CARDIOPULMONARY PROTECTIVE ROLE OF THE ACE2-ANG-(1-7)-MAS AXIS IN LUNG AND HEART DISEASES

By

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To my Family and Friends, for their constant support and love

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Pulmonary hypertension and pulmonary fibrosis are lung diseases with poor diagnosis and limited therapeutic options. Current therapies are ineffective in improving the quality of life and reducing mortality rates. Thus, there is an unmet medical need to treat these diseases, which necessitates the discovery of novel therapeutic targets/agents for a safe and effective therapy. An altered renin-angiotensin system (RAS) comprising of the ACE-AngII-AT_1R axis has been implicated as a causative factor in the pathogenesis of these pulmonary diseases. Recently, a new member of the RAS, angiotensin converting enzyme 2 (ACE2) was discovered, which metabolizes active Angiotensin II to Angiotensin-(1-7). Angiotensin-(1-7) stimulates the Mas receptor to produce biological actions that are opposite to that of angiotensin II. Thus, ACE2-Ang-(1-7)-Mas form a protective axis that counteracts the deleterious effects of the ACE-AngII-AT_1R axis that appears to be responsible for the pathogenesis of a variety of diseases and end-organ damage. The studies presented in this dissertation were designed to characterize the cardio-pulmonary protective actions of the ACE2- Ang-(1-7)-Mas axis on lung diseases, particularly, pulmonary hypertension and pulmonary fibrosis. A monocrotaline model of pulmonary hypertension and a rat model of

bleomycin-induced pulmonary fibrosis were used to evaluate the protective effects of this axis. Lentiviral mediated lung overexpression of ACE2 and Ang-(1-7) rendered beneficial effects against both pulmonary hypertension and pulmonary fibrosis.

Next, we studied the intra-cardiac modulation of the RAS by 17β-estradiol to produce anti-cardiac remodeling actions in the deoxy-corticosterone (DOCA)-salt model of hypertension. Cardiac remodeling plays a detrimental role in the progression of cardiovascular diseases, leading to heart failure. There are abundant data to demonstrate that men are more prone to cardiovascular diseases (CVD) than premenopausal women of similar age. The incidence of CVD, however, increases substantially following menopause, suggesting that female sex hormones may render some degree of protection against heart diseases. Here, we report that estradiol replacement rendered cardioprotection via modulation of the local heart RAS to inhibit interstitial fibrosis and prevent cardiomyocyte hypertrophy.

CHAPTER 1
INTRODUCTION

Renin Angiotensin System

The renin angiotensin system (RAS) comprises of a cascade of proteolytic enzymes that play an important role in regulating arterial blood pressure, blood volume and tissue perfusion (Volpe et al., 2002). This RAS is composed of both a local and systemic components (Paul et al., 2006). For the systemic pathway, the cascade begins with the release of renin from the juxtaglomerular cells that line the afferent arteriole of the renal glomerulus. Renin release is regulated by a number of factors, which include decreased renal perfusion pressure, reduced kidney sodium reabsorption, sympathetic nerve stimulation and negative feedback by a direct action of Angiotensin II on the juxtaglomerular cells (Davis, 1973). Upon release into the bloodstream, renin acts on the circulating large molecular weight globulin, angiotensinogen to form the decapeptide, angiotensin I. Formation of angiotensin I is the rate limiting step in the RAS cascade. Angiotensin I is then acted upon by a zinc metalloprotease, angiotensin converting enzyme (ACE). ACE, which is predominantly expressed on the endothelial cells in the pulmonary circulation, cleaves two amino acids from the carboxyterminal of Angiotensin I to form the octapeptide Ang II (Riordan, 2003). Ang II is considered as the main vasoactive peptide of the RAS. Although ACE mediated generation of Ang II is the primary pathway, alternative enzymes of the nonrenin-angiotensin systems, such as cathepsin D, cathepsin G, tonin (Genest et al., 1983), or chymase (Wolny et al., 1997) can also catalyze Ang II formation. The biological actions of Ang II are mediated through stimulation of two types of G-protein coupled receptors, angiotensin type1 receptor (AT_1R) or angiotensin type2 receptor (AT_2R) (de Gasparo et al., 2000). Furthermore, in

rodents but not humans, two subtypes of AT_1R, namely $AT_{1a}R$ and $AT_{1b}R$ have been discovered (Iwai and Inagami, 1992). AT_1R is widely distributed in the vasculature, heart liver, kidneys, adrenal glands and brain (Shanmugam et al., 1996a). On the contrary, AT_2R is sparsely distributed in the adult tissues, found in the adrenal medulla, uterus, ovary, vascular endothelium, vascular smooth muscle and certain regions of the brain (Kambayashi et al., 1993; Tsuzuki et al., 1994; Allen et al., 1999). The expression of this receptor is high in the fetal tissues but decreases rapidly after birth and is present in low copy number in the adult (Shanmugam et al., 1996). However, under certain pathological conditions such as tissue injury following myocardial ischemia/infarction, the levels of AT_2R increase (Nio et al., 1995). Most of the biological actions of Ang II, which include vasoconstriction, release of aldosterone and vasopressin, inflammation and tissue fibrosis are mediated through activation of the AT_1R (Hunyady and Catt, 2006). Ang II is also known to have mitogenic properties, stimulating the release of cytokines and growth factors via the AT_1R (Sekiguchi et al., 2004). In contrast to AT_1R, AT_2R modulates organ development in the fetus and exhibits vasodilatory, anti-hypertrophic, anti-proliferative and anti-fibrotic effects in certain tissue organs, suggesting AT_2R mediates opposing actions to that of AT_1R (Jones et al., 2008). Over the past few years, novel angiotensin enzymes, peptides and receptors have been discovered. In this regard identification of a) Angiotensin-(1-12), an intermediate precursor peptide derived from angiotensinogen (Nagata et al., 2006); b) Angiotensin-(3-8), also called as Angiotensin IV and its putative receptor Ang IV receptor(AT_4R) (Swanson et al., 1992); c) the renin/prorenin receptor (Nguyen, 2006); d) angiotensin converting enzyme 2, a close homologue of ACE (Donoghue et al., 2000; Tipnis et al.,

2000); e) Angiotensin-(1-7) [Ang-(1-7)], the enzymatic product of ACE2 activity (Vickers et al., 2002); f) Mas as a receptor for Ang-(1-7) (Santos et al., 2003); and, g) angiotensin converting enzyme 3 (ACE3) (Rella M et al., 2007), have been seminal and added a new dimension to our understanding of the RAS, from a traditional linear cascade to a more complex non-linear cascade. Of the new members discovered so far, the ACE2-Ang-(1-7)-Mas axis has been a topic of intense basic and clinical research. Over the past several years, the concept of a local or tissue based RAS has emerged (Kumar et al., 2008; Paul et al., 2006). Support for this concept comes from the cloning of various components of the RAS genes (renin, angiotensinogen, angiotensin converting enzyme and angiotensin type 1 receptor) in multiple tissues (Paul et al., 1996; Tahmasebi et al., 1999). Also, studies from transgenic mice and rodent knock-out models of the RAS genes have highlighted the function and importance of a tissue based RAS (Lake-Bruse and Sigmund, 2000; Bader et al., 2001; Cole et al., 2003).

Angiotensin Converting Enzyme 2

In the year 2000, a close homologue of ACE, called as Angiotensin converting enzyme 2 (ACE2) was discovered by two independent research groups (Donoghue et al., 2000; Tipnis et al., 2000). The ACE2 gene maps to the X chromosome and encodes an 805 amino acid protein. ACE2 is a zinc metallopeptidase and shares 42% sequence homology with ACE. However, unlike ACE, which is a dipeptidyl-carboxypeptidase, ACE2 is a mono-carboxypeptidase and cleaves a single c-terminal amino acid of targeted peptides. ACE2 hydrolyzes the decapeptide, Ang I to form Ang-(1–9) and metabolizes Ang II, an octapeptide to Ang-(1–7). It was observed that the catalytic efficiency of ACE2 for metabolizing Ang II is 400-fold greater than that of Ang I (Vickers et al., 2002), indicating Ang II as the preferred substrate for ACE2 enzymatic

activity. ACE2 also acts on various other vasoactive peptides such as apelin-13, apelin-36, [des-Arg^{10}] kallidin, [des-Arg^{9}] bradykinin, neurotensin and the opioid peptide, dynorphin A (1–13). ACE2 expression is widely distributed in the whole body and has been found in the heart, kidneys, testes, gastrointestinal tract, brain and lungs (Crackower et al., 2002; Donoghue et al., 2000; Harmer et al., 2002). ACE2 is predominantly confined to the vascular endothelium and the renal tubular epithelium, and to a lesser extent on vascular smooth muscle cells (VSMC) (Crackower et al., 2002; Donoghue et al., 2000). The expression of ACE2 is significantly altered during pathological conditions such as hypertension, diabetes, cardiovascular and kidney diseases. Genetic knock-out of the ACE2 gene was associated with induction of hypoxia-response genes, increased circulating Ang II levels and impaired cardiac contractility in aged mice (Crackower et al., 2002). However, loss of the ACE gene in the ACE2 knock-out background reversed this cardiac phenotype, suggesting that ACE2 acts a negative regulator of ACE (Crackower et al., 2002). Overexpression of ACE2 has been associated with decreased systolic pressure (Yamazato et al., 2007), attenuation of cardiac remodeling (Díez-Freire et al., 2006) and stabilization of the atherosclerotic plaque (Dong et al., 2008). However, adverse events such as lethal cardiac arrhythmia in transgenic mice overexpressing ACE2 (Donoghue et al., 2003) and increased ventricular fibrosis with cardiac specific overexpression of ACE2 have been reported (Masson et al., 2009). The possible explanation for these adverse phenotypes may be related to an undefined role of ACE2 during development and the beneficial/deleterious effects of ACE2 against ventricular fibrosis could be a dose-dependent phenomenon. The significance of this enzyme in lung diseases gained

importance during the severe acute respiratory syndrome (SARS) outbreak when ACE2 was identified as a receptor for the SARS coronavirus (Li et al., 2003; Imai et al., 2005). SARS infection leads to a decrease in lung ACE2 levels followed by acute lung injury, resulting in respiratory failure and death. Recent studies have revealed that ACE2 might have a protective role against other lung diseases. Studies of Li et al. (2008) have demonstrated decreased expression of ACE2 in the lungs of patients with fibrotic lung diseases. Also, in an animal model of bleomycin–induced pulmonary fibrosis, ACE2 was shown to be downregulated, suggesting that this enzyme might have a protective role against lung fibrosis (Li et al., 2008). Studies from our own lab have established a beneficial role for ACE2 against pulmonary hypertension (Yamazato et al., 2009). In addition, our group has also demonstrated that activation of the endogenous ACE2 enzyme, using a synthetic molecule, prevented pulmonary hypertension and associated cardio-pulmonary remodeling in the monocrotaline model of PH (Ferreira et al., 2009).

Angiotensin-(1-7)

Angiotensin-(1–7) is a short, seven amino acid peptide formed by the enzymatic action of ACE2 on Angiotensin II (Vickers et al., 2002). Though ACE2 mediated formation of Ang-(1-7) is the major pathway, alternative routes of Ang-(1-7) generation do exist. Neprylysin, a neutral endopeptidase or thimet oligopeptidase can directly act on Angiotensin I to produce Ang-(1-7) (Yamamoto et al., 1992; Chappell et al., 2000). Another pathway of Ang-(1-7) production involves ACE2-mediated conversion of Ang I to Ang-(1-9), which is further acted upon by ACE to form Ang-(1-7). Studies of Schiavone et al. (1988) were the first to discover a biological role for Ang-(1-7) wherein, they reported infusion of Ang-(1-7) in the brain stimulated vasopressin release. Over the

years, a substantial amount of work has been carried out that has resulted in identifying several physiological actions of Ang-(1-7) and they include: a) release of nitric oxide (NO) from the vascular endothelium to mediate vasodilation (Seyedi et al., 1995); b) reduction in cellular growth and proliferation of vascular smooth muscle cells (Freemann et al., 1996); c) inhibition of cardiac remodeling, by preventing myocyte hypertrophy and interstitial fibrosis (Grobe et al., 2007); d) antiarrhythmic effects on the heart via activation of the sodium pump (De Mello, 2004); and e) attenuation of proteinuria in diabetic rats (Benter et al., 2007). The role of RAS in cardiovascular and kidney diseases is well established. Use of ACE inhibitors and Angiotensin receptor blockers (ARBs) are successful pharmacological therapies in the treatment of hypertension, heart failure, diabetes and coronary heart disease. It was observed that treatment with ACE inhibitors and ARBs increased circulating Ang-(1-7) levels (Allred et al., 2000; Igase et al., 2005; Iyer et al., 1998), suggesting that the protective effect of these agents may be in part mediated via Ang-(1-7). Also, Ang-(1-7) is known to counter-balance the deleterious actions of Ang II in the systemic circulation (Kucharewicz et al., 2002), as well as in tissues involved in cardiovascular regulation (Ferreira and Santos, 2005; Dilauro and Burns, 2008). Though Ang-(1-7) has potential therapeutic implications, there are certain limitations. Being a peptide, it cannot be administered orally. Also, it has a short biological half life as it undergoes rapid proteolytic degradation by circulating peptidases (Yamada et al., 1998). However, the recent discovery of AVE-0991, a novel orally active, nonpeptide mimetic of Ang-(1-7) represents an important step in this regard (Wiemer et al., 2002). Efforts are also being directed at making Ang-(1-7) analogues that would be resistant to proteolytic degradation (Kluskens et al., 2009;

Silva-Barcellos et al., 2001). Ang-(1-7) mediates its biological effects through stimulation of a G-protein receptor called Mas (Santos et al., 2003).

Mas Receptor

The Mas gene maps to chromosome 6 and codes for a G-protein cell surface receptor. The gene is highly conserved between species and was originally described as a proto-oncogene as it could render NIH 3T3 cells tumorigenic in nude mice (Young et al., 1988). High expression levels of the Mas protein are observed in the adult rodent testis while lower levels are found in the brain, kidney, heart, liver, lung and skeletal muscle (Metzger et al., 1995). Mas is predominantly confined to the vascular endothelium, where it mediates vasodilation through release of nitric-oxide (NO) (Sampaio et al., 2007). Knock-out of the Mas gene impairs endothelial function, lowers nitric oxide synthase expression and subsequently, decreases NO production. All these changes eventually lead to an elevation in blood pressure (Xu et al., 2008). Studies of Santos et al. (2003) established Ang-(1-7) as an endogenous ligand for the Mas receptor. Mas-deficient mice demonstrated decreased kidney binding of Ang-(1-7) and a blunted vasodilatory response of aortic rings to Ang-(1-7) treatment. Also, the Mas knock-out mice failed to evoke an anti-diuretic action of Ang-(1-7) to an acute water load. Furthermore, AVE0991, the synthetic mimetic of Ang-(1-7), which induces vasorelaxation and produces anti-diuresis in wild type mice, failed to evoke such responses in the Mas knock mice (Pinheiro et al., 2004). Collectively, these studies indicated that Ang-(1-7) mediates its effects through activation of the Mas receptor. Evidence from *in vitro* studies also confirmed that Ang-(1-7) is a Mas ligand, since Ang-(1-7) treatment of Mas transfected CHO-cells elicited arachidonic acid release (Santos

et al., 2003). The Mas receptor has been shown to activate a number of signaling pathways. *In vitro* studies using NIH 3T3 cells, Mas activated the GTP-binding protein Rac1, a member of the Rho family proteins (Zohn et al., 1998), while expression of Mas in HEK293 cells activated phospholipase C, suggesting that Mas couples to the $G\alpha_{q/11}$ family of heterotrimeric G proteins (Canals et al., 2006). However, in the heart, activation of the Mas by Ang-(1-7) was shown to phosphorylate protein kinase B, also known as Akt (Giani et al., 2007). The Ang-(1-7) stimulated cardiac Akt phosphorylation was blunted by A-779, a specific Ang-(1-7) antagonist. Over the years, interaction of Ang-(1-7) with the Mas receptor has uncovered important functional implications for vascular regulation and blood pressure control. It is becoming clear that the RAS is represented by two major arms; ACE-Ang II-AT_1R and ACE2-Ang-(1-7)-Mas, which act counter-regulatory to each other. The ACE-Ang II-AT_1R is responsible for vasoconstriction, proliferation and tissue fibrosis while the ACE2-Ang-(1-7)-Mas mediates vasodilatory, antiproliferative and antifibrotic effects.

Pulmonary Hypertension

Pulmonary hypertension (PH) is defined as a vascular disease wherein the mean pulmonary arterial pressure is more than 25mmHg at rest or greater than 30mmHg during exercise (Runo and Loyd, 2003). In a normal human, the mean pressure in the pulmonary artery is about 14mm Hg at rest. Pulmonary arteries and arterioles carry de-oxygenated blood from the right ventricle to the lungs for gas exchange. Oxygenated blood is then returned to the left atrium via the pulmonary venules and veins. The reasons for the abnormal elevation in pulmonary arterial pressure could be primary or secondary in nature. In case of primary PH, the etiology is unknown, whereas,

secondary PH could be as a result of other lung diseases or related diseases in other organs such as collagen vascular diseases, systemic lupus erythematosus or human immunodeficiency virus infection (Rabinovitch, 2008). Genetic and epigenic risk factors have also been identified for PH. Whatever may be the initial cause, PH is associated with vasoconstriction and vascular remodeling of the blood vessels supplying the lungs. Narrowing of the pulmonary vasculature increases resistance and disrupts normal blood flow, thus making it harder for the right ventricle to pump blood. Also, over time, the affected blood vessels undergo remodeling characterized by proliferation of arterial smooth muscle cells and accumulation of extracellular matrix, which further increases the blood pressure within the lungs. To compensate for the increased resistance and maintain normal blood flow, the right ventricle enlarges as an adaptive response. But prolonged period of increased workload on the right ventricle leads to maladaptive cardiac remodeling, making the heart less able to pump blood through the lungs and ultimately, culminating in right heart failure and death (Bogaard et al., 2009).

Angiotensin II (Ang II) plays a major role in the pulmonary vascular remodeling process. In vitro studies have demonstrated a hypertrophic or proliferative response of pulmonary artery smooth muscle cells to Ang II treatment, which is mediated via stimulation of the AT_1R (Morrell et al., 1999). Furthermore, *in vivo* studies have demonstrated increased expression of angiotensin converting enzyme (ACE) on the endothelial cells lining the pulmonary arteries (Morrell et al., 1995). ACE is the main enzyme involved in Ang II synthesis. Human studies have revealed an association between an ACE insertion/deletion polymorphism and PH (Kanazawa et al., 2000), though this association has been debated (Hoeper et al., 2003). The human ACE gene

contains an insertion or deletion of a 287-base pair in the intron 16. Although the insertion-deletion polymorphism is located in a noncoding region of the ACE gene, the deletion allele is associated with increased ACE activity and higher circulating levels of Ang II, while the insertion allele is associated with decreased ACE activity and lower Ang II levels. Abraham et al. (2003) have reported that the ACE DD genotype is significantly increased in patients with severe primary PH, suggesting that certain individuals may be genetically predisposed to developing PH. However, another study by van Suylen et al. (1999) observed a negative association. Nevertheless, pharmacological treatments with ACE inhibitors (Molteni et al., 1985) or AT_1R blockers (Kato et al., 2008) have shown protective effects in experimental models of PH. With the discovery of ACE2, its enzymatic product Ang-(1-7) and Mas, the receptor for Ang-(1-7), a new dimension has been added to the RAS. The ACE2-Ang-(1-7)-Mas axis has been shown to counterbalance the vasoconstrictive, proliferative and fibrotic axis of the ACE-AngII-AT_1R axis in several tissue organs.

Pulmonary Fibrosis

Pulmonary fibrosis (PF) is the end-result of lung injury characterized by architectural remodeling and excessive scarring of the lung tissue (Chua et al., 2005). Pulmonary fibrotic diseases have varied etiology, with an array of triggers that include infections, environmental toxins, radiation, allergens, chemicals and pollutants. However, the most common form of fibrotic lung diseases is idopathic pulmonary fibrosis, in which the inciting agent is not known. The alveolar-capillary membrane forms the basic structural unit of the lung and is the site for gaseous exchange. The alveolar-capillary membrane is composed of alveolar epithelium, the epithelial basement membrane, the interstitial stroma, the endothelial basement membrane and the capillary

endothelium. Following injury to the lung, a cascade of events ensue, that result in the release of chemokines, recruitment of leukocytes, inflammation and a dysregulated healing response that gradually evolves into a pathogenic fibrotic lesion with increased accumulation of extracellular matrix (ECM) components. These fibrotic conditions make the lungs rigid and incapable of carrying out normal gaseous exchange, ultimately resulting in death. The median survival time following diagnosis of idiopathic PF is 3-5 years. Although there are various mechanisms involved in the fibrotic process, a common observation is the terminal proliferation and progressive accumulation of the connective tissue replacing normal functional parenchyma. *In vivo* animal models have demonstrated a role for RAS in the fibrotic lung diseases (Antoniu, 2008; Uhal et al., 2007). Elevated levels of angiotensinogen and Ang II have been observed in pulmonary fibrotic patients (Molina-Molina et al., 2008; Li et al., 2007). Also, patients with fibrotic lung diseases have high concentration of ACE in their bronchoalveolar lavage fluid (Kelley, 1988; Specks et al., 1990). Use of ACE inhibitors (Wang et al., 2000; Uhal et al., 1998) or AT_1R blockers (Yao et al., 2006; Otsuka et al., 2004) ameliorated lung collagen deposition in both bleomycin and radiation mediated lung injury. A recent study of Li et al. (2008) also has reported a decrease in lung ACE2 expression and activity in both human and animal model of pulmonary fibrosis, suggesting a protective role for this enzyme against lung fibrosis.

