its capacity to enter cells (Siddappa et al., 2006). In addition, more recent studies indicate that the redox state of Tat alters protein uptake by macrophages and hinders Tat biological activity due to protein aggregation (Pierleoni et al., 2010). Research also indicates that Tat transactivation is regulated by cellular redox state (Fanales-Belasio et al., 2002). Normally, Tat stimulates transcriptional elongation from the viral LTR through a specific interaction with a 59-residue stem-loop on RNA known as the transactivation-response element (TAR). Yet, recent studies demonstrate that 1 hour of NAC exposure reduces Tat-induced HIV-1 LTR transactivation in MAGI cells to 39.3% of the Tat-alone levels (Zhang et al., 2009). Selenium administration also inhibits Tat-dependent LTR activity in human MDM and 100nM selenium also significantly reduces Tat-dependent transcription in U937 cells when compared to untreated controls (Kalantari et al., 2008).

These studies provide remarkable evidence of the independent effects of Tat on ROS levels and antioxidant availability. Recent studies examining novel anti-AIDS therapies have attempted to target Tat activity and binding. However, the available data regarding the effectiveness of these agents is controversial. Although further investigation is needed to better understand the mechanism underlying its effects, Tat clearly alters cellular function and likely contributes to the vascular dysfunction and disease associated with HIV-1 infection.

Nef

Several studies implicate the HIV-1 protein Nef as a potential mediator in HIV-induced vascular injury. Nef, "the negative factor" is an HIV viral accessory protein with a molecular weight ranging between 27-34 kDa. Although normally found within the cytoplasm, Nef associates with the cellular membrane upon activation via myristoylation. Nef expression has been shown to down-regulate the cell-surface levels of both CD4 and MHC-1 molecules. It also interferes with numerous intracellular pathways, leading to the dysregulation of cellular signaling and activation (Geyer M, 2001). *In vitro* studies indicate that Nef influences HIV-1 pathogenesis through its ability to increase viral replication and infectivity in primary lymphocytes and macrophages. In addition, i*n vivo* studies show that Nef is essential for high virus replication and disease progression to AIDS in HIV-infected individuals (Harris, 1999).

Although studies demonstrate that Nef plays a significant role in HIV function, research investigating Nef-induced endothelial dysfunction and ROS release is limited. In 2002, it was shown that Nef protein expression does not independently induce

microglial NADPH oxidase (Villhardt F, 2002). However, Nef significantly enhanced superoxide release by NADPH oxidase following challenge with the calcium ionophore, formyl peptide or lipopolysaccharide (LPS) (Villhardt F, 2002). Other studies reveal that Nef regulates superoxide production in a biphasic manner. Research by Olivetta et al demonstrates that human monoblastic cells (U937) stably transfected with a vector expressing a Nef-ER fusion protein produce greater ROS than controls at 1- and 4- hours post-transfection. However, Nef-expressing cells exhibit a complete ablation in ROS production at later time points. In more recent studies, exposure of neutrophils from healthy donors to Nef for one hour increased superoxide production. DPI administration significantly reduced the Nef-induced superoxide production, implicating activation of a flavin-containing enzyme. Also, studies performed with neutrophil cellular lysates demonstrate that Nef associates with p22-phox, but not any other NADPH oxidase subunits (Salmen et al., 2010). Similarly, in *ex vivo* studies, exposure of porcine pulmonary arteries or pulmonary artery endothelial cells (HPAEC) to Nef markedly increases superoxide release by 54% and 70%, respectively. In addition to these effects on ROS, Nef also concomitantly decreased eNOS expression and NO production in porcine arterial rings and HPAEC (Duffy et al., 2009).

Collectively, these reports implicate Nef as a mediator of HIV-induced injury and vascular dysfunction. Although *in vitro* data suggest a potential cell-type dependent effect, *ex vivo* studies underscore the potential physiological relevance of Nef in HIV-related vascular disease. Moreover, studies performed by the Flores group demonstrate that HIV-1 Nef contributes to HIV-associated PH by promoting vascular remodeling (Almodovar et al., 2011b; Marecki et al., 2006; Sehgal et al., 2009). Altogether, HIV-1 Nef

may contribute to the vascular dysfunction documented in the HIV-1 population via increased ROS production and effects on the nitric oxide synthase pathway.

gp120

The HIV-1 protein, gp120 also induces vascular cell injury. The envelope glycoprotein gp120 is expressed on the surface of HIV-1 virions and facilitates the receptor binding and subsequent membrane fusion required for HIV-1 infection (Freed and Martin, 1995). In addition, soluble gp120, estimated to exist between 12-92 ng/mL in the serum of HIV-1 patients (Oh SK, 1992), can be shed from virus particles or infected cells into the circulation. As a result, gp120 can cause extensive cellular damage by stimulating inflammatory cytokine release (Gendelman et al., 1994), apoptosis (Huang et al., 2001; Singhal et al., 2000), tight junction injury (Annunziata et al., 1998; Kanmogne et al., 2005a), and oxidative stress pathways (Bagetta et al., 2004; Holguin et al., 2004). Several studies indicate that gp120 stimulate ROS release in numerous cell types. For example, exposure to gp120 for 24 hours causes an almost 6-fold elevation in MDA levels in astroglial cell homogenates. This effect was significantly antagonized by pretreatment with the antioxidant, NAC (Visalli et al., 2007). gp120 exposure also induces marked increases in human retinal epithelial cell MDA and NO production, as well as inducible nitric oxide synthase expression over a 72-hour interval when compared to untreated controls (Yu QR, 2008). Additionally, gp120 induces marked staining for HNE, an indicator of lipid peroxidation, in cells expressing the endothelial cell marker, CD31. These increases in ROS production were associated with elevations in MMP-9, and gene delivery of the antioxidant enzymes GPx and SOD1 returned MMP-9 to control levels

(Louboutin et al., 2010). Research also demonstrates that low concentrations of gp120 promote ROS release. Recombinant gp120 at a concentration of 340 nM increases ROS production in human monocyte-derived macrophages (Pietraforte et al., 1994). Also, gp120 concentration of 40 nM was also shown to increase intracellular H_2O_2 in lymphoid cells (Shatrov, 1996). Picomolar concentrations of gp120 induce ROS release in U937 cells, whereas co-administration of catalase and SOD decreased the gp120-induced oxidative damage by 81% (Foga IO, 1997). Also, *in vivo* studies also established that injecting 500 ng of gp120 significantly increases MDA levels.

In addition to the increases in ROS, gp120 has also been shown to alter antioxidant regulation. Seventy two hours of gp120 exposure significantly decreases the mRNA expression of the Nrf2 transcription factor in the L2 epithelial cell line (Fan et al., 2011). The decrease in Nrf2 mRNA expression, however, did not produce a significant reduction in Nrf2 protein expression. Moreover, HIV-1 transgene expression in rat alveolar epithelial cells produced a 30% attenuation in Nrf2 mRNA expression (Fan et al., 2011). Overall, these data suggest a role of gp120 in ROS release. In addition, the effects of gp120 on Nrf2 expression may act as a possible mechanism underlying HIV-induced antioxidant depletion.

While it is unclear whether the concentrations of HIV-1 proteins tested *in vitro* are physiologically relevant or appropriately effective to induce these changes, it is clear that specific HIV-1 proteins significantly impact ROS levels. Research indicates that HIV-infected monocytes and macrophages serve as reservoirs for HIV. Whether these reservoirs have the ability to continuously secrete HIV-1 proteins into the bloodstream remains controversial and more studies are needed to determine the effect of active HIV-1

infection on circulating HIV-1 proteins. Additionally, further investigation to ascertain if these proteins potentiate the effect of HIV-1 infection is warranted. Nonetheless, these data confirm that HIV-1 proteins may contribute to HIV-vascular disorders by disrupting vascular cell signaling pathways and/or altering ROS generation and antioxidant activity.

HIV-associated Vascular Disorders: Human immunodeficiency virus type 1 (HIV-1) infection and acquired immunodeficiency syndrome (AIDS) pose one of the greatest challenges to global public health. Since the development of highly active antiretroviral therapies (HAART), mortality and the incidence of opportunistic infections in people living with HIV-1 have declined substantially (Corey et al., 2007; Palella et al., 2006). As HIV/AIDS patients live longer, however, serious non-AIDS events occur and are associated with a greater risk of death than opportunistic AIDS-related events (Neuhaus et al., 2010). As such, vascular complications including coronary heart disease, pulmonary hypertension (PH), and atherosclerosis (Barbaro, 2002; Hsue et al., 2011; Hsue et al., 2004; Krishnaswamy, 2000) are some of the most widely recognized (Crum et al., 2006; Seaberg et al., 2010) non-AIDS diseases recorded in HIV-infected patients. In addition to the increase in susceptibility, clinical data also reveal that vascular complications in HIV-1 patients progress much more rapidly than in non-infected individuals (Guaraldi et al., 2011; Hsue et al., 2004). The exact mechanisms by which HIV-1 promotes the development and progression of these disorders remain unknown and are likely multi-factorial. Current research has identified endothelial dysfunction and increased vascular injury as a contributing underlying pathways.

Coronary Heart Disease and Atherosclerosis: HIV-1 positive patients have a higher prevalence of atherosclerotic lesions (Fontas et al., 2004; Maggi et al., 2004; Meng et al., 2002; Spieker et al., 2005), and elevated markers of subclinical atherosclerosis including increased carotid artery intima-media thickness (Chironi et al., 2003; Currier et al., 2005; Hsue et al., 2009a; Hsue et al., 2004; Johnsen et al., 2006; MasiÃ¡ et al., 2009; McComsey et al., 2007; Mercié et al., 2005; van Vonderen et al., 2009b), increased arterial stiffness (Bonnet et al., 2004b; Sevastianova K, 2005) and endothelial dysfunction (Blanco et al., 2006; Hsue et al., 2009b; Kristoffersen et al., 2009; Nolan et al., 2003). Clinical studies examining cardiovascular disease in HIV-1-positive people prior to the era of HAART are relatively few, yet there is evidence of serious cardiovascular anomalies in these patients. Seminal work by Joshi revealed coronary arteriopathy in 3 of 6 HIV-1-infected children at autopsy; it also described vasculitis and perivasculitis with infiltration of lymphocytes and mononuclear cells in vessel walls (Joshi et al., 1987). Other post-mortem analyses described major atherosclerotic lesions in proximal coronary arteries in 6 out of 8 HIV-infected patients who were 23-32 years of age (Paton et al., 1993). The high frequency of abnormalities in these early studies is striking considering that cardiovascular pathologies are normally rare in these age groups. Vasculitis in small blood vessels (Cebrian et al., 1997; Mandell and Calabrese, 1998), aneurysms in medium or large arteries (Maniker and Hunt, 1996), and significantly lower levels of high density lipoprotein cholesterol (HDLc) in the bloodstream (Grunfeld et al., 1992) of untreated HIV-1-positive individuals indicate that HIV-1 infection increases cardiovascular complications. These findings provide initial evidence of vascular dysfunction in HIV-1 patients, and support the premise that HIV-1 viral proteins have a role in the development of cardiovascular disease in this population.

Antiretroviral-naïve HIV-1-positive patients are found to have markers of endothelial activation including elevated plasma levels of von Willebrand factor, plasminogen activator inhibitor-1 antigen, and tissue-type plasminogen activator (Lafeuillade et al., 1992; Schved et al., 1992). These elevations in markers of endothelial dysfunction were found to correlate with anti-p24 antibodies and disease severity (Schved et al., 1992). Antiretroviral-naïve HIV-1-positive people have also been found to have higher levels of soluble vascular cell adhesion molecule-1 (VCAM-1) (Wolf et al., 2002), intracellular adhesion molecule-1 (ICAM-1) (Greenwood et al., 1998), and E-selectin (Greenwood et al., 1998; Lafeuillade et al., 1992; Seigneur et al., 1997) compared to healthy controls. This up-regulation of cell adhesion markers suggests that HIV-1 increases endothelial cell activation and dysregulation. These derangements may contribute to the increased incidence of pulmonary and systemic vascular disease.

There is also indirect clinical evidence showing that the presence of the HIV-1 virus increases cardiovascular risk. An ongoing retrospective analysis by the Kaiser Permanente Medical Care Program of Northern California has determined hospitalization rates for coronary heart disease and myocardial infarction in 4159 HIV-1-positive male members (Klein et al., 2002). The authors did not find a correlation between antiretroviral therapies (ARTs) and hospitalization rates in the HIV-1 positive group after a 4 year follow-up. However, they demonstrated significantly higher hospitalization rates in the infected group when comparing them to age- and sex-matched HIV-1-negative controls during this same timeframe. They were unable to establish correlations between the increase in hospitalization rate with other known risk factors (i.e. smoking, hypertension, diabetes, and hyperlipidemia) in the HIV-1-positive

group, thus concluding that HIV-1 infection itself increases the hospitalization rate for coronary heart disease and myocardial infarction. This conclusion has been supported by other clinical studies as well. A 2007 study examined acute myocardial infarction in patients (3851 HIV-1-positive and 1,044,589 HIV-1-negative) at 2 large Massachusetts hospitals (Triant et al., 2007). The authors found a significantly increased risk for heart attack in the HIV-1-positive population at all ages examined, even when adjusted for the presence of other traditional risk factors in this group. Interestingly, the risk for myocardial infarction was roughly tripled in HIV-1-positive women compared to uninfected women, a group generally considered to be at lower risk of developing cardiovascular disease compared to men. However, the impact of HAART on this observation could not be adjusted for due to insufficient data. Another long-term multi-institution analysis, the Strategies for Management of Antiretroviral Therapy (SMART) Study Group, concluded that cessation of antiretroviral therapy (ART) in HIV-1-positive patients increases their short-term risk of developing cardiovascular disease (El-Sadr et al., 2006). Because prolonged ART has been associated with major metabolic and cardiovascular disorders, the authors of the study had hoped to evaluate the effectiveness of episodic ART in 2,720 HIV-1-positive patients using a treatment paradigm that administered HAART to maintain CD4+ lymphocyte levels. Unfortunately, interruption of antiretroviral therapy did not benefit this cohort and actually increased the incidence of major cardiovascular events.

It is suggested that HIV-1 infection elicits endothelial dysfunction in patients, as measured by flow-mediated dilation (FMD) of the brachial artery. A controlled case-study of 4 HIV-1-positive patients suggested that viral load inversely correlated with

endothelium-dependent FMD without any relation to antiretroviral regimens (Blum et al., 2005). Solages monitored FMD in 75 HIV-1-positive and 223 control subjects, and found significantly impaired endothelial function in the infected population. This study also found that viral load was a significant predictor of FMD (Solages et al., 2006b). The authors did not observe an association between endothelial dysfunction and the use of HAART, which could potentially be explained by the small sample size and unrepresentative demographic characteristics of this specific population. A smaller study in patients from 3.5-19.5 years of age also showed that HIV-1-infected children had significantly reduced FMD, increased wall stiffness, and lower cross-sectional compliance and distensibility of the carotid artery than non-infected children (Bonnet et al., 2004a). Interestingly, no differences in these parameters were observed when comparing HIV-1-positive children on HAART with those who were HAART-naïve, suggesting that the HIV-1 viral infection increased endothelial dysfunction. Other data also suggest that HIV, independent of HAART, can induce vascular dysfunction (Hsue et al., 2009b; Oliviero et al., 2009). However, a 2007 study published by Lorenz concluded that HIV-1 infection and HAART are both independent risk factors for the development of atherosclerosis in adults (Lorenz et al., 2007). They found that intima media thickness of the carotid bifurcation, a predictor of subclinical atherosclerosis, was 24.8% higher in an HIV-1-positive/antiretroviral-naïve group compared to an uninfected control group. They also observed significantly greater IMT of the carotid bifurcation and the common carotid artery associated with HAART treatment in HIV-1-positive individuals. This effect of HIV-1 proteins was confirmed in a 2009 study which demonstrated that HIV-1 infection is independently associated with carotid intima media thickening, a measure of sub-clinical atherosclerosis (van Vonderen et al., 2009a).

HIV-associated Pulmonary Arterial Hypertension: First identified in 1987 (Kim KK, 1987), HIV-associated PAH is an acquired form of PAH (Category I) and is being diagnosed with increasing frequency. Similar to IPAH, the pathogenic mechanisms that predispose HIV patients to develop PAH are unclear. However, studies show that HIV-PAH occurs in the absence of any apparent lung disease and PAH disease severity does not correlate with CD4+ lymphocyte count (Seoane et al., 2001; Speich et al., 1991). In addition, although HIV is present in inflammatory cells in the lungs, HIV has not been found in endothelial cells of patients who develop PAH (Humbert et al., 1998a; Mette SA, 1992) nor has HIV DNA, RNA, or p24 antigen been detected in the pulmonary vessels of HIV-PAH patients (Kanmogne et al., 2001; Klings ES, 2003; Pellicelli et al., 2004). These data suggest the pathogenesis of HIV-PAH is unrelated to infection or immune dysfunction and may be partially attributable to the indirect actions of HIV-1 proteins on the vasculature (Mette SA, 1992).

Although histologic characteristics of HIV-PAH are similar to idiopathic PAH (Rubin, 1997), numerous studies report an increased occurrence of PAH in the HIV-infected population, with a prevalence of approximately 1 case per 200 (0.5%). More recent studies suggest, however, that this number is increasing and estimate that up to 1.0% of HIV-1 patients will develop PAH (Nunes et al., 2003). This increase in incidence is likely underestimated as HIV-1 positive patients are not routinely examined for PAH, and PAH is often misdiagnosed resulting in an inaccurate assessment of incidence among HIV-1 patients (Petrosillo et al., 2006). Overall, the current data suggests that more than 10,000 HIV-1-infected individuals in the U.S. alone will develop PH. This incidence of PH in the HIV-1-infected population is extremely high compared to the 1 to 2 cases per million recorded in the general population (Opravil et al., 1997).

HIV-PAH also progresses more rapidly (Seoane et al., 2001) and is associated with a poorer prognosis than PAH in the general population (Opravil et al., 1997). Epidemiological studies demonstrate that the survival of patients with HIV-PAH is significantly less than other patients classified with Group I PAH (McLaughlin et al., 2004). In a recent Swiss HIV Cohort Study, the median survival of patients with HIV-PAH was reported to be 3.6 years (Opravil and Sereni, 2008). Additionally, death of HIV-PAH patients is causally related to PAH rather than HIV-related events (Petitpretz et al., 1994). Studies also reveal that the average age range of patients with HIV-PAH is 38-41 (Degano et al., 2010; Zuber et al., 2004). Additionally, men are slightly more likely to develop HIV-PAH than women (Degano et al., 2010; Nunes et al., 2003; Zuber et al., 2004).

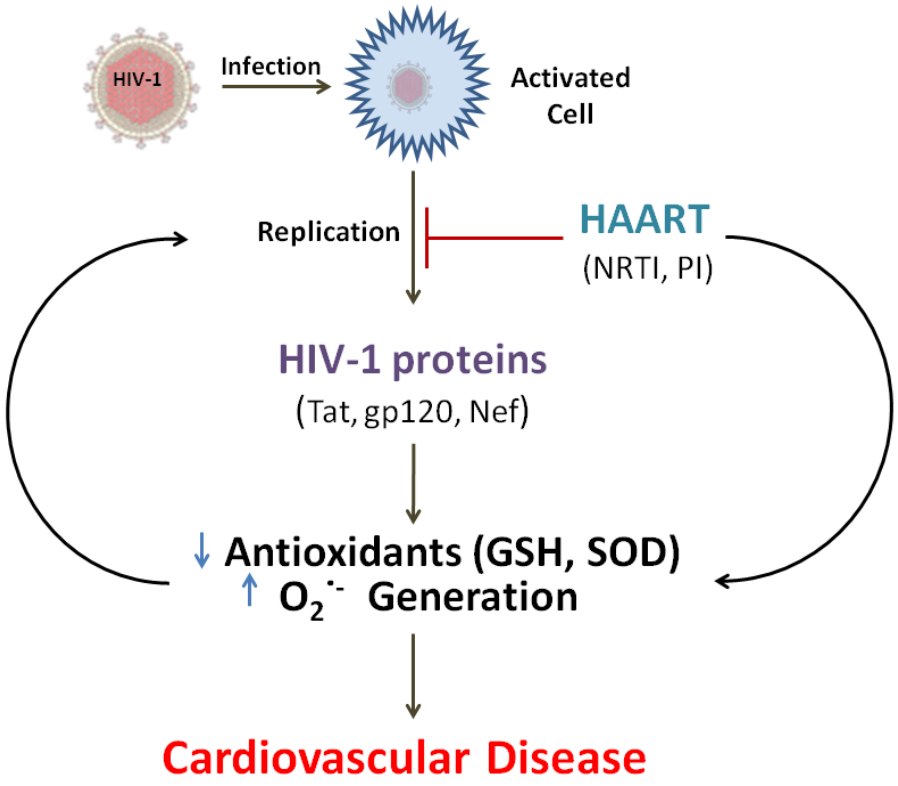
The primary symptoms of HIV-PAH are nonspecific and are often attributed to underlying conditions associated with HIV-1 infection. However, the symptoms most-commonly associated with HIV-PAH and hospital visits occur as a result of right ventricular dysfunction such as dyspnea (85%), pedal edema (30%) and non-productive cough (19%) (Nunes et al., 2003). Right heart catherization is the most effective method for PAH diagnosis as well as for hemodynamic evaluation and treatment response. This method of diagnosis is considerably more important for HIV-PAH patients as the false-positive rate from other diagnosis tools such as Doppler-echocardiography are reported to be as high as 72% (Sitbon et al., 2008).

Current treatments for HIV-PAH are largely based upon data from IPAH patients. Studies demonstrate that intravenous prostacyclin and prostacyclin analogs (Nunes et al., 2003) as well as oral endothelin receptor antagonists (Sitbon et al., 2004) improve the exercise capability and hemodynamic parameters of HIV-PAH patients.

Recent studies investigating HIV-PAH suggest that HAART fails to prevent the development of HIV-PH or improve the hemodynamic parameters in HIV-PAH patients (Degano et al., 2010; Simonneau et al., 2009). Also, although HAART regulates viral replication and improves survival, patients with well-controlled HIV infection still develop PAH (Degano et al., 2009). Additionally, HAART may potentially contribute to or exacerbate HIV-PAH development by inducing endothelial dysfunction via ROS release, ET-1 production, and endothelial proliferation (Hebert et al., 2004; Jiang et al., 2006). These observations underscore the complex etiology and severity of this disorder and highlight the need for further investigation of this disease. Therefore, the purpose of our research is to identify the mechanism by which HIV-1 promotes PAH pathogenesis. We hypothesize that HIV-1 promotes PAH development and progression by altering the expression of genes that regulate pulmonary vascular tone. We demonstrate that HIV-1 proteins promote endothelial cell proliferation and vascular remodeling by stimulating the 5-lipoxygenase pathway.

Summary: One of the hallmark characteristics of human immunodeficiency virus-1 (HIV-1) is the enhanced susceptibility to opportunistic infections, such as *Pneumocystis pneumonia*, *Mycobacterium tuberculosis*, and *Haemophilus influenzae*. Recent studies indicate that HIV-infected persons also have a heightened risk of developing non-infectious lung disorders such as, HIV-related pulmonary arterial hypertension (HIV-PAH), a disorder characterized by increased pulmonary vessel tone and vascular remodeling. Patients living with HIV-1 have an increased susceptibility to develop severe PAH irrespective of their CD4+ lymphocyte counts. Additionally, HIV has not been found in endothelial

Figure 1.6. Contribution of HIV- and HAART-induced ROS to Cardiovascular Disease



cells of patients who develop PAH nor has HIV DNA, RNA, or p24 antigen been detected in the pulmonary vessels of HIV-PAH patients. While the underlying cause of HIV-PAH remains unknown, the interaction of HIV-1 proteins with the pulmonary vascular endothelium may play a critical role in HIV-PAH development by altering pathways that regulate vascular tone and remodeling such as arachidonate 5-lipoxygenase (ALOX5). ALOX5 is the enzyme that catalyzes the production of leukotrienes, which stimulate cellular proliferation and pulmonary vasoconstriction. Altogether, we hypothesize that HIV-1 proteins promote HIV-PAH development and progression by stimulating pulmonary endothelial ALOX5 expression and activity.

