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INTERACTION OF TUMOR NECROSIS FACTOR-ALPHA AND THE RENIN ANGIOTENSIN SYSTEM IN THE PATHOGENESIS OF HYPERTENSION

A Dissertation

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by
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ABSTRACT

Hypertension is a major predisposing factor for the development of cardiovascular and renal diseases. The renin-angiotensin system (RAS) plays a pivotal role in the pathogenesis of cardiovascular diseases such as hypertension, myocardial infarction, heart failure, and stroke. Angiotensin II (Ang II), the effector peptide of the RAS, activates a wide spectrum of signaling responses via the Ang II type-I receptor that mediate its physiological control of blood pressure, thirst and sodium balance. For the past two decades, increasing evidence has demonstrated that the circulatory RAS and local/tissue RAS components (heart and brain) may contribute to the development of hypertensive response. Currently, hypertension is considered a low-grade inflammatory condition induced by interaction of the RAS with various pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α). Several *in vitro* and *in vivo* studies suggest the existence of a cross-talk between Ang II and TNF-α, implying an important role for TNF-α in blood pressure regulation. However, the functional importance of TNF-α in Ang II-induced response is unclear. In this dissertation, we examined the hypothesis that TNF-α is involved in the Ang II-induced hypertensive response and explored the interaction of Ang II and TNF-α in the heart and brain in the pathogenesis of hypertension. To examine this interaction, the effects of chronic administration of Ang II was evaluated in TNF-α knockout mice to dissect out the role played by TNF-α in the Ang II-induced effects. Additionally, the role of reactive oxygen species and the transcription factor nuclear factor-kappaB (NF-κB), were examined in this interaction between Ang II and TNF-α. Furthermore, to understand the role of central control of blood pressure via the hypothalamic paraventricular nucleus, an important cardiovascular regulatory center in the brain, we studied the effect of TNF-α blockade and Angiotensin Converting Enzyme 2 overexpression within the brain on blood pressure control.

Overall, these studies demonstrate a functional interaction between the RAS and TNF-α in hypertension and the possible roles of oxidative stress and NF-κB in mediating the Ang II-induced hypertensive response. These findings provide an important clue in our quest for understanding the pathophysiology of hypertension and other cardiovascular diseases.

CHAPTER 1

BACKGROUND AND LITERATURE REVIEW

HYPERTENSION

Hypertension is the single most important predisposing factor for the development of pathological cardiovascular events. Untreated hypertension leads to coronary heart disease, congestive heart failure, renal failure and stroke in patients. Hence, one of the primary goals in the management of hypertension is the end organ protection of the kidneys, heart and brain. Several prospective clinical trials using calcium channel antagonists, renin inhibitors, angiotensin converting enzyme inhibitors, and angiotensin receptor blockers have documented efficacy in treating hypertension; however, the clinical course of hypertension is progressive, resulting in end organ damage. Consequently, new and innovative approaches for treating hypertension are required.

The pathogenesis of essential hypertension is multifactorial and highly complex. Numerous factors, including, humoral mediators, local vascular reactivity, circulating blood volume, vascular caliber, blood viscosity, cardiac output, blood vessel elasticity, and neural stimulation, modulate blood pressure for adequate tissue perfusion and nutrient delivery. A possible pathogenesis of essential hypertension has been proposed in which multiple factors, including genetic predisposition, excess dietary salt intake and adrenergic tone, may interact to produce hypertension. Although genetics appear to contribute to hypertension, the exact mechanism has not been established. Despite the enormous amount of research carried out in recent years to understand the pathogenesis of hypertension, the molecular mechanisms underlying hypertension are still relatively unknown.

The renin-angiotensin system (RAS) plays a pivotal role in the pathogenesis of hypertension, myocardial infarction, stroke, renal failure, and diabetic vascular complications.

Besides the classical regulatory effects on blood pressure and volume, sodium excretion and aldosterone secretion, evidence is now accumulating that the RAS may also induce an inflammatory response and oxidative stress in hypertension. Recent studies suggest that hypertension is a low grade inflammatory condition induced by interaction of RAS with various inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α). Thus, it is imperative that further emphasis should be focused on complete elucidation of the interactive role of the RAS and inflammatory cytokines to increase our understanding of how cardiovascular diseases are linked with inflammatory processes.

RENIN ANGIOTENSIN SYSTEM AND HYPERTENSION

Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system (RAS), plays a key role in regulation of body fluid homeostasis, the development of hypertension, and the maintenance of cardiovascular function.