Estrogen and Cardiac Remodeling

Cardiac remodeling, also known as ventricular remodeling refers to the changes in size, shape, and function of the heart. The remodeling may be physiological or pathological in nature. Physiological remodeling is observed in athletes and is characterized by increased ventricular cavity size and volume, but with normal systolic

and diastolic function (McMullen and Jennings, 2007). On the contrary, pathological remodeling is associated with diminished cardiac function and as the name suggests, pathological remodeling is associated with tissue injury, more commonly myocardial infarction (MI). Cardiac remodeling following MI is associated with myocyte hypertrophy, collagen deposition, and left ventricular dilatation (Sutton and Sharpe, 2000). The initial remodeling process is an adaptive response to replace the area of myocyte necrosis with myocardial scarring and compensate for the hemodynamic overload with enlarged myocytes. However, over a period of time the heart undergoes structural changes from elliptical to a more spherical shape with increased ventricular mass that contributes to the development of heart failure. Gender related differences in the incidence of heart failure are well documented (Mendelsohn and Karas, 2005). Premenopausal women are less prone to cardiovascular diseases than men of similar age. However, following menopause there is a sharp increase in women (Crawford and Johannes, 1999). These observations suggest that estradiol, the female hormone, might have a protective role to play against heart diseases. Estrogens have been known to modulate many hormonal systems involved in the regulation of cardiac structure and function. The RAS plays a key player in the cardiac remodeling process. Ang II, the main effector peptide contributes to ventricular fibrosis by promoting collagen synthesis of fibroblasts (Kumar et al., 2008). Also, Ang II stimulates the generation of the profibrotic cytokine, TGF-β (Lim and Zhu, 2006). Pharmacological inhibitors of ACE or AT_1R blockers have rendered protective actions against cardiac fibrosis and hypertrophy implicating the significance of Ang II in the remodeling process (Dendorfer et al., 2005). Estradiol has been shown to regulate several components of the RAS. Estrogen increases levels of

angiotensinogen (Healy et al., 1992) and AT_2R (Macova et al., 2008) while ACE (Turner and Hooper, 2002), prorenin (Szilagyi et al., 1995) neprylysin (Pinto et al., 1999), aminopeptidases (Seli et al., 2001) and AT_1R (Nickenig et al., 1998) are all decreased. Together, these findings indicate that estrogen modulates tissue and circulating RAS to exert cardioprotective effects.

Aims

Overall, the goal of these studies were to evaluate if: a) lentiviral mediated overexpression of Ang-(1-7) in the lungs would exert protective effects in a rat model of monocrotaline-induced pulmonary hypertension; b) Ang-(1-7) would attenuate bleomycin-induced pulmonary fibrosis and; c) 17β-Estradiol would prevent cardiac remodeling in the DOCA-salt model of hypertension through modulation of the intracardiac RAS. Together, these studies will demonstrate cardio-pulmonary protective actions of ACE2 and Ang-(1-7) in lung diseases, particularly pulmonary fibrosis and pulmonary hypertension and establish the intracardiac modulatory role of estradiol on the RAS in mediating anti-remodeling effects.

CHAPTER 2
LENTIVIRAL MEDIATED GENE DELIVERY OF ANGIOTENSIN-(1-7) ATTENUATES MONOCROTALINE-INDUCED PULMONARY HYPERTENSION IN RATS

Abstract

Recent studies from our lab revealed that overexpression of Angiotensin converting enzyme 2 (ACE2) or its activation using a synthetic molecule renders protective effects against monocrotaline (MCT)-induced pulmonary hypertension (PH). ACE2 is an ectopic enzyme that converts Angiotensin II to Angiotensin-(1-7) [Ang-(1-7)]. The present study was carried out to determine if overexpression of Ang-(1-7), the enzymatic product of ACE2 activity, would exert beneficial effects against PH. Lentivirus producing the Ang-(1-7)-fusion protein (lenti-Ang-(1-7)) was administered intra-tracheally into the lungs of male SD rats. Two weeks following lentiviral administration, PH was induced by a single subcutaneous injection of monocrotaline (MCT, 50mg/Kg). A subset of animals treated with lenti-Ang-(1-7) received the Mas antagonist (A-779, 2.5µg/h via osmotic pumps) along with MCT administration, to determine if the beneficial effects of Ang-(1-7) were mediated through stimulation of the Mas receptor. After 28 days of MCT administration, right ventricular systolic pressure (RVSP), a surrogate marker for pulmonary arterial pressure was measured, followed by heart excision to examine right ventricular hypertrophy (RVH) and right ventricular fibrosis (RVF). MCT administration resulted in a significant increase in RVSP, accompanied by development of RVH and RVF. Overexpression of Ang-(1-7) significantly attenuated MCT-induced elevations in RVSP, RVH and RVF. However, the beneficial effects of Ang-(1-7) against MCT-induced PH were completely abolished with the co-administration of A-779. Furthermore, MCT challenged rats exhibited increased

pulmonary vessel wall thickening and enhanced lung mRNA levels of the pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), which was significantly attenuated by Ang-(1-7) overexpression. Our observations demonstrate that Ang-(1-7) attenuates MCT-induced PH and the associated disease pathophysiology by stimulating the Mas receptor, suggesting that targeting the ACE2- Ang-(1-7)-Mas axis may be a potential therapeutic approach for treating and controlling PH.

Introduction

Pulmonary hypertension (PH) is a devastating disease characterized by increased pressure in the pulmonary circulation that progressively leads to heart failure and death (Runo and Loyd, 2003). Genetic and environmental factors play a crucial role in the etiology of this disease (Humbert et al., 2004). One of the causal factors implicated in the development of this disease is an activated renin angiotensin system (RAS) comprising of the ACE-AngII-AT_1R axis (Cargill and Lipworth, 1995). Increased activity of intrapulmonary ACE and its enzymatic product, Ang II have been reported in pulmonary hypertensive patients (Morrell et al., 1995; Orte et al., 2000). Ang II acts on the angiotensin type 1 receptor (AT_1R) present on the cardio-pulmonary system to cause deleterious effects such as vasoconstriction, proliferation, inflammation and fibrosis, which are all characteristic features of PH. Recently, a homologue of ACE, called the angiotensin converting enzyme 2 (ACE2) was discovered (Donoghue et al., 2000; Tipnis et al., 2000) and has been shown to counteract most of the detrimental actions of Ang II in various tissue organs possibly through generation of its enzymatic product, Angiotensin-(1-7) [Ang-(1-7)] (Santos et al., 2008). Studies from our own lab have demonstrated that overexpression of ACE2 or its activation using a synthetic molecule renders protective effects against monocrotaline (MCT) induced PH

(Yamazato et al., 2009; Ferreira et al., 2009) in rodents. Furthermore, higher ACE2 activity with increased Ang-(1-7) formation has been reported in the heart ventricles of patients suffering from primary pulmonary hypertension suggesting a cardio-pulmonary protective role for ACE2 and Ang-(1-7) (Zisman et al., 2003). Collectively, these observations led us to propose that overexpression of Ang-(1-7) might have beneficial effects against PH. Administration of MCT, a plant-derived alkaloid is used as a standard animal model for studying PH. MCT is metabolized in the liver by cytochrome P450 3A to a highly reactive pyrrole derivative (Kasahara et al., 1997), which selectively damages the pulmonary endothelial lining to cause infiltration of mononuclear cells and muscularization of pulmonary arteries resulting in PH (Lame et al., 2000).

Materials and Methods

Animals

Male Sprague Dawley rats, 5 weeks of age were used in this study. All animals were housed in a temperature-controlled room (25 ± 1^{o}C) and were maintained on a 12:12 hour light: dark cycle with free access to water and food. All procedures involving experimental animals were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Florida and complied with NIH guidelines.

Cloning of Ang-(1-7)-fusion Gene in Lentiviral Vector and Production of Lenti-Ang-(1-7) Viral Particles:

The Ang-(1-7)-fusion transgene construct used in our study (Fig 2-1A) has been described elsewhere (Santos et al., 2004). Briefly, the human prorenin signal peptide and the immunoglobulin fragment from mouse IgG2b were linked to a portion of the human prorenin prosegment and the *Bgl*II site after the prorenin segment was used to insert a furin cleavage site and the coding sequence for Ang-(1-7). This construct (Fig

1A) was cloned into the PCR-Blunt TOPO vector (Invitrogen, Carlsbad, CA) and then subcloned from this vector into pTY-EF1α to set it under the control of human elongation factor1α. Vesicular stomatitis virus G protein (VSV-G) pseudotyped lentiviral particles were prepared as previously described (Coleman et al., 2003). The virus containing supernatant was collected, concentrated, and titered. Concentration of viral particles was determined using the HIV-1 p24 antigen ELISA assay (Beckman Coulter, Fullerton, CA), following the manufacturer's instructions. A control virus without the gene construct was prepared employing similar methodology.

Measurements of Efficacy of lenti-Ang-(1-7):

Rat cardiac myoblasts (H9C2) from American Type Culture Collection (Manassas, VA) were used to determine the efficacy of lenti-Ang-(1-7). Ang-(1-7) is fused to the murine IgG2b using a furin cleavage site. The furin enzyme cleaves the fusion protein at this site to release equal amounts of Ang-(1-7) and IgG2b. Efficacy of the lentivirus in producing Ang-(1-7) was indirectly tested by measuring the secreted levels of murine IgG2b isotype. The cardiac myoblasts were maintained in DMEM supplemented with 4 mM L-glutamine and 10% fetal bovine serum. Cells were grown to 80% confluency (24 well culture plate), when they were transduced with lenti-Ang-(1-7) at a concentration of 10 multiplicities of infection (MOI) in the presence of 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO). After 6 hours of viral transduction, the viral media was replaced with fresh growth medium. After 72h, the secreted levels of murine IgG2b isotype was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). For the *in vivo* experiments, six weeks after intra-tracheal administration of the lentivirus, Ang-(1-7) levels in the rat lungs were measured by radioimmunoassay (RIA), as previously described (Santos et al., 2004).

Monocrotaline-Induced Pulmonary Hypertension:

Two weeks following gene transfer, PH was induced by single subcutaneous (s.c) injection of monocrotaline (50mg/kg; Sigma-Aldrich). Control rats received saline (500µl, s.c). At the same time of MCT administration, a subset of lenti-Ang-(1-7) animals was implanted with osmotic minipumps (model 2ML4) releasing 60µg/day (1mg/mL stock solution) of A-779, a Mas antagonist, for 28 days.

Systemic and Right Ventricular Blood Pressure Measurements:

Weekly systemic blood pressure was measured in conscious rats by the tail-cuff method. Briefly, rats were warmed using an infra-red lamp for 5 minutes before restraining them in a temperature controlled Plexiglas cage. A pneumatic pulse sensor was attached to the tail distal to an occluding cuff controlled by a Programmed Electro-Sphygmomanometer (Narco Bio-Systems, Austin, TX). Voltage output from the cuff and the pulse sensor were recorded and analyzed by a PowerLab signal transduction unit and associated Chart software (AD Instruments, Colorado Springs, CO). Systolic blood pressure values from each animal were determined by averaging a minimum of five separate indirect pressure measurements. All pressures were performed in the morning between 9 AM and noon by the same individual. For measurement of RVSP, rats were anesthetized with subcutaneous injection of a rodent cocktail mixture of ketamine (30mg/kg), xylazine (6 mg/kg) and acepromazine (1mg/kg), after which they were placed in a supine position, breathing room air. The RVSP was measured using a silastic catheter inserted into the right descending jugular vein and forwarded to the RV. The data was recorded after stabilization of the tracing using a liquid pressure transducer, which was interfaced to a PowerLab (AD Instruments) signal transduction unit. The waveform was used to confirm the positioning of the catheter in the RV. Data

were analyzed by using the Chart program that was supplied with the PowerLab system. After RVSP was measured, the mice were euthanized, and the hearts and lungs were harvested.

Hypertrophy and Histological Analysis:

The RV was separated from the left ventricle (LV) plus septum and the wet weights were determined. RV hypertrophy was assessed from the weight ratio of RV to LV plus septum (RV/LV+S). The right ventricle was then fixed in 10% formalin for 24 hrs and transferred to 70% ethanol until processed. The lungs were excised along with the trachea. The right primary bronchus was tied off and the four lobes of the right lung, distal to the ligature were cut and snap frozen in liquid nitrogen. The left lungs were perfused through the trachea with PBS followed by 4% para-formaldehyde, after which the left primary bronchus was tied off. The lungs were kept in 4% para-formaldehyde for 24 hrs and then replaced with phosphate buffered saline (PBS) until processed. After fixation and paraffin embedding, 5µm-thick sections of right ventricle and lung tissues were obtained. The lung tissue was stained with anti-α smooth muscle actin (SMA) (1:600, clone 1A4, Sigma, St Louis, Mo), as described previously (Jones et al., 1999). The external diameter and medial wall thickness were measured in 30 muscular arteries per lung section for analysis of the medial wall thickness of the pulmonary arterioles. The medial thickness was calculated as follows: percent wall thickness = [(medial thickness x 2)/external diameter] x100 (n=4-5 rats per group). Picro-sirius red staining was carried out to assess the extent of collagen deposition in the right ventricles. Quantification of interstitial fibrosis was carried out by performing percent area analysis using the ImageJ software from the NIH.

RNA Isolation and Real-time PCR:

Total RNA was extracted from frozen lung tissues using the RNaqueous-4RCP Kit (Ambion, Foster City, CA). cDNA samples obtained by reverse transcription reactions were amplified by quantitative real-time polymerase chain reaction, to determine the expression levels of ACE2, GP91phox, IL-1b, IL-6, TNF-α, and IL-10 using Taqman probes and primer pairs in an ABI Prism 7900 sequence detection system. mRNA levels of target genes were normalized to 18s RNA from the same samples. The $2^{-\Delta\Delta}$CT method was used to calculate relative changes in target gene expression. 18s RNA was used as an endogenous control to normalize expression levels of target genes.

Statistical Analysis:

Data are presented as means ± SEM. Statistical differences were evaluated by either one-way ANOVA or two-way ANOVA wherever applicable, followed by the Newman-Keuls test. p values <0.05 were considered statistically significant.

Results

Efficacy of the Lentivirus to Overexpress Ang-(1-7)

Infection of cardiac myoblasts with 10 multiplicity of infection (MOI) of lenti-Ang-(1-7) fusion protein resulted in a robust expression of IgG2b isotype (Fig. 2-1B). Furthermore, *in vivo* experiments demonstrated increased levels of Ang-(1-7) peptide in the lenti-Ang-(1-7) infected rat lungs compared to control animals (Fig. 2-1C). These data establish that lenti-Ang-(1-7) is active and effective in secreting Ang-(1-7).

Effect on RVSP and Associated Cardiac and Lung Remodeling

A single subcutaneous administration of MCT in rats resulted in massive increase in RVSP in 4 weeks (30.8 ± 2.6 mmHg in control vs 67.5 ± 7.3 mmHg in MCT, $p<0.05$,

n=4-7, Fig. 2-2). This increase was associated with the development of right ventricular hypertrophy as measured by the RV/LV+S weight ratio (0.29 ± 0.004 in control vs 0.55 ± 0.05 in MCT, $p<0.05$, n=4-7, Fig. 2-3). Overexpression of Ang-(1-7) significantly attenuated MCT-induced elevations in RVSP (47 ± 7 mmHg, $p<0.05$, n=9, Fig. 2-1) and RVH (0.41 ± 0.04, $p<0.05$, n=9, Fig. 2-3) when compared to the MCT group. Furthermore, MCT treatment resulted in increase in the medial wall thickness, which also was significantly attenuated by Ang-(1-7) overexpression (Fig. 2-4). Rats treated with MCT also exhibited increased interstitial right ventricular fibrosis (5.3 ± 0.6% in control vs. 13.0 ± 0.7% in MCT, $p<0.05$, n=5-7, Fig. 2-5). This effect was partially attenuated with Ang-(1-7) treatment (10.1 ± 0.4 in MCT +lenti-Ang-(1-7), n=5, Fig. 2-5). Co-administration of A-779 in the Ang-(1-7) treated MCT animal completely blocked the beneficial effects of Ang-(1-7) (Fig. 2-2, 2-3, 2-5). In contrast to RVSP, the systemic blood pressure did not change with MCT treatment or overexpression of Ang-(1-7) in any of the groups (Control, 120$\pm$7 mmHg, n=4; MCT, 119$\pm$3 mmHg, n=8; MCT+lenti-Ang-(1-7), 115+2mmHg, n=9).

Protective Actions of Ang-(1-7), Possibly Mediated through Modulation of Lung Pro- And Anti-Inflammatory Cytokines.

Next, we studied the effects of lenti-Ang-(1-7) on cytokines since they have been shown to play a crucial role in the pathology of PH. Table 2-1 shows relative fold-change in the lung cytokine mRNA levels of control, MCT and MCT+ lenti-Ang-(1-7) treated rats. MCT treatment resulted in significant increases in the mRNA levels of ACE and pro-inflammatory cytokines (TNF-α, IL-1b, IL-6). GP91(phox), a subunit of the NADPH oxidase, tended to be increased by MCT treatment, but this increase did not reach statistical significance (Table 1). On the other hand, Ang-(1-7) treatment of MCT-

challenged rats resulted in the reversal of this pattern with decreased levels of TNF-α, IL-1b, IL-6, GP91(phox) and ACE. In contrast, there was a significant increase in the anti-inflammatory cytokine (IL-10) and ACE2 mRNA levels by Ang-(1-7) treatment of the MCT-challenged rats.

Discussion

The novel finding of the present study is that overexpression of Ang-(1-7) through lentiviral mediated gene delivery prevented the development of MCT-induced PH in rats. Administration of MCT resulted in elevated right ventricular systolic pressure, right ventricular enlargement, right ventricular fibrosis and structural changes of lung vasculature. Lenti-Ang-(1-7) treatment, prior to induction of PH, resulted in amelioration of the elevated pulmonary pressure and attenuation of the remodeling effects on the lung and heart. Stimulation of the ACE-AngII-AT_1R axis mediates vasoconstriction (Touyz, 2003), proliferation (Schelling et al., 1991) inflammation (Marchesi et al., 2008) and tissue fibrosis (Lijnen et al., 2001). On the contrary, the recently discovered counter-regulatory arm of RAS comprising of ACE2-Ang-(1-7)-Mas has been shown to exert vasodilatory, antiproliferative, anti-inflammatory and antifibrotic effects (Santos et al., 2008). Studies from our lab demonstrated that lung overexpression of ACE2 prevented and reversed MCT-induced PH (Yamazato et al., 2009) in mice. Also, use of a synthetic activator of the endogenous ACE2 enzyme exerted protective effects against PH (Ferreira et al., 2009). In the current study, we observed that overexpression of Ang-(1-7), the catalytic product of ACE2 enzyme, ameliorated elevation in RVSP induced by MCT administration. The beneficial actions of Ang-(1-7) against PH were also associated with anti-cardiac remodeling effects, which may be secondary to the decrease in pulmonary pressure.

Right ventricular remodeling characterized by hypertrophy and fibrosis are risk factors for right heart failure in pulmonary hypertensive patients (Haworth, 2007). Ang-(1-7) overexpression prevented right ventricular hypertrophy and fibrosis. These cardio-protective effects of Ang-(1-7) are consistent with previous findings in the left ventricle of hypertensive rats (Grobe et al., 2007). Structural changes in the pulmonary vasculature, characterized by medial wall thickening of the pulmonary arterioles is a prominent feature of human PH. Thickening of the pulmonary arterioles increase pulmonary arterial resistance contributing to the elevated pressure (Morrell et al., 2009). MCT-challenged animals exhibited marked increase in the wall thickness of pulmonary resistant vessels. Overexpression of Ang-(1-7) resulted in near normal vessel morphology. It is likely that attenuation of vascular remodeling by Ang-(1-7) is due to inhibition of pulmonary arterial smooth muscle proliferation, as *in vitro* studies have demonstrated anti-proliferative properties of Ang-(1-7) in cultured vascular smooth muscle cells (Clark et al., 2001; Freeman et al., 1996). The beneficial effects of lenti-Ang-(1-7) against MCT-induced PH were abolished by co-administration of A-779, a Mas antagonist, suggesting that Ang-(1-7) mediates its effects through stimulation of this receptor.

Increase in the expression of pro-inflammatory cytokines is a characteristic hallmark of PH and correlates with the disease severity (Dorfmüller et al., 2003). In line with these findings, elevated lung mRNA levels of pro-inflammatory cytokines were observed in the MCT alone group. On the otherhand, overexpression of Ang-(1-7) was associated with a decrease in the mRNA levels of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) but an increase in the anti-inflammatory cytokine (IL-10). XNT, an ACE2 activator was

also shown to increase lung IL-10 expression in the MCT model of PH (Ferreira et al., 2009). Anti-inflammatory therapy is currently being investigated for the treatment of PH. A recent report by Ito et al. (2007) demonstrated that overexpression of IL-10 exerts beneficial effects against animal model of PH. One reason for the observed favorable changes in cytokine levels with Ang-(1-7) overexpression may be due to modulation of intra-pulmonary RAS, which is known to be a potent regulator of cytokines and inflammation (Marchesi et al., 2008). However, a direct effect of Ang-(1-7) on the immuno-modulatory system may not be ruled out. Ang-(1-7) treatment prevented the MCT-induced increase in ACE mRNA levels, but significantly upregulated ACE2 levels. It is surprising that ACE2 expression was increased by Ang-(1-7) treatment. A similar increase in ACE2 expression was also observed with XNT treatment. Also, cardiac-specific overexpression of Ang-(1-7) resulted in increased ACE2 levels in the heart (unpublished data). It is purely speculative that a positive feedback mechanism may be responsible for this observed phenomenon. Nevertheless, this explanation clearly warrants further evaluation.