Proposed Research: More than 10,000 HIV-1-infected individuals in the U.S. alone will develop PAH. The underlying mechanism of HIV-PAH remains unclear and effective treatments are limited. The purpose of this research is to examine whether HIV-1 protein expression contributes to HIV-PAH pathogenesis by stimulating the ALOX5 pathway. We also examine the independent effects of HIV-1 proteins and/or hypoxia exposure on vascular endothelial ALOX5 expression and activity. *In vitro* and *in vivo* studies utilizing human pulmonary artery endothelial cells and an HIV-1 transgenic animal model, respectively were utilized to study the effects of HIV in chronic hypoxia conditions. Altogether, our evidence demonstrates that HIV-1 protein expression is associated with an increased susceptibility to hypoxia-induced PAH and promotes biochemical changes that are associated with PAH pathogenesis. These studies provide a better understanding of PAH pathogenesis and may yield additional therapies for the treatment of PAH.

CHAPTER 2

MATERIALS AND METHODS

Reagents. Endothelin-1 and TGF-β ELISA kits were purchased from R&D Systems (Minneapolis, MN) and Promega, respectively. Trypan blue, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), PEG-Catalase, and gelatin were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant full-length, Tat was obtained from Immuno Diagnostics (Woburn, MA). Recombinant HIV-1 Nef (11478) was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD). Zileuton was obtained from Patheon Pharmaceuticals (Cincinatti, OH). MK-886 was purchased from EMD Biosciences (San Diego, CA).

Cell Culture: Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza (Walkersville, MD). HPAEC were maintained in 10% fetal bovine serum (FBS) endothelial basal medium (Clonetics) supplemented with EGM-2 SingleQuots (Clonetics) at 37°C in a humidified incubator with a 5% CO_2 atmosphere. For co-culture studies, HPAEC were grown in EGM-2 medium and maintained at 37°C with 5% CO_2. Confluent HPAEC were incubated with 10 x 10^6 peripheral blood monocytes (PBMC) prestimulated with PMA. HPAEC and/or PBMC were inoculated with or without HIV-1 virus. Following 1 hr incubation at 5% CO_2, cells were spun down to remove excess inoculum then placed in 5 ml of FBS-free EGM-2 medium. Twenty-four hours following medium replacement, cells and supernatant were separated and stored for analysis.

Monocyte-derived Macrophages: Medium from HIV-infected and control monocyte-derived macrophages were obtained from Dr. William Tyor of the Atlanta VA Medical

Center. Primary human monocyte-derived macrophages (MDM) were cultured at 37°C with 5% CO_2 in DMEM containing 10% human serum, L-glutamine, penicillin-streptomycin and macrophage colony stimulating factor (M-CSF) for 7 days. MDM (5 x 10^6) were infected with HIV-1_{ADA} (clade B) at a multiplicity of infection (MOI) of 0.1 for 1 hour. Following infection, MDM were resuspended in medium devoid of M-CSF and cultured for 14 days with media changes every 3 days (Rao et al., 2008). HIV-1 p24 levels were measured in media (1:10,000 dilution) by ELISA (Advanced BioScience Laboratories, Kensington, MD).

***In Vitro* Hypoxia Exposure**. HPAEC, passages 3-8, were exposed to hypoxia in a Biospherix exposure chamber (Lacona, NY). Confluent cultures were trypsinized at a ratio of 1:6, plated in 10 cm^3 dishes, and allowed to adhere overnight. Following incubation, cells were placed in normoxic or hypoxic conditions for 24, 48, or 72 hours. For normoxic conditions, HPAEC were either placed into a standard incubator maintained at 37°C and 5% CO_2 levels. For hypoxic conditions, HPAEC were placed in a hypoxia chamber maintained at 37°C, 1% oxygen, and 5% CO_2 levels. HPAEC were cultured under normoxic or hypoxic conditions for 24-, 48-, or 72 hours. In studies assessing the role of ROS levels in hypoxia-induced HPAEC proliferation and ALOX5 expression, PEG-Catalase was administered during the final 24 hours of the 72 hours of hypoxia exposure. To determine the role of 5-Lipoxygenase in HPAEC proliferation, Zileuton (10-50μM) was administered either throughout the entire exposure period or during the final 24 hours of the 72 hour exposure.

Animals. Male Fischer 344 wild-type and HIV-1 Transgenic (Tg) rats were obtained from Harlan (Indianapolis, Indiana) and bred in the animal facility at the Atlanta VA under a 12:12 light-dark cycle. All studies were completed in compliance with protocols approved by the Atlanta VA Animal Care and Use Committee. The HIV-1 Tg rats for this study were generated from established lines of an HIV provirus. This HIV-1 Tg rat model was developed at UMD using the NL4-3 *gag/pol* HIV-1 transgene (Reid et al., 2001). The HIV Tg rat line has proviral DNA with deleted *gag* and *pol* but intact *env* and *tat*, *nef*, *rev*, *vif*, *vpr*, and *vpu* accessory genes (Dickie et al., 1991; Kopp et al., 1992). HIV-1 transgenic rats have dense cataracts at birth but otherwise appear healthy and develop normally. However, by 6 months of age, they begin to display evidence of systemic disease including poor weight gain and muscle atrophy that progresses over time (Reid et al., 2001). HIV-1 transgene expression has been detected in the intestines, and at low levels in kidney, lymph nodes, lung and spleen (Bruggeman et al., 1994; Reid et al., 2001). Hemizygous rats, ages 7-9 months, were used for this study.

Isolated Perfused lung: Rats were anesthetized with isoflourane and mechanically ventilated after tracheal cannulation at a rate of ~60 strokes per minute with a tidal volume of 2.5 mL/breath. The heart and lungs were exposed by thoracotomy and heparin was administered. The pulmonary artery was cannulated with a 14G cannula connected to a pressure transducer (ADInstruments, Colorado Springs, CO). The pulmonary artery was then perfused with 37^{o}C Hanks' Balanced Salt Solution (Sigma Chemical, St Louis, MO) at a rate of 7ml/min for 5 min while the right atrium was incised to allow removal of the perfusate. Pressure/volume relationships were generated using a calibrated

peristaltic pump at flow rates of 7, 16, 26 and 35 mls/min. Data were collected using the PowerLab digital acquisition and analyzed using Chart software (ADInstruments, Colorado Springs, CO).

***In Vivo* Chronic Hypoxia Model**. A chronic hypoxia model previously used by our lab to induce experimental PH, RVH, and pulmonary vascular remodeling was employed in the current study (Nisbet et al., 2010). Rats were housed in normoxic (21% O_2) or hypoxic (10% O_2) conditions. Hypoxic conditions were created by infusing nitrogen gas into an enclosed chamber until the desired oxygen tension was reached. Rats placed in the hypoxia chamber remained there for four weeks to induce experimental pulmonary hypertension. Food and water were provided *ad libitum*.

Enzyme-linked Immunoassays: Leukotriene-specific ELISA kits (Cayman Chemical) were employed to measure HPAEC leukotriene levels and leukotriene levels in animal bronchoalveolar lavage fluid (BALF). Endothelin-1 (ET-1) ELISA kits (R&D Systems) were used to assess ET-1 levels in serum samples. Serum and BALF levels of transforming growth factor-beta (TGF-β) were determined using the Promega TGF-β1 Emax ImmunoAssay System (Madison, WI) ELISA kit. HIV-1 p24 ELISA (Advanced BioScience Laboratories, Inc, Kensington, MD) were used to measure p24 levels in media from HIV-infected monocyte derived macrophages (MDM). HIV-MDM samples were diluted 1:10,000 to ensure accurate concentration extrapolation. All ELISAs were performed according to manufacturer's instruction.

Real-time PCR Analysis: Real-time PCR was used to measure gene expression alterations in rat lung homogenates and HPAEC lysates. Total RNA was isolated from HPAEC and rat lungs using RNA-Bee (Tel-Test Inc., Friendswood, TX) followed by purification with serial chloroform, isopropanol and ethanol extractions. The mRNA was reverse transcribed (Invitrogen, Carlsbad, CA) to cDNA using random nanomer primers and quantification of the genes of interest was accomplished using a Roche Lightcycler Real-Time PCR detection system. All transcripts were detected using SYBR Green I (Molecular Probes, Inc, Carlsbad, CA). The genes of interest were normalized to the housekeeping gene β-globin to determine a gene of interest to β-globin ratio. Relative expression was calculated using the Delta-Delta C_T method.

Western Blotting: HPAEC lysates and rat lung homogenates were homogenized and re-suspended in ice cold cell lysis buffer. Protein concentrations were determined by the BioRad method, comparing samples versus a BSA standard. 30 micrograms of protein were loaded per lane and subjected to SDS-PAGE. Protein samples were then transferred to nitrocellulose membranes using the Fast Semi-Dry Blotter according to manufacturer's instruction (Thermo Scientific). After blocking in 5% non-fat dried milk (NFDM), membranes were placed in antibody solutions against HIF-1alpha (Santa Cruz) or PCNA (Abcam). Membranes were incubated overnight at 4°C, washed with TBST and placed in solutions containing fluorescent anti-goat or anti-rabbit secondary antibodies for 1 hour. Immunoreactive bands were detected using the Licor system and

the proteins of interest were quantified by densitometry and normalized to beta-actin levels within the same sample.

Right Ventricular Systolic Pressure: Right ventricular systolic pressures (RVSP) were assessed using a 0.8 F micro-tip pressure transducer. Rats were anesthetizied with isoflurane. A 0.8 F microtip pressure transducer (Millar Instruments) was inserted into the right jugular vein and advanced to the right ventricle. Right ventricular pressure was continuously monitored for a period of 10 minutes. Data was analyzed using a Powerlab system (ADInstruments, Denver, CO).

Right Ventricular Hypertrophy Measurements: Right ventricular hypertrophy was assessed by right ventricle/left ventricle + septum gross weight ratios. Rat hearts were removed following sacrifice. The right ventricle was dissected from the left ventricle and septum, the weights for the right ventricle and left ventricle + septum were determined, and the ratio of these values was calculated.

Histology: The pulmonary circulation was perfused with PSS to remove red blood cells and followed by perfusion with calcium-free PSS. Lungs were simultaneously inflated with 4% paraformaldehyde via the trachea. The perfused lung was immersed in 4% paraformaldehyde, embedded in paraffin and sectioned for analysis. Medial thickness of pulmonary arteries was quantified by measuring wall thickness, as delineated by the internal and external elastic lamina, and expressing it as a percentage of the vessel

diameter. In addition, the percentage of smooth muscle within the media was also determined by staining with antibodies to smooth muscle α-actin (Sigma, St Louis, MO). Immunohistochemistry samples were quantitated using Scion software (Scion Corporation, Frederick, MD). Photomicrographs were obtained using a Leica DM4000B microscope.

Cell Proliferation Assays. Human pulmonary arterial endothelial cell (HPAEC) proliferation was assessed using MTT Cell Proliferation Assay (ATCC). Briefly, proliferating cells reduce the tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) resulting in intracellular formazan. Detergent reagent was added to cell to solubilize formazan. Supernatants were then collected and quantified using a spectrophotometer at 562nm. HPAEC were seeded at 10,000 cells/well in 24-well plates. Selected cells were cultured in an incubator under normoxic conditions (21% O_2, 5% CO_2), and others were exposed to hypoxic conditions (1% O_2, 5% CO_2) in a hypoxia chamber for 72 hours. Following exposure to normoxic or hypoxic conditions, cells were subjected to MTT assay according to the manufacturer's protocol. MTT results were also confirmed by Trypan Blue Dye Exclusion and Cell Counting.

Exosome Isolation. Exosomes were isolated from HIV-MDM medium using the ultra-centrifugation method as previously described (Théry et al., 2001). Briefly, medium was subjected to repeated centrifugations to remove dead cells and debris at 200- and 3000rpm at 4°C, respectively. Medium was then spun down at 100,000 rpm for 70 minutes at 10°C using a Beckman XL-90 ultracentrifuge and Type 50.4 TI rotor.

Supernatants and exosome-containing preparations were carefully collected and stored at 4°C.

Hydrogen Peroxide (H_2O_2) Analysis. Hydrogen Peroxide (H_2O_2) release was quantified using the Amplex Red Assay. Cells were incubated in a solution containing the Amplex Red reagent (Molecular Probes), horseradish peroxidase and a buffer solution for 30 minutes at 37°C. Supernatants were then collected and fluorescence measured at 560nm. H_2O_2 concentrations were determined through standard curve extrapolation.

DCF Staining. Overall reactive oxygen species (ROS) and reactive nitrogen species were detected using the ROS-sensitive fluorescent probe 2',7'-dihydrodichlorofluorescein diacetate (DCF-DA; Invitrogen, Carlsbad, CA). Confluent HAEC monolayers were loaded with 25 µg/mL DCF-DA for 1 hour at 37°C in Krebs–Ringer Phosphate Buffer (KRPG; 145 mM NaCl, 5.7 mM KH_2PO_4, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, and 5.5 mM glucose, pH 7.35). A laser-scanning confocal microscope (Olympus, Center Valley, PA) and fluorimeter were used to detect DCF fluorescence at excitation and emission wavelengths of 488 nm and 520 nm, respectively. For quantification, DCF fluorescence intensity was measured on a Victor plate reader (PerkinElmer, Waltham, MA).

Statistical Analysis. A student's t-test analysis was used for comparison of two groups. One-way ANOVA with Tukey's posttest was used for the comparison of multiple groups. All experiments using cell cultures were repeated at least twice, and samples were run in duplicate or triplicate. Statistical significance was defined as $P< 0.05$, and all graphs are expressed as mean ± SEM.

CHAPTER 3

HIV-1 Transgene Expression Exacerbates Hypoxia-induced Pulmonary Hypertension Development

Introduction

An estimated 1.2 million United States residents are infected with human immunodeficiency virus-1 (HIV-1) and nearly 600,000 people with acquired immune deficiency syndrome (AIDS) have died since the epidemic began (Source: CDC, 2011). The advent of highly active antiretroviral therapy (HAART) has greatly reduced the incidence of infectious pulmonary complications such as bacterial pneumonia and tuberculosis and improved survival. However, noninfectious complications of HIV-1, such as pulmonary hypertension (PH) are now being recognized with increasing frequency.

Pulmonary arterial hypertension is defined as a sustained elevation of pulmonary arterial pressure greater than 25 mm Hg and a pulmonary capillary wedge pressure or left ventricular end diastolic pressure less than 15 mmHg (McGoon et al., 2004). Pathogenic vascular alterations in PH are characterized by abnormal muscularization of small pulmonary arteries and progressive intimal hyperplasia. Patients with severe PAH may develop obstructive plexiform lesions in the distal pulmonary circulation (Rabinovitch, 2008). These occlusive lesions are associated with decreased lumen cross-sectional area and progressive increases in pulmonary vascular resistance, which leads to the development of right ventricular hypertrophy (RVH) and PAH. Although the underlying cause of PH remains unknown, endothelial dysfunction and proliferation are implicated as major contributors to PAH pathogenesis and progression (Huang et al., 2010; Sakao et al., 2009; Tuder et al., 2001). Significant endothelial alterations have been identified in the pulmonary arteries of PH patients (Rabinovitch et al., 1986). Abnormal endothelial cell growth patterns have also been documented in the vascular wall and in cultured

pulmonary artery endothelial cells isolated from patients with idiopathic pulmonary arterial hypertension (IPAH) (Masri FA, 2007).

PAH is associated with HIV-1 infection (Lederman MM, 2008). HIV-associated pulmonary arterial hypertension (HIV-PAH) was first identified in 1987 (Kim KK, 1987) and has been increasingly diagnosed thereafter (Nunes et al., 2003; Petrosillo et al., 2006). According to the most updated clinical classification of PAH, HIV-PAH is designated as a Group I form of pulmonary arterial hypertension (PAH) (Simonneau et al., 2009). The Group I classification of PAH refers to a chronic disease caused by increased vascular obstruction and resistance of the small pulmonary arteries as opposed to a general elevation of pulmonary pressures, or PH. In 2001, the incidence of PH in HIV-1 patients was estimated to be 1:200, whereas PH in the general population occurred in approximately 1-2 patients per every million (Speich et al., 1991). More recent studies, estimate that up to 1.0% of HIV-1 patients will develop PH (Nunes et al., 2003). This increase is still likely to underestimate the true incidence of HIV-PAH because the complication is not routinely evaluated in patients and is often misdiagnosed (Petrosillo et al., 2006). HIV-PAH also progresses more rapidly (Seoane et al., 2001) and is associated with a poorer prognosis than PH in the general population (Opravil et al., 1997). Furthermore, epidemiological studies demonstrate that the survival of patients with HIV-PAH is significantly less than other patients classified with Group I PAH, including IPAH-, collagen vascular disease- and congenital heart disease-associated PAH (McLaughlin et al., 2004).

The pathogenic mechanisms that predispose HIV patients to develop PAH are unclear. However, studies show that HIV-PAH occurs in the absence of any apparent lung disease and that there is no correlation between PAH disease severity and CD4+

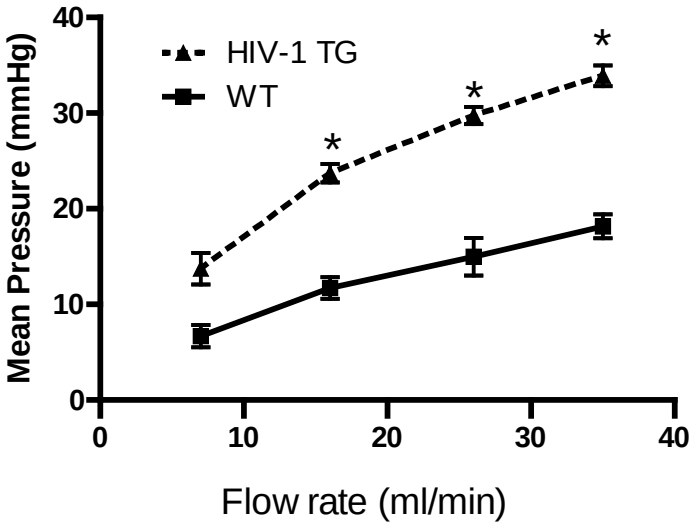
lymphocyte count (Seoane et al., 2001; Speich et al., 1991). In addition, HIV has not been found in endothelial cells of patients who develop PAH (Humbert et al., 1998b; Mette SA, 1992) nor has HIV DNA, RNA, or p24 antigen been detected in the pulmonary vessels of HIV-PAH patients (Kanmogne et al., 2001; Klings and Farber, 2003; Pellicelli et al., 2004). These data suggest that the pathogenesis of HIV-PAH is unrelated to the direct infection of the pulmonary vasculature or to immune dysfunction and may be partially attributable to the indirect actions of HIV-1 proteins on the vasculature (Mette SA, 1992). The endothelium is continually exposed to actively secreted viral proteins due to its position between the blood and the vascular wall (Chang et al., 1997) and the HIV proteins Tat, Nef, and gp120 are able to enter the endothelium (Gujuluva C, 2001; Liu et al., 2002) and alter cell function, further suggesting that HIV-1 proteins may play a role in the development of HIV-PAH development. Alternatively, HIV-1 proteins may "prime" vascular endothelial cells by enhancing their susceptibility to a "second hit" necessary for PH development (Almodovar et al., 2011a; Cota-Gomez et al., 2011; Voelkel et al., 2008; Zietz et al., 1996) Altogether, this evidence demonstrates that HIV-1 infection contributes to pulmonary dysfunction through indirect mechanisms that may be related to secreted HIV-1 proteins and their impact on endothelial function.

Therefore, to determine the role of HIV-1 proteins in the development of HIV-PAH, we examined the direct effect of HIV-1 proteins on pulmonary vascular function. Using an HIV-1 transgenic (Tg) rat model, we show HIV-1 protein expression exacerbates the development of hypoxia-induced pulmonary hypertension.

RESULTS

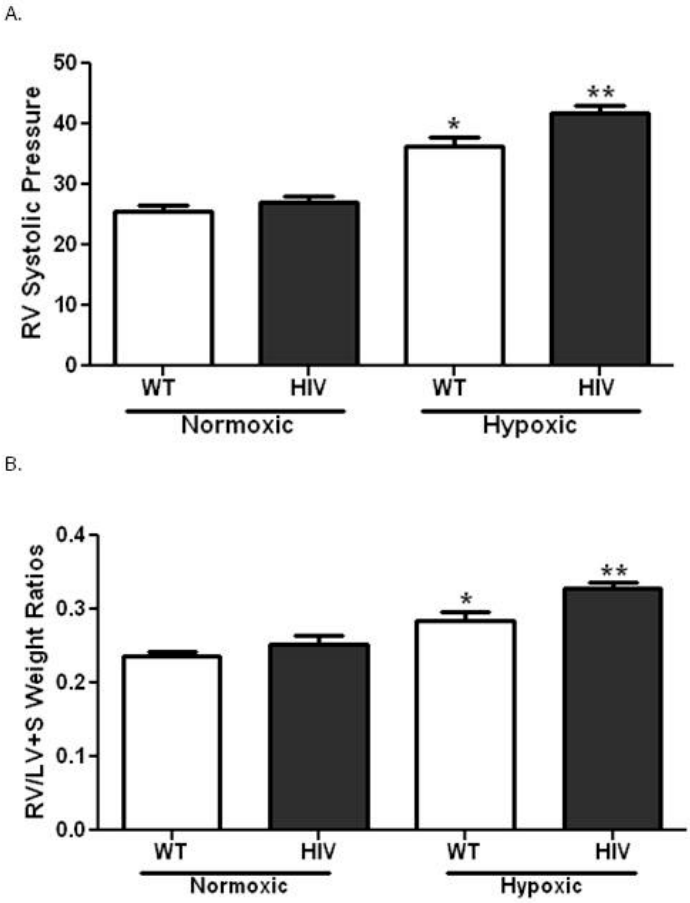
HIV-1 protein expression alters vascular resistance: HIV-1 proteins are associated with significant vascular injury. To determine whether HIV-1 protein expression affects pulmonary vascular reactivity, pressure-flow measurements were obtained to examine pulmonary vascular reactivity in wild-type and HIV-1 Tg pulmonary arteries. In response to increases in pulmonary flow, lungs from HIV-1 Tg rats showed significantly greater elevations in pressure when compared to wild-type controls (Figure 1). These data indicate that vessels from HIV-1 Tg animals are less able to regulate pressure responses due to alterations in blood flow ($n = 4\text{-}5$; $p < 0.0001$). Furthermore, this data indirectly demonstrates that HIV-1 proteins can affect pulmonary vascular resistance.

FIG 3.1. Pulmonary arteries from HIV-1 Tg rats (dotted line) show marked elevations in pressure in response to increased flow when compared to pulmonary arteries from WT rats (solid line). ($n = 4\text{-}5$). To assess pressure-volume relationships in wild-type and HIV-1 Tg animals, rats were anesthetized with isoflourane and mechanically ventilated after tracheal cannulation at a rate of ~60 strokes per minute with a tidal volume of 2.5 mL/breath. The pulmonary artery was cannulated with a 14G cannula connected to a pressure transducer and pressure/volume relationships were generated using a calibrated peristaltic pump at flow rates of 7, 16, 26 and 35 mls/min. * denotes $p < 0.0001$ when compared to pulmonary arteries of wild-type controls.