Classically angiotensinogen, produced by liver, is hydrolyzed by renin, from the juxtaglomerular cells in the kidney to produce the decapeptide angiotensin I, which is then converted by angiotensin converting enzyme (ACE) into the biologically active octapeptide, angiotensin II. The circulating Ang II stimulates the angiotensin receptors present in the kidney and the vasculature, which leads to elevations in blood pressure by promoting vasoconstriction, renal sodium and water reabsorption, increased cardiac output, sympathetic tone, arginine vasopressin release, and stimulation of thirst in the central nervous system (Phillips, 1987; Reid et al., 1978). Originally, the RAS was considered to be an endocrine system with circulating Ang II as functional effector hormone. However, in the recent decade with the advent of new molecular techniques, there have been significant changes in this view of the RAS. For instance,

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recent studies have made clear that a local RAS is present in many tissues and are implicated in the classical effects of this system. It has been shown that brain, kidney, heart, vasculature, adipose tissue, pancreas and gonads all have fully functional components of local tissue RAS components (Lavoie and Sigmund, 2003). With the development of many tissues-specific transgenic animal models the roles of these local RAS components have become clear. Ang II, the effector peptide of the RAS, exerts its actions by binding to its receptors, called type I (AT_1R) and type II (AT_2R), located on the plasma membrane of target cells throughout the body.
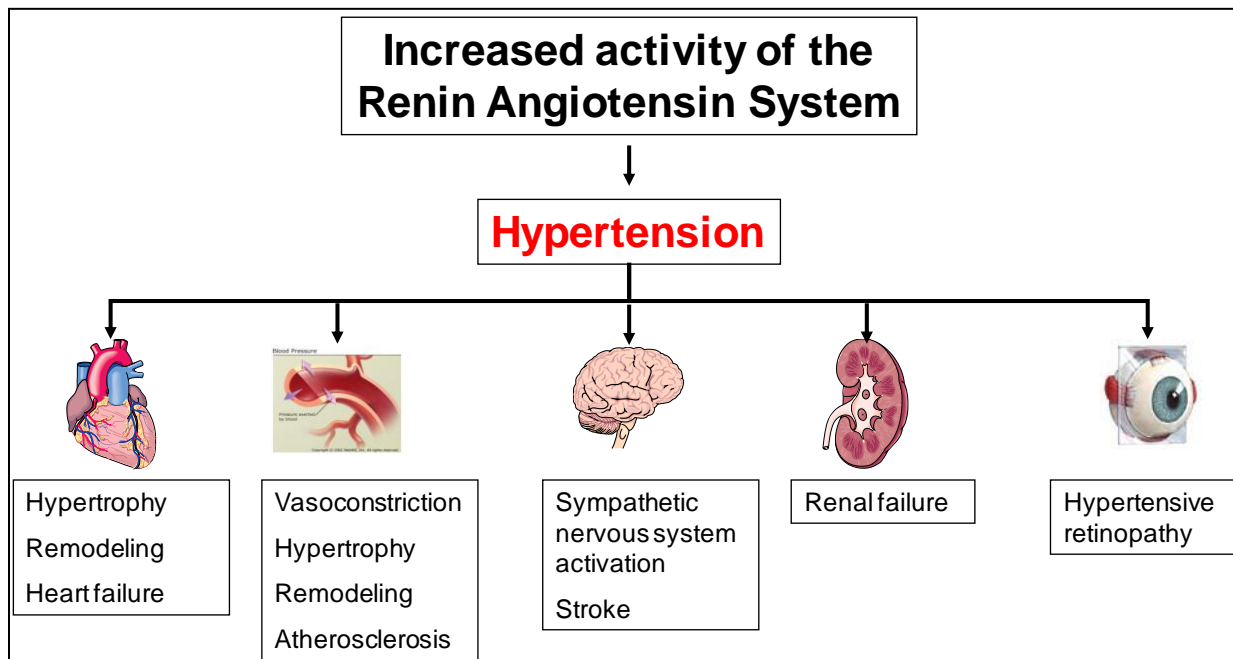
Figure 1.1. Increased activity of renin angiotensin system is critical in development and maintenance of hypertension and may lead to end organ damage.