Lung overexpression of Ang-(1-7) did not lower basal systemic arterial pressure (BP), proving selectivity of this approach for pulmonary circulation. This is an important aspect to be considered during treatment, since patients in advanced stages of right ventricular overload would be already hypotensive and any further decrease in pressure, due to treatment could prove detrimental. Our earlier studies involving pulmonary overexpression of ACE2 (Yamazato et al., 2009) or a synthetic ACE2 activator (Ferreira et al., 2009) rendered protective effects against MCT-induced PH. Collectively, these studies indicate that targeting of the ACE2-Ang-(1-7)-Mas axis could

provide novel therapeutic strategy in the treatment of PH and its associated complications. In summary, our data demonstrate that overexpression of Ang-(1-7) attenuates MCT-induced PH. These beneficial effects involve inhibition of pro-inflammatory cytokines and are mediated by Mas, the receptor for Ang-(1-7). Our results suggest that Ang-(1-7) gene transfer holds novel potential for the treatment of PH.

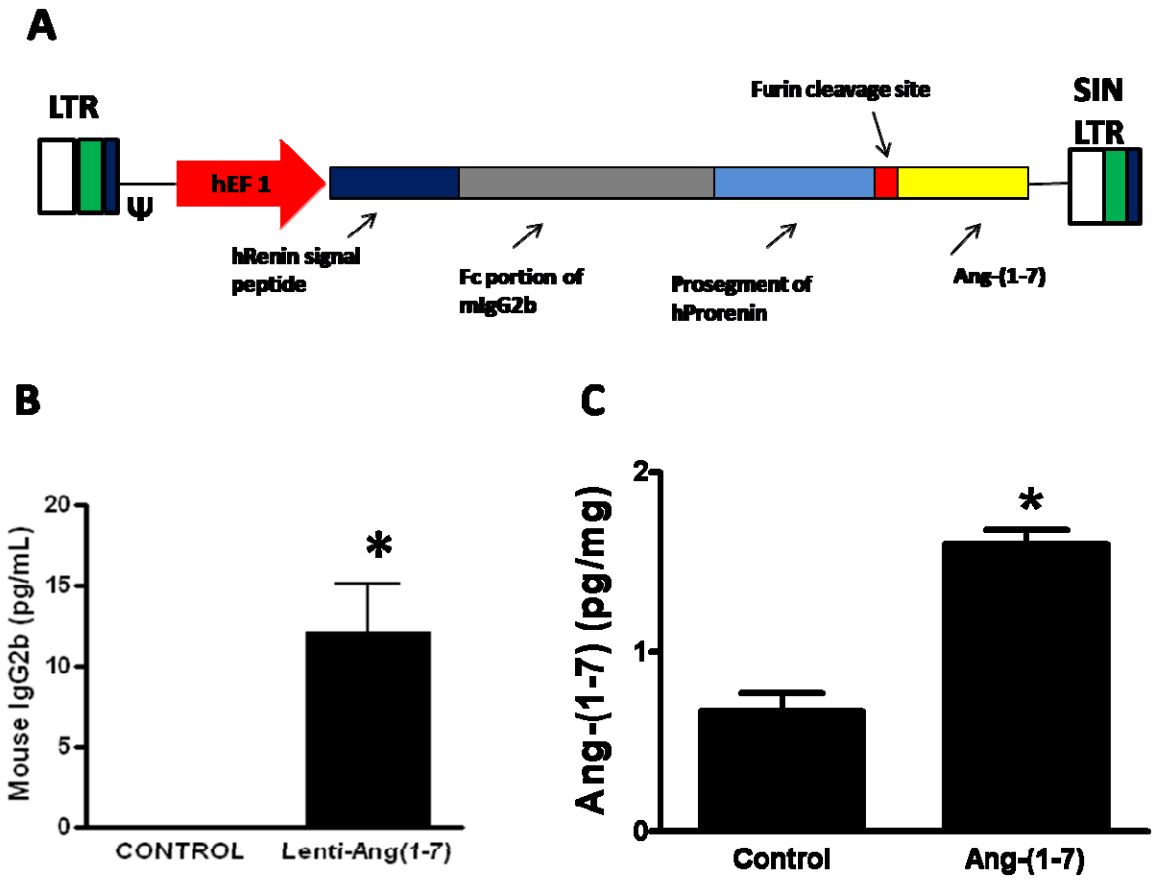

Figure 2-1: Construct and efficacy of the lenti-Ang-(1-7). (A) Lentiviral construct of Ang-(1-7). (B) Lenti-Ang-(1-7) caused a significant increase in the levels of mouse IgG2b after 72 hrs of infection in the H9C2 myoblasts. (C) In vivo administration of lenti-Ang-(1-7) significantly increased the levels of Ang-(1-7) in the rat lungs after 6 weeks of gene transfer. Data are represented as mean ± SEM. * $p<0.05$ vs control, (n=4-5 per group).

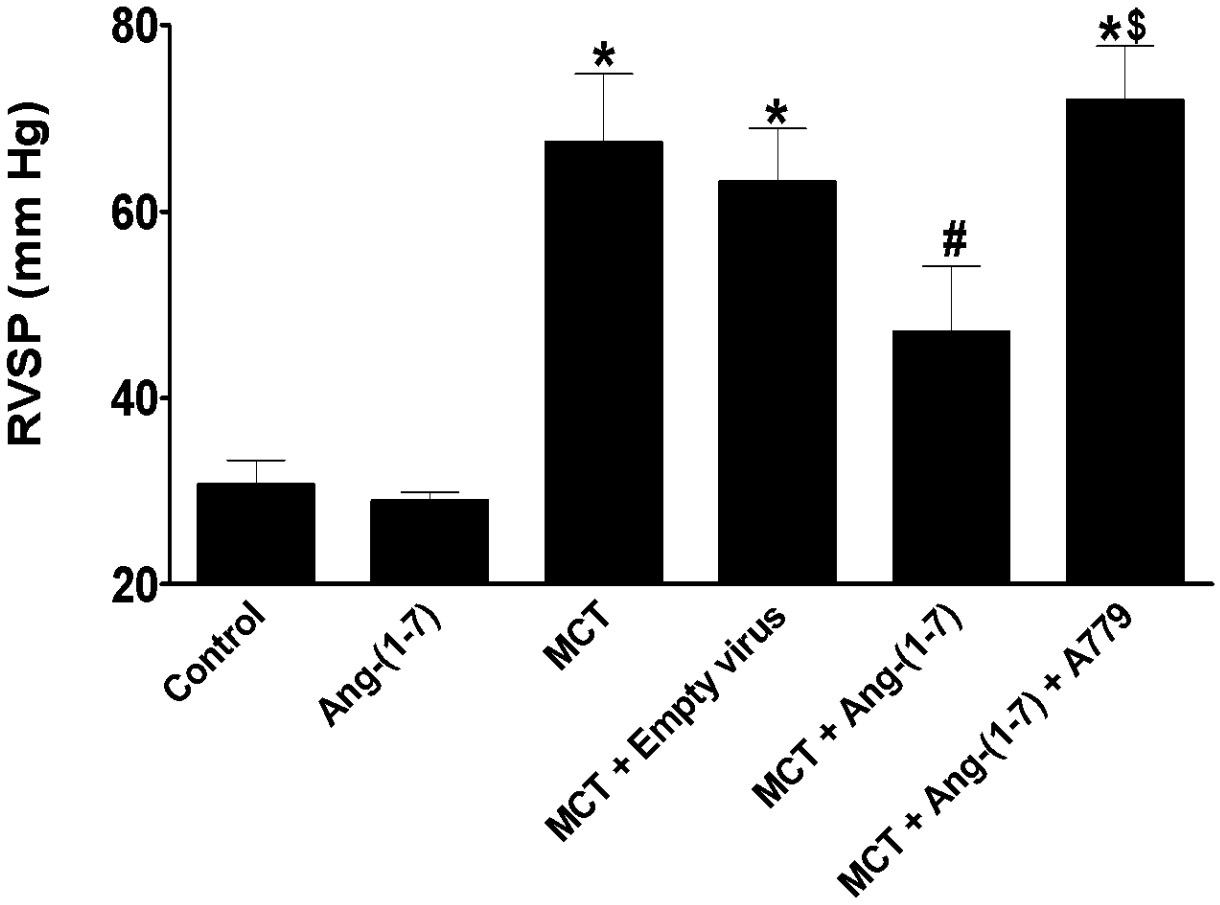

Figure 2-2: Effects of Lenti-Ang-(1-7) on MCT-induced PH.MCT administration caused a significant increase in the RVSP after 4 weeks, which was significantly attenuated by lenti-Ang-(1-7) treatment. This beneficial effect of Lenti-Ang-(1-7) treatment was lost upon blockade of the Mas receptor with A-779. Data are represented as mean ± SEM. * $p<0.05$ vs control, # $p<0.05$ vs MCT, $ vs MCT + Ang-(1-7), (n=3-9 per group).

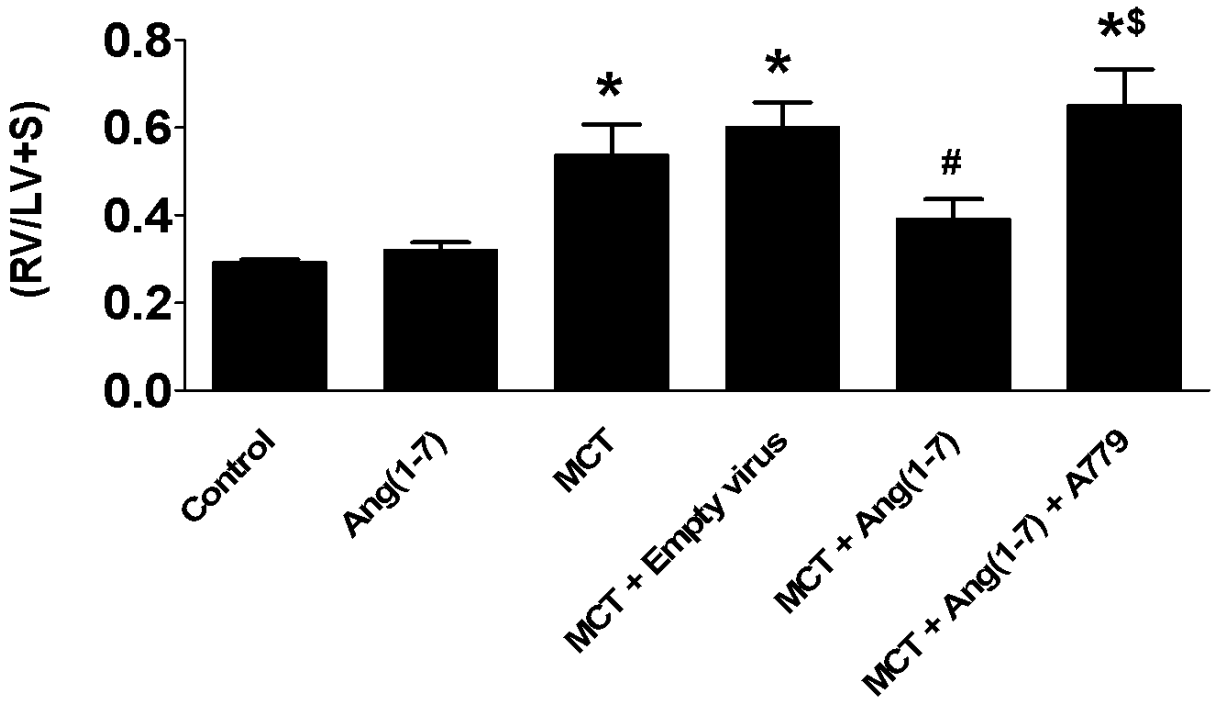

Figure 2-3: Effects of Lenti-Ang-(1-7) on MCT-induced RV hypertrophy. MCT administration resulted in the development of right ventricular hypertrophy as indicated by a significant increase in the RV/LV+S ratio. This increase in ratio was prevented by Ang-(1-7) overexpression. Blockade of the Mas receptor with A-779 resulted in the loss of the beneficial effect of Ang-(1-7) on right ventricular enlargement. Data are represented as mean ± SEM. * $p<0.05$ vs control, # $p<0.05$ vs MCT group, $ vs MCT + Ang-(1-7), (n=3-9 per group).

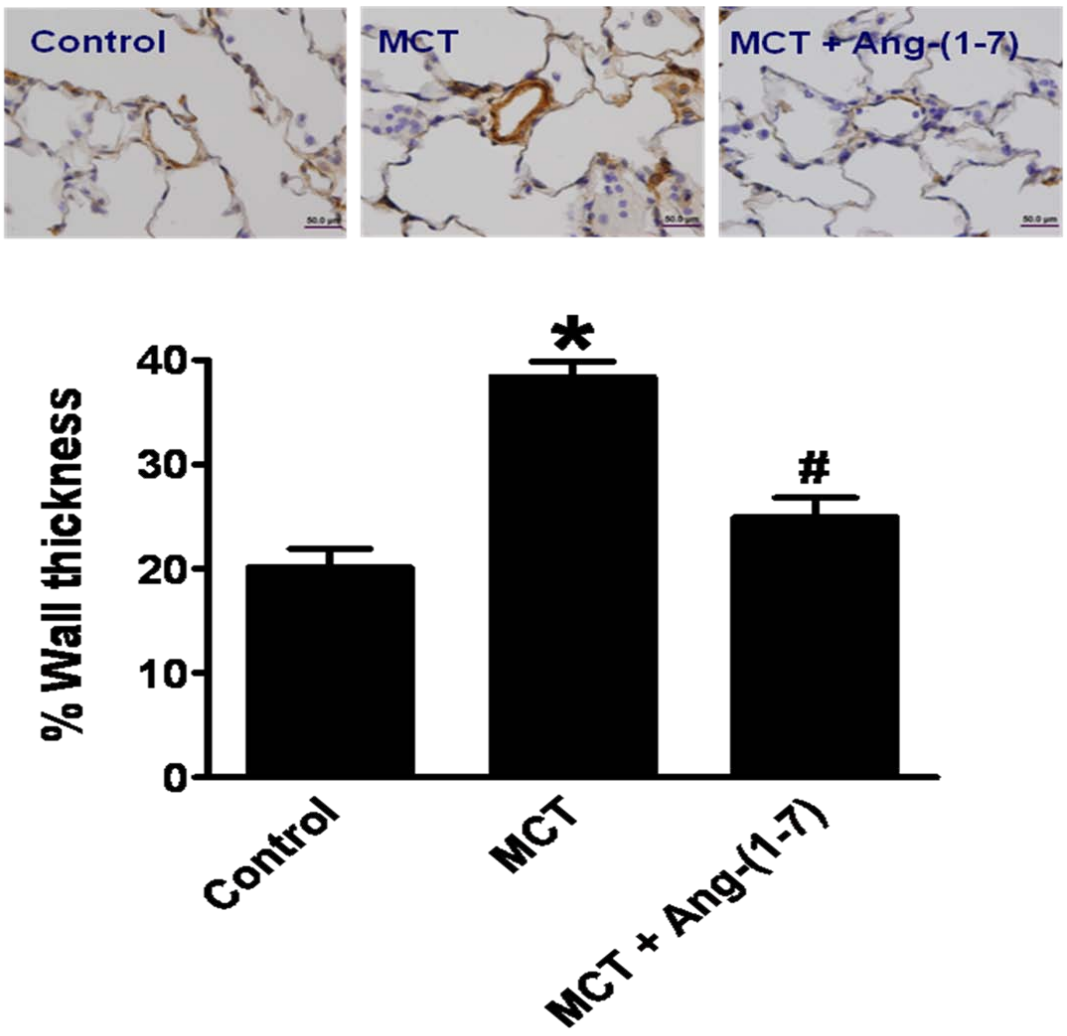

Figure 2-4: Effect of Lenti-Ang-(1-7) on pulmonary vessel wall thickness.The vascular hypertrophic effect of MCT, indicated by the brown staining of the pulmonary vasculature, was significantly blunted by lenti-Ang-(1-7) treatment. Data are expressed as mean ±SEM. * $p<0.05$ vs. control, # $p<0.05$ vs MCT, (n=4-5 per group).

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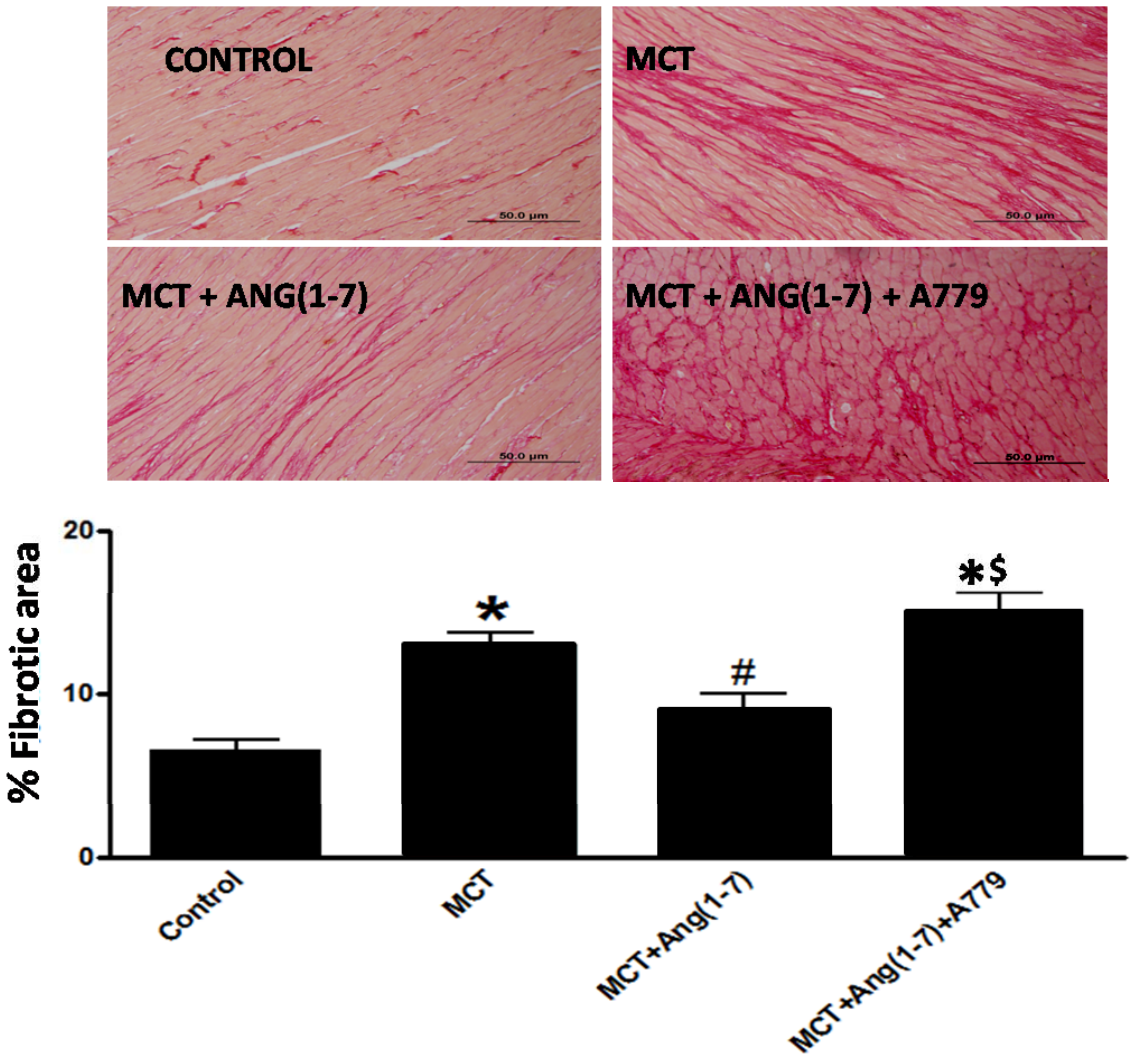

Figure 2-5: Effect of lenti-Ang-(1-7) treatment on right ventricular fibrosis. MCT administration caused a significant increase in the right ventricular fibrosis, which was partially blocked by lenti-Ang-(1-7). This beneficial effect was totally lost on co-administration of A-779, a Mas antagonist. Data are represented as mean ± SEM. * $p<0.05$ vs control, # $p<0.05$ vs MCT, $ vs MCT + Ang-(1-7), (n=3-5 per group).

Table 2-1. Relative fold-change in lung RAS and cytokine mRNA levels.

Gene	Control	MCT	MCT + Ang-(1-7)
ACE	0.85 ± 0.37(4)	2.89 ± 0.45*(7)	1.22 + 0.1#(4)
ACE2	0.82 ± 0.28(4)	3.18 ± 0.69(6)	4.89 ± 1.93*(4)
IL-10	1.22 ± 0.38(4)	2.68 ± 0.44(6)	4.13 ± 0.33*(5)
IL-1b	1.6 ± 0.57(4)	4.62 ± 1.00*(7)	1.02 ± 0.41#(4)
IL-6	1.63 ± 0.74(4)	16.37 ± 4.59*(7)	2.1 ± 0.61#(4)
GP91(Phox)	1.96 ± 0.97(4)	3.6 ± 1.22(7)	1.78 ± 0.84(4)
TNF-α	1.08 ± 0.53(4)	6.7 ± 1.53*(7)	2.34 ± 0.65#(6)

* Signifies $p<0.05$ vs control, # $p<0.05$ vs MCT. Numbers within parenthesis refer to the sample size.