HIV-1 TG
WT
Mean Pressure (mmHg)
Flow rate (ml/min)
0
10
20
30
40
*
*
*

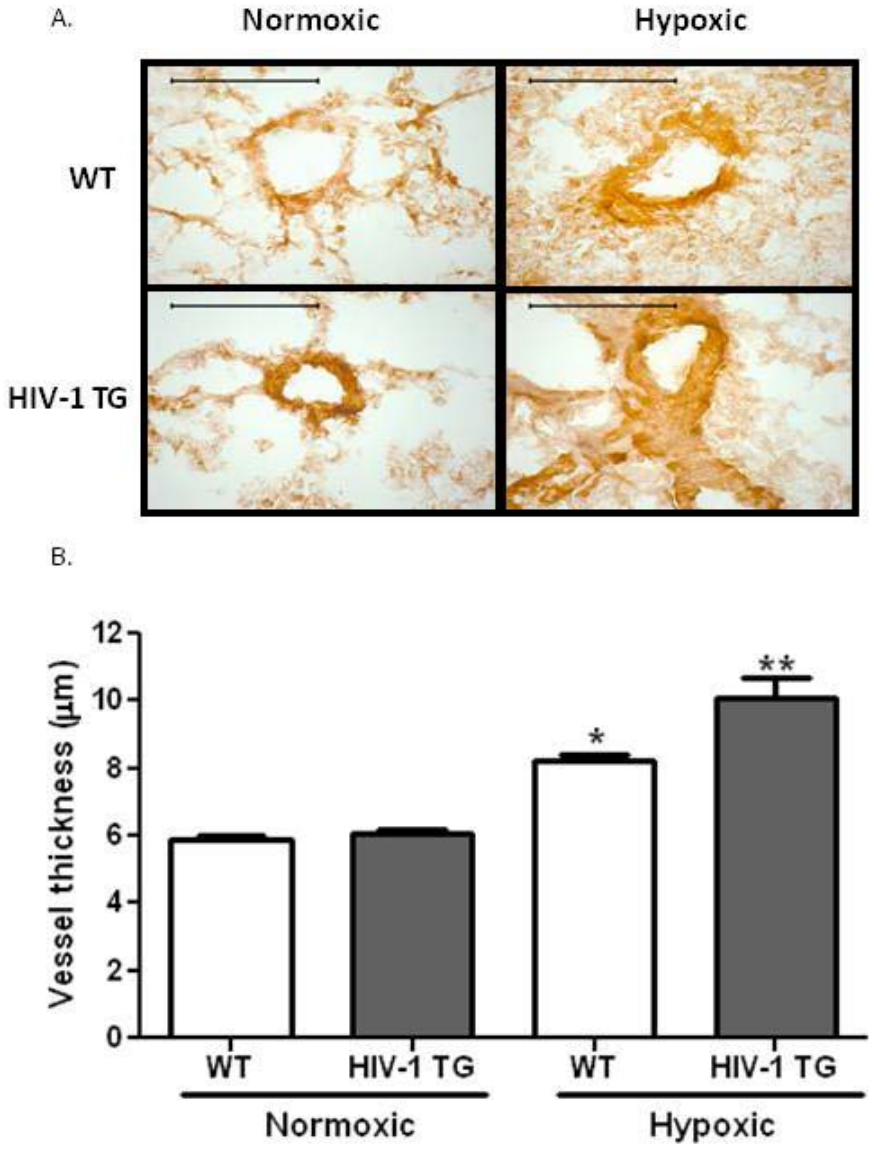
HIV-1 protein expression exacerbates hypoxia-induced pulmonary hypertension: Pulmonary arterial hypertension is defined as a sustained elevation of pulmonary arterial pressure (McGoon et al., 2004). Pathogenic vascular alterations in PH are characterized by abnormal muscularization of small pulmonary arteries and progressive intimal hyperplasia. Patients with severe PH may develop obstructive plexiform lesions in the distal pulmonary circulation (Rabinovitch, 2008). These occlusive lesions are associated with decreased lumen cross-sectional area and progressive increases in pulmonary vascular resistance, which leads to the development of right ventricular hypertrophy (RVH). Therefore, right ventricular systolic pressures (RVSP) and right ventricular hypertrophy (RVH) are indices used to confirm the presence of PH. To determine whether HIV-1 protein expression exacerbates hypoxia-induced PH, we assessed RVSP and RVH following 4 weeks of hypoxia exposure in wild-type and HIV-1 Tg rats. HIV-1 Tg rats ($P<0.0001$) exhibit greater elevations in RVSP in response to hypoxia when compared to hypoxic wild-type and normoxic control rats (Figure 3.2A). Hypoxic HIV-1 Tg rats also exhibit marked increases in RVH when compared to hypoxic wild-type and normoxic control rats (Figure 3.2B). Collectively, these results demonstrate that the expression of HIV-1 proteins causes exaggerated pulmonary vascular responses to hypoxia and structural alterations to the right ventricle.

FIG 3.2. **HIV-1 protein expression exacerbates hypoxia-induced right ventricular pressures and hypertrophy**. Hypoxic HIV-1 rats have significantly greater RVSP (A, n = 6-11) and right ventricular hypertrophy (B, n=7-9) than normoxic controls and hypoxic wild-types. Wild-type and HIV-1 Tg rats were housed in either normoxic or hypoxic conditions for four weeks. Rats were then anesthetized with isoflourane then the microtip pressure transducer was inserted into the right jugular vein and advanced to the right ventricle to monitor right ventricular pressures. There is no significant difference between normoxic groups. * denotes $p < 0.0001$ when compared to normoxic groups. ** denotes a $p < 0.01$ when compared to hypoxic wild-types.



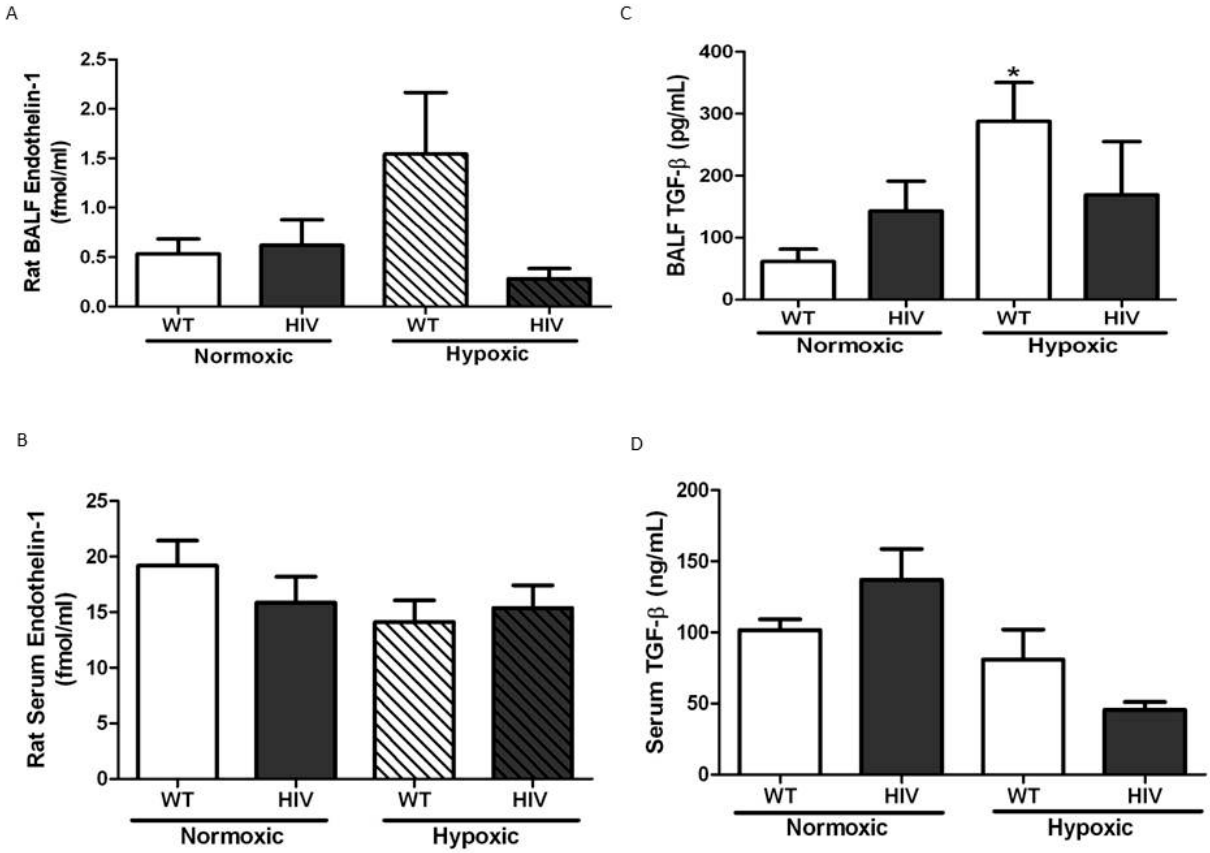
HIV-1 protein expression exacerbates hypoxia-induced vessel muscularization: PH is also associated with enhanced vascular wall cell proliferation and vascular remodeling. These alterations progressively narrow the vascular lumen and increase pulmonary vascular resistance. To determine if HIV-1 protein expression promotes vascular remodeling as evidenced by increased vessel wall thickness, the smooth muscle actin-alpha (α-SMA) content was measured in rat pulmonary arteries by immunostaining. In representative images showing alpha smooth muscle actin (Figure 3.3A), pulmonary arteries from hypoxic HIV-1 Tg rats exhibit a greater staining intensity when compared to normoxic HIV-1 or WT pulmonary arteries. Figure 3.3B shows a graphical representation of smooth muscle alpha actin staining in pulmonary vessels. Pulmonary arteries from hypoxia-exposed HIV Tg rats demonstrated significant increases in vessel thickness when compared to all other groups. There was no difference in pulmonary artery vessel thickness between normoxic wild-type and HIV-1 Tg rats.

FIG 3.3. HIV-1 protein expression exacerbates hypoxia-induced vessel muscularization. Hypoxic HIV-1 Tg rats show greater smooth muscle alpha actin staining when compared to normoxic controls and hypoxic wild-type (A, n = 3). Rats were exposed to normoxia or hypoxia for 4 weeks. Rat lungs were isolated, pressure perfused, inflated and immersed with 4% paraformaldehyde then embedded in paraffin for sectioning. The percentage of smooth muscle within the media was determined by staining with antibodies to smooth muscle α-actin (α-SMA). Scale bar in each image = 50 μm. Hypoxia exposure increases HIV-1 Tg rat vessel thickness (B). There is no significant difference between normoxic groups. ** denotes $p < 0.0001$ when compared to normoxic groups. * denotes $p < 0.01$ when compared to hypoxic controls.


A.
Normoxic
Hypoxic
WT
HIV-1 TG
B.
Vessel thickness (µm)
12
10
8
6
4
2
0
*
**
WT
HIV-1 TG
WT
HIV-1 TG
Normoxic
Hypoxic

HIV-1 protein expression does not alter endothelin-1 or TGF-β levels: Endothelin-1 (ET-1) and transforming growth factor-beta (TGF-β) are both implicated in pulmonary hypertension pathogenesis. ET-1 is a potent vasoconstrictor involved in the regulation of vascular tone and cell proliferation. TGF-β is a multifunctional cytokine known to contribute to the proliferation, migration, and differentiation of smooth muscle cells and fibroblasts in the systemic (Khan et al., 2007) and pulmonary arteries (Long et al., 2009). To determine whether HIV-1 transgene expression potentiates hypoxia-induced pulmonary hypertension by altering ET-1 or TGF-β levels, we assessed ET-1 and TGF-β levels in the serum and bronchoalveolar lavage fluid (BALF) of normoxic and hypoxic wild-type and HIV-1 transgenic animals.

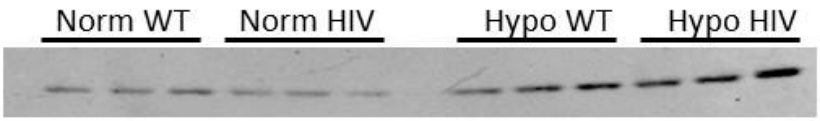
FIG 3.4. HIV-1 transgene expression does not alter endothelin-1 or TGF-β levels. Following hypoxia exposure, wild-type and HIV-1 transgenic rats were sacrificed. Serum was collected via cardiac puncture and BALF was obtained via tracheotomy. ET-1 and TGF-β ELISAs were used according to manufacturer's instruction to measure ET-1 and TGF-β levels, respectively.



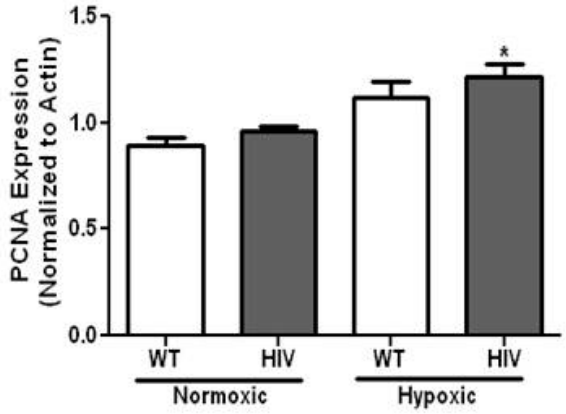
HIV-1 protein expression increases PCNA expression: The excessive proliferation of pulmonary endothelial and smooth muscle cells is believed to contribute to the abnormal vascular phenotype characteristic of PH. To examine whether HIV-1 proteins exacerbate hypoxia-induced cellular proliferation *in vivo*, the proliferating cell nuclear antigen (PCNA) content in lung homogenates from HIV-1 Tg rats was assessed by western blot. Representative western blot images show that lungs from hypoxic HIV-1 Tg rats express significantly greater PCNA than normoxic WT or HIV-1 Tg animals (Figure 3.5).

FIG 3.5. HIV-1 protein exposure exacerbates pulmonary cellular proliferation. Lung homogenates from HIV-1 Tg rats express significantly greater PCNA than normoxic WT or HIV-1 Tg animals ($n = 6$). Following four weeks of normoxic or hypoxic conditions, rats were sacrificed and lungs removed for protein expression analysis. Lung homogenates (40 µg) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were then exposed to anti-PCNA antibodies overnight at 4°C, rinsed, and incubated in anti-rabbit fluorescent antibody solution. * denotes $p < 0.0001$ when compared to normoxic controls. ** denotes $p < 0.0001$ when compared to hypoxic wild-types.

A


Norm WT
Norm HIV
Hypo WT
Hypo HIV

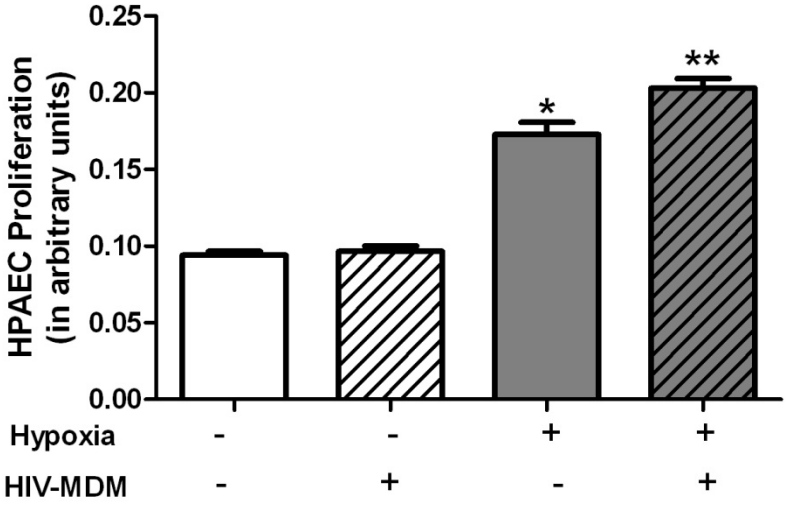
B


PCNA Expression
(Normalized to Actin)
1.5
1.0
0.5
0.0
*
WT
HIV
WT
HIV
Normoxic
Hypoxic

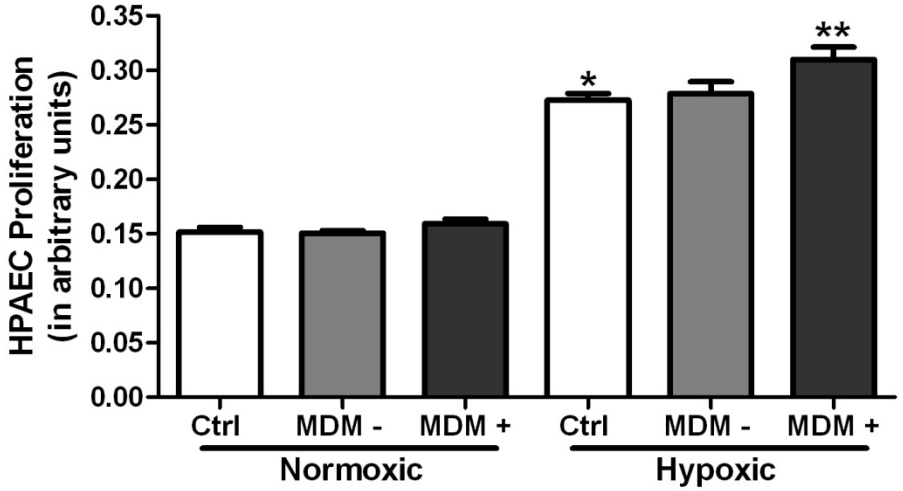
HIV-1 proteins potentiate hypoxia-induced endothelial proliferation: The HIV-1 proteins gp120 (Patke and Shearer, 2000; Singhal et al., 1999), Nef (Husain et al., 2002), and Tat (Bergonzini et al., 2004; Bettaccini et al., 2005; Campioni et al., 1995; Seve et al., 1999) are linked to increased cell proliferation. To assess whether HIV-1 proteins exacerbate hypoxia-induced proliferation *in vitro*, we exposed HPAEC to hypoxia (1% oxygen) for 72 hours in the presence or absence of medium of HIV-infected monocyte derived macrophage (HIV-MDM). Medium was diluted to clinical concentrations of p24 levels observed in serum of HIV-infected patients, or 50 pg/mL (Reddy et al., 1988). Our data indicate that hypoxia-exposed HPAEC cultured in HIV-MDM medium exhibit significant increases in cell proliferation when compared to all other treatment groups (Figure 3.6A).

FIG 3.6. HIV-1 proteins potentiate hypoxia-induced endothelial proliferation. Medium from HIV-infected Monocyte derived Macrophages (HIV-MDM) exacerbates hypoxia-induced proliferation of human pulmonary artery endothelial cells (HPAEC). HPAEC were exposed to 1% oxygen for 72 hours in the presence or absence of medium of HIV-infected monocyte derived macrophage (HIV-MDM). Following exposure to normoxic or hypoxic conditions, cells were subjected to MTT assay to assess cell proliferation ($n = 4$). * denotes $p < 0.0001$ when compared to treated and untreated normoxic groups. ** denotes $p < 0.001$ when compared to untreated hypoxic groups. Exosome-containing fractions of HIV-MDM (MDM+) potentiate hypoxia-induced HPAEC proliferation when compared to HIV-MDM devoid of exosomes (MDM-) (B, $n = 4$). * $p < 0.0001$ when compared to treated and untreated normoxic groups. ** $p < 0.01$ when compared to control hypoxic and hypoxic MDM- groups.

A.

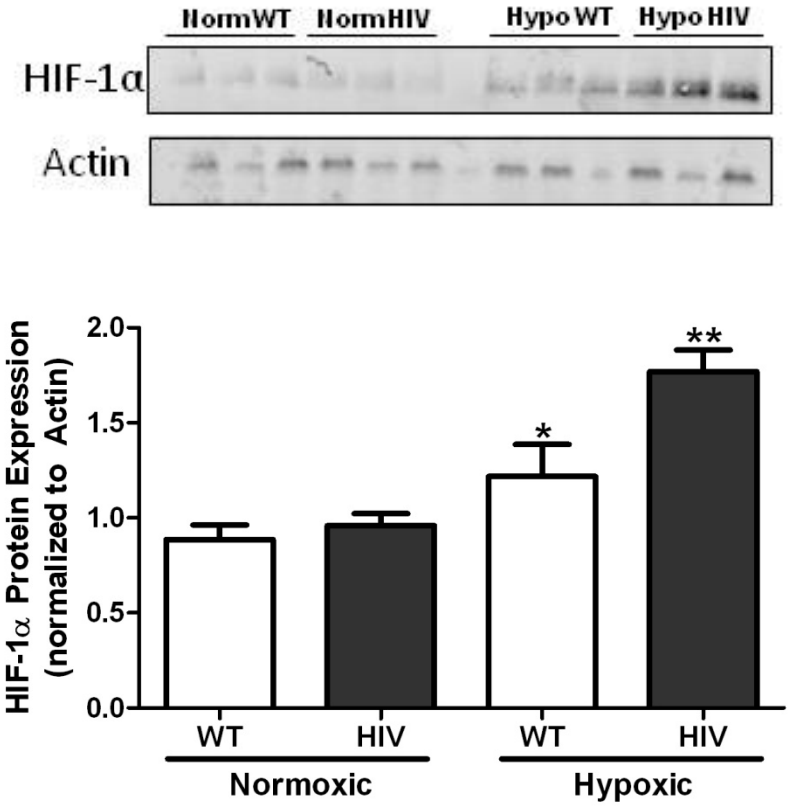

HPAEC Proliferation
(in arbitrary units)
0.25
0.20
0.15
0.10
0.05
0.00
*
**
Hypoxia - - + +
HIV-MDM - + - +

B.


HPAEC Proliferation
(in arbitrary units)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
**
Ctrl
MDM -
MDM +
Ctrl
MDM -
MDM +
Normoxic
Hypoxic

HIV-1 proteins potentiate hypoxia-induced HIF-1α expression: HIF-1α is an oxygen-sensing molecule (Semenza, 2007) that mediates an adaptive response to low oxygen conditions by activating genes associated with energy metabolism, erythropoiesis, vasomotor tone, and angiogenesis (Semenza, 2003). Hypoxia stabilizes HIF-1α by inhibiting prolyl hydroxylation and thereby inducing the upregulation of genes that promote survival in low oxygen environments. In fact, recent research demonstrates that inhibition of HIF-1α by the cardiac glycoside digoxin attenuates hypoxia-induced increases in RV pressures, RV hypertrophy, and vascular remodeling (Abud et al., 2012). Due to these effects, HIF is implicated in PAH pathogenesis. To determine whether HIV-1 protein expression modulates hypoxia-induced HIF-1α, HIF-1α expression was assessed in lung homogenates from wild-type and HIV-1 Tg rats following normoxic and hypoxic conditions. Results demonstrate that hypoxia exposure stimulates a significant increase in lung HIF-1alpha expression in HIV-1 Tg rats when compared to normoxic animals and hypoxic wild-type controls (Figure 3.7). No differences in lung HIF-1alpha expression were found between WT or HIV-1 Tg animals exposed to normoxic conditions.

FIG 3.7. Hypoxia exposure increases HIF-1alpha expression. Rat Lung homogenates from hypoxic HIV-1 Tg animals exhibit greater HIF-1alpha expression than all other groups (n = 3). Wild-type and HIV-1 Tg rats were housed in either normoxic or hypoxic conditions for four weeks. Lung homogenates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and exposed to anti-HIF-1alpha antibodies overnight at 4°C, rinsed, and incubated in anti-rabbit fluorescent antibody solution. * denotes $p < 0.01$ when compared to normoxic groups. ** denotes $p < 0.05$ when compared to hypoxic wild-types.



DISCUSSION

In this study, we demonstrate that HIV-1 protein expression promotes PH development following prolonged hypoxia exposure. Using an HIV-1 transgenic rat model, we show that HIV-1 protein expression alters pulmonary vascular resistance and exacerbates hypoxia-induced increases in right ventricular systolic pressures, right ventricular hypertrophy, and vessel muscularization. In addition, *in vivo* and *in vitro* studies demonstrate that HIV-1 protein exposure exacerbates pulmonary cell proliferation following prolonged hypoxia exposure.

Our experimental design employs the NL4-3Δ *gag/pol* HIV-1 transgenic (Tg) rat model. The HIV-1 transgenic model allows the study of the physiological effects of HIV-1 proteins *in vivo* in a noninfectious and relevant manner. Due to the deletion of *gag* and *pol* regions of the viral genome, this animal model expresses a nonreplicative HIV-1 provirus under the viral promoter. The transgene encodes for the viral genes *env*, *tat*, *nef*, *rev*, *vif*, *vpr*, and *vpu* (Reid et al., 2001). These levels of viral gene products are consistent with those observed in the blood and lymphoid tissue of HIV-1 patients. Moreover, HIV-1 gp120 has been detected in bronchoalveolar lavage fluid (BALF) and serum of HIV-1 Tg rats at approximate levels of 10 ng/ml and 28 ng/ml, respectively (Joshi et al., 2008). The transgenic rat is also a useful model for studying HIV-1-associated neurological and cardiac pathologies because it displays clinical manifestations that resemble those seen in HIV-1 patients (Reid et al., 2001). Thus, the transgenic HIV-1 model is a useful and appropriate tool for examining the physiological consequences of HIV-1 proteins on cells of the vasculature.

In these studies, HIV-1 Tg rats were exposed to normobaric hypoxia to examine the contribution of HIV-1 proteins to PH development. These studies are clinically relevant as chronic hypoxia occurs as a result of pulmonary parenchymal disease, sleep disordered

breathing, and severe chronic obstructive pulmonary disease (COPD) (Stenmark et al., 2009). Additionally, pulmonary hypertension is usually of mild to moderate severity in COPD patients but is nonetheless associated with increased risk of exacerbations and decreased survival (Chaouat et al., 2008). Consistent with clinical studies and statistics, our results show that the presence of HIV-1 exacerbates PH development following hypoxia exposure. HIV-1 Tg rats demonstrate increased systolic pressures and right heart hypertrophy when compared to wild-type controls. In addition, pulmonary arteries of hypoxic HIV-1 Tg rats exhibited a 65% increase in vessel muscularization and cellular proliferation when compared to normoxic controls.