CHAPTER 3
CARDIO-PULMONARY PROTECTIVE EFFECTS OF LENTIVIRAL MEDIATED OVEREXPRESSION OF ANGIOTENSIN-(1-7) IN BLEOMYCIN INDUCED LUNG FIBROSIS

Abstract

An activated vasoconstrictive, proliferative and fibrotic axis of the renin angiotensin system (RAS; ACE-AngII-AT_1R) has been implicated in the pathophysiology of pulmonary fibrosis (PF). The recent discovery of a counter-regulatory axis of the RAS comprising of ACE2-Ang-(1-7)-Mas has led us to examine the role of this vasoprotective axis on fibrotic lung diseases. We hypothesized that lentiviral mediated overexpression of Angiotensin converting enzyme 2 (ACE2) or its enzymatic product , Angiotensin-(1-7) [Ang-(1-7)], in the rat lungs would exert protective effects against bleomycin-induced PF. Lentiviral packaged ACE2 or Ang-(1-7)-fusion gene ($3x10^8$ transducing units, TU) was intra-tracheally administered into the lungs of male SD rats. Two weeks following gene transfer, animals were administered with bleomycin (2.5mg/kg). After 15 days of bleomycin administration, we observed significant increase in right ventricular systolic pressure (RVSP) followed by development of right ventricular hypertrophy (RVH) in the bleomycin alone group. The lungs of these bleomycin treated animals also exhibited excessive collagen deposition, decreased expression of ACE and ACE2, increased mRNA levels for TGF-β, pro-inflammatory cytokines and increased protein levels of the AT_1R. Overexpression of Ang-(1-7) significantly prevented all the above mentioned pathophysiological conditions. Similar protective effects against lung fibrosis were also obtained with lenti-ACE2 overexpression. In conclusion, our data demonstrate that lentiviral mediated overexpression of ACE2 or Ang-(1-7) ameliorates bleomycin-induced

pulmonary fibrosis and cardiac remodeling in rats and thus, may have a therapeutic potential to treat fibrotic lung diseases

Introduction

Pulmonary fibrosis (PF) is a progressive and ultimately fatal lung disease characterized by excessive accumulation of matrix proteins, thickened alveolar walls and development of rigid lung causing an irreversible loss of the tissue's ability to carry out normal gaseous exchange (Gross and 2001). Existing therapies with corticosteroids and immunosuppressants are ineffective with limited efficacy and long-term side effects. As a result, the overall median survival time is 3 years from the time of diagnosis. Thus, the discovery of novel therapeutic targets/agents is critical for the treatment of PF. Animal and human studies have implicated the involvement of the renin-angiotensin system (RAS) in the pathogenesis of PF. Evidence for this conclusion includes the following: a) Angiotensinogen (AGT) has been found to be one of the most overexpressed genes in PF patients (Selman et al., 2006); b) polymorphism in the promoter region of the AGT gene has been associated with the disease progression (Molina-Molina et al., 2008); c) patients with fibrotic lung diseases have high concentration of angiotensin-converting enzyme (ACE) in their bronchoalveolar lavage fluid (Kelley, 1988; Specks et al., 1990); d) experimental models of PF have demonstrated increased concentrations of angiotensin II (Ang II) in lung tissue homogenates (Song et al., 1998); e) use of ACE inhibitors (Wang et al., 2000; Uhal et al., 1998) and angiotensin receptor blockers (Yao et al., 2006; Otsuka et al., 2004) have been shown to attenuate PF in animal models; f) Angiotensin converting enzyme 2 (ACE2) is significantly decreased in lung biopsies of patients with idiopathic PF (Li et al., 2008); and, g) decreased lung ACE2 activity has been reported in animal model of

bleomycin-induced pulmonary fibrosis (Li et al., 2008). Collectively, these observations suggest an important role for local RAS in lung fibrosis.

ACE2 is en ectopic enzyme that degrades Ang II to Angiotensin-(1-7) [Ang-(1-7)]. Since the level of this enzyme is decreased during lung fibrosis, our aim was to investigate whether overexpression of ACE2 or its enzymatic product, Ang-(1-7), would produce beneficial effects against bleomycin-induced pulmonary fibrosis. Ang-(1-7), is a heptapeptide that counteracts many of the detrimental effects of Ang II, including heart failure, kidney diseases and diabetic complications (Katovich et al., 2008; Iusuf et al., 2008). However, the role of Ang-(1-7) in pulmonary disorders, especially in PF has not yet been investigated. So, we decided to probe whether Ang-(1-7) may offer beneficial effects against this lung disease. Ang-(1-7) is a peptide that undergoes rapid proteolytic degradation in circulation, to have a very short biological half life (Yamada et al., 1998). To overcome this limitation, we decided to adopt a novel strategy of overexpressing this peptide locally in the lung tissue through a lentiviral mediated gene delivery approach. Thus, in this study we propose that lentiviral mediated pulmonary overexpression of Ang-(1-7) exerts cardio-pulmonary beneficial effects against experimental model of lung fibrosis.

Methods

Cloning of Murine ACE2 and Ang-(1-7)-Fusion Gene in Lentiviral Vector and Production of Lenti-Ang-(1-7) Viral Particles

The Ang-(1-7)-fusion transgene construct used in our study has been described elsewhere (Santos et al., 2004). Briefly, the human prorenin signal peptide and the immunoglobulin fragment from mouse IgG2b were linked to a portion of the human prorenin prosegment and the *Bgl*II site after the prorenin segment was used to insert a

furin cleavage site and the coding sequence for Ang-(1–7). This construct (Fig 1A) was cloned into the PCR-Blunt TOPO vector (Invitrogen, Carlsbad, CA) and then subcloned from this vector into pTY-EF1α to set it under the control of human elongation factor1α. Vesicular stomatitis virus G protein (VSV-G) pseudotyped lentiviral particles were prepared as previously described (Coleman et al., 2003). The virus containing supernatant was collected, concentrated, and titered. Concentration of viral particles was determined using the HIV-1 p24 antigen ELISA assay (Beckman Coulter, Fullerton, CA), following the manufacturer's instructions. A control virus without the gene construct was prepared employing similar methodology. The ACE2 lentivirus was prepared using murine ACE2 cDNA as previously described (Huentelman et al., 2005).

Measurements of Efficacy of Lenti-Ang-(1-7)

Rat cardiac myoblasts (H9C2) from American Type Culture Collection (Manassas, VA) were used to determine the efficacy of lenti-Ang-(1-7). Ang-(1-7) is fused to the murine IgG2b using a furin cleavage site. The furin enzyme cleaves the fusion protein at this site to release equal amounts of Ang-(1-7) and IgG2b. Efficacy of the lentivirus in producing Ang-(1-7) was indirectly tested by measuring the secreted levels of murine IgG2b isotype. The cardiac myoblasts were maintained in DMEM supplemented with 4 mM L-glutamine and 10% fetal bovine serum. Cells were grown to 80% confluency (24 well culture plate), when they were transduced with lenti-Ang-(1-7) at a concentration of 10 multiplicities of infection (MOI) in the presence of 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO). After 6 hours of viral transduction, the viral media was replaced with fresh growth medium. After 72h, the secreted levels of murine IgG2b isotype was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). For the *in vivo* experiments, six weeks after intra-tracheal

administration of the lentivirus, Ang-(1-7) levels in the rat lungs were measured by radioimmunoassay (RIA), as previously described (Santos et al., 2004).The efficacy of lenti-ACE2 was evaluated by measuring the mRNA levels of murine ACE2 in the infected rat lungs after 6 weeks of infection.

Animal Procedures and Treatment of Rats with Lenti-Ang-(1-7)

Male Sprague Dawley (SD) rats, 5 weeks of age were used in this study. All animals were housed in a temperature-controlled room (25 ± 1^{o}C) and were maintained on a 12:12 hour light: dark cycle with free access to water and food. All procedures involving experimental animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida and complied with NIH guidelines. For gene delivery into the lungs, rats were anesthetized with isoflurane and a midline incision was made to expose the trachea. Empty virus (control) or lenti-Ang-(1-7) viral particles (3x10^{8}TU in 100µl of PBS) were injected into the trachea followed by 300µl of air so as to enhance the spread of virus in the rat lungs. Two weeks following lentivirus treatment, animals were subjected to bleomycin administration.

Bleomycin-Induced Pulmonary Fibrosis

Animals under the influence of isoflurane anesthesia received a single intratracheal instillation of 2.5mg/kg dose of bleomycin sulfate (Calbiochem Labs) in 100 µl of sterile saline. The 100µl solution was instilled at end expiration, and the liquid was immediately followed by 300µl of air to increase delivery to the distal airways. Control animals received an equal volume of sterile saline.

Systemic and Right Ventricular Systolic Pressure (RVSP) Measurements

Weekly systemic blood pressure was measured in conscious rats using the non-invasive tail-cuff method (Narco Bio-Systems, Austin, TX). The animals were briefly

warmed under infra red light to increase blood flow to the tail. A pneumatic pulse sensor and an occluding cuff were attached to the tail. Voltage output from the cuff and the pulse sensor were recorded and analyzed using a PowerLab signal transduction unit and associated Chart software (AD Instruments, Colorado Springs, CO). Systolic blood pressure values from each animal were determined by averaging a minimum of three separate indirect pressure measurements. For measurement of RVSP, rats were anesthetized with subcutaneous injection of a mixture of ketamine (30mg/kg), xylazine (6mg/kg) and acepromazine (1mg/kg) and were placed in a supine position, breathing room air. The RVSP was measured using a silastic catheter inserted into the right descending jugular vein and forwarded to the RV. The data was recorded after stabilization of the tracing using a liquid pressure transducer, which was interfaced to a PowerLab (AD Instruments) signal transduction unit. The waveform was used to confirm the positioning of the catheter in the RV. Data were analyzed by using the Chart program that was supplied with the PowerLab system. After RVSP was measured, the rats were euthanized, and the hearts and lungs were harvested.

Hypertrophy and Histological Analysis

The right ventricle (RV) was separated from the left ventricle plus ventricular septum (LV+S), and their wet weights were recorded. RV hypertrophy was determined by calculating the ratio of RV to LV plus ventricular septum (RV/LV+S) weights. The RV was further processed for histological analysis of collagen content using picro-sirius red staining**.** The left lung was perfused with phosphate-buffered saline (PBS) followed by 4% para-formaldehyde at perfusion pressure of 22cm of water. For assessing inflammatory and fibrotic injury of bleomycin treated animals, lung sections were stained with haematoxylin-eosin (H&E) and picro-sirius red. Severity of lung fibrosis was scored

on a scale from 0 (normal lung) to 8 (completely fibrotic) as described previously (Ashcroft et al., 1988). Picro-sirius red quantification of collagen deposition was carried out by performing percent area analysis using the ImageJ software from the NIH as described elsewhere (Rasband, 1997).

Biochemical Studies for measuring lung hydroxyproline Content

Lung tissue was homogenized in 1.8 ml of glacial acetic acid (0.5mol/L) and dried in a speed vacuum. The dried sample was weighed, dissolved in 2ml of 6N HCl and hydrolyzed at 110°C overnight. The acid hydrolysates (10µl) and standards were applied to an ELISA plate along with 10µl of citric/acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% NaOH, 1.2% glacial acetic acid, pH 6.0) and 100µl of chloramine T solution (564mg chloramine T, 4ml H2O, 4ml n-propanol, 32ml of the citrate/acetate buffer). The plates were incubated for 20 minutes at room temperature and then 100µl of Ehrlich's solution (4.5g of 4-dimethylaminobenzaldehyde, 18.6ml n-propanol, 7.8ml 70% perchloric acid) were added and incubated further at 65°C for 15 min. Reaction product was read at 550nm. Solutions of 0-1000µg/ml hydroxyproline (Sigma, St. Louis, MO) were used to construct the standard curve.

Quantitative Real-Time RT-PCR for Studying Gene Expression

High-quality total RNA was isolated from frozen lung tissues using the RNAqueous-4PCR kit (Ambion, Austin, TX). DNAse I-treated RNA (1µg) was reverse transcribed (High Capacity cDNA kit, Applied Biosystems, Foster City, CA) and the first-strand complementary DNA so obtained was amplified by quantitative real-time polymerase chain reaction using an ABI PRISM 7000 HT Detection system (Applied Biosystems, Foster City, CA) using Taqman probes.and primer pairs The comparative $2^{-\Delta\Delta}C_t$ method was used to calculate the relative fold change in gene expression. 18s

ribosomal RNA was used as an endogenous control to normalize expression levels of target genes.

Western Blot Analysis

To determine the protein expression of AT_1R and ACE, right lung tissues from control and bleomycin treated animals were homogenized in radioimmuno-precipitation assay buffer (RIPA buffer). Equal amounts of protein (30µg total protein) were separated on 12% SDS-polyacrylamide gels and transferred electrophoretically onto nitrocellulose membrane (Bio-Rad Laboratories, USA). The membranes were blocked with 5% non-fat milk solution in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) for 1 hour and incubated either with AT_1-receptor rabbit polyclonal antibody (SantaCruz Biotechnology; 1:400) or ACE rabbit polyclonal antibody (SantaCruz Biotechnology; 1:1000) overnight at 4^oC. Mouse monoclonal anti-β-actin antibody (Sigma Aldrich; 1:1000) was used to confirm equal loading. After overnight incubation with the primary antibody, the membranes were washes thrice for 5 minutes in TBS-T, and then incubated with secondary antibody conjugated with horseradish peroxidase (GE Healthcare, New Jersey, USA; anti-rabbit IgG 1:2500 or anti-mouse IgG 1:5000) for 1 hour. Finally, the membranes were subjected to chemiluminescence detection system and exposed to a photographic film.

Statistical Analysis

Data are presented as means ± SEM. Statistical differences were evaluated by either one-way ANOVA or two-way ANOVA wherever applicable, followed by the Newman-Keuls test. p values <0.05 were considered statistically significant.

Results

Efficacy of the Lentivirus to Overexpress Ang-(1-7) and ACE2

Infection of cardiac myoblasts with 10 multiplicity of infection (MOI) of lenti-Ang-(1-7) fusion protein resulted in a robust expression of IgG2b isotype (Fig. 3-1B). Furthermore, *in vivo* experiments demonstrated increased levels of Ang-(1-7) peptide in the lenti-Ang-(1-7) infected rat lungs compared to control animals (Fig. 3-1C). These data establish that lenti-Ang-(1-7) is active and effective in secreting Ang-(1-7). As for lenti-ACE2 efficacy, murine ACE2 mRNA was detected only in the lungs of rats treated with lenti-ACE2. There was no murine ACE2 expression in the control animals.

Attenuation of Bleomycin-Induced RVSP and RVH

Two weeks of bleomycin administration resulted in a significant increase in the RVSP (Fig. 3-2) followed by development of right ventricular hypertrophy (0.27 $\pm$ 0.01 in control vs 0.49 $\pm$ 0.02 in bleomycin). Gene therapy treatment with either ACE2 or Ang-(1-7) significantly attenuated bleomycin-induced increase in RVSP (Fig. 3-2) and right ventricular hypertrophy (0.37 $\pm$ 0.02 in bleomycin + ACE2 and 0.37 $\pm$ 0.03 in bleomycin + Ang-(1-7).

Pathological Score and Hydroxyproline Content of Lung Tissue:

Lung sections of different groups were stained with H&E and evaluated using Ashcroft scores. The Ashcroft score for the bleomycin alone group was significantly higher than the controls, indicating a fibrotic response to bleomycin administration. However, overexpression of ACE2 or Ang-(1-7) resulted in lower scores, demonstrating an antifibrotic treatment effect (Fig 3-3C). Along similar lines, considerably lower levels of lung hydroxyproline, an index of collagen accumulation, was observed with ACE2 or Ang-(1-7) treatment compared to the bleomycin alone group (Fig. 3-3B).

Effects on the mRNA Expressions of TGF-β and ACE2

As bleomycin administration has been shown to upregulate TGF-β to influence lung fibrogenesis, we analyzed the expression of TGF-β at the mRNA level. The mRNA expression of TGF-β was significantly elevated in the bleomycin alone group compared to normal controls ($p<0.05$). On the contrary, TGF-β expression was markedly suppressed by both ACE2 and Ang-(1-7) treatments (Fig.3-4). On the other hand, mRNA levels of endogenous ACE2, a membrane bound enzyme, were significantly decreased in bleomycin-treated animals (Fig. 3-5). This decrease was completely prevented by overexpression of ACE2. Ang-(1-7) treatment resulted in an intermediate response in that these animals were not different from either controls or the bleomycin treated groups.

Western Analysis for Comparative Quantification of AT_1R and ACE

The protein levels of AT_1R and ACE were measured in the lung homogenates by Western blot. Bleomycin significantly increased the expression of AT_1R, which was attenuated by Ang-(1-7) treatment (Fig. 3-6). However, ACE2 gene therapy did not shown any reduction in lung AT_1R protein levels. A significant decrease in lung ACE protein ($p<0.05$ vs. controls), was observed in the bleomycin alone group, which was significantly prevented by ACE2 and Ang-(1-7) treatment (Fig. 3-7).

Discussion

In this study we provide evidence that lentiviral mediated overexpression of ACE2 or Ang-(1-7) prevents bleomycin-induced lung fibrosis. Ang-(1-7) is not a gene product but an enzymatic breakdown metabolite of Ang II. Here, we devised a synthetic gene for Ang-(1-7) and packaged it into a lentivirus. Lentiviral mediated overexpression of Ang-

(1-7), or ACE2, in the lungs provided protective effects against bleomycin-induced pulmonary fibrosis.

PF is the final pathological outcome of lung injury characterized by accumulation of excessive extracellular matrix deposition in the lung parenchyma. Idiopathic PF has an unknown etiology while other fibrotic lung diseases may be related to connective tissue disorders, environmental toxins or occupational hazards (Wilson and Wynn, 2009). Irrespective of the cause, a dysregulated pulmonary RAS has been implicated in the initiation and progression of this disease. ACE inhibitors (Wang et al., 2000; Uhal et al., 1998) or AT_1R blockers (Yao et al., 2006; Otsuka et al., 2004) have provided beneficial effects against various animal models of pulmonary fibrosis, lending support for the pathological role of RAS in the fibrotic process. With the recent discovery of ACE2 and its alleged counter-regulatory role to that of ACE, made us to hypothesize that ACE2 and its enzymatic product Ang-(1-7) may have a protective role against lung fibrosis.

In the present study, endotracheal instillation of bleomycin evoked severe fibrotic response, characterized by inflammation and interstitial lung collagen accumulation. Collagen deposition was significantly decreased by overexpression of Ang-(1-7) as analyzed by biochemical (hydroxyproline content) and histological studies (Ashcroft score). The major source of collagen in the lungs is from the fibroblasts. They proliferate and differentiate into myofibroblasts which have higher collagen-synthesis activity, in response to Ang II via AT_1R stimulation (Marshall et al., 2004)**.** An increase in AT_1R levels may thus be critically important to the fibrotic process. Lung AT_1R was significantly increased following bleomycin administration, which was consistent with

previous reports (Otsuka et al., 2004). This effect of bleomycin was significantly attenuated by pulmonary overexpression of Ang-(1-7). Downregulation of AT_1R by Ang-(1-7) has also been reported in other tissues (Clark et al., 2001). However, no reduction in lung AT_1R protein levels was observed in the ACE2 treatment group. We anticipated that ACE2 overexpression would result in a similar reduction in AT_1R levels as observed with Ang-(1-7) since both transgenes had similar beneficial effects on lung fibrosis. Thus, it is possible that each of these components of the RAS may have somewhat different effects on the lung. We can speculate that ACE2 overexpression either inhibits the AT_1R signaling pathway, thereby, preventing AT_1R stimulated collagen synthesis or that it stimulates the degradation of collagen, resulting in the observed beneficial effects. Lung protein analysis revealed a decrease in ACE levels in bleomycin treated rats. Decreased pulmonary ACE activity, associated with bleomycin injury has been reported in animal and human studies (Chen et al., 2007; Newman et al., 1980; Orfanos et al., 2001), which confirm our current findings. The mRNA levels of ACE2 were also significantly lower with bleomycin treatment, in agreement with published data (Li et al., 2008). Accordingly, the observed decreases in ACE and ACE2, which are predominantly expressed on the endothelial cells, may be attributed to endothelial injury. The endothelium of pulmonary arteries and veins has been identified as the primary site of injury following bleomycin toxicity (Lazo, 1986). Thus, both ACE2 and Ang-(1-7) overexpression may have a protective effect on the endothelium as they prevented bleomycin-induced lowering of endogenous ACE and ACE2 levels. Yet to be determined is how the ratio of ACE/ACE2 may be responsible for the pathophysiological consequences in this disease and its therapy.

Transforming growth factor-β (TGF-β) is an important cytokine that plays a key role in fibrogenesis (Goodwin and Jenkins, 2009). Both *in vitro* and *in vivo* studies have demonstrated increased TGF-β levels with bleomycin treatment (Breen et al., 1992; Azuma et al., 2005). In line with these studies, we also observed an increase in lung mRNA levels of this pro-fibrotic cytokine after bleomycin instillation, which was significantly reduced by ACE2 and Ang-(1-7) treatments. Downregulation of TGF-β may be an important aspect of the antifibrotic effect of Ang-(1-7).

Clinically, presence of PH secondary to fibrotic lung diseases, termed as cor-pulmonale indicates poor prognosis with compromised cardiac function. A part of the reason is that the elevated vascular resistance and pulmonary hypertension following lung fibrosis subjects the right ventricle to increased work-load leading to maladaptive hypertrophy and heart failure. In our study, we did detect pulmonary hypertension and right ventricular hypertrophy following bleomycin administration. However, treatment with ACE2 and Ang-(1-7) prevented both the development of pulmonary hypertension and right ventricular hypertrophy.

Over the past few years, evidence is accumulating to indicate a protective role for ACE2. Kuba et al. (2005) demonstrated the beneficial role of ACE2 against acute lung injury, importantly against SARS-CoV infections. Our own studies established that pulmonary overexpression of ACE2 (Yamazato et al., 2009) or its activation using a chemical entity (Ferreira et al., 2009) rendered protective effects against PH. Similarly, studies by Li et al. (2008) showed that ACE2 is protective but is downregulated in experimental and human lung fibrosis. ACE2 is an ectopic enzyme that catalyzes the conversion of Ang II to Ang-(1-7), which can interact with the Mas receptor to produce

beneficial effects. Taken together, all these studies indicate that targeting of the pulmonary ACE2-Ang-(1-7)-Mas axis could provide novel therapeutic strategy in the treatment of lung diseases, particularly those diseases involving pulmonary fibrosis and pulmonary hypertension.

Perspectives

We have demonstrated that Angiotensin-(1-7) has a cardio-pulmonary protective role against recognized animal models of lung fibrosis. Clinical trials of Ang-(1-7) are currently underway for treating cancer patients (Petty et al., 2009), so the use of Ang-(1-7) as a new therapy for pulmonary fibrosis is plausible. The medical use of bleomycin as a tumor suppressant is limited due to induction of PF. Thus, it is possible to have a combination therapy with Ang-(1-7) to enhance the therapeutic efficacy of bleomycin by preventing pulmonary toxicity.

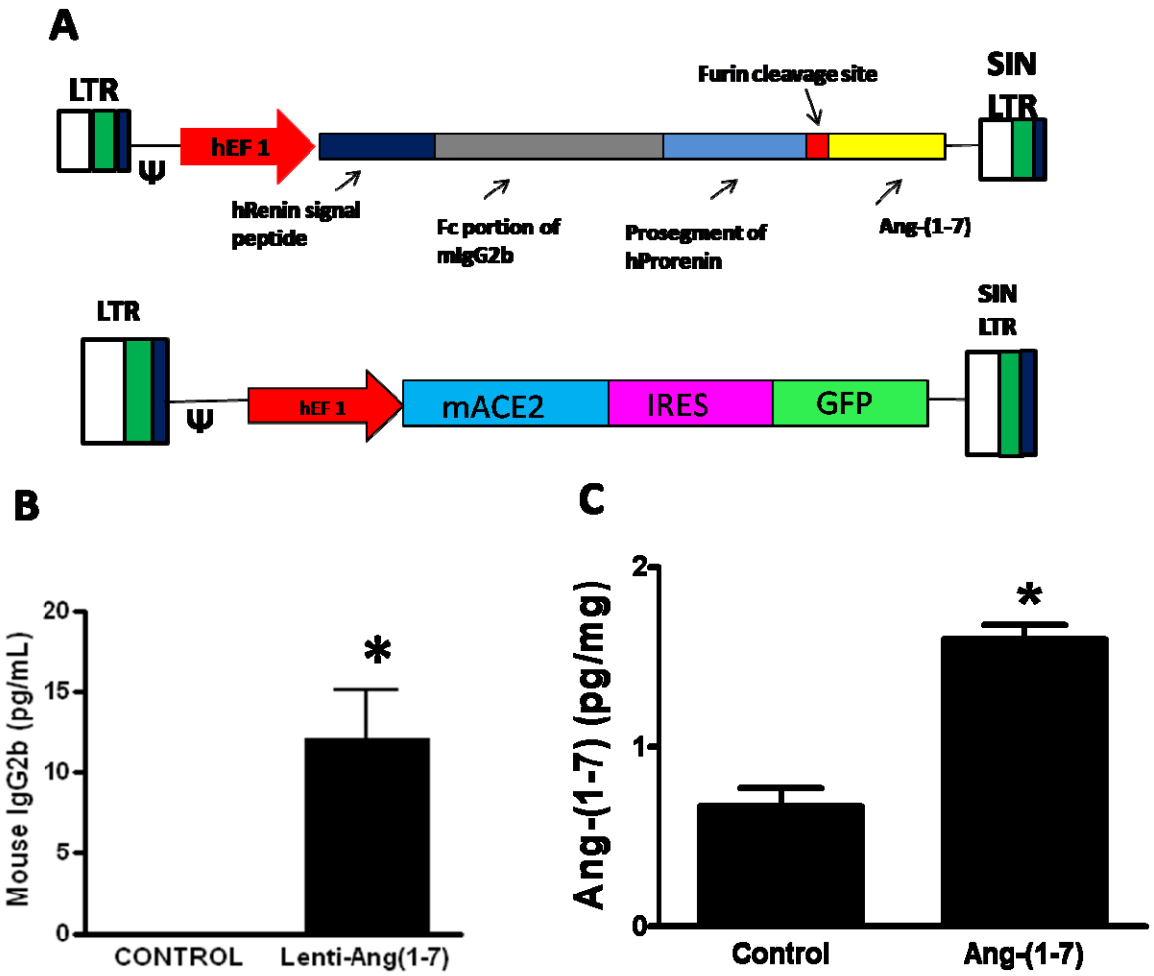

Figure 3-1: Construct and efficacy of the lenti-Ang-(1-7) and lenti-ACE2. (A) Lentiviral construct of Ang-(1-7) and ACE2. (B) Lenti-Ang-(1-7) caused a significant increase in the levels of mouse IgG2b after 72 hrs of infection in the H9C2 myoblasts. (C) In vivo administration of lenti-Ang-(1-7) significantly increased the levels of Ang-(1-7) in the rat lungs after 6 weeks of gene transfer. Data are represented as mean ± SEM. * $p<0.05$ vs control group (n=4-5 per group)

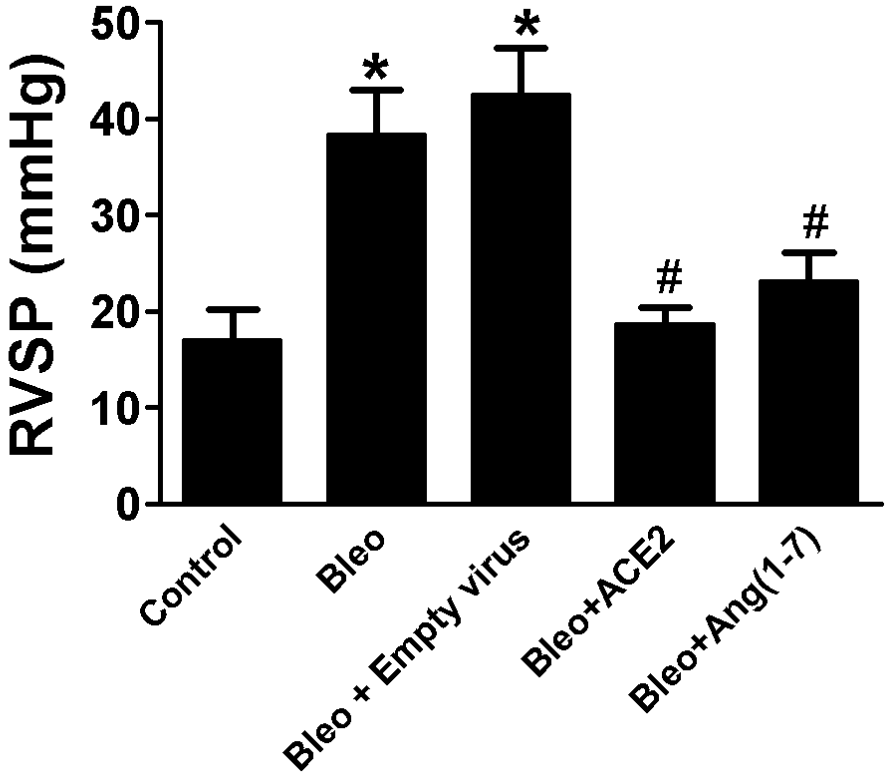

Figure 3-2: Effects of lentiviral mediated overexpression of ACE2 and Ang-(1-7) on bleomycin-induced PH. Bleomycin administration caused a significant increase in the RVSP after 2 weeks, which was completely attenuated by lenti-ACE2 and lenti-Ang-(1-7) treatment. Data are represented as mean ± SEM. * $p<0.05$ vs Control group, # $p<0.05$ vs bleomycin (n=5-6 per group)

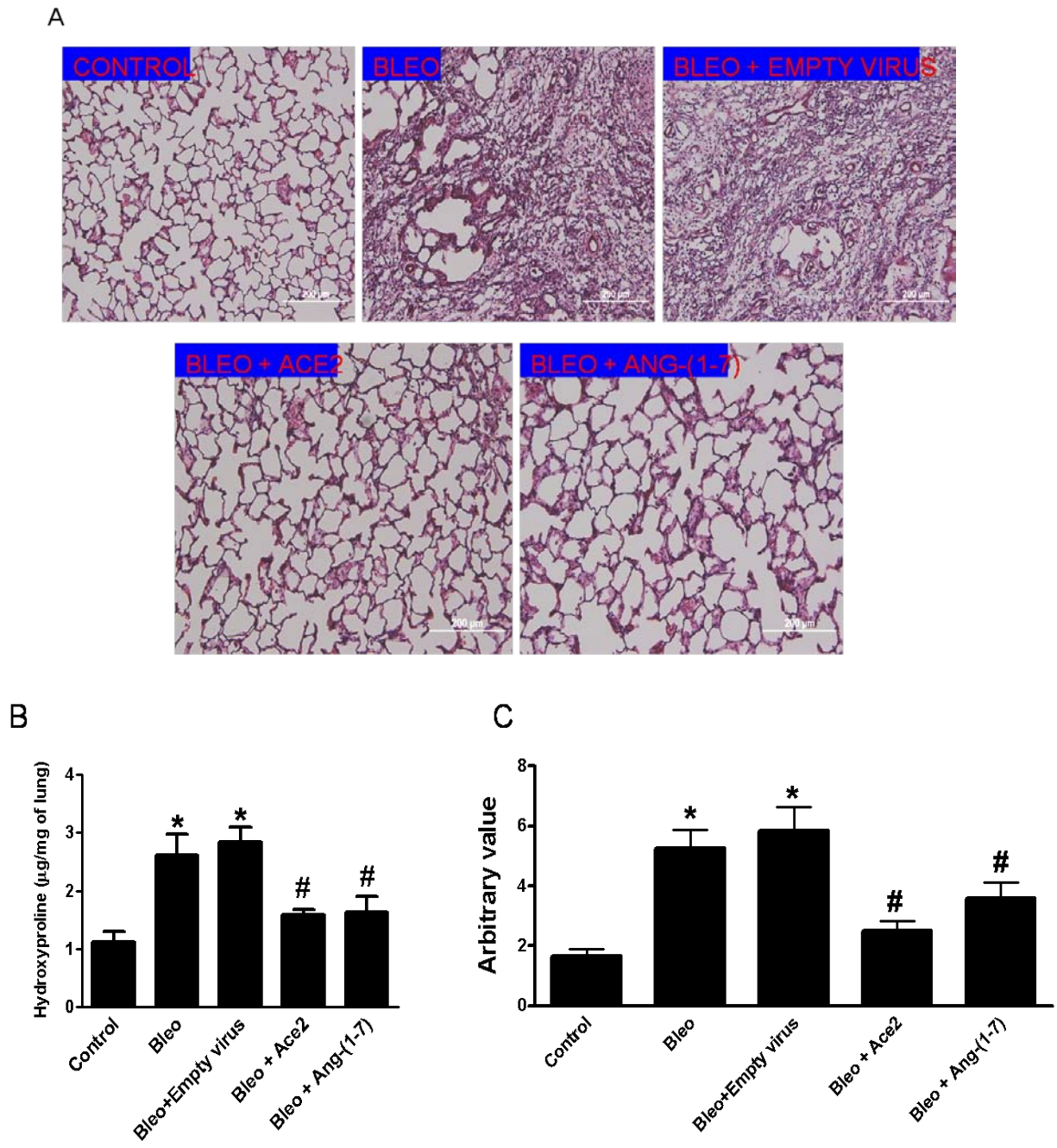

Figure 3-3: Histological analysis and quantitative fibrosis scoring of lung sections. (A) Representative photographs of the lung tissue stained with H&E. (B) Lung hydroxyproline content, in the various treatment groups. (C) Morphological changes in fibrotic lungs quantified using the Ashcroft score Data are represented as mean + SEM * $p<0.05$ vs. Control; # $p<0.05$ vs. bleomycin (n=4 per group)

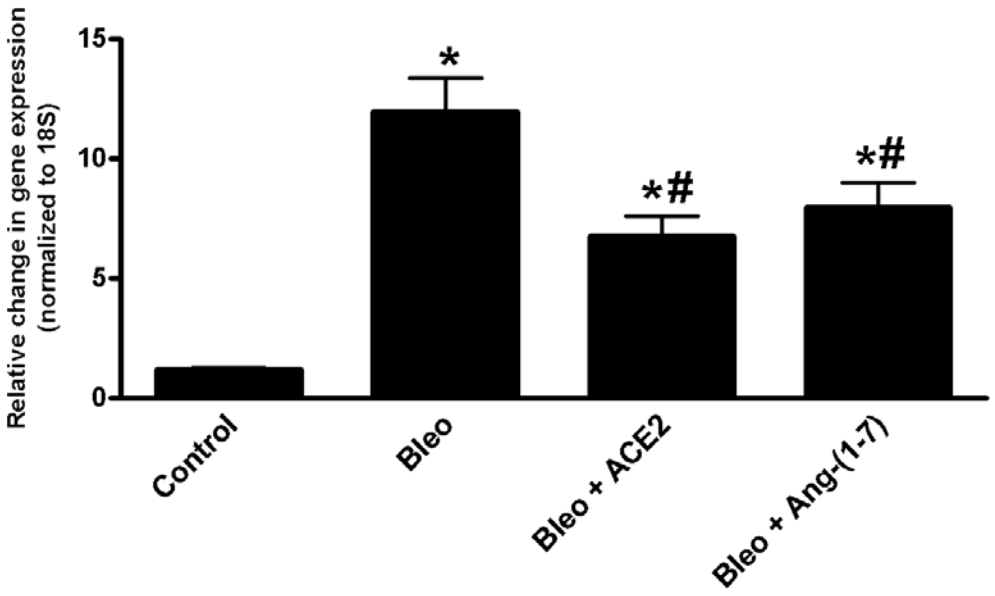

Figure 3-4: Effect of ACE2 and Ang-(1-7) overexpression on bleomycin-induced increases in TGF-β levels: Lung TGF-β mRNA levels were significantly increased by bleomycin administration. Overexpression of murine ACE2 and Ang-(1-7) prevented the bleomycin-induced increase in TGF-β levels. * $p<0.05$ vs. Control; # $p<0.05$ vs. bleomycin (n=4-5 per group)

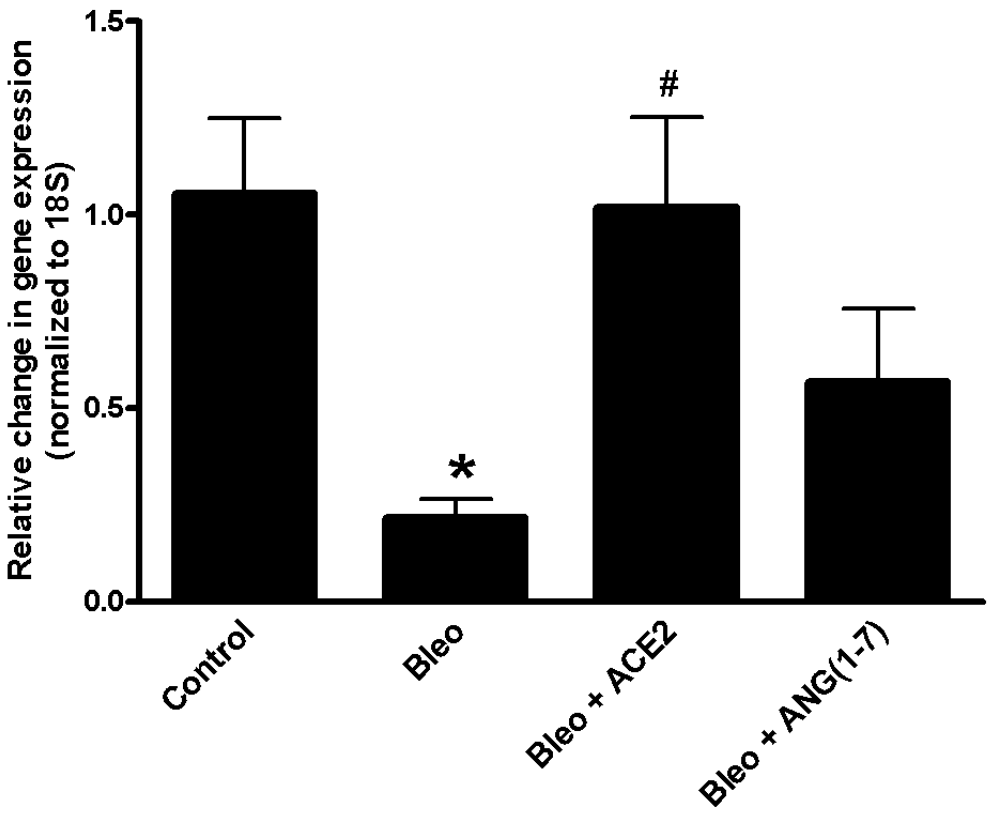

Figure 3-5: Downregulation of ACE2 mRNA by Bleomycin. ACE2mRNA levels in the lungs were significantly decreased by bleomycin administration. Overexpression of murine ACE2 and Ang-(1-7) prevented the bleomycin-induced decrease in ACE2 mRNA. * $p<0.05$ vs. Control; # $p<0.05$ vs. bleomycin (n=4-5 per group)

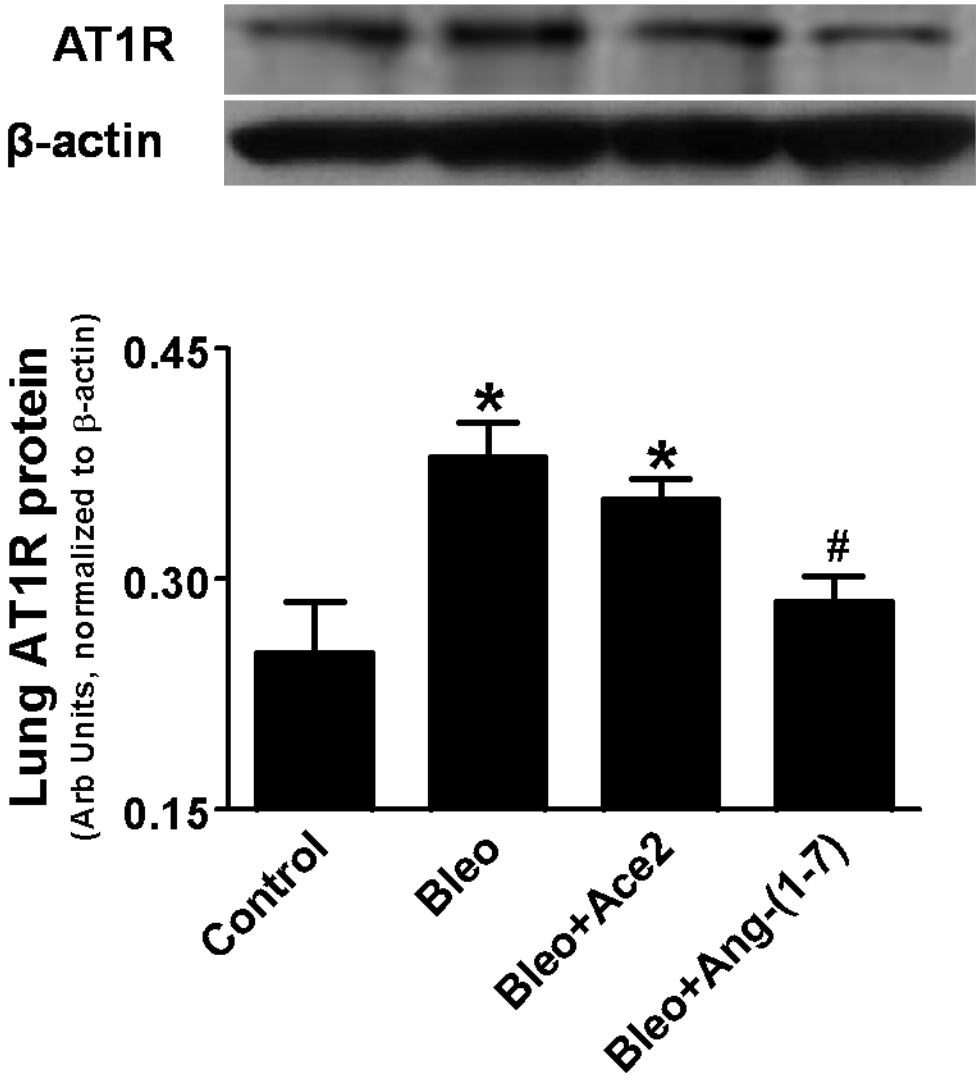

Figure 3-6: Effect of bleomycin treatment on lung AT_1R protein levels. Bleomycin administration increased lung AT_1R protein level which was significantly decreased by Ang-(1-7). However, ACE2 overexpression failed to decrease the elevated AT_1R levels induced by bleomycin * $p<0.05$ vs. Control; # $p<0.05$ vs. bleomycin (n=3 per group)