Interestingly, in our model, the expression of HIV-1 proteins failed to induce PH under normoxic conditions. Yet, hypoxia exposure markedly exacerbates PH development in wild-type and HIV-1 Tg animals. These results conflict with data recently published demonstrating that HIV-1 Tg animals spontaneously exhibit PH (Lund et al., 2011; Mermis et al., 2011). Several potential factors may provide an explanation for these conflicting results. Studies published by Lund et al were performed in Albuquerque, NM, which is located at an elevation of 5312 feet above sea level. This altitude could result in hypoxic challenge and is an altitude associated with altitude-related lung diseases such as chronic obstructive pulmonary disease (COPD). Additionally, other research indicates that HIV-1 Tg animals display increases in pulmonary vessel wall thickness and HIF-1α expression independent of hypoxia exposure (Mermis et al., 2011). However, strain differences may account for the varied results (Aguirre et al., 2000; Bonnet et al., 2006; Pan et al., 1993; Sato et al., 1992). The animals used in the Mermis et al studies were completed in HIV Tg rats in the Sprague Dawley strain, whereas our studies were completed in rats on the Fischer 344 background. Research demonstrates that this strain is more resistant to hypoxia-induced PH. For example, rats on the Wistar-Kyoto background exhibit an exacerbated PH following

prolonged hypoxia exposure compared to those on the Fischer 344 background (Aguirre et al., 2000). Moreover, isolated perfused lung preparations from Fischer 344 rats demonstrate a reduced pulmonary vascular response to alveolar hypoxia when compared to lungs from Sprague Dawley rats (He et al., 1991). These studies suggest that Fischer 344 rats may be more resistant to vascular injury and likely explain the differences in outcome.

The underlying mechanism of HIV-related PH is unknown. However, research suggests that endothelial cell dysfunction and excessive proliferation may contribute to HIV-PH pathogenesis. Studies show that the endothelium is continually exposed to actively secreted viral proteins due to its position between the blood and the vascular wall (Chang et al., 1997). Clinical studies reveal that antiretroviral-naïve HIV-1 patients display markers of endothelial activation. Plasma levels of von Willebrand factor, plasminogen activator inhibitor-1 antigen, and tissue-type plasminogen activator are significantly elevated in HIV-1 positive patients (Lafeuillade A, 1992; Schved JF, 1992). In addition, comparison of flow-mediated dilation (FMD) of the brachial artery in 75 HIV-1 positive and 223 control subjects revealed significantly impaired endothelial function in the HIV-1-infected population (Solages et al., 2006a). A smaller study also showed that both treated and HAART-naïve HIV-1 infected children between 3.5 to 19.5 years old have significantly reduced FMD compared to non-infected age- and sex- matched controls (Bonnet et al., 2004b). These studies suggest that HIV-1 significantly affects endothelial cell function in the absence of cardiovascular risk factors and irrespective of age or duration of infection.

In addition to the cardiovascular effects, HIV-1 also significantly impairs lung function (Morris et al., 2012). Despite the availability of combination antiretroviral therapies, respiratory complications and chronic lung disease remain common among HIV-infected individuals (Diaz et al., 2003; George et al., 2009; Gingo et al., 2010) and rates of hospitalization and deaths from pulmonary obstructive diseases are growing (Grubb et al.,

2006; Louie et al., 2002). Lung cancer (Sigel et al., 2012) and COPD (Crothers et al., 2006; Magalhaes et al., 2007) diagnoses are significantly higher in the HIV-1 population. Recent studies also report that HIV-1 patients are almost three times more likely to develop asthma than the general population, with no correlation between the risk of asthma and viral load or CD4 lymphocyte count (Gingo et al., 2012). These studies highlight the targeted effects of HIV-1 on the lung and the contribution of HIV-1 to pulmonary disease.

Although we utilize a noninfectious, replication-incompetent animal model, we report that HIV-1, particularly HIV-1 proteins, contributes to PH pathogenesis. These data also indicate that HIV-1 protein exposure potentiates hypoxia-induced endothelial proliferation. Although previous studies report that hypoxia exposure fails to promote endothelial cell proliferation (Yu and Hales, 2011), our findings demonstrating that 72 hours of hypoxia promotes endothelial cell proliferation and are consistent with published data (Kang et al., 2011; Schaefer et al., 2006). Furthermore, our studies suggest that exosomes contribute to the HIV-MDM-induced potentiation of pulmonary artery endothelial proliferation following hypoxia exposure. We postulate that the increase in cellular proliferation is linked to the dysregulation of HIF-1α, as hypoxia significantly increases HIF-1alpha expression in HIV-1 Tg animals. This finding is interesting as HIF-1alpha is suggested to regulate the metabolic shift to glycolysis in pulmonary hypertensive endothelial cells associated with abnormal proliferation and apoptosis-resistance (Fijalkowska et al., 2010).

At this time, it is unclear how HIV-1 proteins mediate the effects seen in this study. However, the HIV-1 proteins, tat, gp120 and/or Nef have been shown to affect endothelial cell function and are suggested to mediate PH pathogenesis and progression in HIV-1 patients. The HIV-1 protein, Tat is a transcriptional transactivator with the ability to upregulate viral gene expression by increasing the rates of transcription initiation and elongation (Rice and Mathews, 1988). As a secreted protein from HIV-1 infected cells, Tat

has been shown to enter non-infected cells from the circulation and alter normal cellular function (Chang et al., 1997). With this capacity, Tat is known to induce endothelial activation (Urbinati et al., 2005a; Urbinati C, 2005), inflammation (Toborek et al., 2003), cell growth (Barillari et al., 1999) and injury. Porcine coronary arteries exhibit significant decreases in eNOS expression and alterations in endothelium-dependent relaxation when incubated in HIV-1 Tat (Paladugu et al., 2003). Tat transgenic mice also exhibit marked increases oxidant levels and oxidative stress genes when compared to wild-type mice (Cota-Gomez et al., 2011). Additionally, HIV-1 Tat stimulate the production of proinflammatory cytokines, such as IL-6, which has been found in the lungs of patients with severe PAH (Humbert M, 1995).

Another potential mediator is the HIV-1 accessory protein, Nef. Nef is found in HIV-infected patients at a level of 10ng/mL (Fujii et al., 1996) and may mediate PH development by altering pulmonary morphology. For example, Marecki reports that simian HIV carrying a functional Nef protein promotes vascular remodeling (Marecki et al., 2005; Marecki et al., 2006) and induces PAH in macaques. The same study also showed an enhanced accumulation of Nef in endothelial cells in plexiform lesions in lungs of patients with HIV-PAH. In addition, Nef administration is shown to alter endothelium-dependent relaxation, decrease HPAEC eNOS mRNA levels and increase superoxide production in porcine pulmonary arteries (Duffy et al., 2009).

The HIV-1 surface protein gp120 is also implicated in the development of pulmonary vascular disorders. gp120 is actively secreted into the bloodstream and is readily detectable in sera of HIV-1 infected patients at concentrations ranging from 0.24 ng/mL to 92 ng/mL (Oh SK, 1992). In 2005, Kanmogne showed that gp120 significantly increases the secretion of endothelin-1 by human lung endothelial cells (Kanmogne et al., 2005b). Similarly to Tat, gp120 induces monocyte adhesion and increased ICAM-1 gene expression, but not VCAM-1

or E-selectin in human endothelial cells (Ren Z, 2002). Furthermore, gp120-induced generation of reactive oxygen species (ROS) has been implicated in endothelial cell toxicity (Price TO, 2005).

Exosomes released by HIV-infected cells may also contribute to vascular injury as evidenced by the HIV-MDM-induced potentiation of hypoxia-induced proliferation. Exosomes are released by most cell types and act as intercellular signaling molecules influencing the physiology of neighboring cells (Ludwig and Giebel, 2012). Although the mechanism of action remains unclear, exosomes are thought to alter cellular function by binding to cell-surface receptors of nearby cells or by transferring both mRNA and microRNAs to recipient cells (Mathivanan et al., 2010). Further studies are needed to determine the role of exosomes in HIV-associated pathologies.

Oxidative stress may also mediate the development of pulmonary hypertension in our HIV-1 model. Our group has previously shown that aortas from HIV-1 Tg demonstrate increases in superoxide and 3-nitrotyrosine due to a diminished antioxidant capacity particularly GSH and Cu/Zn superoxide dismutase activity (Kline et al., 2008). Similarly, lung lavage fluid from HIV-1 Tg rats shows decreases in GSH and increased ratios of glutathione disulfide (GSSG) to GSH (Lassiter et al., 2009). In addition, hydrogen peroxide (H_2O_2) was increased in lung tissue from HIV-1 Tg rats (Lassiter et al., 2009). These results are consistent with clinical data showing lung homogenates from severe PH patients demonstrate decreased total SOD activity, reduced MnSOD protein levels and increased nitrotyrosine expression (Bowers R, 2004).

Although HIV-1 transgene expression induces significant oxidative stress, HIV-1 Tg animals do not show signs of chronic inflammation. Previous studies have shown no differences in HIV-1 Tg cytokine levels when compared to wild-type controls. Lassiter el al (Lassiter et al., 2009) showed similar concentrations of interleukin-2, TNF-α, and

interleukin-4 in lung lavage fluids of HIV-1 Tg rats and wild-type controls. These results are consistent with previous studies performed by our group showing no differences in GM-CSF, IL-12, IL-6, IL-10, IL-4, IL-2, IL-1β or TNF-α in HIV-1 Tg mouse serum when compared to wild-type controls (Jacob et al., 2006).

In summary, our results establish that expression of HIV-1 proteins impairs pulmonary vascular function and exacerbates hypoxia-induced PH development. Ultimately, these studies underscore the potential contribution of HIV-1 proteins to HIV-PAH.

Chapter 4

HIV-1 Increases Arachidonate 5-Lipoxygenase Expression and Activity

INTRODUCTION

Arachidonate 5-Lipoxygenase (ALOX5) catalyzes the production of leukotrienes (LT) from arachidonic acid (Peters-Golden, 2000). These leukotrienes include LTB_4 and the cysteinyl leukotrienes (CysLT) LTC_4, LTD_4 and LTE_4. ALOX5 expression and activity is linked to pulmonary artery endothelial cell proliferation (Walker et al., 2002). CysLT also induce constriction of the pulmonary arteries. Furthermore, overexpression of ALOX5 both exacerbates and accelerates the development and progression of pulmonary hypertension in monocrotaline (MCT)-treated rats (Jones, 2004). These studies suggest that increases in ALOX5 may contribute to PH pathogenesis. However, the role of ALOX5 in HIV-PAH is unknown.

Previous research suggests that HIV-1 stimulates the 5-lipoxygenase pathway. *In vitro* studies demonstrate that monocytes infected with HIV-1 exhibit significant elevations in leukotriene release. The administration of the HIV inhibitor, Avarol induces a 50% reduction in virus release and leukotriene production (Schroder et al., 1991). Similarly, *in vivo* studies demonstrate that HIV-1 transgene expression augments ALOX5 expression and activity. Brains from HIV-1 Tg rats exhibit significant increases in ALOX5 mRNA and protein expression when compared wild-type controls (Rao et al., 2011), and HIV-1 stimulates leukotriene release in the brain of HIV-1 Tg animals (Basselin et al., 2011). These data highlight a potential role for HIV-1 proteins in HIV-induced ALOX5 expression and activity. Studies performed in human neuroblastoma cells corroborate this hypothesis as exogenous gp120 administration increases intracellular leukotriene concentration by stimulating ALOX5 activity and expression (Maccarrone et al., 1998). Altogether, these studies suggest that HIV-1 proteins alter ALOX5 expression and activity. However, the effect of HIV-1 proteins on pulmonary endothelial ALOX5

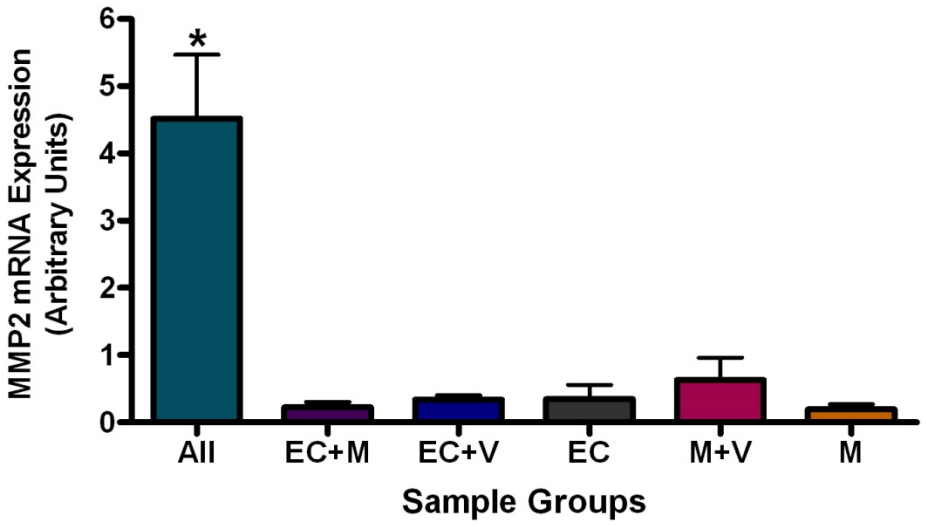
expression and activity has not been defined. We hypothesize that HIV-1 proteins stimulate pulmonary endothelial ALOX5 and leukotriene production. In our study, we employ both *in vitro* and *in vivo* models to assess the effect of HIV-1 and HIV-1 proteins on pulmonary endothelial ALOX5. These studies underscore the potential role of HIV-1 protein-induced ALOX5 in pulmonary disorders such as HIV-associated pulmonary hypertension.

RESULTS

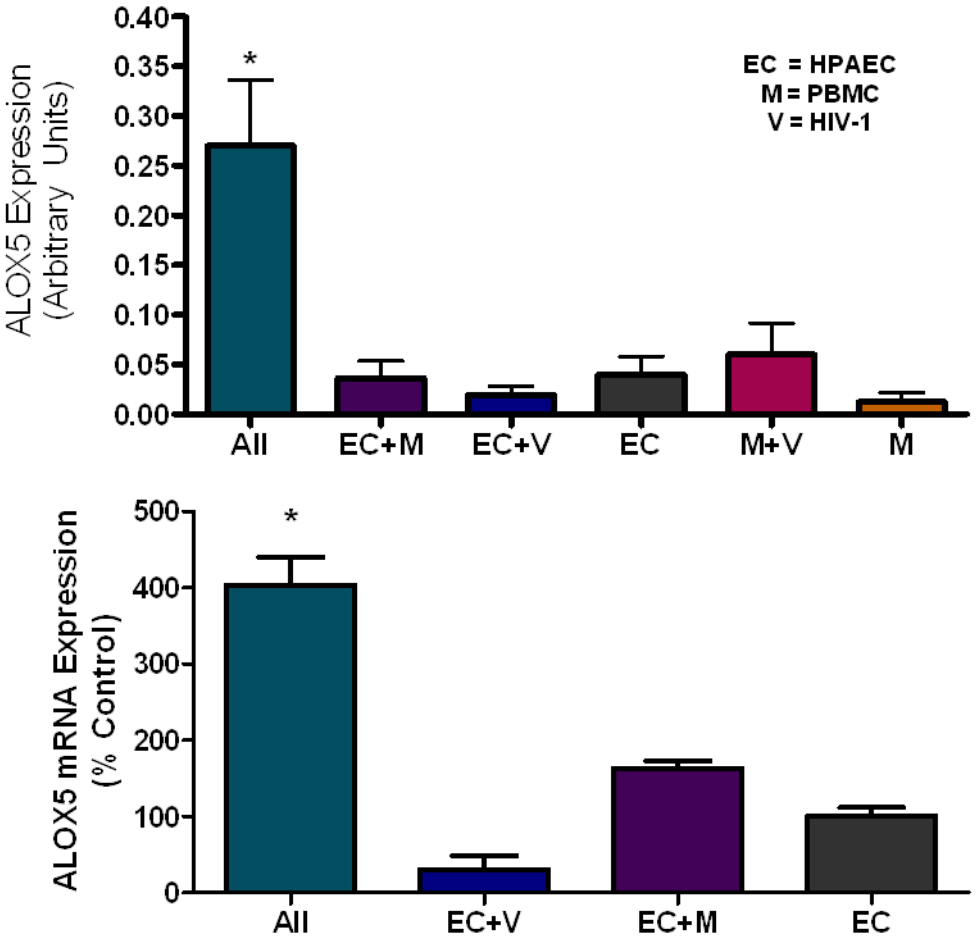
HIV-1 alters endothelial expression of vasoactive mediators: Although HIV-1 is largely believed to be incapable of infecting endothelial cells *in vivo*, multiple studies demonstrate that HIV-1 significantly impairs endothelial cell function (Price TO, 2005; Toborek M, 2003). To determine whether HIV-1 induces these effects by altering endothelial gene expression, human pulmonary artery endothelial cells (HPAEC) cultured alone or in the presence of peripheral blood mononuclear cells (PBMCs) were inoculated with HIV-1 for 1 hour. Following exposure, the HIV-1 inoculum was removed and the cells were allowed to grow for 24 hours. To assess the effect of HIV-1 on HPAEC expression, we performed a gene array to assess over 90 genes associated with vascular biology signaling pathways such as angiogenesis, proliferation, remodeling, and vessel tone. The results reveal that HIV-1 exposure increases the expression of endothelial matrix metalloproteinase (MMP)-2 (Figure 4.1A). Gene array results also reveal that co-culture groups containing HPAEC, PBMC and HIV-1 demonstrate a 5-fold increase in ALOX5 when compared to all other groups (Figure 4.1B). Quantitative real time PCR analysis confirms that ALOX5 is increased (Figure 4.1). These data demonstrate that HIV-1 increases endothelial ALOX5 expression. These results also suggest that the combination of HPAEC, PBMCs and HIV is required for HIV-induced endothelial alterations.

FIG 4.1. HIV-1 exposure increases endothelial matrix metalloproteinase (MMP)-2 and 5-lipoxygenase (ALOX5). HPAEC and/or peripheral blood mononuclear cells (PBMC) were cultured in the presence or absence of HIV-1 for one hour. Following exposure, the inoculum was removed by centrifugation and cells were cultured for 24 hours (n = 2-4). Total RNA was isolated for gene expression analysis. 96 Human endothelial cell biology genes were examined using a GEArray Q Series gene array blots from SuperArray, Inc. Quantitative real time PCR was used to confirm the gene array results (n = 4). * $p < 0.05$ when compared to all other groups.

A.



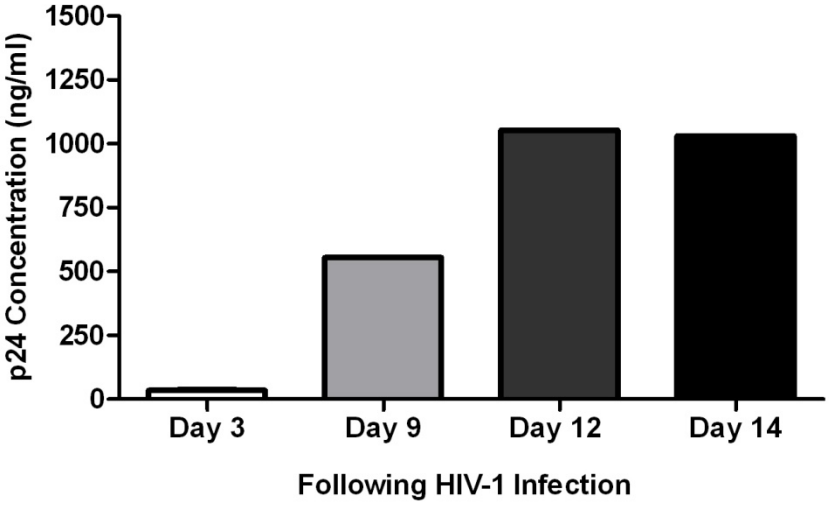
B.


ALOX5 Expression
(Arbitrary Units)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
EC = HPAEC
M = PBMC
V = HIV-1
All
EC+M
EC+V
EC
M+V
M
ALOX5 mRNA Expression
(% Control)
500
400
300
200
100
0
*
All
EC+V
EC+M
EC

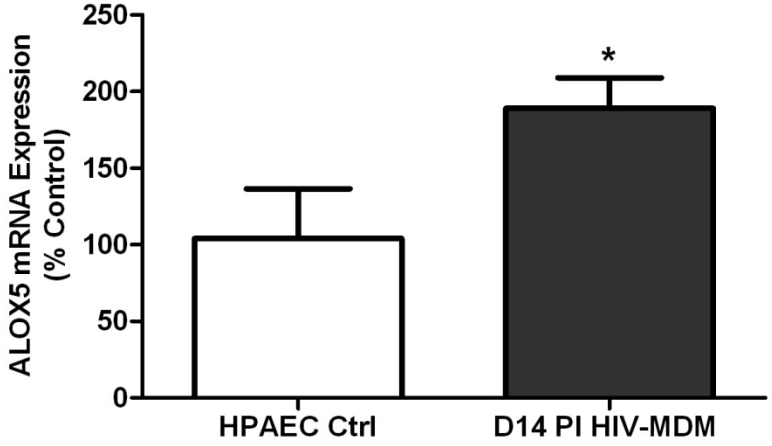
Secreted mediators from HIV-infected monocyte-derived macrophages (HIV-MDM) stimulate ALOX5 expression and activity: Co-culture studies implicate a potential contribution of secreted mediators from HIV-infected PBMCs in HIV-induced endothelial ALOX5. To investigate the role of secreted mediators in HIV-induced ALOX5, HPAEC were exposed to medium from HIV-infected monocyte derived macrophages (HIV-MDM) for 24 hours. HIV-MDM medium was diluted to clinically-relevant levels of p24 or 50 pg/ml (Reddy et al., 1988). HPAEC exposed to medium from HIV-1 infected monocyte-derived macrophages (HIV-MDM) for 24 hours display significant increases in ALOX5 expression when compared to HPAEC controls (Figure 4.2B). Medium from HIV-MDM also stimulates an almost 4-fold increase in HPAEC CysLT release measured by ELISA (Figure 4.2C). These data suggest that secreted mediators from HIV-infected monocytes/macrophages alter ALOX5 expression.

FIG 4.2. Exposure to HIV-MDM medium augments HPAEC ALOX5 expression and CysLT release. HIV-1 p24 levels in HIV-MDM medium were measured over time using ELISA (Figure 4.2A). HPAEC were exposed to medium from uninfected or HIV-infected monocyte derived macrophage (HIV-MDM) for 24 hours. Following exposure, medium was collected and cells were harvested for gene expression analysis via quantitative real time PCR. Results demonstrate that exposure to medium from HIV-MDM augments HPAEC ALOX5 expression (Figure 4.2B; $n = 5$) and stimulates CysLT release (Figure 4.2C; $n = 5$). * $p < 0.05$ when compared to control HPAEC groups. ** $p < 0.01$ when compared to control HPAEC groups.