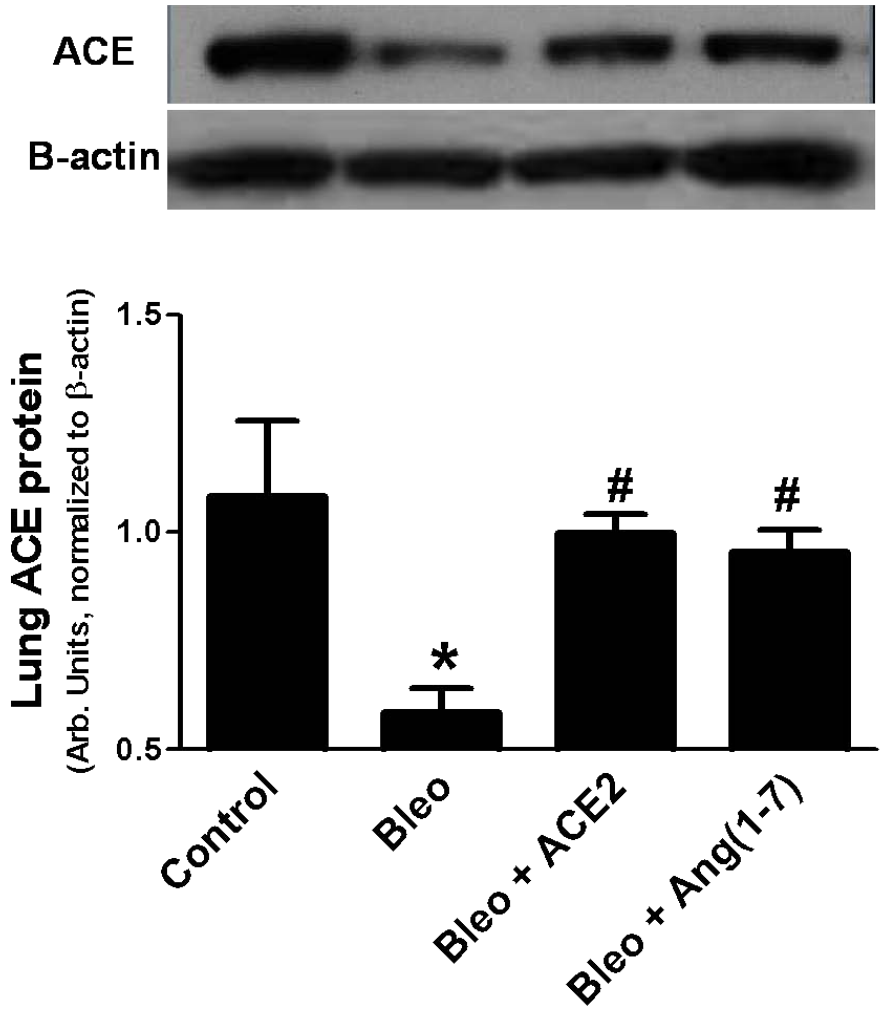

Figure 3-7: Effect of bleomycin treatment on lung ACE protein levels. Bleomycin administration decreased lung ACE protein level which was significantly increased by ACE and Ang-(1-7) overexpression. * $p<0.05$ vs. Control; # $p<0.05$ vs. bleomycin (n=3 per group)

CHAPTER 4
17BETA-ESTRADIOL MODULATES LOCAL CARDIAC RENIN-ANGIOTENSIN SYSTEM TO PREVENT CARDIAC REMODELING IN THE DOCA-SALT MODEL OF HYPERTENSION IN RATS

Abstract

Ventricular remodeling can play a detrimental role in the progression of cardiovascular diseases, leading to heart failure. The current study was designed to investigate the effects of 17β-estradiol (E2) on cardiac remodeling. Cardiac fibrosis and hypertrophy were examined in deoxycorticosterone acetate (DOCA)-salt treated rats with chronic, six-week administration of two different doses of E2. Bilaterally ovariectomized (Ovex) female Sprague-Dawley rats were randomly assigned to one of the following groups: Ovex-control; Ovex-DOCA; Ovex-DOCA + Low-dose E2 (1.66μg/day); or Ovex-DOCA + High-dose E2 (2.38μg/day). All DOCA treated rats were uninephrectomized and drinking water was replaced by 0.15 M NaCl solution for the remainder of the study period. DOCA-salt treatment resulted in a significant increase in blood pressure, which was not altered by estrogen replacement. Histological examinations revealed marked cardiac remodeling (both ventricular hypertrophy and interstitial fibrosis) with DOCA treatment, which was attenuated in animals receiving estrogen therapy. Western blot analysis demonstrated increased cardiac levels of angiotensin converting enzyme (ACE) with DOCA treatment, which was attenuated by E2 replacement. Furthermore, increased levels of cardiac angiotensin converting enzyme 2 (ACE2) protein were observed in animals receiving high-dose E2 replacement. These findings suggest that physiologically relevant estrogen replacement therapy has blood pressure-independent cardioprotective effects, which are possibly mediated through modulation of the cardiac renin-angiotensin system.

Introduction

There are abundant data to demonstrate that men are more prone to cardiovascular disease (CVD) than premenopausal women of similar age (Pilote et al., 2007). Gender related differences in aspects of CVD have been attributed to several factors that include differences in endothelial function, lipid profile, functions of the vascular smooth muscle, and blood pressure (Mendelsohn and Karas, 2005). The incidence of CVD, however, increases substantially following menopause, suggesting that female sex hormones may render some degree of cardioprotection. Similar effects have also been observed in animal studies as ovariectomy abolished the female-associated cardioprotection following ischemia/reperfusion injury or volume overload (Gabel et al., 2005; Brower et al., 2003). Conversely, cardiac protection was restored with exogenous estradiol administration in a pressure overloaded mouse model (van Eickels et al., 2001).

Though observational studies in post-menopausal women have found consistent, powerful protection by hormone replacement therapy (HRT) against CVD (Hu and Grodstein, 2002), results from the Women's Health Initiative (WHI) (Writing Group for the Women's Health Initiative investigators, 2002) and the Heart and Estrogen-progestin Replacement study (HERS) (HERS Research Group, 2002) showed no reduction in cardiovascular risk with HRT. There could be several possible explanations, both methodological and biological, such as: a) the mode of estrogen administration (transdermal versus oral) (John and Malcolm 2007); b) type of estrogen utilized (natural versus synthetic) (Mashchak et al., 1982); c) age and duration of menopause before treatment (Harman et al., 2005); d) the dose and duration of estrogen administration

(Lobo, 2004); and, e) the overall health of the women studied, that may account for the apparent discrepant results.

In view of the ongoing debate over the use of HRT, we investigated whether administration of 17β-estradiol (E2), immediately after ovariectomy, can offer cardioprotective effects in the deoxycorticosterone acetate (DOCA)-salt model of hypertension. In the DOCA model, initial volume overload and elevated sympathetic drive lead to chronic increases in blood pressure, causing end-organ damages. We have previously found that this particular model produces marked cardiac remodeling (Grobe et al., 2006). Among the most perilous forms of end-organ damage produced by chronic hypertension is ventricular remodeling, characterized by cardiomyocyte hypertrophy, apoptosis, and increased deposition of extracellular matrix (ECM) proteins, contributing to interstitial and perivascular fibrosis (Remme, 2003). Cardiac remodeling leads to ventricular dysfunction, heart failure and ultimately death. Therefore, prevention or reversal of the remodeling process may have significant impact on organ function and survival.

The renin-angiotensin system (RAS) has been shown to play a pivotal role in producing structural alterations of the heart in response to hemodynamic overload. Activation of the cardiac angiotensin type 1 receptor (AT_1R) by angiotensin II (Ang II), the main effector peptide of the RAS, stimulates myocyte growth, fibroblast proliferation, collagen synthesis and expression of ECM proteins (Lijnen and Petrov, 2003). Previous studies in animals have demonstrated that DOCA-induced activation of local RAS leads to cardiac damage, and this can be prevented by administration of angiotensin converting enzyme (ACE) inhibitor or AT_1R blocker (Brown et al., 1999).

In the current study, we investigated the effects of two doses (1.66µg/day and 2.38µg/day) of estrogen replacement would prevent or attenuate cardiac remodeling in DOCA-salt treated animals, and further, that this protective effect of E2 is mediated through modulation of the cardiac RAS. We chose to use two different doses that mimic physiologically relevant levels of circulating E2 since Zhan et al. (2008), recently reported detrimental effects with higher levels of E2 treatment.

Materials and Methods

Animals

Female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing between 180 and 250 g were used for this study. Animals were housed in a temperature and humidity-controlled room maintained on a 12:12-h light-dark schedule with free access to food and water. All animal procedures were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee conforming to US National Institutes of Health guidelines.

DOCA-Salt Model of Hypertension

Animals were anesthetized with an intraperitoneal injection of a ketamine, xylazine, and acepromazine mixture (30, 6 and, 1 mg/kg, respectively). Eighteen animals underwent uninephrectomy and bilateral ovariectomy (Ovex) with subcutaneous implantation of a 40 mg pellet of DOCA (Sigma Aldrich, USA). Immediately after removal of the ovaries, some of the animals were additionally implanted subcutaneously with either 0.05 mg [21 day release pellet delivering 2.38 µg/day, (n=6)] or 0.1 mg [60 day release pellet delivering 1.66 µg/day, (n=6)] of E2 (Innovative Research of America Florida, USA). Pellets were replaced after three weeks in the higher dose group. A separate group of age-matched rats (n=6) underwent

bilateral ovariectomy alone (without uninephrectomy), to serve as normotensive controls. After surgery (and for the remainder of the experiment), normotensive controls received plain drinking water while all DOCA implanted animals received 0.9% (0.15 M) sodium chloride (NaCl) solution.

Indirect Blood Pressure Measurement

Systolic blood pressure was determined weekly by the indirect tail cuff method as described previously (Grobe et al., 2006). Briefly, animals were lightly heated for five minutes under a 200 W heat lamp before placing into a temperature-controlled restrainer to which the animals had previously become acclimated. A pneumatic pressure sensor was attached to the tail, distal to a pneumatic pressure cuff, both under the control of a programmed Electro-Sphygmomanometer (Narco Bio Systems, Austin, TX). Voltage outputs from the pressure sensor bulb and inflation cuff were recorded and analyzed electronically using a Power Lab signal transduction unit and associated Chart software (AD Instruments, Colorado Springs, CO). Systolic blood pressure values from each animal were determined by averaging a minimum of five separate indirect pressure measurements. All pressures were recorded in the morning between 9 AM and noon by the same individual.

Isolated Heart Preparation

After six weeks of treatment, the animals were decapitated 10-15 minutes after an intraperitoneal administration of heparin (400 IU). Their hearts were excised and mounted onto a Langendorff apparatus. Hearts were perfused using Krebs-Ringer solution (118.4mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 2.5mM $CaCl_2$, 26.5mM $NaHCO_3$and 11.7mM glucose) at a constant flow rate of approximately 8mL/min. The perfusate temperature was maintained at 37°C and bubbled with 95%

O_2/5% CO_2. To measure the intra-ventricular pressure, a balloon catheter was inserted into the left ventricle and connected to a pressure transducer (AD Instruments), which was interfaced to a PowerLab data acquisition unit (AD Instruments). Coronary perfusion pressure was measured by means of a pressure transducer connected to the aortic cannula and coupled to the recording system. After 30 minutes of stabilization, functional parameters were recorded for an additional period of 30 minutes. Data were analyzed using the Chart software.

Tissue Collection and Cardiac Remodeling Analysis

Following experiments on the Langendorff apparatus, the hearts were blotted dry and weighed. Cross-sections of the ventricles were obtained and fixed in 10% neutral-buffered formalin solution for 24 hrs, after which they were moved to 70% ethanol until processed. The hearts were embedded in paraffin, sectioned at 5μm and sections were stained for collagen content using Picro-sirius red stain. Single sections from each animal were then viewed and photographed with a Moticam 1000 digital camera (Motic; Richmond, BC, Canada) under x100 magnification for interstitial fibrosis. The collagen content of the left ventricular free wall was quantified using the ImageJ program from the National Institutes of Health (Rasband, 1997), as described elsewhere (Grobe et al., 2006). Quantifications were carried out as the mean observations from three individuals blinded to the treatment groups. For collagen quantification, each observer examined a minimum of five separate images per section from different (non-overlapping) regions of the left ventricular free wall. The results for each animal from each of the three observers were then averaged for subsequent statistical analysis. The heart weights were normalized to tibial length to determine myocardial hypertrophy.

Estradiol Measurement

Venous blood from the inferior vena cava was collected at the time of sacrifice into EDTA-coated collection tubes and centrifuged at 800 x g for five minutes. Plasma was separated and stored at -80^{o}C until assayed. Plasma estradiol levels were measured using an ELISA kit as per manufacturer's instructions (ALPCO, USA). The kit could measure E2 levels ranging from 10pg/ml to 3200pg/ml.

Western Blot Analysis

Heart tissue was homogenized in radioimmuno-precipitation assay buffer (RIPA buffer) and the concentration of the protein extracted was measured using Bradford assay. The homogenates were suspended in loading buffer and heated in boiling water for 5 min. Equal amounts of protein (30µg total protein) were separated on 12% SDS-polyacrylamide gels and transferred electrophoretically onto nitrocellulose membrane (Bio-Rad Laboratories, USA). The membranes were blocked with 5% non-fat milk solution in TBS with 0.1% Tween 20 (TBS-T) for 1 hour and then incubated either with AT_1-receptor rabbit polyclonal antibody (SantaCruz Biotechnology; 1:400), ACE rabbit polyclonal antibody (SantaCruz Biotechnology; 1:1000) or ACE2 rabbit polyclonal antibody (SantaCruz Biotechnology; 1:1000). Mouse monoclonal anti-GAPDH antibody (Sigma Aldrich; 1:20000) was used to confirm equal loading. After washing 3 times for 5 minutes in TBS-T, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (GE Healthcare, New Jersey, USA; anti-rabbit IgG 1:2500 or anti-mouse IgG 1:5000) for 1 hour. The membrane was then subjected to a chemiluminescence detection system and exposed to photographic film.

Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM) with $P<0.05$ considered statistically significant. Interstitial fibrosis, cardiac hypertrophy and western blot data were analyzed by One-way ANOVA followed by Newman-Keuls post-hoc test. Repeated measures ANOVA were used for analyzing body weight and blood pressure data.

Results

Effect of Treatments on Blood Pressure

DOCA-salt treatment resulted in a significant increase in blood pressure in ovariectomized rats over the six-week study period, as compared to Ovex-controls (Ovex-control, 121 ± 9mmHg; Ovex-DOCA, 174 ± 23mmHg, n=6, $p<0.05$). Neither dose of estrogen supplementation significantly altered the elevated blood pressure (Low-dose E2, 173 ± 22mmHg; High-dose E2, 178 ± 13mmHg, n=6), although there was an initial trend for the high dose E2 to attenuate the rise in blood pressure in DOCA-treated animals (Fig. 4-1).

Plasma estradiol Levels

Estradiol replacement resulted in a dose-dependent increase in the circulating levels of this steroid, which was within the physiological range (Fig. 4-2). Significant increase in the plasma levels of estrogen was observed with both the doses of E2 replacement when compared to Ovex-DOCA ($p<0.05$). Also, elevated levels of E2 was obtained with the higher dose when compared to the low-dose E2 therapy ($p<0.05$).

Effect of 17ß-Estradiol on Body and Uterine Weights

Ovariectomized normotensive and hypertensive rats without estrogen replacement had significantly higher body weights compared to estrogen-treated animals starting at

the third week of estrogen replacement ($p<0.05$). Estradiol treated animals showed a dose-dependent decrease in body weight when compared to Ovex-control or Ovex-DOCA groups (Fig. 4-3A). Animals with the low-dose estrogen treatment showed an 11% reduction, while the higher dose of estrogen resulted in a larger (29%) reduction in body weight when compared to the Ovex-DOCA group. Uterine weight was used as an indicator for effectiveness of ovariectomy. The ratio of uterine weight/tibial length was lower in the estradiol deficient animals, suggesting an atrophied uterus, which was reversed by estradiol replacement (Fig. 4-3B).

Effect of 17ß-Estradiol on Interstitial Fibrosis and Cardiac Hypertrophy

Cardiac hypertrophy was evaluated by normalizing wet heart weight to tibial length. Tibial length was used for normalization as body weights varied significantly among the different treatment groups, whereas, tibial length was similar among all groups. The heart weight/tibial length ratio was significantly increased in DOCA-treated animals when compared to Ovex-controls (Ovex-control, 0.026 ± 0.002; Ovex-DOCA, 0.037 ± 0.01, $n=6$, $p<0.05$), indicating development of cardiac hypertrophy. Both doses of estrogen prevented the development of cardiac hypertrophy to a similar degree (Low-dose E2, 0.029 ± 0.003; High-dose E2, 0.029 ± 0.002, $n=6$, Fig. 4-4). DOCA-salt treatment also resulted in a significant increase in myocardial interstitial fibrosis when compared to Ovex-controls ($P<0.05$) and this increase was also prevented by both the doses of E2 replacement ($p<0.05$ vs. Ovex-DOCA), with a tendency for the higher dose of estrogen to induce a greater reduction in cardiac fibrosis (Fig. 4-5).

AT_1R, ACE and ACE2 Protein Levels

There were no significant changes in the protein levels of cardiac AT1-receptor among the different groups as analyzed by Western blot (Fig. 4-6A). Significant

increases in cardiac ACE protein ($p<0.05$ vs. Ovex-control), however, were observed in the Ovex-DOCA group and this increase was significantly attenuated by both doses of E2 replacement (Fig. 4-6B). Cardiac ACE2 protein levels were unchanged by DOCA-salt treatment (Fig. 4-7). While the low-dose E2 replacement had no appreciable effect on cardiac ACE2 levels, high-dose E2 replacement significantly elevated cardiac ACE2 protein ($p<0.05$ vs. Ovex-control) (Fig. 4-7).

Myocardial Function

No significant changes were observed among the different groups with regard to intraventricular end-diastolic pressure, myocardial contractility (+dP/dt, –dP/dt) and heart rate as analyzed by an isolated heart preparation (Fig. 4-8). DOCA-salt animals treated with low dose of E2 presented a significant increase in the intraventricular systolic pressure when compared with Ovex-control rats.

Discussion

In the present study, we examined the effects of 17β-estradiol (E2) replacement on cardiac remodeling in bilaterally ovariectomized rats treated with DOCA-salt. The primary findings of the study were that E2 replacement significantly prevented both cardiac hypertrophy and interstitial fibrosis induced by DOCA-salt administration. Further, while DOCA-salt treatment appears to promote the production of cardiac Ang II through increased expression of ACE, E2 replacement appears to prevent this elevation. Moreover, higher physiologically relevant levels of E2 appear to have the additional benefit of increasing cardiac ACE2 protein, which we have previously demonstrated to be cardioprotective (Huentelman et al., 2005).

Estradiol has been reported to provide protective effects against hypertensive cardiac remodeling by a variety of potential mechanisms that have been summarized by Deschepper and Llamas (2007). Several of these potential mechanisms directly or indirectly involve actions mediated by components of the RAS. Activation of the ACE-Ang II-AT_1R axis plays a crucial role in mediating vasoconstriction, proliferation and fibrosis. *In vitro* studies have demonstrated the protective effects of E2 against Ang II induced cardiac fibroblast proliferation (Stewart et al., 2006) and differentiation (Stramba-Badiale, 2009), which are critical steps in the pathogenesis of cardiac fibrosis. It is well established that hemodynamic overload activates local RAS, contributing to cardiac remodeling and dysfunction. ACE, the key enzyme involved in the generation of the pro-remodeling peptide Ang II and degradation of the anti-remodeling peptide angiotensin-(1-7) [Ang-(1-7)] is expressed by myocardial tissue and increased expression has been shown to be associated with myocyte hypertrophy and collagen deposition (Higaki et al., 2000). Previous studies have reported that chronic estrogen treatment decreases serum and tissue ACE activity and down-regulates tissue ACE mRNA (Gallagher et al., 1999; Schunkert et al., 1997). In this study, we observed that DOCA-salt treatment of ovariectomized rats significantly increased cardiac ACE levels. Further, E2 replacement prevented this increase, and thus it is reasonable to conclude that this reduction in ACE protein may contribute to the cardioprotective effects of E2 replacement.

Another component of RAS that is actively involved in the maladaptive process is the AT_1R. This receptor appears to mediate many of the deleterious effects of RAS. Prolonged activation of AT_1R has been shown to cause cardiac hypertrophy and

interstitial fibrosis (González et al., 2004; Iwai et al., 1995). Fareh et al. (1997) have demonstrated increased AT_1R density on the myocytes of DOCA-salt treated rats, though, no increase was observed on fibroblasts. However, our data shows no change in the protein levels of cardiac AT_1R among the different treatment groups. The discrepancy between the studies may be due to the methodology and the technique employed. We analyzed whole heart homogenate (which predominantly comprises of fibroblasts) using immunoblotting, while Fareh et al. (1997), studied particular cell types using receptor binding assay. van Eickles et al. (2001), also, did not observe any reduction in AT_1R with E2 treatment in a mouse model of pressure overload cardiac hypertrophy, using a dose of E2 that approximated the low dose utilized in the current study. A recent study of Ricchiuti et al. (2009), have demonstrated increased levels of cardiac AT_1R in E2 replaced ovariectomized rats receiving a combination of Ang II and nitric-oxide synthase (NOS) inhibitor while, previous studies have reported reduced tissue levels of AT_1R with E2 treatment (Nickenig et al., 1998; Wassmann et al., 2001; Brosnihan et al., 2008). Therefore, the effects of E2 on the modulation of cardiac AT_1R may depend on the experimental model and requires further evaluation. However, experimental studies using combination of E2 and AT_1R blocker or ACE inhibitor demonstrated enhanced cardiovascular protection, than either of the individual agents in spontaneously hypertensive rats, suggesting estradiol favorably modulates the RAS to bring out a synergistic effect (García et al., 2006; Giménez et al., 2006).

A recent study by Ji et al. (2008), observed that E2 replacement protected against tubulointerstitial fibrosis and glomerulosclerosis by preventing the decrease in renal ACE2 and the consequent decrease in Ang-(1-7) production induced by renal

wrap hypertension. We have also previously demonstrated that overexpression of ACE2 or increased circulating levels of Ang-(1-7) reduce cardiac fibrosis in different models of hypertension (Huentelman et al., 2005; Grobe et al., 2006; Grobe et al., 2007). In the current study, we observed no decrease in cardiac ACE2 levels with DOCA-salt treatment. However, a significant increase in ACE2 protein was noted with the higher dose of E2 therapy, suggesting that E2 induced increase in ACE2 levels could be dose dependent.

Cardiomyocyte hypertrophy is a strong predictor of cardiovascular morbidity and mortality. Both doses of estrogen replacement utilized in the current study prevented the ventricular hypertrophy as measured by heart weight/tibial length ratio. Our finding is consistent with previous findings reported in the literature (Donaldson et al., 2009). The beneficial effects of E2 replacement appear to be independent of blood pressure changes, which also are in agreement with previous reports (David et al., 2001; Khazaei and Nematbakhsh, 2006]. Similar results for anti-fibrotic effects, independent of blood pressure changes have been reported with Ang-(1-7) treatment in different animal models (Grobe et al., 2006; Grobe et al., 2007) suggesting that myocardial fibrosis may be a humorally-mediated event independent of blood pressure.

Though we demonstrated cardiac hypertrophy and increased fibrosis in DOCA-salt treated rats, no signs of cardiac dysfunction were observed with isolated heart studies. During the initial stages of volume overload, cardiac remodeling is an adaptive and compensatory response to increased wall stress. Thus, it is conceivable that the time point when we terminated the study (after 6 weeks of DOCA-salt treatment), these animals might have been in an adaptive phase with no signs of gross cardiac

dysfunction. We speculate, however, that longer periods of treatment with DOCA should result in a decompensatory stage, ultimately leading to heart failure.

Although we and others (Kolodgie et al., 1997; Gabel et al., 2005) have suggested preventative or protective cardiovascular effects, the results obtained from some clinical trials, such as the WHI and those in some animal studies have reported detrimental effects of estrogen treatment. Stevenson (2009) and Stramba-Badiale (2009) recently, have discussed inconsistencies in the findings of these studies and subsequent publications (Hsia et al., 2006; Rossouw et al., 2007) have cast doubts on the WHI recommendation that HRT increases the risk of cardiovascular disease. It is essential that factors such as dosages, route of administration and duration of HRT need to be critically assessed before a final verdict can be rendered as to the protective or detrimental effects of estrogen on cardiovascular risks. In a recent animal study, Zhan et al. (2008), demonstrated that low dose estrogen ($\leq$ 200 pg/ml and similar to those used in the current study) were protective, whereas high doses ($\geq$ 400pg/ml) increased mortality, enhanced fibrosis, and decreased cardiac function in a myocardial infarct mouse animal model. The treatment period in that study also began at ovariectomy, but lasted for 16 weeks.

In the current study, though both the doses of E2 treatment rendered cardioprotective effects, there existed some differences in terms of dose-related effects on cardiac function and renin-angiotensin system. High dose E2 therapy increased cardiac ACE2 protein levels while the low dose therapy increased intra-ventricular systolic pressure (IVSP). However, ventricular protein levels of AT_1R/ACE and other parameters of cardiac function were similar between the two doses. This indicates that

lower E2 levels would be sufficient enough in bringing out protective effects. This finding is in line with the recent treatment guidelines issued by the Board of the International Menopause Society (Board of the International Menopause Society, 2007) and the North American Menopause Society (North American Menopause Society, 2007), suggesting the use of the lower dose of estrogen immediately after menopause can render beneficial effects.

Timing of hormone initiation relative to the onset of menopause also appears to be an important issue that may have biased previous clinical trials (Grodstein et al., 2006). Although the present study was not designed to study this aspect, we noted that hormone replacement immediately after ovariectomy did result in cardioprotective effects, suggesting that hormone replacement is beneficial when started early and kept at moderate dosing.

In summary, our results indicate that early treatment with physiologically relevant doses of 17β-estradiol exerts blood pressure-independent cardioprotective effects, by preventing hypertrophy and decreasing accumulation of ECM proteins. These effects are likely mediated, at least in part, through modulation of the local cardiac production of the pro-remodeling peptide, Ang II, and the anti-remodeling peptide, Ang-(1-7). Future studies will be directed at more closely examining the mechanisms of estrogenic modulation of the enzymes and peptides of the intra-cardiac RAS, with the goal of identifying improved hormone replacement methodologies.

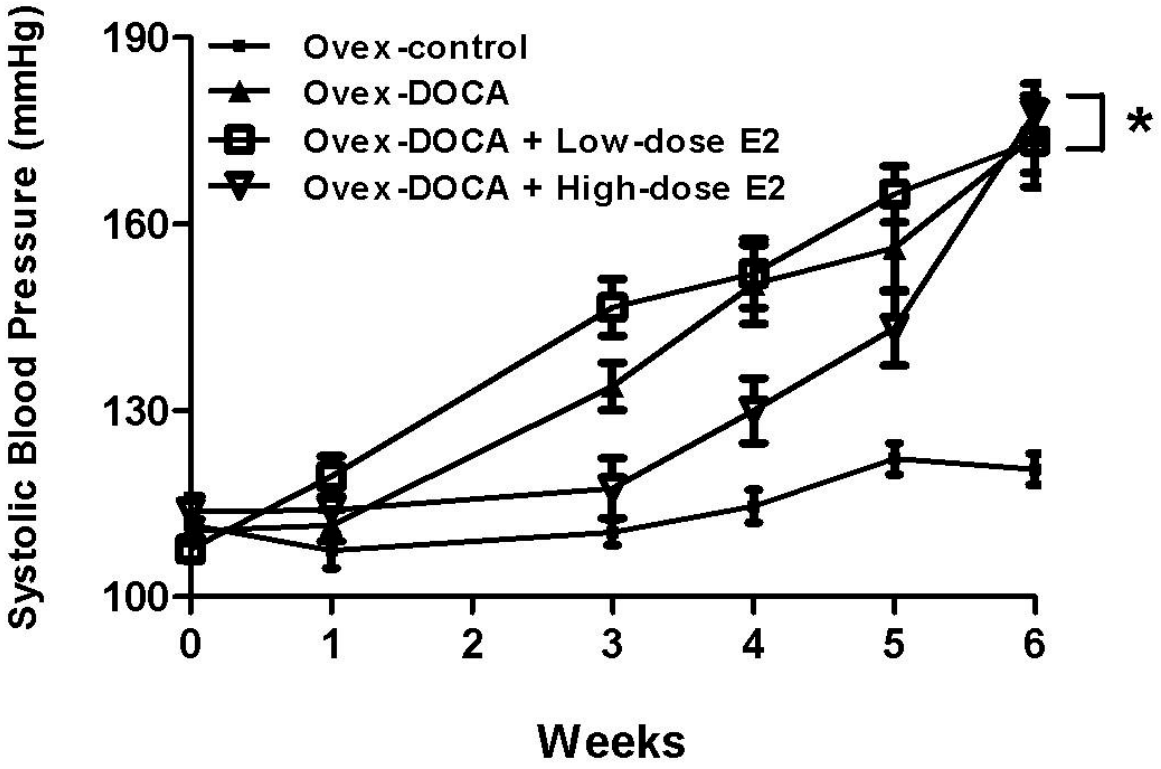

Figure 4-1: Systolic blood pressure (SBP) in ovariectomized rats. SBP was significantly elevated by DOCA-salt treatment. Estradiol replacement had no effect on the elevated SBP observed in DOCA-salt treated animals. Data are presented as mean ± SEM. * $p<0.05$ vs. Ovex-control, (n=6 per group).

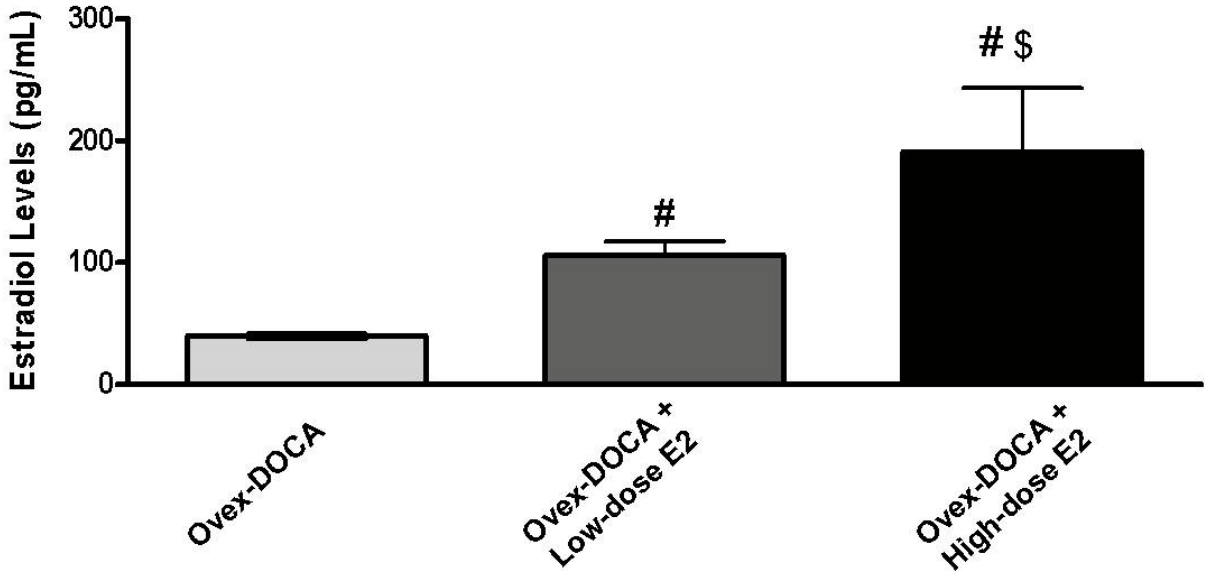

Figure 4-2: Effect of ovariectomy, low (E2, 1.66µg/day) and high doses of 17β -estradiol (E2, 2.38µg/day) on plasma estrogen levels. Data are presented as mean ± SEM. # $p<0.05$ vs. Ovex-DOCA; $ $p<0.05$ vs. Ovex-DOCA+low-dose E2, (n=4-6 per group).

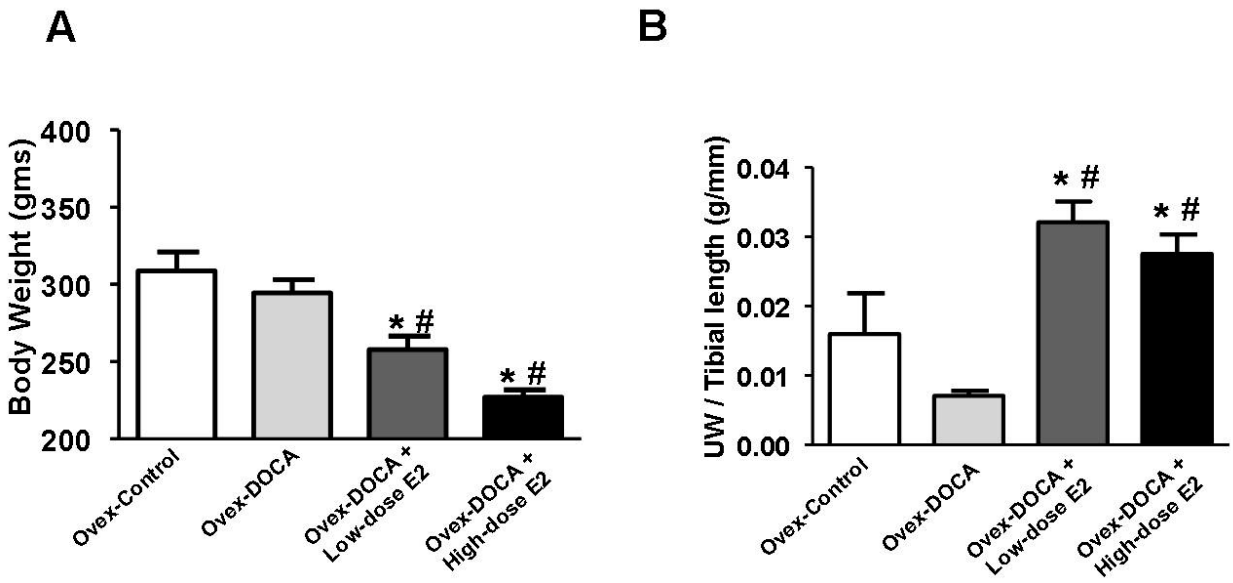

Figure 4-3: Effects of estradiol treatment on body and uterine weights. (A) Estrogen exposure caused dose-dependent decreases in body weights. (B) Uterine weights of estradiol-deficient animals were significantly lower than those on E2 replacement therapy. Data are presented as means ± SEM. * $p<0.05$ vs. Ovex-control; # $p<0.05$ vs. Ovex-DOCA, (n=6 per group)

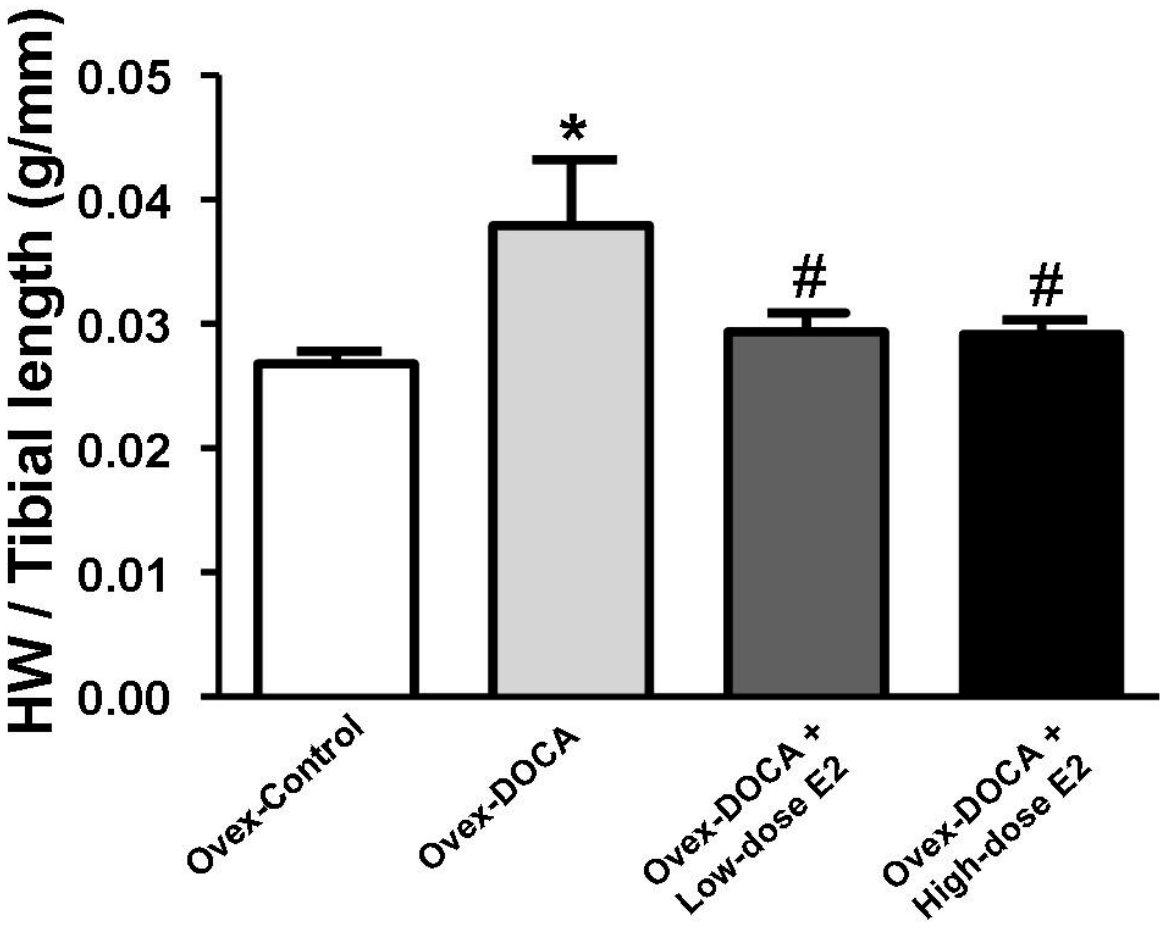

Figure 4-4: Cardiac hypertrophy. DOCA-salt treatment significantly increased heart mass. Estradiol replacement prevented the hypertrophic effect produced by DOCA-salt administration. Data are presented as means ± SEM. * $p<0.05$ vs. Ovex-control; # $p<0.05$ vs. Ovex-DOCA, (n=6 per group).

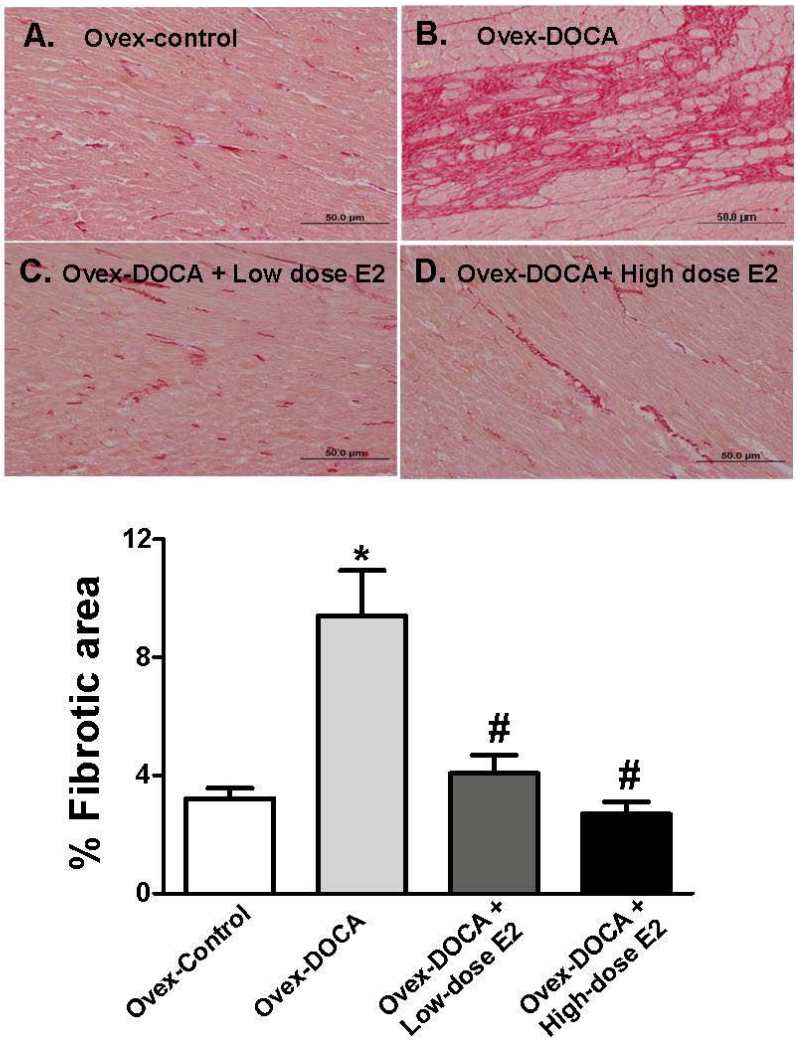

Figure 4-5: Left ventricle wall fibrosis. Upper: Representative images of mid-myocardial interstitial fibrosis at 100x magnification, stained with Picro-Sirius red (scale bars = 50 microns), where intense red color indicates a buildup of extracellular matrix (A) Ovex-control; (B) Ovex-DOCA; (C) Ovex-DOCA+ low-dose E2; (D) Ovex-DOCA+ high-dose E2. Lower: Mean % of left ventricular fibrosis. Quantitative analysis of images by ImageJ reveals a significant increase in collagen deposition in left ventricle wall by DOCA-salt treatment, which was prevented by both doses of estradiol replacement. Data are presented as means ± SEM. * $p<0.05$ vs. Ovex-control; # $p<0.05$ vs. Ovex-DOCA, (n=4-6 per group).