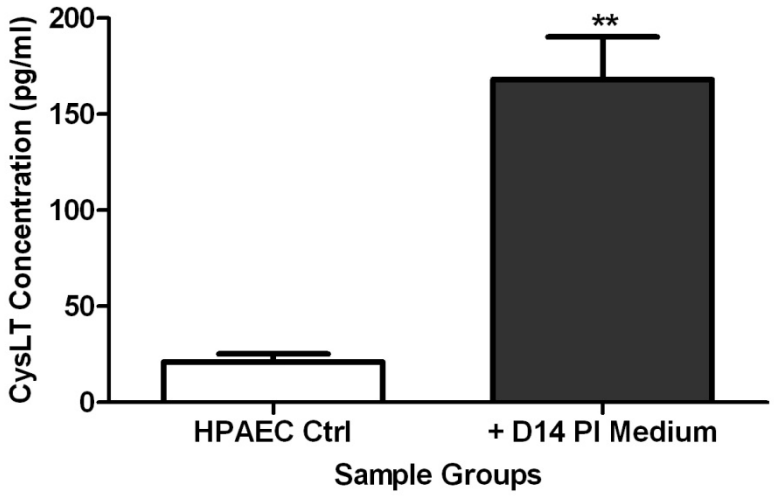
A.


p24 Concentration (ng/ml)
1500
1250
1000
750
500
250
0
Day 3
Day 9
Day 12
Day 14
Following HIV-1 Infection

B

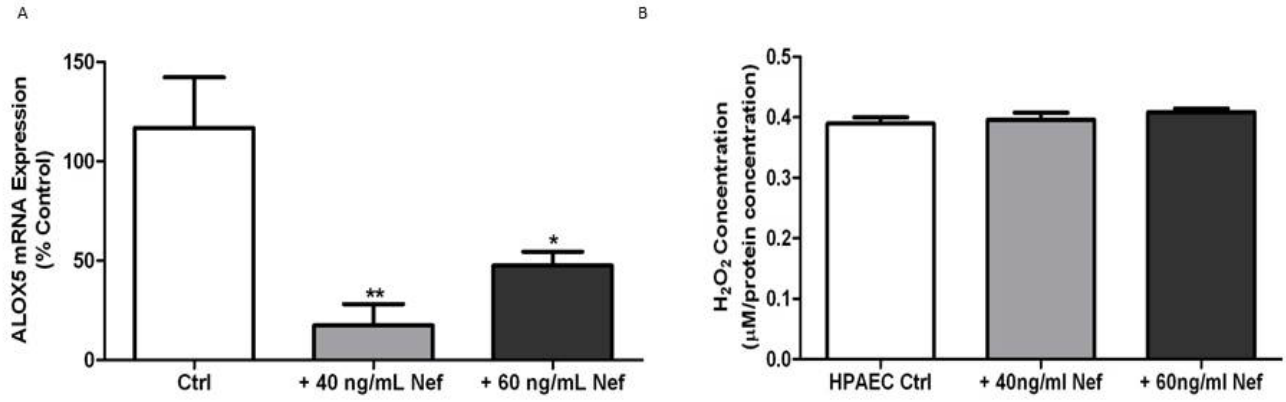

ALOX5 mRNA Expression
(% Control)
250
200
150
100
50
0
*
HPAEC Ctrl
D14 PI HIV-MDM

C

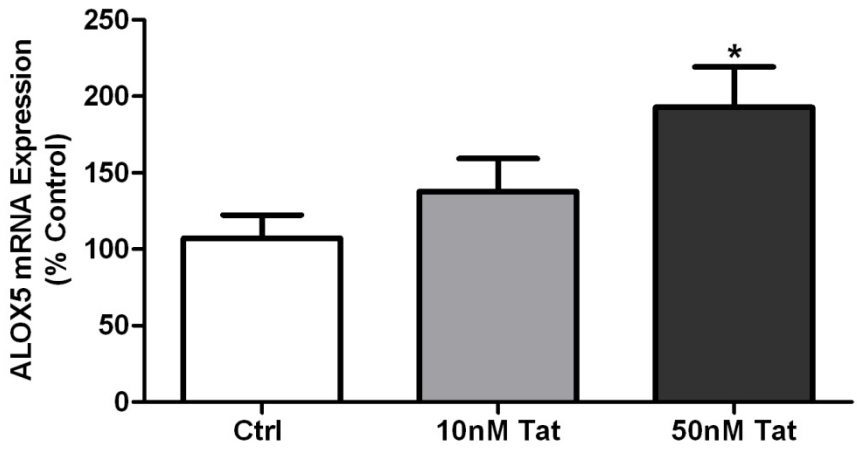

CysLT Concentration (pg/ml)
200
150
100
50
0
**
HPAEC Ctrl
+ D14 PI Medium
Sample Groups

HIV-1 Tat exposure, but not Nef, promotes endothelial ALOX5 expression: The results from our co-culture and HIV-MDM studies indicate that secreted mediators increase ALOX5. We hypothesize that HIV-1 proteins such as Nef and Tat may mediate the HIV-induced alterations in ALOX5. To examine whether HIV-1 proteins alter endothelial ALOX5 expression, we exposed HPAEC to the HIV-1 proteins Tat or Nef for 24 hours. Following exposure, cells were collected for gene expression analysis by quantitative real time PCR, H_2O_2 generation, and cell proliferation. Our studies reveal that HIV-1 Nef significantly attenuates ALOX5 expression (Figure 4.3A) and has no effect on endothelial H_2O_2 release (Figure 4.3B) following 24 hours of exposure when compared to untreated controls. Conversely, HIV-1 Tat induces a dose-dependent increase endothelial ALOX5 expression (Figure 4.3C) and H_2O_2 release (Figure 4.3D) following 24 hours of exposure. These data demonstrate that HIV-1 proteins contribute to HIV-induced alterations in ALOX5 expression and activity. In addition, our results suggest that Tat-induced ALOX5 may mediate the concomitant increases in HPAEC proliferation (Figure 4.3E).

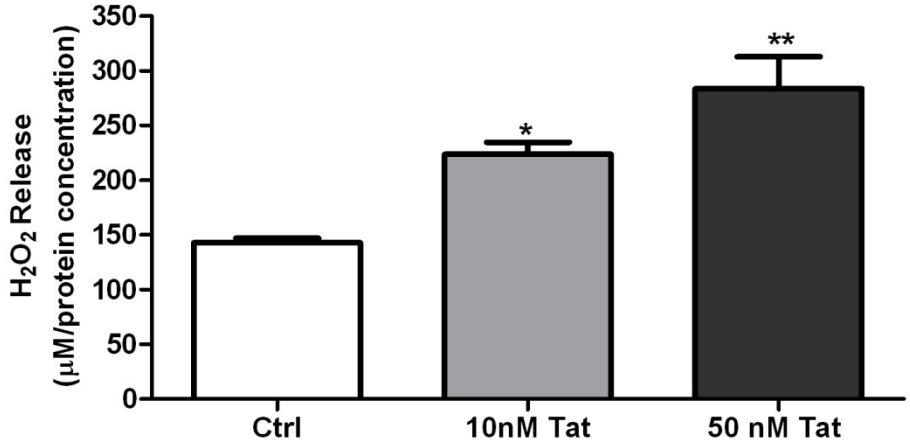
FIG 4.3. Exposure to HIV-1 Tat, not Nef, promotes endothelial ALOX5 expression. HPAEC were exposed to varying concentrations of recombinant Nef or Tat for 24 hours. Following exposure, cells were collected, total RNA was isolated, and mRNA was reverse transcribed (Invitrogen, Carlsbad, CA) to cDNA using standard protocols and quantification of the genes of interest using a Roche Lightcycler Real-Time PCR detection system. ALOX5 was normalized to the housekeeping gene β-globin. Relative expression was calculated using the Delta-Delta C_T method and values were expressed as percent of control (n = 8) * $p < 0.01$ when compared to untreated HPAEC.



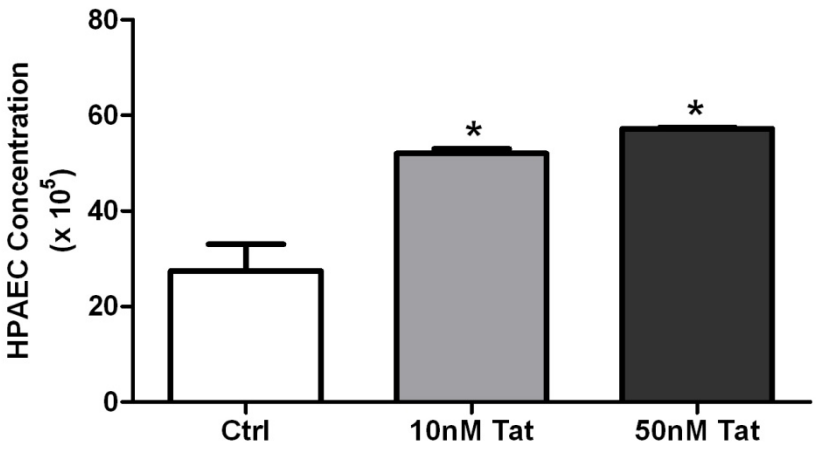
C.


ALOX5 mRNA Expression
(% Control)
250
200
150
100
50
0
*
Ctrl
10nM Tat
50nM Tat

D.

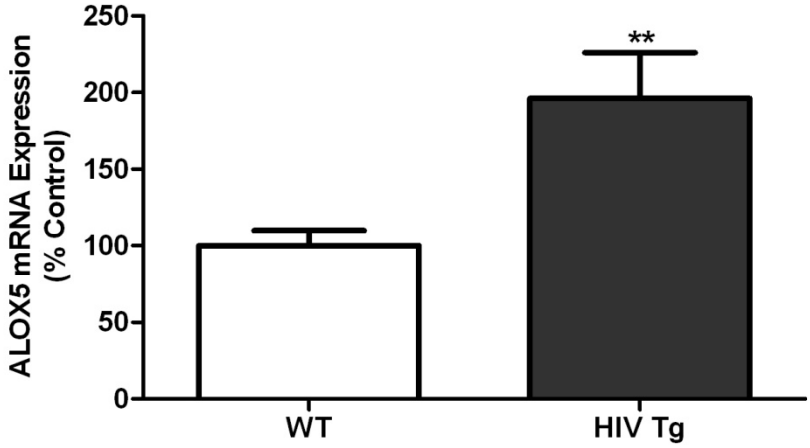

H_2O_2 Release
(μM/protein concentration)
350
300
250
200
150
100
50
0
*
**
Ctrl
10nM Tat
50 nM Tat

E.



HIV-1 protein expression increases pulmonary ALOX5 expression: *In vitro* studies demonstrate that monocytes infected with HIV-1 exhibit significant elevations in leukotriene release (Schroder et al., 1991). Recent studies also reveal that rat brains from HIV-1 Tg rats exhibit significant increases in ALOX5 mRNA and protein expression when compared wild-type controls (Rao et al., 2011). Our data demonstrate that lung homogenates from HIV-1 Tg animals exhibit a 2-fold increase in ALOX5 expression when measured using quantitative real-time PCR (Figure 4.4A).

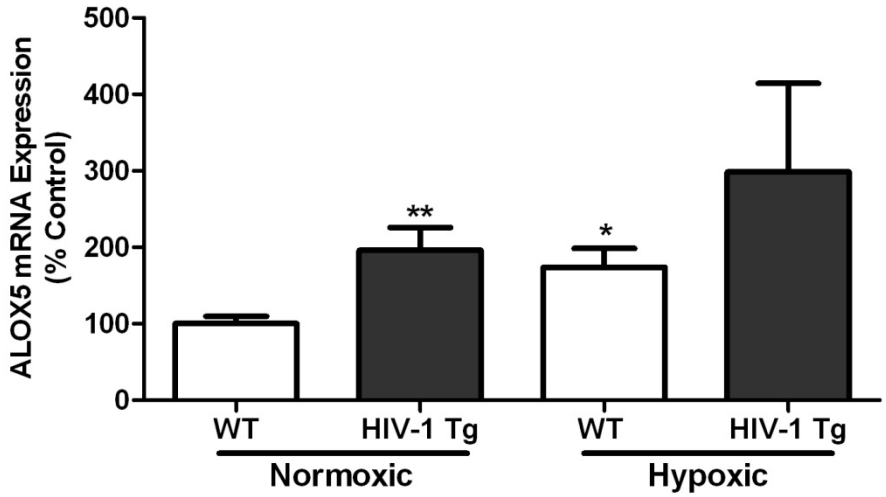
FIG 4.4. HIV-1 transgene expression increases ALOX5 expression. Rat lungs were collected and total RNA was isolated using RNA-Bee (Tel-Test, Inc) followed by purification with serial chloroform, isopropanol and ethanol extractions. mRNA was reverse transcribed (Invitrogen, Carlsbad, CA) to cDNA and the genes of interest were quantified using a Roche Lightcycler Real-Time PCR detection system. ALOX5 was normalized to the housekeeping gene β-globin. Relative expression was calculated using the Delta-Delta C_T method and values were expressed as percent of control. N = 5-8. ** $p < 0.01$ when compared to wild-type animals.


ALOX5 mRNA Expression
(% Control)
250
200
150
100
50
0
**
WT
HIV Tg

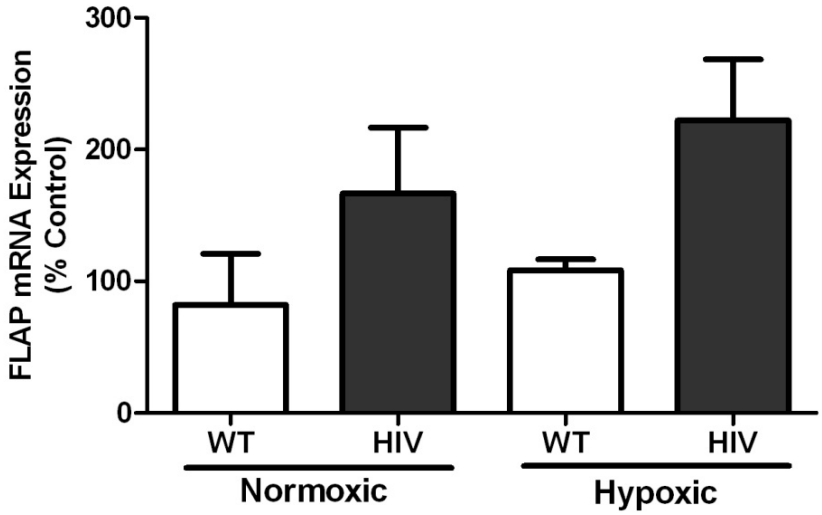
Hypoxia exposure augments 5-lipoxygenase and FLAP expression in HIV-1 Tg rats: Data examining the effect of hypoxia on ALOX5 and FLAP expression are variable and highly-dependent on cell type. However, ALOX5 and FLAP are significantly increased in rat pulmonary arteries following 28 days of hypoxia exposure (Burke et al., 2009). Similarly, *in vitro* studies in human pulmonary vascular endothelial cells and transformed human brain endothelial cells demonstrate that hypoxia (1% oxygen) enhances FLAP mRNA and protein expression (Gonsalves and Kalra, 2010). To determine whether HIV-1 transgene expression exacerbates ALOX5 and its required cofactor, FLAP in our experimental PH model, ALOX5 and FLAP gene expression levels were measured in normoxia- and hypoxia-exposed wild-type and HIV-1 Tg animals. Our data demonstrate that HIV-1 transgene expression significantly augments ALOX5 (Figure 4.5A). FLAP is similarly, although not significantly, increased in response to combined hypoxia exposure and HIV-1 transgene expression (Figure 4.5B).

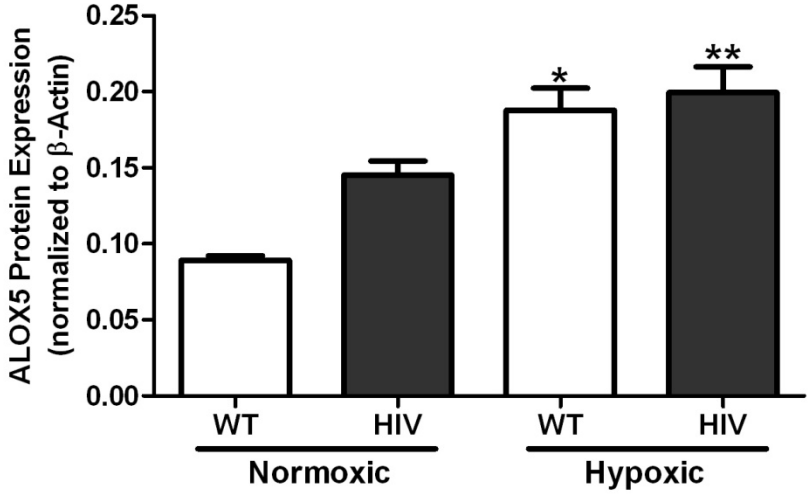
FIG 4.5. HIV-1 transgene expression potentiates hypoxia-induced 5-lipoxygenase expression. Following 4 weeks of hypoxia exposure, rats were sacrificed, lungs were collected and total RNA was isolated using RNA-Bee (Tel-Test, Inc) followed by purification with serial chloroform, isopropanol and ethanol extractions. mRNA was reverse transcribed (Invitrogen, Carlsbad, CA) to cDNA using standard protocols and quantification of the genes of interest using a Roche Lightcycler Real-Time PCR detection system. ALOX5 and FLAP were normalized to the housekeeping gene β-globin. Relative expression was calculated using the Delta-Delta C_T method and values were expressed as percent of control. N = 5-8. ** $p < 0.01$ when compared to wild-type animals. * $p < 0.05$ when compared to normoxic wild-type animals.

A.



B.

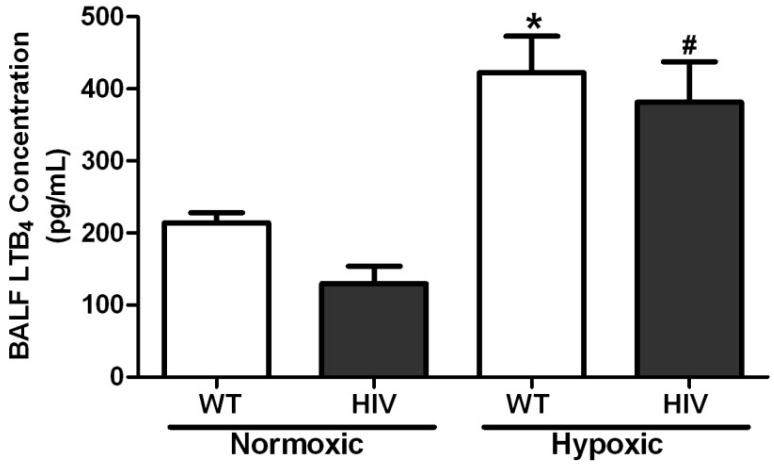

FLAP mRNA Expression
(% Control)
300
200
100
0
WT
HIV
WT
HIV
Normoxic
Hypoxic


ALOX5 Protein Expression
(normalized to β-Actin)
0.25
0.20
0.15
0.10
0.05
0.00
*
**
WT
HIV
WT
HIV
Normoxic
Hypoxic

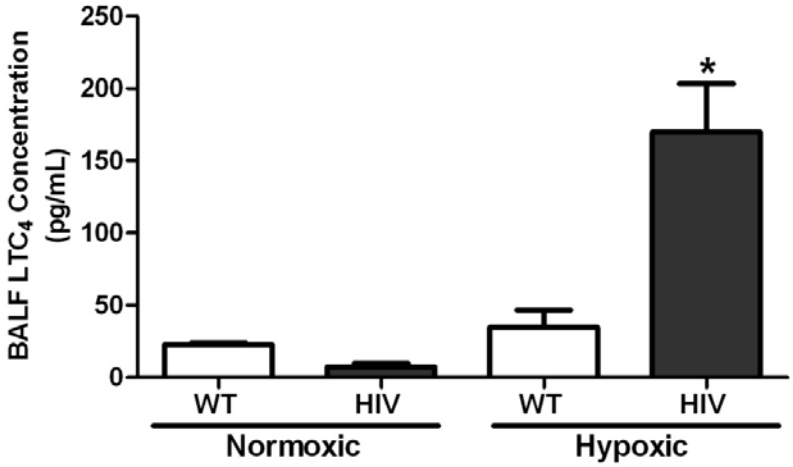
Hypoxia induces LTC_4 release in HIV-1 Tg mice: HIV-1 stimulates leukotriene release in the brain of HIV-1 Tg animals (Basselin et al., 2011). However, the effect of hypoxia exposure on leukotriene production is controversial. Early studies indicate that hypoxia exposure has no effect on LTB_4 and LTC_4 production in rat alveolar macrophages (Ohwada et al., 1990). However, other research indicates that LTE_4 levels are increased in the urine of obstructive sleep apnea patients (Stanke-Labesque et al., 2009). Additionally, an *in vitro* ischemic-like injury following five hours of oxygen-glucose deprivation stimulates a 2.5 fold increase in CysLT release from rat pheochromocytoma cells (PC12) (Li et al., 2009). Therefore, to determine whether hypoxia alters leukotriene production/release in our HIV-1 Tg model, we measured LTB_4 (Figure 4.6A) and LTC_4 (Figure 4.6B) levels in wild-type and HIV-1 Tg mouse bronchoalveolar lavage fluid (BALF) following normoxia or hypoxia exposure.

FIG 4.6. Hypoxia induces bronchoalveolar lavage fluid LTC_4 levels in HIV-1 Tg mice. Leukotriene ELISAs (Cayman Chemical) were performed to assess leukotriene (LT) B_4 ($n = 4$) and C_4 ($n = 3$-5) levels in mouse bronchoalveolar lavage fluid (BALF). To obtain BALF, mice were euthanized via CO_2 inhalation and subjected to a tracheotomy. One milliliter of cold PBS was slowly released into the trachea and lungs then retrieved. LTB_4 and LTC_4 ELISA were performed according to manufacturer's instruction. * $p < 0.05$ when compared to normoxic wild-type animals. # $p < 0.05$ when compared to normoxic HIV-1 Tg animals.

A.

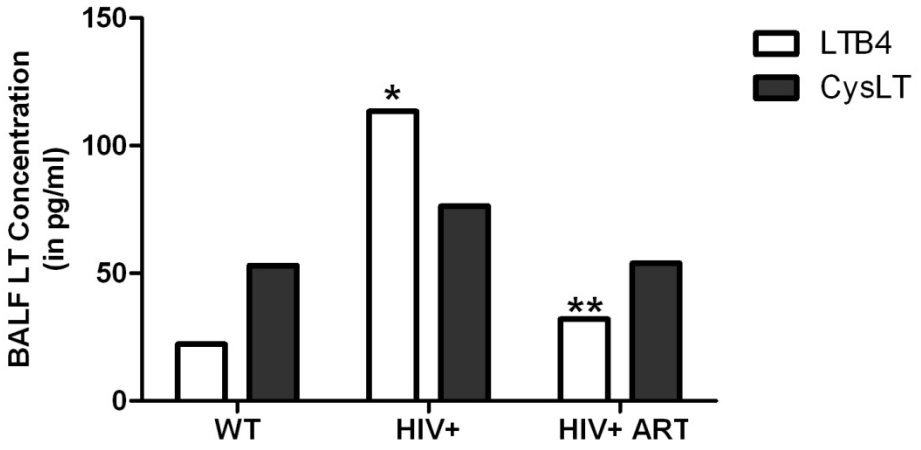

BALF LTB4 Concentration (pg/mL)
500
400
300
200
100
0
*
#
WT
HIV
WT
HIV
Normoxic
Hypoxic

B.


BALF LTC4 Concentration (pg/mL)
250
200
150
100
50
0
*
WT
HIV
WT
HIV
Normoxic
Hypoxic

Leukotriene levels are increased in bronchoalveolar lavage fluid of HIV-1 patients: Our *in vitro* evidence suggests that HIV-1 proteins increase LT levels (Figure 4.2C). Yet, *in vivo* studies performed in HIV-1 Tg animals yielded contradictory results. Moreover, studies examining leukotriene levels in HIV-1 patients are limited. Therefore, to determine whether HIV-1 infection alters lung leukotriene levels, we measured LTB_4 and CysLT in bronchoalveolar lavage fluid from uninfected (Control) subjects, HIV-1 positive patients (HIV+), and HIV-1 positive patients on a HAART treatment regime (HIV+ART).

FIG 4.7. HIV-1 infection increases bronchoalveolar lavage fluid (BALF) leukotriene levels. Leukotriene ELISAs (Cayman Chemical) were performed to assess leukotriene (LT) B_4 ($n = 3$-4) and CysLT ($n = 6$-10) levels in bronchoalveolar lavage fluid (BALF) from uninfected (Control), HIV-1 positive (HIV+), and HIV-1 positive patients on a HAART treatment regime (HIV+ ART). Results demonstrate that HIV-1 infection increases BALF LTB_4 levels when compared to BALF from uninfected subjects. Results also indicate that HAART reduce BALF LTB_4 levels caused by HIV-1 infection. HIV-1 infection produced a slight, yet insignificant change in CysLT levels. * $p < 0.01$ when compared to BALF from control, uninfected subjects. ** $p < 0.01$ when compared to HIV-1 positive subjects.


150
100
50
0
BALF LT Concentration (in pg/ml)
*
**
WT
HIV+
HIV+ ART
LTB4
CysLT

DISCUSSION

Our studies demonstrate that HIV-1 increases pulmonary artery endothelial ALOX5 expression and activity. These data also suggest that HIV-induced elevation in ALOX5 expression and LT release may in part be mediated by HIV-1 proteins such as Tat. Overall, our studies implicate a potential contributing role of HIV-induced ALOX5 in the development and progression of HIV-PH.

Results from our co-culture studies reveal that HIV-1 can significantly alter endothelial gene expression associated with vascular signaling and reactivity. These data are also consistent with clinical and experimental studies showing that HIV-secreted mediators have significant effects on endothelial cell biology (de Larranaga et al., 2003; Liu et al., 2005a; Louboutin et al., 2010; Zietz et al., 1996) independent of cellular infection. This belief was evident in co-culture results as the group containing HPAEC and HIV-1 failed to demonstrate increases in ALOX5, whereas the HPAEC, PBMC, and HIV group exhibited marked elevations in ALOX5. Additionally, a 24 hour exposure to medium from HIV-infected monocytes significantly stimulated HPAEC ALOX5 expression and CysLT release when compared to cells exposed to medium from control, uninfected monocytes. These data indicate that secreted effectors such as HIV-1 proteins stimulate ALOX5 expression and activity independent of direct monocyte-endothelial cell contact.

Numerous studies implicate HIV-1 proteins as key effectors of and contributors to HIV-induced cellular injury and vascular disease development. To determine whether HIV-1 proteins independently alter endothelial ALOX5, HPAEC were exposed to varying concentrations of recombinant HIV-1 Nef or Tat for 24 hours. Full length (86 amino acids), HIV-1 Tat (Immuno Diagnostics, Woburn, MA) was administered at nanomolar

concentrations and within a range found in serum of HIV-1 patients, or 2-40 ng/mL (Westendorp, 1995; Xiao et al., 2000). Similar to results obtained from the co-culture and HIV-MDM medium studies, exogenous administration of HIV-1 Tat dose-dependently increased endothelial ALOX5 gene expression. Our data also demonstrate that HIV-1 Tat exposure stimulates endothelial ROS release and cell proliferation. These results are consistent with previous studies investigating the effects of Tat exposure on vascular endothelial cells (Wu et al., 2010; Wu RF, 2007). Conversely, exposure to recombinant HIV-1 Nef for 24 hours caused a significant and dose-dependent reduction in endothelial ALOX5 expression. Although we can provide no clear reason for this effect, we believe that Tat-induced ROS release may mediate ALOX5 expression alterations. If Tat-induced ROS indeed contributes to endothelial ALOX5 expression, this provides a potential explanation as to why HIV-1 Nef has opposite effects on ALOX5 expression than those seen in our co-culture, HIV-MDM medium, and exogenous Tat study results.

We also examined whether HIV-1 transgene expression alters ALOX5 expression and activity via the NL4-3Δ *gag/pol* HIV-1 transgenic rat model. These animals express a nonreplicative HIV-1 provirus due to the deletion of the *gag* and *pol* regions of the viral genome. Yet, the transgene encodes for the viral genes *env*, *tat*, *nef*, *rev*, *vif*, *vpr*, and *vpu* (Reid et al., 2001). Therefore, this animal model allows the study of the physiological effects of HIV-1 proteins *in vivo* in a noninfectious manner. Interestingly, lung homogenates from HIV-1 Tg animals exhibit a 2-fold increase in ALOX5 gene expression when compared to wild-type controls. Although further studies are needed to determine

a direct mechanism, our results implicate HIV-1 proteins in HIV-induced elevations of pulmonary ALOX5.

Additionally, *in vivo* studies indicate that HIV-induced ALOX5 expression and LTC_4 release is further increased in experimental PH. Previous studies indicate that ALOX5 and the ALOX5 cofactor, FLAP are significantly increased in experimental and clinical PAH (Burke et al., 2009; Wright et al., 1998). In addition, ALOX5 overexpression accelerates and exacerbates pulmonary hypertension development in monocrotaline-treated rats (Jones et al., 2004). *In vivo* agents that inhibit ALOX5 activation and leukotriene release attenuate disease development and severity (Jones et al., 2004; Morganroth et al., 1985; Voelkel et al., 1996). Furthermore, ALOX5 is shown to mediate pulmonary artery endothelial proliferation and pulmonary vasoconstriction via LTC_4. These data suggest that increases in ALOX5 expression and activity by HIV-1 or HIV-1 proteins may contribute to the development and/or progression of HIV-PAH. In addition, the combined effect of HIV- and hypoxia-induced elevations in ALOX5 may contribute to the increased severity and rapid progression associated with clinical HIV-PAH.

HIV-PAH occurs more frequently and progresses more rapidly than PH in the general population. HIV-PAH also occurs in the absence of any apparent lung disease, and there is no correlation between PAH disease severity and CD4+ lymphocyte count (Seoane et al., 2001; Speich et al., 1991). In addition, HIV has not been found in endothelial cells of patients who develop PAH (Humbert et al., 1998b; Mette SA, 1992) nor has HIV DNA, RNA, or p24 antigen been detected in the pulmonary vessels of HIV-PAH patients (Kanmogne et al., 2001; Klings and Farber, 2003; Klings ES, 2003; Pellicelli et al., 2004). These data suggest the pathogenesis of HIV-PAH is unrelated to direct

infection of the pulmonary vasculature or to immune dysfunction and may be partially attributable to the indirect actions of HIV-1 proteins on the vasculature (Mette SA, 1992). Similar to clinical HIV-PAH, HIV-1 Tg animals develop an exacerbated form of PH in response to hypoxia exposure (data previously shown) as evidenced by RV systolic pressures, RV hypertrophy, and PA muscularization. Our data demonstrate the HIV-induced ALOX5 may contribute to PH development and progression by promoting pulmonary vasoconstriction and vascular remodeling.

The endothelium is continually exposed to actively secreted viral proteins due to its position between the blood and the vascular wall (Chang et al., 1997). Supporting evidence demonstrates that HIV proteins are able to enter the endothelium (Gujuluva C, 2001; Liu et al., 2002) and alter cell function, further suggesting that HIV-1 proteins may play a role in the development of HIV-PAH development. Additionally, studies demonstrate that vascular remodeling is increased in a simian HIV model that expresses functional Nef protein (Marecki et al., 2005; Marecki et al., 2006). The same study also showed an enhanced accumulation of Nef in endothelial cells in plexiform lesions in lungs of patients with HIV-PAH. Research also indicates that HIV-1 Tat represses bone morphogenic protein receptor-2 (BMPR2) promoter activity by more than 50% and induces a 75% reduction in BMPR2 gene expression in U937 monocytic cells (Caldwell et al., 2006). HIV-1 Tat exposure also stimulates platelet-derived growth factor (PDGF) mRNA and protein expression in rat and human astrocytes (Bethel-Brown et al., 2011). These studies indicate that HIV-1 Tat alters key signaling pathways associated with PH pathogenesis (Perros et al., 2008; Schermuly et al., 2005).

Indeed, HIV-1 causes the release of numerous mediators such as cytokines, chemokines, and ROS into the supernatant of infected cells or serum of infected patients.

Alveolar macrophages from AIDS patients constitutively release TNF-α, GM-CSF, and IL-6 (Agostini et al., 1992; Agostini et al., 1991; Trentin et al., 1992). In addition, BALF from asymptomatic HIV-1 subjects exhibits elevated levels of IL-8 and IL-10 (Denis and Ghadirian, 1994). *In vitro* studies in macrophages also indicate the HIV-1 infection stimulates the sustained release of IL-1 and IL-6 (Berman et al., 1994). Additionally, the oxidative stress marker, d-ROM (derivatives of reactive oxygen metabolites) was shown to be greater in serum of HIV-1 patients than that of healthy controls (Mandas et al., 2009). Malondialdehyde (MDA), an index of lipid peroxidation, was also significantly elevated in the serum from both symptomatic and asymptomatic HIV-1 patients (Revillard JP, 1992; Sonnerborg A, 1988; Suresh et al., 2009). Alternatively, it is possible that both HIV-1 infection and HIV-1 proteins act in concert to increase these molecules to alter pulmonary vascular cell signaling and function in an either independent or complementary manner. Nonetheless, our studies demonstrate that HIV-1 increases ALOX5 expression. In addition, hypoxia exposure further increases ALOX5 expression and activity. Altogether, HIV-induced ALOX5 may contribute to PH development by promoting cellular proliferation and vasoconstriction.

Chapter 5

Hypoxia Stimulates Pulmonary Endothelial Cell Proliferation via Arachidonate 5-Lipoxygenase

Introduction

Pulmonary Hypertension (PH) is a progressive disorder characterized by sustained increases in pulmonary arterial pressures and vascular remodeling. Although the mechanism underlying PH remains unknown, hypoxia causes PH in experimental models and in humans suffering from chronic hypoxic lung diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, and asthma (Orr et al., 2012; Wright et al., 1983). Hypoxic conditions are associated with significant alterations in endothelial cell function (Tuder et al., 1994) which may contribute to PH pathogenesis These alterations include abnormal endothelial cell growth which has been documented in lung sections and pulmonary artery endothelial cells from IPAH patients (Masri FA, 2007; Rabinovitch et al., 1986).

Arachidonate 5-lipoxygenase (ALOX5), the enzyme that catalyzes the production of vasoactive leukotrienes from arachidonic acid, is associated with endothelial proliferation and PH development. Research reveals that patients with primary pulmonary hypertension exhibit increased ALOX5 expression in the lung tissue particularly within infiltrating perivascular alveolar macrophages and in small pulmonary artery endothelial cells. Research also demonstrates that the inhibition of ALOX5 or its required cofactor, 5-lipoxygenase activating protein (FLAP) attenuate hypoxia- or monocrotaline (MCT)-induced PH (Morganroth et al., 1985; Stenmark et al., 1985). Conversely, the over-expression of ALOX5 accelerates and exacerbates PH in MCT-treated rats (Jones et al., 2004). The exact role of ALOX5 in PH is not fully understood. However, studies demonstrate that ALOX5 metabolites, cysteinyl leukotrienes (CysLT) induce vasoconstriction in the distal segments of pulmonary arteries (Friedman et al., 1984). Additionally, inhibitors of cysteinyl leukotriene (CysLT)

production attenuate proliferation of pulmonary artery endothelial cells (Walker et al., 2002).

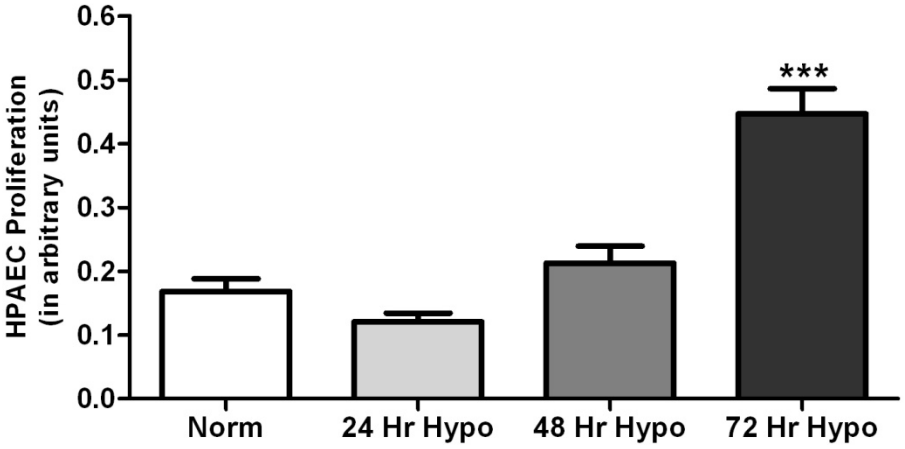
Furthermore, *in vitro* studies demonstrate that ALOX5 is activated in conditions that promote lipid peroxidation (Riendeau et al., 1989) particularly following glutathione depletion (Hatzelmann et al., 1989; Hatzelmann and Ullrich, 1987). Similarly, stimulation of endogenous ROS release by antimycin A causes an almost 4-fold increase in leukotriene formation in transformed B lymphocytes (Werz et al., 2000b). These studies suggest that ROS-mediated ALOX5 expression may mediate PH development and progression. However, the effect of hypoxia on endothelial ALOX5 is unknown. In this study, we investigate whether chronic hypoxia exposure alters endothelial ALOX5 expression and the effects of hypoxia-induced ALOX5 expression on endothelial cell proliferation.

RESULTS

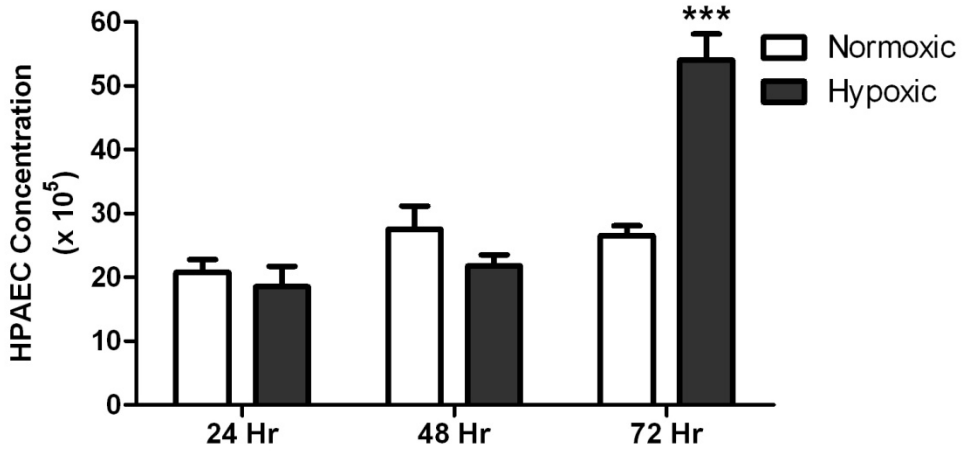
Chronic hypoxia promotes HPAEC proliferation: Hypoxia is associated with significant endothelial alterations which are thought to contribute to PH development and progression (Tuder et al., 1994). Research reveals significant endothelial alterations in pulmonary arteries of PH patients (Sakao et al., 2009) and abnormal endothelial cell growth is evident in lung sections and pulmonary artery endothelial cells from IPAH patients (Masri FA, 2007; Rabinovitch et al., 1986). To determine whether hypoxia alters HPAEC function, we assessed HPAEC proliferation following 24, 48, and 72 hours of hypoxia. MTT assay demonstrates that 72 hours of hypoxia increases cellular proliferation when compared to all other groups (Figure 5.3A). These results were confirmed by counting cells (Figure 5.3B).

FIG 5.1. Chronic hypoxia promotes HPAEC proliferation. Human pulmonary artery endothelial cells (HPAEC) were exposed to 24-, 48-, or 72 hours of normoxic or hypoxic (1% O_2) conditions (n=4). Following exposure, cells were subjected MTT assay or Trypan Blue Dye Exclusion Assay to assess HPAEC proliferation. Results indicate that while 24- or 48- hours of hypoxia has no effect of HPAEC proliferation, 72 hours of hypoxia exposure stimulates a 2-fold increase in cellular proliferation when compared to all other groups (Figure 5.1).

A


HPAEC Proliferation
(in arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
Norm
24 Hr Hypo
48 Hr Hypo
72 Hr Hypo

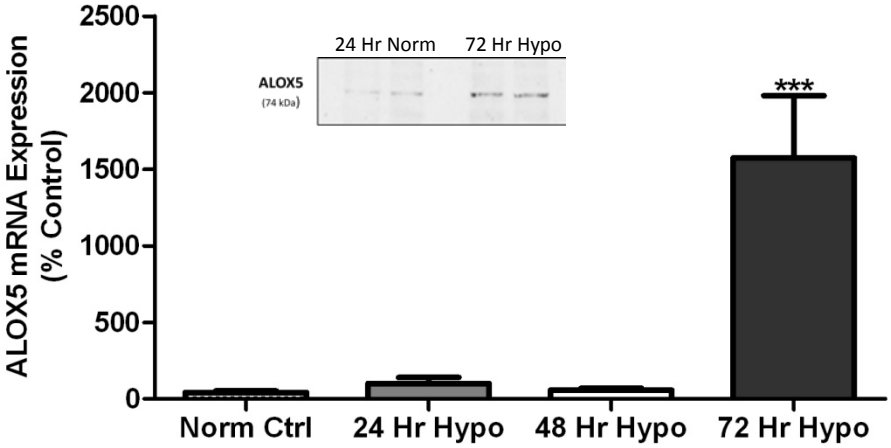
B

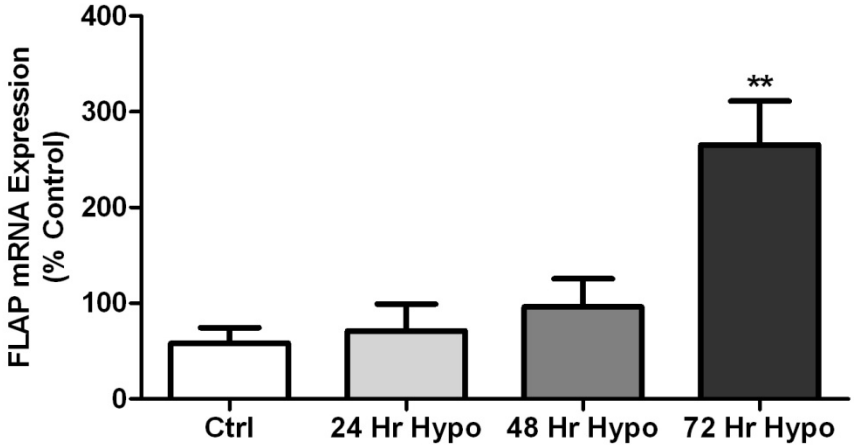

HPAEC Concentration
(x 10^5)
60
50
40
30
20
10
0
***
Normoxic
Hypoxic
24 Hr
48 Hr
72 Hr

Chronic hypoxia exposure stimulates HPAEC 5-Lipoxygenase and FLAP expression: *In vivo* studies demonstrate that both hypoxia exposure and MCT administration upregulate 5-Lipoxygenase (ALOX5) (Jones et al., 2004; Voelkel et al., 1996). To specifically investigate the effect of hypoxia on pulmonary artery endothelial ALOX5 and its required cofactor, FLAP, HPAEC were exposed to hypoxia (1% oxygen) for 24-, 48-, or 72-hours. Quantitative RT-PCR analysis shows that 72 hours of hypoxia exposure significantly increases HPAEC ALOX5 mRNA expression (Figure 5.2A).

FIG 5.2. Hypoxia exposure stimulates endothelial ALOX5 and FLAP expression. HPAEC were exposed to 24-, 48-, or 72 hours of normoxic or hypoxic conditions. Following exposure, cells were collected and total RNA was isolated for gene expression analysis via quantitative real time PCR. Results indicate that 72 hours of hypoxia increases HPAEC ALOX5 (Figure 5.2A; n = 3-8) and FLAP expression (Figure 5.2B; n = 3-8). ** $p < 0.0001$ when compared to all other groups.

A

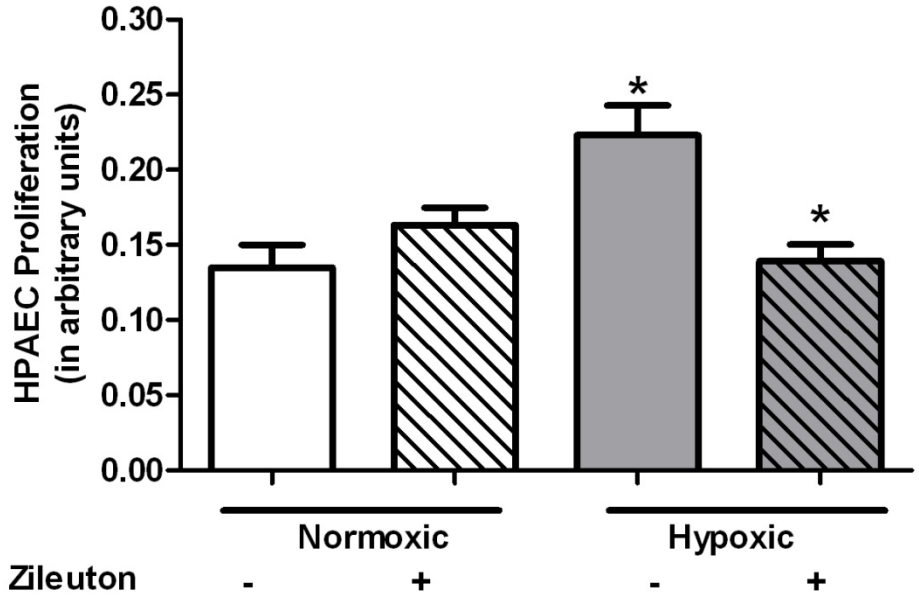

ALOX5 mRNA Expression (% Control)
2500
2000
1500
1000
500
0
24 Hr Norm
72 Hr Hypo
ALOX5
(74 kDa)
***
Norm Ctrl
24 Hr Hypo
48 Hr Hypo
72 Hr Hypo


FLAP mRNA Expression (% Control)
400
300
200
100
0
**
Ctrl
24 Hr Hypo
48 Hr Hypo
72 Hr Hypo

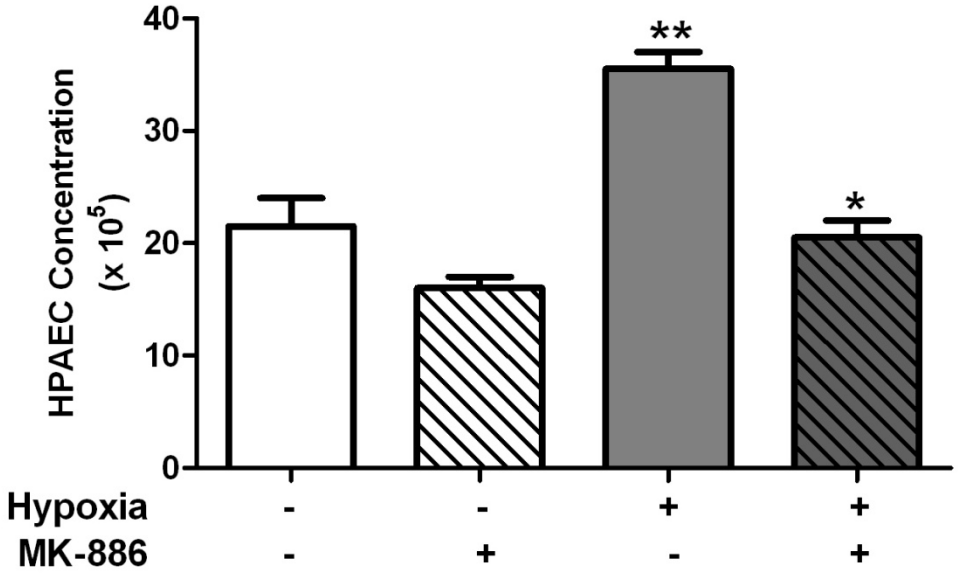
ALOX5 inhibition prevents hypoxia-mediated endothelial proliferation: Studies demonstrate that 5-lipoxygenase mediates HPAEC proliferation (Walker et al., 2002). To determine whether 5-lipoxygenase promotes hypoxia-induced HPAEC proliferation, we measured cellular proliferation in response to hypoxia in the presence or absence of two 5-lipoxygenase inhibitors, Zileuton and MK-886 (3-[1-(p-chlorobenzyl)-5-(isopropyl)-3-tert-butylthio-indol-2,2-dimethylpropanoic acid). Zileuton is an iron-ligand inhibitor which is approved for the therapy of asthma in the U.S. Zileuton is a selective and potent redox inhibitor of ALOX5. MK-886 (Gillard et al., 1989) is a potent and specific inhibitor of leukotriene synthesis (Rouzer et al., 1990). Our results indicate that inhibition of ALOX5 by zileuton (Figure 5.3A; n = 3-4) or MK-886 (Figure 5.3B, n = 3-4) attenuates hypoxia-induced increases in HPAEC proliferation.

FIG 5.3. ALOX5 inhibition attenuates hypoxia-mediated cell proliferation. HPAEC were exposed to 72 hours of normoxia or hypoxia. ALOX5 inhibitors, Zileuton (10 μM) and MK-886 (0.5 μM) were administered during the final 24 hours of normoxia or hypoxia exposure. Following exposure, cells were subjected to a MTT assay or cell counts via the Trypan Blue method to assess proliferation. ** $p<0.0001$ when compared to normoxic groups. * $p< 0.01$ when compared to untreated hypoxic groups.

A


0.30
0.25
0.20
0.15
0.10
0.05
0.00
HPAEC Proliferation (in arbitrary units)
*
*
Normoxic
Hypoxic
Zileuton
-
+
-
+

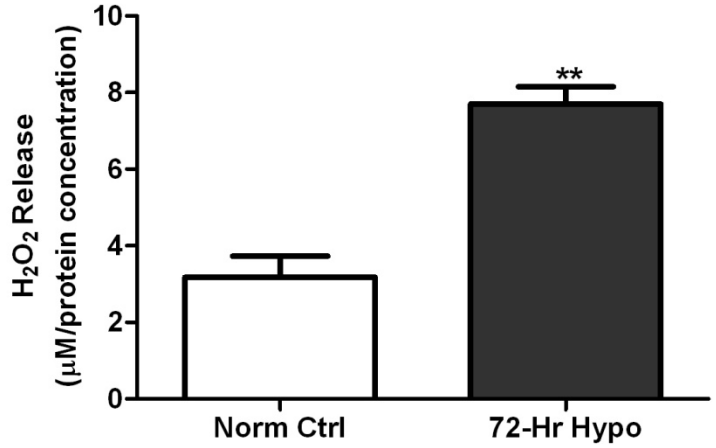
B.