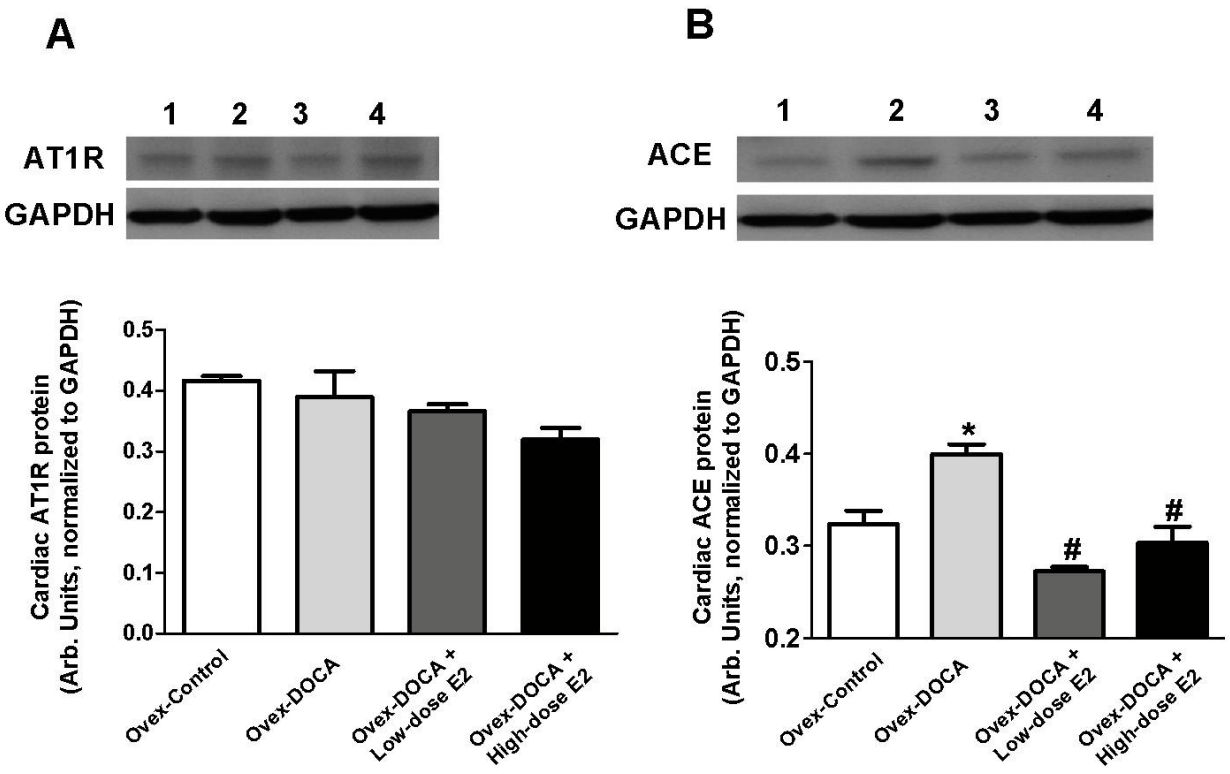

Figure 4-6: Effect of DOCA and E2 treatment on cardiac AT_1R and ACE protein levels. (A) No difference in cardiac AT_1R protein levels were observed among the different treatment groups. (B) Cardiac ACE levels were increased with DOCA-salt treatmentand this effect was prevented by E2 replacement. * $p<0.05$ vs. Ovex-control; # $p<0.05$ vs. Ovex-DOCA, (n=3 per group).

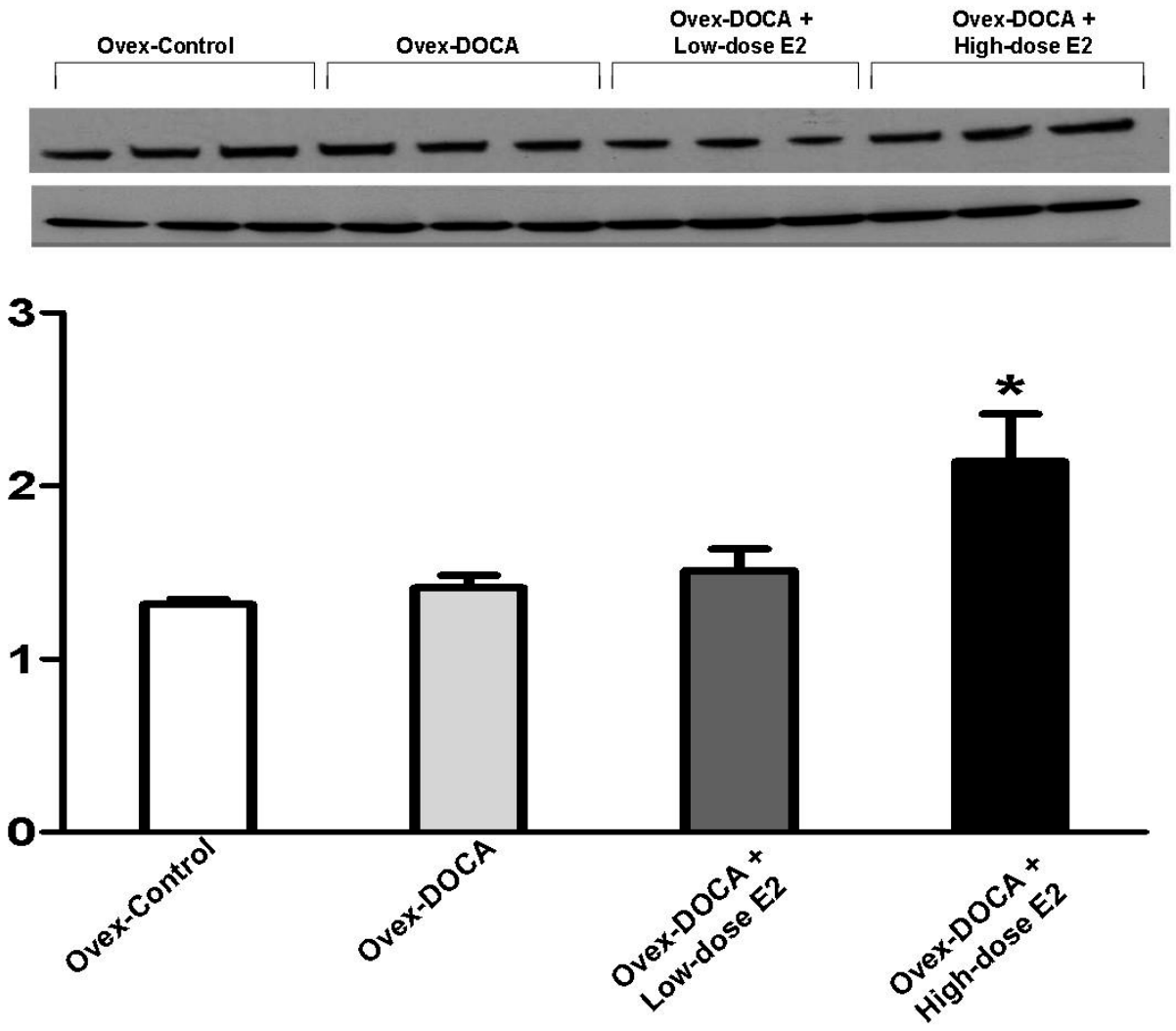

Figure 4-7: Effect of DOCA and E2 treatment on cardiac ACE2 protein levels. High-dose E2 replacement increased the levels of cardiac ACE2 protein. * $p<0.05$ vs. Ovex-control, (n=3 per group).

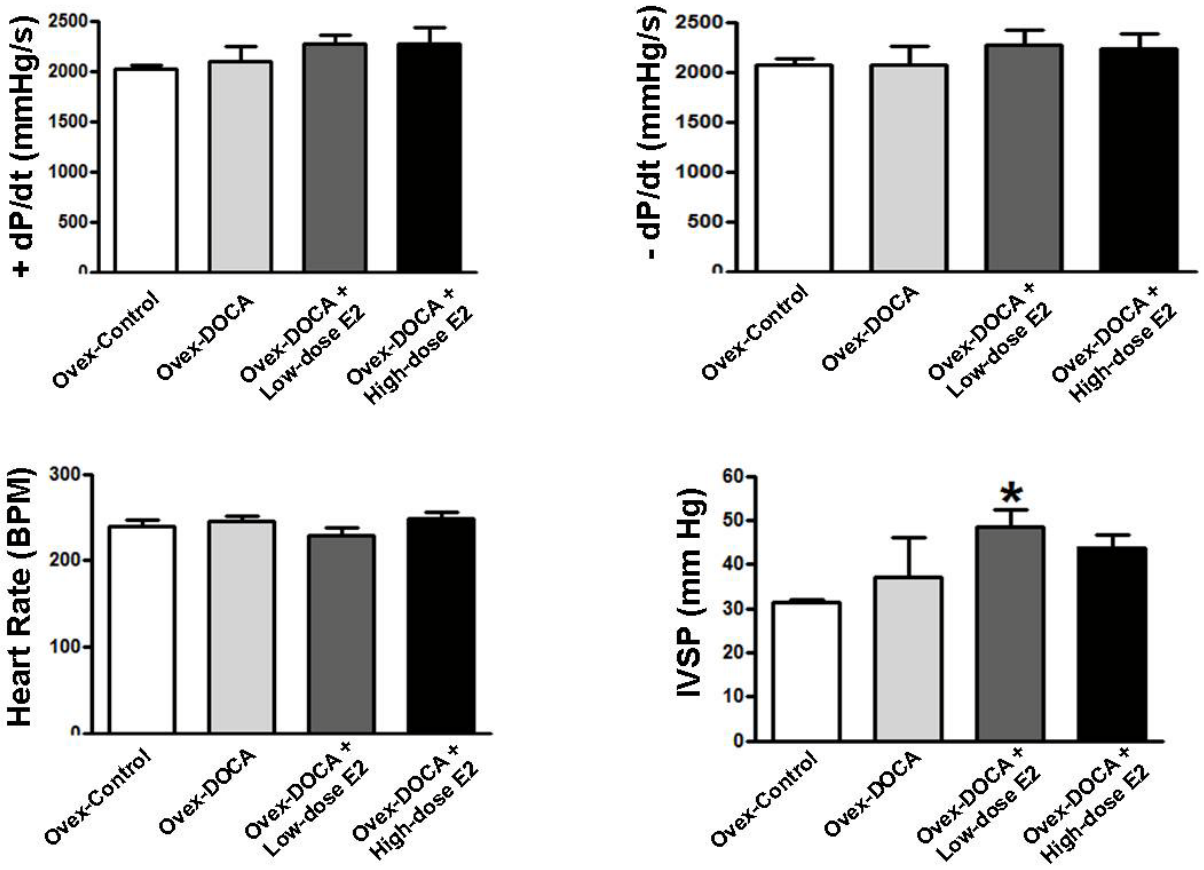

Figure 4-8: Effects of DOCA and E2 treatment on cardiac function. (A) +dP/dt, (B) -dP/dt, (C) heart rate and (D) intraventricular systolic pressure (IVSP), in isolated rat hearts perfused according to the Langendorff technique. The values for each animal were obtained by averaging 7 readings (each reading was collected at 5 min interval during experimental period of 30 min). * $p<0.05$ vs. Ovex-control, (n=4-6 per group).

CHAPTER 5
OVERALL CONCLUSIONS

The overall goal of our lab is to study the various components of the endocrine renin-angiotensin system (RAS) and their effects on several disease states and associated end-organ damage. Pulmonary hypertension and pulmonary fibrosis are lung diseases with poor diagnosis and limited therapeutic options. The available therapies are ineffective in improving the quality of life and reducing mortality rates. Thus, there is an unmet medical need to treat these diseases, which necessitates the discovery of novel therapeutic targets/agents for a safe and effective therapy. An altered RAS has been implicated in the pathogenesis of lung diseases including pulmonary hypertension (PH) and pulmonary fibrosis (PF). Over the past few years, new components of the RAS have been discovered, of which the ACE2-Ang-(1-7)-Mas axis has gained significant importance, due to its beneficial effects on kidney, liver and cardiovascular diseases. Accumulating evidence suggest that this axis may also exert a protective role against lung diseases. Here, we investigated the role of ACE2-Ang-(1-7)-Mas axis on PH and PF. The studies presented here indicate a cardio-pulmonary protective role for this axis against PH and PF. Lentiviral mediated overexpression of ACE2 and Ang-(1-7) were carried out to study their protective roles in lung disorders. Ang-(1-7) is an enzymatic breakdown product of Angiotensin II; hence, there is no endogenous gene that encodes for this peptide. For the studies presented in this dissertation work, we designed a synthetic gene that would produce a secretory form of Ang-(1-7), which was packaged into a lentiviral vector. Lentiviral mediated overexpression of Ang-(1-7) had a therapeutic effect against pulmonary hypertension and pulmonary fibrosis, which confirm that gene delivery to the lungs is an effective and

viable option for treating pulmonary diseases. Also, site directed overexpression of the therapeutic gene in the lungs helps overcome the associated systemic side effects. In our case, systemic administration of the Ang-(1-7) leads to rapid proteolytic degradation of the heptapeptide resulting in a short biological half life. However, local overexpression of this peptide in the lungs helped overcome this limitation. Studies described in chapter 2 demonstrate that Ang-(1-7) attenuated monocrotaline-induced pulmonary hypertension. This study reveals several important findings. First and foremost, it indicates that the predominant mechanism by which ACE2 mediates its beneficial actions is through the generation of Ang-(1-7) rather than just degradation of Ang II as previous studies from our lab had reported that the overexpression of ACE2 or its activation using a synthetic molecule leads to protective effects against PH (Yamazato et al., 2009; Ferreira et al., 2009). Furthermore, it provides evidence that the observed protective effects of Ang-(1-7) are mediated via activation of the G-protein coupled Mas-receptor, as blockade of this receptor completely abolished the beneficial effects of Ang-(1-7).

Next, we went on to evaluate the protective actions of ACE2 and Ang-(1-7) against pulmonary fibrosis. Bleomycin-induced pulmonary fibrosis is a well established animal model that mimics several features of the human fibrotic lung diseases. Studies described in chapter 3 establish the anti-fibrotic effects of ACE2 and Ang-(1-7) against bleomycin-induced lung fibrosis.

Heart failure happens to be the leading cause of death in patients suffering from pulmonary hypertension and lung fibrosis. One of the causal factors for heart failure is the development of right ventricular remodeling which is invariably associated with

these types of pulmonary diseases. Overexpression of Ang-(1-7) prevented right ventricular hypertrophy and fibrosis in the monocrotaline model of pulmonary hypertension. Also, lung overexpression of ACE2 and Ang-(1-7) prevented the development of secondary pulmonary hypertension and right ventricular hypertrophy in the bleomycin model of lung fibrosis. These results obtained from two different models of lung diseases support the fact that ACE2-Ang-(1-7)-Mas axis mediates cardiopulmonary beneficial actions. An interesting and important aspect of our studies is that the beneficial effects of ACE2 and Ang-(1-7) overexpression was associated with no effects on systemic blood pressure. This is relevant since systemic hypotension can be counter-productive in patients with pulmonary disorders, as they are already at a high risk of developing hypotension due to right ventricular overload.

Study presented in chapter 4 determined the role of 17β-estradiol replacement in ovariectomized animals on cardiac remodeling (cardiac hypertrophy and interstitial fibrosis) in the DOCA-salt model of hypertension. Administration of DOCA-salt represents a volume overload model of hypertension, associated with the development of cardiac hypertrophy and fibrosis. 17β-estradiol replacement exerted anti-cardiac remodeling effects via modulation of the local heart RAS. The protective effect of 17β-estradiol was devoid of any reduction in the elevated blood pressure induced by DOCA-salt administration, suggesting that estrogen may have direct effects on the heart. There are functional estrogen receptors on the heart, which might mediate these beneficial effects, independent of blood pressure changes. Dosage and timing of hormone initiation relative to the onset of menopause appears to be an important issue, which might have a role to play in the observed adverse effects of estrogen replacement

therapy in the recent clinical trials. Although the present study was not designed to study the timing aspect, we noted that hormone replacement immediately after ovariectomy did result in cardioprotective effects, suggesting that hormone replacement is beneficial when started early and kept at moderate dosing.

Shortcoming and pitfalls: Our studies presented here have certain limitations. In the studies described in chapters 2 and 3, we used a single dose of lentivirus for gene delivery to the lungs. This dose of lentivirus was selected based on previous studies carried out in the Raizada lab. Future studies should certainly be carried out with different doses to probe into the dose-effect relationship between ACE2/Ang-(1-7) and their protective actions against lung diseases.

Though we injected ACE2 and Ang-(1-7) lentivirus into the lungs, we did not study the whole body tissue distribution or cellular localization of the lentivirus within the heart or lungs (our two organs of interest). ACE2 is involved in degrading other peptides apart from Ang II, and Ang-(1-7) has been reported to potentiate the actions of bradykinin. We did not investigate the levels of other peptides metabolized by ACE2 enzyme nor did we analyze the role of bradykinin in the protective actions of Ang-(1-7). Also, the cytokine measurements in these pulmonary studies were only performed at the level of mRNA. It is not necessary that the mRNA reflects protein levels. Therefore, evaluation of both types of gene product will be desirable in future work.

In the DOCA-salt model, the animals were treated with estradiol for a period of 6 weeks. The 6 week timeframe was selected for practical and financial reasons, particularly, the subcutaneous implantation of DOCA and estradiol pellets. Timeframes longer than 6 weeks would require replacement of the DOCA and estradiol pellets. As

anesthesia and subcutaneous surgery are unnecessary risks for these animals, it was decided not to increase the timeframe. However longer endpoints would give us a better understanding of the protective effects of estrogen on cardiac function and heart failure, as presumably, the DOCA alone group would have experienced severe cardiac dysfunction and heart failure.

17β- Estradiol was delivered continuously by subcutaneous implantation of estradiol pellets. However, under normal conditions there is always a cyclic change in the hormonal levels of estradiol. By administering at a constant rate, continuously for 6 weeks, we are not mimicking the normal physiological pattern. In addition to estrogens, ovaries also produce other hormones such as progesterone etc, which were not replaced in the present study. Hence, it is difficult to delineate whether the protective effects were solely due to estradiol replacement or the concomitant lack of other endogenous hormones. Also, estrogens have been shown to affect both the synthesis and degradation pathways of collagen. More detailed studies would have to be performed, both *in vitro* and *in vivo* to determine the interaction between estrogen and the RAS in mediating these observed cardiovascular protective effects.

APPENDIX
PRODUCTION OF LENTIVECTOR

Transfection

293FT cells (Invitrogen Corporation, #R70007) were seeded in a T-225cm^2 culture flask at a density of 3 x 10^3/cm^2 and grown in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum and antibiotics (100U/ml penicillin and 100µg/ml streptomycin; growth medium). When the cultures reached 100% confluency, the flask was split into 1:5. When all the 5 flasks were 100% confluent, cells from 4 of the flasks were harvested and distributed into 9 T-225cm^2 culture flasks while the remaining one flask was again split into 1:5. Prior to cell seeding, all the 9 T-225cm^2 culture flasks were coated with poly-D-lysine in PBS for 3hours at 37°C. The cultures were maintained at 37°C in 5% CO_2 throughout the virus production period. When the cultures reached 90-95% confluency, the transfection was performed. For one large-scale preparation of virus, 9 T-225 flasks of 293FT cells were transfected as follows: Transfection mixture for all 9 T-225cm^2 flasks was prepared by gently adding 192 µg pHP, 95µg pTYF-transgene 76µg pHEF.VSVG and 16µg of Tat plasmid DNA into 8.0ml of plain DMEM (with no antibiotics) in one 50ml polystyrene tube. After mixing, 756µl of Superfect (Qiagen) was added to the DNA solution. The contents of the tube were gently mixed and incubated at room temperature for 10 min. Next, these transfection complexes were diluted into 140ml of pre-warmed plain growth medium (with no antibiotics). The media from the 9 flasks was aspirated and replaced with 17ml of growth medium containing the transfection complexes. The flasks were then incubated for 4-6 hours in a 37°C/5% CO_2 incubator. Following the incubation period, the medium containing the transfection mixture was replaced with 17.0ml of fresh growth medium.

After 24 hours, the media containing the first batch of virus was harvested from each flask and 11.0ml of fresh growth medium was added to the cells. This should result in two collections of vector, one at ~30 hours post-transfection and a second at ~45 hours.

Lentiviral Concentration

The two collections of vector are handled independently. Each collection of vector is performed in 50 ml polystyrene tubes on ice. Each tube is then centrifuged at 2000 x g for 10 minutes at 4^0C then filtered through a 0.45 micron low protein binding membrane (Nalgene, PES). The two collections are then mixed together and subjected to ulrafilteration. For ultrafiltration, the virus stock was divided into two 60ml aliquots and centrifuged through two separate Centricon-70 ultrafilteration columns (Millipore) for 1 h in 4°C at 2500 x g. The retentate was retrieved by centrifuging the inverted column for 1 min in 4°C at 990 x g. The virus-containing retentate was then added to 20 ml of virus-containing media in a 30ml Beckman conical-bottom tube and mixed thoroughly. The samples were then centrifuged at 45,000 x g for 2.5 h at 4^0C using a Beckman SW-28 swinging bucket rotor. The resulting supernatant was removed and discarded. 30-50µl of phosphate buffered saline (PBS) was added on top of the viral pellet. After 24 hours, the viral pellet was gently mixed by pipetting, aliquoted and stored at -80°C until use

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BIOGRAPHICAL SKETCH

Vinayak Shenoy Katapadikar was born on 30th November in Mumbai, India. Immediately upon graduating high school, Vinayak began his undergraduate studies, in Pharmacy from the College of Pharmaceutical Sciences, Manipal, India. Vinayak completed the Bachelor of Pharmacy degree in 1999 and began working as a Marketing Executive with Pharmacia and Upjohn Company.

After working for a year, he decided to pursue his master's degree and enrolled at Birla Institute of Technology and Sciences, Pilani, India. As a part of his master's degree, he interned at the Torrent Research Laboratories, Gandhinagar, India where he was exposed to the exciting field of cardiovascular research. After graduating with a Master of Science degree, he joined the drug discovery team at Nicholas Piramal Research Center, Mumbai, India as a research scientist. He worked for 2 years before he got accepted into the doctorate program in the Department of Pharmacodynamics. He chose Dr. Katovich as his major professor and studied the protective role of ACE2-Ang-(1-7)-Mas axis against heart and lung diseases. Vinayak is married to Anitha Shenoy who is a graduate student at the University of Florida and they are proud parents of Abhinav Shenoy (Sai). Vinayak looks forward to beginning post-doctoral work in the lab of Dr. Mohan Raizada in the College of Medicine, also at the University of Florida. Following hir post-doctoral work, Vinayak hopes to pursue a career in pre-clinical research at the industrial level and later return to academia as a teacher and mentor to students interested in a career in research.