40
30
20
10
0
HPAEC Concentration (x 10^5)
**
*
Hypoxia
MK-886
-
-
+
+
-
+
-
+

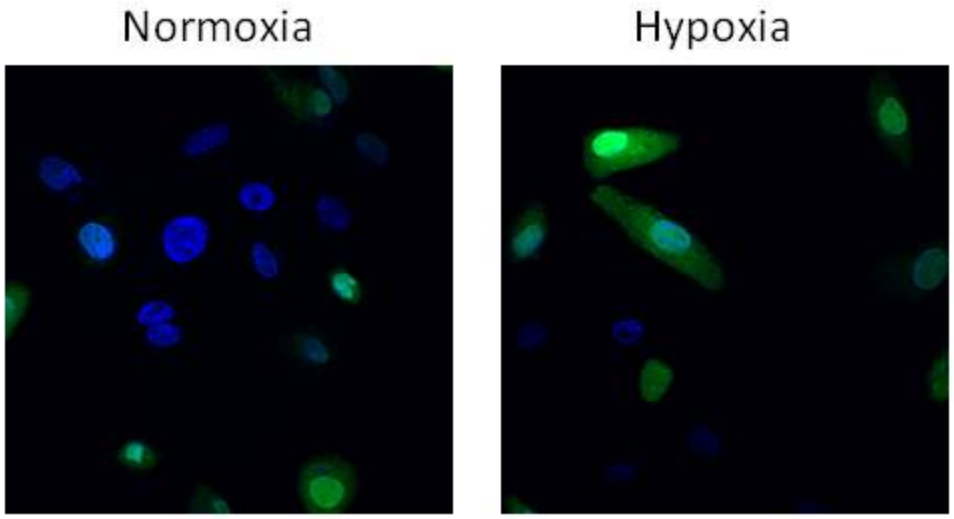
Hypoxia exposure stimulates endothelial ROS release: Previous studies demonstrate that acute (Strasser et al., 1997), intermittent (Dopp et al., 2011; Hitomi et al., 2003; Zhou et al., 2012) and chronic (Hartney et al., 2011; Kolamunne et al., 2011; Resta et al., 2010) hypoxia exposure induces ROS generation. To determine the effect of hypoxia exposure on HPAEC hydrogen peroxide (H_2O_2) release, cells were placed in normoxic or hypoxic conditions for 24, 48, or 72 hours. 72 hours of hypoxia significantly increases H_2O_2 over all other groups (Figure 5.1A). Dichlorofluorescein (DCF) fluorescence staining (Figure 5.4B) confirms that 72 hours of hypoxia exposure increase HPAEC ROS. In addition to increased ROS production, quantitative real-time PCR reveals that hypoxia exposure significantly decreases Nrf2 expression (Figure 5.4B).

FIG 5.4. Hypoxia exposure stimulates endothelial H_2O_2 release. Human pulmonary artery endothelial cells were exposed to normoxic or hypoxic (1% O_2) conditions for 24-, 48- or 72-hours. Following exposure, ROS release was assessed by Amplex Red assay (5.2A; n = 4) or DCF staining (5.2B; n = 3) ** $p < 0.01$ when compared to normoxic controls.

A.


H_2O_2 Release
(μM/protein concentration)
10
8
6
4
2
0
**
Norm Ctrl
72-Hr Hypo

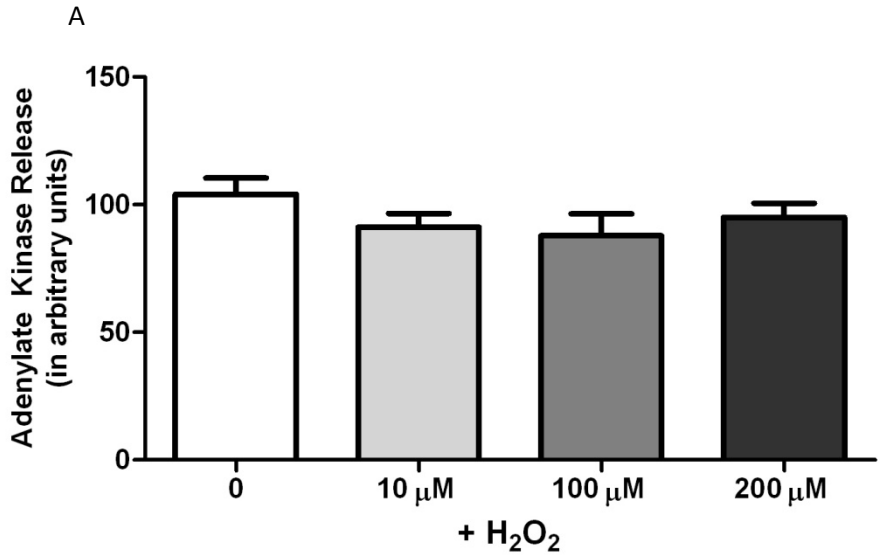
B.

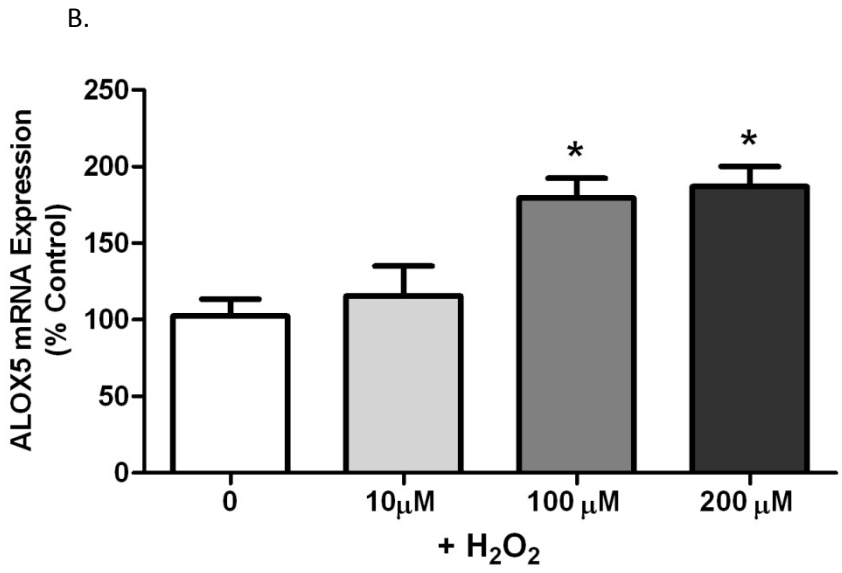

Normoxia
Hypoxia

Hydrogen Peroxide (H_2O_2) increases endothelial 5-Lipoxygenase expression: To examine if an increases in H_2O_2 affects ALOX5 expression, HPAEC were exposed to 0-200μM concentrations of hydrogen peroxide (H_2O_2) for 24 hours. Results demonstrate that 10-200 μM concentrations of H_2O_2 produce no significant changes in cell toxicity or cell death as indicated by adenylate kinase release (Figure 5.5A). H_2O_2 exposure produced marked increases in endothelial ALOX5 mRNA (Figure 5.5B) and protein (Figure 5.5C) expression. These data suggest that reactive oxygen species (ROS) mediate increases in endothelial ALOX5 expression.

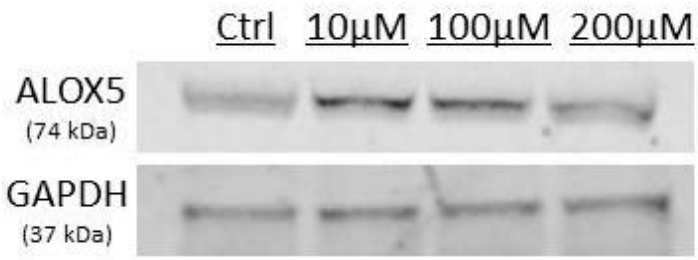
FIG 5.5. Hydrogen peroxide increases ALOX5 expression. Human pulmonary artery endothelial cells (HPAEC) were exposed to 0, 10, 100, and 200 μM hydrogen peroxide (H_2O_2) for 24 hours. Following exposure, supernatants were collected to assess adenylate kinase release. Results demonstrate no significant changes in cell death as indicated by adenylate kinase release (Figure 5.5A; n = 4-6). ALOX5 expression was analyzed by qRT-PCR and western blot. Total RNA was collected, mRNA was reverse transcribed to cDNA, and ALOX5 was quantified using a Roche Lightcycler Real-Time PCR detection system. ALOX5 was normalized to the housekeeping gene β-globin. Relative expression was calculated using the Delta-Delta C_T method and values were expressed as percent of control (Figure 5.5B; n = 3-5). * $p < 0.05$ when compared to untreated controls. For ALOX5 protein expression analysis (Figure 5.5C; n = 3-5), HPAEC lysates (30 μg) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and incubated in antibodies against ALOX5 overnight at 4°C. Immunoreactive bands were detected using the Licor system and the proteins of interest

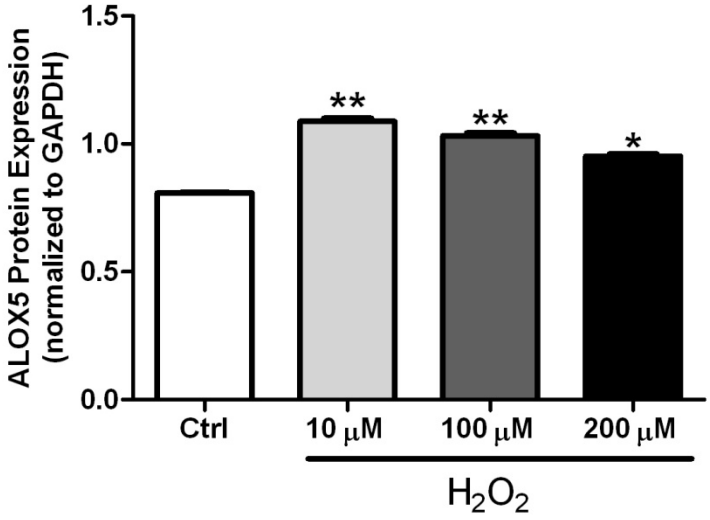
were quantified by densitometry and normalized to GAPDH levels within the same sample. ** p<0.0001 when compared to control, untreated groups. *p<0.001 when compared to control, untreated groups.





C.

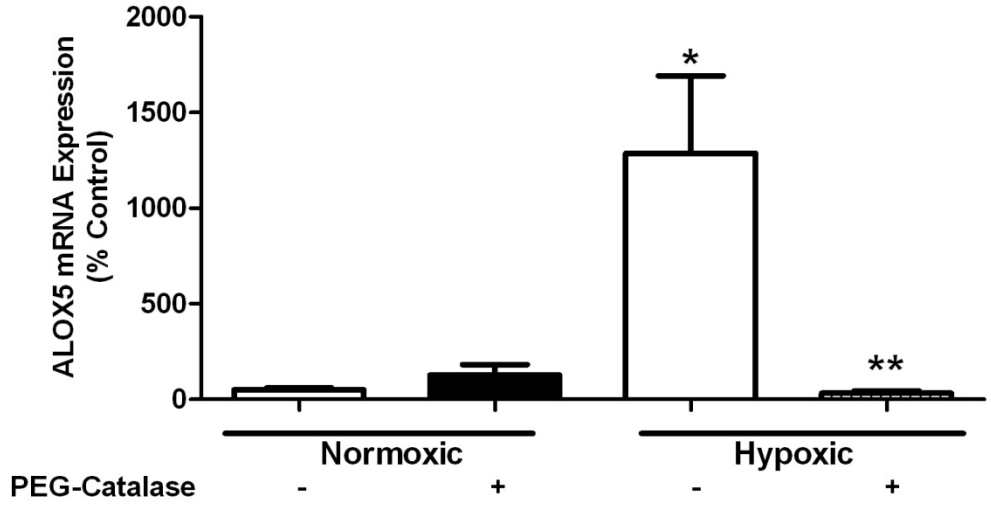

Ctrl
10μM
100μM
200μM
ALOX5
(74 kDa)
GAPDH
(37 kDa)


ALOX5 Protein Expression
(normalized to GAPDH)
1.5
1.0
0.5
0.0
**
**
*
Ctrl
10 μM
100 μM
200 μM
H2O2

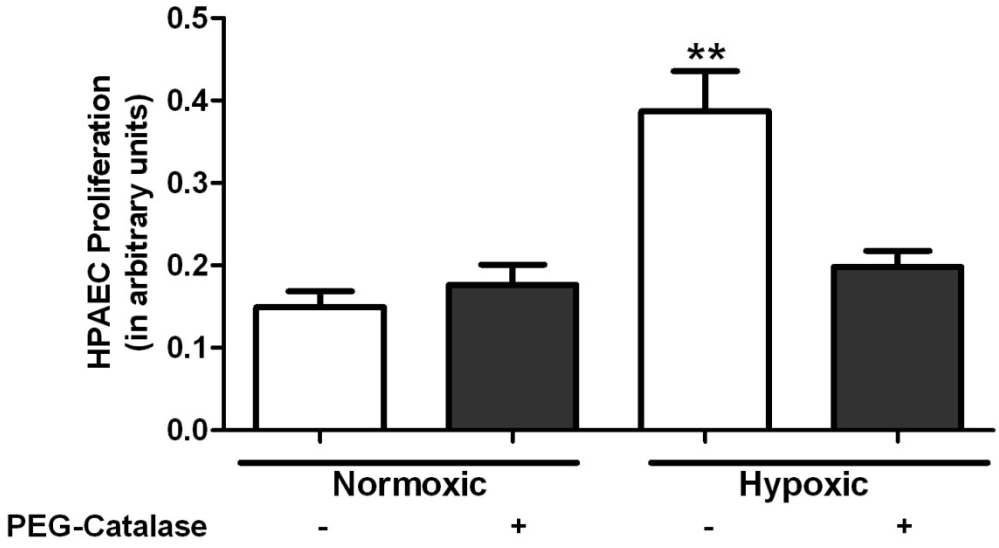
Catalase attenuates hypoxia-induced HPAEC 5-Lipoxygenase and cell proliferation. To further confirm that hypoxia-induced ROS mediate alterations in HPAEC ALOX5 expression and cell proliferation, we assessed HPAEC proliferation and ALOX5 expression in the presence or absence of PEG-Catalase (100 U/mL) during the final 24 hours of the 72 hour exposure period. Results indicate that catalase administration significantly reduces hypoxia-induced HPAEC ALOX5 gene expression (Figure 5.6A, * $p<0.01$ when compared to normoxic groups; ** $p<0.01$ when compared to hypoxic controls) and proliferation (Figure 5.6B, ** $p < 0.001$ when compared to normoxic groups).

FIG 5.6. Catalase attenuates hypoxia-induced HPAEC 5-Lipoxygenase and cell proliferation. HPAEC were exposed to normoxic or hypoxia conditions for 72 hours. PEG-Catalase was administered during the final 24 hours of the 72 exposure period. Following exposure, cells were collected and total RNA was isolated for gene expression analysis via quantitative real time PCR.

A.


ALOX5 mRNA Expression
(% Control)
2000
1500
1000
500
0
*
**
Normoxic
Hypoxic
PEG-Catalase
-
+
-
+

B.


HPAEC Proliferation
(in arbitrary units)
0.5
0.4
0.3
0.2
0.1
0.0
**
Normoxic
Hypoxic
PEG-Catalase
-
+
-
+

DISCUSSION

Our studies indicate that hypoxia exposure promotes pulmonary artery endothelial proliferation by stimulating ALOX5 and its required co-factor, FLAP. We also demonstrate that hypoxia mediates these events by increasing ROS release as PEG-catalase attenuates hypoxia- induced proliferation and ALOX5 expression. Overall, these studies suggest that ALOX5 contributes to hypoxia-induced endothelial proliferation in a ROS-dependent manner.

Previous research demonstrates that chronic hypoxia significantly increases endothelial cell proliferation. *In vivo* studies indicate that endothelial cells in the main pulmonary artery and in the small muscular arteries are increased in chronically hypoxic rats (Howell et al., 2003; Meyrick and Reid, 1979). Endothelial proliferation is also increased in neonatal calves following exposure to 8% oxygen for 14 days (Stiebellehner et al., 1998). Additionally, excessive endothelial proliferation leads to plexiform lesion formation in idiopathic PAH patients (Voelkel and Tuder, 1997). Our research, performed *in vitro*, similarly demonstrates that hypoxia promotes endothelial proliferation. We also indicate that hypoxia-induced endothelial proliferation is ROS-dependent as the administration of PEG-catalase attenuates these events. These hypoxia-induced ROS are likely produced by NADPH oxidases (Noxes) as previous studies indicate that Nox4 expression is elevated in low oxygen environments (Nisbet et al., 2009). Interestingly, previous studies also suggest that NADPH oxidase activity is required for endothelial cell proliferation (Abid et al., 2000). Additionally, studies have implicated Nox4 in the hypoxia-induced proliferation of adipose-derived stem cells (Kim et al., 2012) and human pulmonary artery smooth muscle cells (Ismail et al., 2009).

Though it is widely-accepted that hypoxia promotes pulmonary vascular cell proliferation, the underlying mechanism remains unclear. Endothelial cells contribute to hypoxic pulmonary remodeling by increasing the release of pro-proliferative mediators such as ET-1 and angiotensin II (AngII) and reducing the release of anti-proliferative agents such as nitric oxide (NO). Similarly, hypoxia stimulates cell proliferation by increasing the production of pro-proliferative stimuli from smooth muscle cells, platelets, fibroblasts, and endothelial cells (Humar et al., 2002; Kourembanas et al., 1990; Kourembanas et al., 1991; Mukhopadhyay et al., 1995). Our data indicate that hypoxia increases endothelial cell proliferation by stimulating the ALOX5 pathway as the administration of the two ALOX5 pathway inhibitors with distinct mechanisms of action, Zileuton and MK-886 significantly attenuate endothelial proliferation. Zileuton inhibits ALOX5 by binding to the iron atom needed for catalytic function (Carter et al., 1991). MK-886, however, inhibits leukotriene synthesis (Rouzer et al., 1990) by binding to the membrane-bound FLAP and preventing translocation of ALOX5 from the cellular cytoplasm to the plasma membrane for activation. These data implicate a major contributing role of leukotrienes in hypoxia-induced endothelial proliferation.

Although our evidence indicates that hypoxia increases ALOX5 in a redox-sensitive manner, we are unable to demonstrate the mechanism underlying these events. However, it is likely that the transcription factor hypoxia inducible factor (HIF) mediates the hypoxia-induced increases in endothelial ROS release and ALOX5 expression. HIF-1α is an oxygen-sensing molecule (Semenza, 2007) that mediates an adaptive response to low oxygen conditions by activating genes associated with energy metabolism, erythropoiesis, vasomotor tone, and angiogenesis (Semenza, 2003).

Hypoxia stabilizes HIF-1α by inhibiting its prolyl hydroxylation and thereby inducing the HIF-1α-dependent upregulation of genes that promote survival in low oxygen environments. HIF-2α also contributes to the vascular response to chronic hypoxia by stimulating the expression of genes involved in pulmonary vascular cell proliferation (Tuder et al., 1995). Due to these effects, HIF is implicated in PH pathogenesis. Recent research demonstrates that inhibition of HIF-1α by the cardiac glycoside digoxin attenuates hypoxia-induced increases in RV pressures, RV hypertrophy, and vascular remodeling (Abud et al., 2012). Moreover, hypoxia-induced increases in HIF-1α promote reductions in Nrf2 expression and activity in human lung endothelial cells (Loboda et al., 2009). These data suggest that hypoxia-induced HIF may contribute to PH pathogenesis via antioxidant depletion and excessive endothelial cell proliferation.

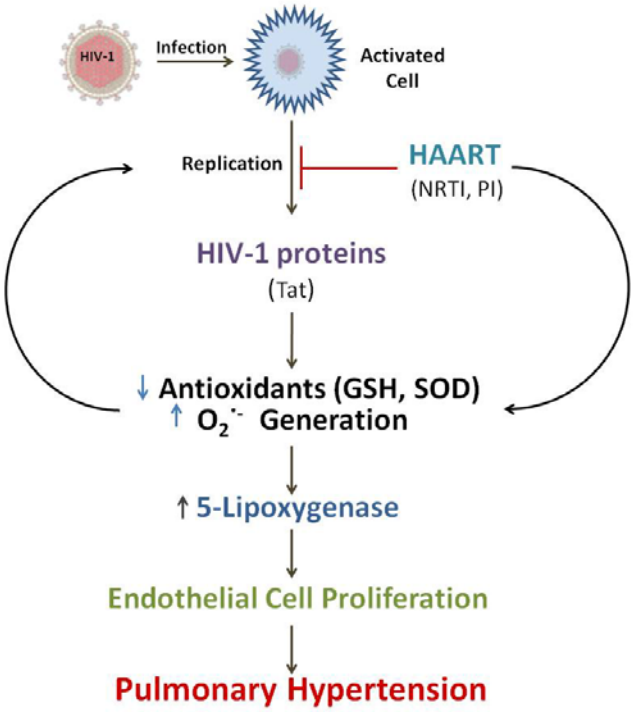
Our research also provides additional evidence of a hypoxia-induced ROS in endothelial injury and proliferation. Additionally, our results suggest that ALOX5 mediates hypoxia-induced endothelial cell proliferation. These studies also implicate ALOX5 in increased vascular remodeling documented in experimental and clinical PAH cases. Overall, our evidence demonstrating that ALOX5 inhibition attenuates hypoxia-induced proliferation suggests that ALOX5 may serve as a potential new therapeutic target for PAH patients.

CHAPTER 6

DISCUSSION

Our data indicate that HIV-1 protein expression impairs pulmonary vascular reactivity and exacerbates hypoxia-induced PH development with potentiated responses in RV systolic pressures and RV hypertrophy as well as pulmonary vessel muscularization and cellular proliferation. These results indicate that our model accurately recapitulate features associated with clinical HIV-PAH cases. First, PAH in HIV-1 patients develop a more severe form of PAH than uninfected individuals. Although Second, PAH development and progression is associated with indirect actions of HIV-1 and unrelated to immune dysfunction caused by HIV-1 infection. Finally, HIV-1 protein expression resulted in a more severe form of PH in response to chronic hypoxia exposure. Evidence also indicates that lung homogenates from HIV-1 Tg animals express significantly more ALOX5 than control animals. Additionally, exposure to chronic hypoxia further increases ALOX5 expression and LTC_4 release. These data implicate ALOX5 in our HIV-PH model. To determine whether HIV-induced ALOX5 contributes to PH development, we independently assessed the effects of hypoxia exposure and HIV-1 proteins such as Tat and Nef on ALOX5 expression and activity. Our *in vitro* studies indicate that ALOX5 mediates pulmonary artery endothelial proliferation in response to chronic hypoxia exposure and ROS release. Results also reveal that medium from HIV-infected MDM exacerbates hypoxia-induced endothelial cell proliferation. These studies highlight the potential contribution of HIV-1 proteins and ALOX5 in HIV-PAH pathogenesis. In additional studies, results demonstrate that the HIV-1 protein, Tat stimulates pulmonary artery endothelial ROS release, ALOX5 expression, and cellular proliferation. Altogether, these results suggest that Tat-induced ALOX5 exacerbates PAH development and progression by stimulating vascular remodeling via increased endothelial proliferation (Figure 6.1).

FIG 6.1. Proposed Role of arachidonate 5-lipoxygenase in HIV-associated pulmonary arterial hypertension.



Our results also suggest that ALOX5 inhibition may attenuate PAH progression since administration of the FDA-approved Zileuton (Zyflo) attenuates hypoxia-induced endothelial cell proliferation.

Our interest in HIV-PAH is due to the disproportionate incidence of PAH diagnoses in HIV-infected persons. The frequency of HIV-PAH is strikingly higher than PAH in the general population. In addition, according to French registries, PAH associated with HIV infection is the fourth-leading cause of PAH (Humbert et al., 2006). The reason HIV-infected patients develop PH at this alarming rate remains unknown. It has been suggested that the increased lifespan of HIV-1 patients on antiretroviral therapy increases the likelihood of exposure to the "multiple hits" believed to be required to develop PH (Yuan and Rubin, 2005). Additionally, HIV infection is associated with 50-60% increased odds of COPD diagnosis (Crothers et al., 2006). These factors, in addition to co-morbidities more common to HIV-infected patients such as cigarette smoking, alcohol disorders, and drug abuse (Crothers et al., 2011) may likely contribute to the PAH prevalence.

We also chose to investigate HIV-PAH because the underlying mechanism of this disease is unknown and effective treatments are few. The pathogenesis of HIV-PAH may rely on the indirect action of HIV-secreted mediators such as HIV-1 proteins on the endothelium. Yet, only limited studies demonstrate a link between HIV-1 proteins and PAH (Almodovar et al., 2011b). Our studies indicate that exposure to hypoxia and/or HIV-1 Tat may contribute to PAH development by increasing endothelial cell proliferation.

The strength of our studies lies in the fact that we are able to study the contribution of HIV-1 proteins to PH development by employing our HIV-1 Tg animal model. This advantage is important as PAH disease severity in HIV-1 patients is unrelated to CD4 lymphocyte levels and may predominantly rely on the action of HIV-1 proteins on the vasculature. Additionally, instead of studying the sole effect of one HIV-1 protein *in vitro*, our HIV-1 Tg model expresses multiple HIV-1 proteins in numerous tissues and organs. As a result, we are able to perform more physiologically-relevant studies by assessing the collective effect of HIV-1 proteins on the pulmonary vasculature.

An additional strength of our study is that similar results were obtained with our transgenic model and human cell culture model. Quantitative real time PCR analyses demonstrate that ALOX5 is significantly increased in lung homogenates from HIV-1 Tg animals as well as human pulmonary artery endothelial cells exposed to medium from HIV-MDM and recombinant HIV-1 Tat. These results confirm that HIV-1 proteins stimulate ALOX5. Also, both our *in vivo* and *in vitro* studies suggest increased endothelial cell proliferation as a potential mechanism by which ALOX5 promotes PAH development. These findings are particularly interesting as the excessive proliferation of endothelial cells resulting from pro-proliferative/anti-apoptotic stimuli are thought to play a major role in PH-associated vascular remodeling (Sakao et al., 2009). Therefore, the contribution of ALOX5 to HIV-PAH development and progression may possess clinical value and should be further explored. We plan to determine whether ALOX5 inhibition attenuates PH development and progression by treating normoxic and hypoxic wild-type and HIV-1 Tg animals with zileuton daily. However, clinical studies

investigating whether the ALOX5 inhibitor, Zileuton (Zyflo) are also needed to determine whether ALOX5 inhibition is a viable option for PAH treatment.

We also employ a normobaric hypoxia chamber as a method to induce experimental PAH in the HIV-1 Tg rat model. This experimental PH method induces many of the phenotypic pathophysiological changes seen in PAH patients. As a result, the true contribution of HIV-1 proteins to HIV-PAH development and progression was assessed. However, a potential weakness of our model is the inability chronic hypoxia exposure to induce plexiform lesion formation in rats. Plexiform lesions are a well-characterized pathology of PAH and remain one of the most common findings of human HIV-PAH (Limsukon et al., 2006). Previous studies have reported that of the available histopathologic findings from HIV-PAH patients, 78% showed plexigenic pulmonary lesions (Mehta, 2000). Due to this disadvantage of the HIV-PAH animal model, we are unable to assess the role of HIV-1 proteins or HIV-induced endothelial injury in plexiform lesion formation. Further studies utilizing an extended chronic hypoxia model or chronic hypoxia exposure and Sugen administration, a combination shown to promote plexiform and obliterative lesion formation, may provide more insight as to the role of HIV-1 proteins in PAH development.

In vivo studies reveal that chronic hypoxia exposure increases ALOX5 mRNA and protein expression in lung homogenates from HIV-1 Tg animals. These studies question which cell type is responsible for increased pulmonary ALOX5 mRNA and protein expression. Since ALOX5 is expressed in monocytes, macrophages, and vascular smooth muscle cells, it is plausible that any of these cell types may be responsible for the increased ALOX5 expression, activity, and leukotriene production. To determine this,

future studies may include co-immunolocalization staining for CD14, von Willebrand, and/or smooth muscle alpha-actin antigen staining to determine if mononuclear, endothelial, or vascular smooth muscle cells are the predominant ALOX5-expressing cell types.

Our studies also demonstrate that exposure of pulmonary artery endothelial cells to medium of HIV-MDM markedly increases cysteinyl LT release. Since cysteinyl LTs are associated with vasoconstriction of the small pulmonary arteries (Friedman et al., 1984) and proliferation of pulmonary artery endothelial cells (Walker et al., 2002), our data suggest that secreted mediators from HIV-infected cells may contribute to PAH pathogenesis by increasing pulmonary artery vessel tone and remodeling. Although the effect of HIV-1 protein-induced LT release on other vascular cell types was not examined in these studies, it is likely that LT release following HIV- and hypoxia-induced ALOX5 may directly alter pulmonary smooth muscle cell function. Recent studies provide evidence that LTB_4 promotes vascular smooth muscle cell migration (Moraes et al., 2010). CysLT are also implicated in the proliferation and migration of murine vascular smooth muscle cells (Kaetsu et al., 2007; Porreca et al., 1996; Porreca et al., 1995). This LT-induced SMC proliferation effect may also mediate the increased vessel muscularization seen in our HIV-PH studies (Figure 3.3). Therefore, it is possible that HIV-induced ALOX5 activity and LT release may promote PAH development by acting in a dual manner and promoting proliferation of both pulmonary endothelial and pulmonary vascular smooth muscle cells.

Previous studies also implicate a role of transcellular biosynthesis of cysteinyl leukotrienes in inflammatory response (Fabre et al., 2002) and various disease states (Di

Gennaro et al., 2004). Transcellular biosynthesis occurs when LT production occurs as a result of the transfer of lipid metabolite intermediates between cells via direct cell-cell interaction. This process requires a donor cell to synthesize and release one component of the biosynthetic cascade and a second cell to take up the required intermediate to produce the final biologically active product. It is unknown whether this process occurs between macrophages and/or vascular SMC and pulmonary artery endothelial cells. However, previous studies provide evidence of direct interactions between purified human leukocytes and myocardial endothelial cells, which resulted in increased LT formation via the transfer of the LTA_4 (Sala et al., 1996). In addition, research reveals that the biosynthesis of LTB_4 is significantly enhanced when macrophages transformed low levels of SMC-released LTA_4 into LTB_4 (Zou and Anges, 1994). This event may explain why the exposure of pulmonary artery endothelial cells to medium from HIV-infected MDM produces such significant leukotriene release. Future experiments may include investigating whether the direct interaction of HIV-infected MDM with pulmonary vascular endothelial potentiates cysteinyl leukotriene release as this event may facilitate pulmonary vasoconstriction and vascular remodeling.

Future Studies

Our current results suggest that HIV-1 increases the susceptibility to develop PH by altering endothelial cell signaling and proliferation. These studies provide a possible explanation of why HIV-1 patients develop a more severe and accelerated form of PH than the general population. As HIV-induced 5-lipoxygenase seems to mediate the significant increases in endothelial cell proliferation, and may worsen vasoconstriction in the pulmonary vasculature, future studies warrant the investigation of ALOX5 inhibition

in HIV-associated PH development and progression. In addition, studies using the administration of the FDA-approved, Zileuton (Zyflo) would be helpful to determine if the ALOX5 inhibition ameliorates HIV-PAH pathologies, such as the right ventricular hypertrophy and elevated RVSP.

Future studies may also include examining the mechanism by which HIV-1 Tat stimulates endothelial ALOX5. HIV-1 Tat may stimulate ALOX5 expression and activity by increasing cellular ROS release. Numerous studies demonstrate that Tat promotes ROS release in a variety of cell types (Paladugu et al., 2003; Price TO, 2006; Toborek et al., 2003). Additionally, previous studies indicate that H_2O_2 stimulates ALOX5 activity in B-lymphocytes (Werz et al., 2000b). Our studies also reveal that H_2O_2 increases ALOX5 in pulmonary artery endothelial cell (Figure 4.2). Furthermore, *in vitro* studies demonstrate that ALOX5 is activated in environments of high oxidative stress (Lee et al., 2010) and conditions that promote lipid peroxidation (Riendeau et al., 1989) particularly following glutathione depletion (Hatzelmann et al., 1989; Hatzelmann and Ullrich, 1987). These studies strongly suggest that Tat may stimulate ALOX5 expression by inducing endothelial ROS release. Future *in vitro* experiments may include the examination of ALOX5 expression following Tat exposure in the presence or absence of an antioxidant such as PEG-Catalase or Tempol. It will also be important to determine if Tat activation of transcription factors is ROS-dependent or independent. Previous studies demonstrate that Tat alters endothelial gene expression by increasing NF-κB expression. It is likely that Tat also increases ALOX5 in an NF-κB-dependent manner as the ALOX5 promoter contains a NF-κB binding site. If Tat directly binds to the ALOX5 promoter, it may also be useful to identify the Tat motif that facilitates this interaction. Previous

research demonstrates that the arginine- and cysteine-rich domains of Tat are needed for NF-κB activation. Therefore, Tat-neutralizing antibodies specific for these regions may inhibit Tat-induced ALOX5, and may attenuate HIV-induced vascular injury.

HIV proteins may also induce ALOX5 in a TGF-β-mediated manner. Although TGF-β is directly associated with PAH progression, it may also contribute to PAH by stimulating other vascular remodeling pathways. TGF-β potently stimulates ALOX5 transcription and expression (Sorg et al., 2006). Studies also indicate that TGF-β mRNA expression and plasma TGF-β levels (Wiercinska-Drapalo et al., 2004) are increased in HIV-infected patients and by hypoxia exposure. Moreover, the HIV-1 proteins, Tat and gp160 are linked to increases in TGF-β expression and activity (Hu et al., 1996; Lotz et al., 1994). Although BALF and serum from HIV-1 Tg animals exhibit no significant increases in TGF-β levels in our studies, concentrations of TGF-β are increased in HIV-1 patients. Thus, clinical studies may be useful in determining whether TGF-β levels affect ALOX5 expression and metabolite release in HIV-PAH patients.

To better understand the role of ALOX5 in HIV-PAH, clinical investigation of ALOX5 expression and LT release in HIV-1 patients is also needed. Evidence from our study and others suggest that ALOX5 is increased in PH (Wright et al., 1998). Elevations in ALOX5 expression and activity are also associated with exacerbated and accelerated forms of PH (Jones et al., 2004). We believe that the increased susceptibility of HIV-1 patients to develop PAH is linked to Tat-induced elevations in ALOX5. In order to determine whether this association exists in PAH patients, studies are necessary to determine ALOX5 expression and LT levels of both treatment-naïve and treatment-

experienced HAIV-1 patients. In addition, ALOX5 expression and circulating LT levels may serve as a biomarker to assess one's susceptibility to develop PH.

Alternative/Contributing Mechanisms

HIV-associated Pulmonary Arterial Hypertension: HIV-PAH is a complex disorder characterized by vasoconstriction of the pulmonary vasculature and increased vascular resistance and remodeling. Previous research demonstrates that HIV-1 and HIV-1 proteins significantly alter vascular function. For example, endothelium-dependent vasorelaxation is significantly reduced in pig coronary artery rings treated with Tat protein whereas arteries treated with Tat protein plus anti-Tat antibody relaxed similarly as control arteries. Endothelial NOS expression in Tat-treated coronary artery rings is also reduced by 73% when compared to control vessels (Paladugu et al., 2003). Additional studies also demonstrate that mice expressing HIV-1 Tat protein targeted to the myocardium experience significant depressions in systolic and diastolic functions (Fang et al., 2009). Therefore, it is likely that chronic exposure to HIV-1 proteins promotes vascular dysfunction and leads to cardiovascular disease development over time.

HIV-1 proteins may also contribute to PAH by stimulating endothelial cell activation and the release of cytokines/chemokines onto nearby vascular smooth muscle cells. For example, 24 hours of exogenous Tat administration dose-dependently increases IL-6 release from human umbilical vein endothelial cells (Hofman et al., 1993). These effects may independently contribute to the HIV-PAH pathogenesis as IL-6 is believed to significantly contribute to PAH development (Golembeski et al., 2005a). Similarly, the independent or combined release of vasoactive cytokines such as ET-1,

TGF-β, and IL-6 may act to increase PAH susceptibility via distinct or common signaling pathways.

Furthermore, it is possible that HIV-PAH develops from the combination of multiple vascular events such as HIV-induced endothelial dysfunction and proliferation, cytokine release, and/or ROS production. This "multiple hit" hypothesis provides a potential explanation as to why HIV-1 patients develop PAH at such an alarming rate. In addition, the activation of multiple PAH-associated pathways such as ALOX5, ET-1, IL-6, and TGF-β by HIV-1 and HIV-1 proteins may also account for the accelerated disease progression and decreased survival in HIV-PAH patients. Therefore, early diagnosis coupled with the use of several combined therapies may be essential for the effective treatment of HIV-PAH patients.

HIV-induced Arachidonate 5-Lipoxygenase: Our studies indicate that exposure to medium of HIV-MDM and recombinant Tat significantly increases pulmonary artery endothelial ALOX5. We believe that Tat induces this effect in a ROS-dependent manner. However, additional mechanisms may contribute to ALOX5 upregulation by HIV-1 Tat. For example, recombinant Tat protein activates mitogen-activated protein kinase (MAPK) ERK (1/2) in human, murine, and bovine endothelial cells (Rusnati et al., 2001). The synthetic peptide Tat (41-60), but not peptides Tat (1-21) and Tat (71-86), also causes ERK phosphorylation (Rusnati et al., 2001). These data suggest that HIV-1 Tat may promote ALOX5 activity by stimulating the MAP and ERK kinases associated with ALOX5 regulation.

Previous studies also indicate that HIV infection causes a 6-fold increase in macrophage arachidonic acid (AA) release (Nokta et al., 1995). It is unknown whether

Tat increases AA release in endothelial cells. Nonetheless, HIV-induced increases in available AA may result in the excessive production of cysteinyl leukotrienes and impaired vascular tone. Furthermore, HIV-1 proteins such as gp120 and Tat increase NF-κB. For example, at HIV-1 entry, the binding of the gp120 viral envelope to CD4 induces NF-κB activity by activation of IKK and pro-caspase 8 (Bren et al., 2009). Additionally, studies indicate that NF-κB is constituitively activated in Jurkat cells stably expressing the Tat gene (Scala et al., 1994). Also Tat gene expression and protein transduction induces IκB kinase (IKK) activity and increases expression of inhibitors of NF-κB degradation, (IκB-α) (Demarchi et al., 1996). These studies provide evidence that HIV-1 and HIV-1 gene products may increase ALOX5 expression by stimulating NF-κB expression and activity.

Potential HIV-PAH Therapies

Nutritional Antioxidants: HIV-1 reduces levels of plasma antioxidants (Halliwell, 1999), such as ascorbate, or vitamin C (Stephenson CB, 2006) and these decreases in antioxidant concentrations persist in HIV-1 patients although their dietary intake is sufficient for healthy individuals (Kruzich et al., 2004). Also, low intakes of vitamin C (Tang AM, 1993) and low plasma concentrations of vitamin E (Tang et al., 1997) are associated with a greater risk of progression to AIDS in HIV-infected US subjects. These studies emphasize the importance of dietary antioxidant vitamins in HIV-1 seropositive individuals and suggest a potential therapeutic benefit for HIV-1 patients. However, research examining the effect of dietary supplementation in HIV-1 patients provides conflicting results. Multi-nutrient supplements containing vitamins C and E led to a lower risk of death due to HIV infection in Tanzanian women (Fawzi et al., 2004) and a

small increase in CD4+ T lymphocyte counts in Kenyan women (McClelland et al., 2004). Vitamin C and E supplements were found to reduce oxidative damage and attenuate disease severity in HIV-positive Canadian adults (Allard et al., 1998). Also, α-tocopherol, or vitamin E, (800 mg/day) administration decreased viral load in HIV-1 patients over a 60-day period (Spada et al., 2002). In addition, research demonstrates that antioxidant molecules such as GSH, glutathione ester, and NAC are able to suppress HIV expression in infected monocytic cells (Garaci et al., 1997; Kalebic et al., 1991) as well as viral replication and disease progression in murine AIDS (Magnani et al., 1997; Palamara et al., 1996). These data argue that GSH administration may provide therapeutic benefit against HIV-1 infection and HIV-associated pulmonary arterial hypertension.

Conversely, daily selenium administration has shown no significant effect on CD4+ cell counts or viral load in pregnant, HIV-infected women (Kupka et al., 2008). However, the results of a 9-month selenium supplementation study performed in 450 HIV-1 seropositive men and women may provide an explanation for the recent conflicting results and highlight the importance of treatment adherence. Study subjects with a selenium change less than or equal to 26.1 microgram/L, indicating poor subject compliance, were found to have an increase in HIV-1 viral load and a decrease in CD4+ lymphocyte counts after the 9-month treatment period. Conversely, subjects with an increase in serum selenium levels greater than 26.1 microgram/L demonstrate no change in viral load and increases in CD4+ cell counts (Hurwitz et al., 2007). The administration of alpha-lipoic acid, a glutathione-replenishing disulfide, three times daily increased total glutathione levels but failed to alter HIV RNA levels or improve

CD4+ lymphocyte counts after 6 months (Jariwalla et al., 2008). These results of these intervention trials may not be completely attributable to the antioxidant actions of the supplements, but the data suggest that proper multi-nutrient and antioxidant supplementation may diminish the severity of HIV disease. Still, the varying outcomes of these studies underscore the need for further research in this area.

Nrf2 Activation: NF-E2 related factor 2 (Nrf2) is a ubiquitously expressed transcription factor that regulates antioxidant enzyme expression by binding to the antioxidant response element (ARE). Due to its function, the Nrf2 pathway is thought to play an essential role in cellular protection against ROS effects and oxidative stress (Kaspar et al., 2009). Recent studies indicate that some vascular protective compounds act via the Nrf2 pathway. For example, resveratrol dose-dependently increases Nrf2 promoter activity and stimulates expression of Nrf2-regulated genes such as heme-oxygenase (HO)-1 in cultured primary human coronary arterial endothelial cells. Resveratrol also reduces mitochondrial and cellular ROS release following high glucose and TNF-α exposure in an Nrf2-dependent manner (Ungvari et al., 2010). Additionally, Nrf2 is linked to the anti-atherogenic effects of the *Ginkgo biloba* extract (GBE). Studies indicate that GBE increases Nrf2 promoter activity and nuclear translocation in human aortic endothelial cells. Also, Nrf2 knockdown abolishes GBE-induced suppression of TNF-α-induced VCAM-1 expression in human aortic endothelial cells (Chen et al., 2011).

In addition, Nrf2 activation protects vascular cells against ROS release and inflammation. For example, Nrf2 activation by sulforaphane reduces VCAM-1 signaling in human umbilical vein endothelial cells (Zakkar et al., 2009) and adenoviral Nrf2 overexpression prevents injury by ROS and inhibits monocyte adhesion in endothelial

cells (Chen et al., 2006). Nrf2 gene transfer via adenoviral transduction inhibits proliferation in human and rabbit smooth muscle cells. AdNrf2 also reduces inflammation and oxidized LDL accumulation following aortic balloon injury in rabbits (Levonen et al., 2007). Dh404 stimulates Nrf2 in primary cardiac myocytes and increases Nrf2 nuclear translocation and transcriptional activity in H9C2 cardiomyocytes. Although Nrf2-deficient mice display significant increases in liver MDA, Nrf2 knockdown in ApoE-deficient mice produced a decrease in aortic stiffness and a 61% reduction in plaque area after 20 weeks when compared to ApoE KO controls. Moreover, macrophages from ApoE/Nrf2 deficient mice demonstrate a reduced uptake of AcLDL, a commonly used indicator for OxLDL (Sussan et al., 2008). Additionally, the Nrf2 activator, Dh404 inhibits basal and Angiotensin II-induced superoxide and peroxynitrite formation (Ichikawa et al., 2009). Also, treatment with the Nrf2 activator, CDDO-Imidazolide (CDDO-Im) prevents cigarette smoke-induced increases in RV pressures and alterations in RV diastolic and systolic functions. Nrf2 activation by CDDO-Im also increases GSH levels and attenuates alveolar apoptosis and pulmonary oxidative damage following cigarette smoke exposure (Sussan et al., 2009). Interestingly, numerous clinical trials are currently investigating the effect of Nrf2 activation and sulforaphane treatment in cystic fibrosis, COPD, asthma, cancer, autism, and cardiovascular disease. As such, Nrf2 activation may serve as an ideal therapeutic for HIV-associated PAH.

Targeting Specific Antioxidant Pathways: Extensive research implicates oxidative mechanisms in the development of vascular diseases. However, studies employing antioxidants as a major disease therapy produce controversial results regarding disease protection and reversal (Antoniades et al., 2003; Lachmanová et al., 2005; Redout et al.,

2010; Violi F, 2004). These outcomes, as well as recent advances in ROS detection, have led researchers to redefine the concept of oxidative stress and direct more attention to the balance of redox signaling, particularly the major thiol/disulfide couples such as glutathione (GSH)/glutathione disulfide (GSSG), reduced thioredoxin [Trx-$(SH)_2$]/oxidized thioredoxin (Trx-SS), and cysteine (Cys)/cystine (CySS) (Jones, 2006). These redox couples regulate numerous biological functions within the cell and evidence suggests that specific redox states may work together to perform distinct functions in various cellular locations (Hansen et al., 2006).

Interestingly, the cellular localization of these redox couples and the resulting biological events may have considerable consequences on vascular function and hence, the development and progression of vascular diseases. For example, the expression and DNA-binding of redox-sensitive transcription factors, including NF-κB and AP-1, may be severely altered in response to modulations in nuclear redox states. Moreover, mitochondrial redox states may regulate mitochondrial permeability transition and thereby, trigger cellular necrosis or apoptosis. Differences in redox states, particularly those across the plasma membrane, are implicated in cell proliferation as well as monocyte adhesion to endothelial cells (Go and Jones, 2005). Variations in extracellular Cys/CySS redox states and those found in human plasma also enhance oxidant-induced apoptosis and mediate decreases in cell number (Jiang et al., 2005). Altogether, these data encourage researchers to obtain a better understanding of redox signaling to allow for more effective antioxidant treatments.

Peroxisome Proliferator-Activated Receptors (PPAR) Agonists: Agents that restore NO levels and reduce ROS generation would likely have a favorable impact on HIV-

mediated vascular disease. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily (Isseman I, 1990). PPARs regulate a variety of physiological processes ranging from lipogenesis to inflammation, and have been implicated in numerous disorders including diabetes and atherosclerosis. There are 3 PPAR isotypes: PPARα, PPAR β/δ, and PPARγ. These PPARs are expressed in multiple tissues including the heart and vasculature. Of particular interest to vascular disorders is PPARγ, which regulates genes involved in characteristic vessel pathologies, such as cellular differentiation and growth, inflammation, ROS regulation, apoptosis, and angiogenesis.

PPARγ agonists and PPARγ overexpression increase endothelial NO release (Calnek et al., 2003). PPARγ agonists also decrease the expression of Nox-2 and -4 and significantly stimulate Cu/Zn SOD expression and activity in human umbilical vein endothelial cells (Hwang et al., 2005). Reduced endothelial PPARγ expression attenuates NO production and produces significant increases in serum d-ROMs, derivatives of reactive oxygen metabolites (Kleinhenz et al., 2009). Overexpression of PPARα and PPARγ reduces HIV-induced dysregulation of tight junction proteins in brain endothelial cells, effects mediated by alterations in matrix metalloproteinase (Huang et al., 2009). Moreover, PPARγ activation via rosiglitazone administration in brain microvascular endothelial cells inhibits adhesion and transendothelial migration of HIV-1 infected monocytes (Ramirez et al., 2008). The PPARγ agonist, rosiglitazone, also attenuates LPS-induced inflammation in vascular smooth muscle cells (Ji et al., 2011). Additionally, PPARs decrease endothelial-leukocyte interactions in atherosclerosis models (Kurebayashi et al., 2005) and PPAR agonists demonstrate antiviral activity.

PPAR agonists, rosiglitazone, PgJ2, ciglitazone, troglitazone and fenofibrate, inhibit HIV replication in HIV-1 infected peripheral blood mononuclear cells (PBMCs). These data indicate that decreases in PPAR activity may mediate endothelial dysfunction, ROS regulation, vascular injury, and HIV-1 replication. Although several have been unsuccessful due to patient safety concerns, PPAR agonists serve as an encouraging therapeutic for preventing HIV-associated vascular disorders.

Conclusion

In these studies, we demonstrate that HIV-1 protein expression exacerbates hypoxia-induced PAH. In addition, these studies implicate a novel pathway that may explain how HIV-1 proteins and specifically Tat may contribute to HIV-PAH development -- activation of the ALOX5 pathway. This pathway induces characteristic PAH pathologies such as pulmonary vascular tone and vascular remodeling by increasing endothelial cell proliferation. Our studies also implicate ALOX5 inhibition as a potential PAH therapy as zileuton and MK-886 administration attenuates endothelial proliferation.

CHAPTER 7

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