Recently a new axis of the RAS has been established. In the year 2000, ACE2, a new member of the ACE family was identified by two groups (Donoghue et al., 2000; Tipnis et al., 2000). ACE2 can cleave the decapeptide Ang I to generate the inactive Ang (1-9) peptide, which then can be converted to the vasodilatory peptide Ang (1-7) by ACE or other peptidases (Donoghue et al., 2000). ACE2 can also directly metabolize Ang II to generate Ang (1-7)

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(Vickers et al., 2002). Ang (1-7), the main product of Ang II degradation by ACE2, has opposite properties to that of Ang II. By acting through the receptor Mas, Ang (1-7) promotes vasodilation, antiproliferation and antihypertrophy (Ferrario et al., 2005; Santos et al., 2003). Accumulating evidence indicate that by cleaving Ang II into Ang (1-7), ACE2 may play a pivotal role in counterbalancing the vasoconstrictive actions of the ACE/Ang II/AT_1R axis and may be beneficial for the cardiovascular system.
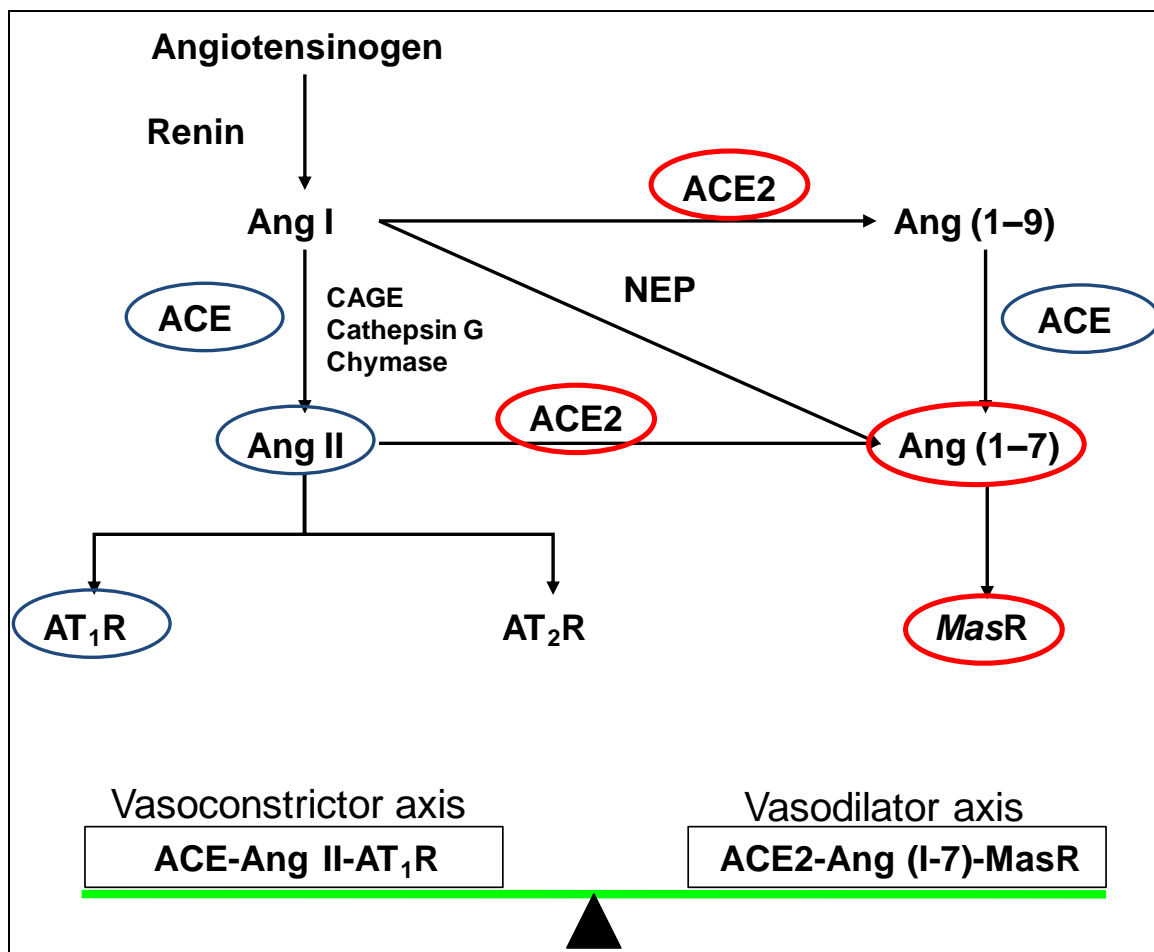
Figure 1.2. The components of the renin angiotensin system. The balance between the vasoconstrictor axis and the vasodilator axis is critical in the maintenance of blood pressure. Ang-Angiotensin, ACE-Angiotensin converting enzyme, NEP-Neutral endopeptidases, ACE2-Angiotensin converting enzyme 2, AT_1R-Angiotensin type 1 receptor, AT_2R-Angiotensin type 2 receptor, MasR-Mas receptor.

Renin Angiotensin System and the Heart. It is now established that all the components of the RAS cascade are expressed in normal cardiac tissue (Bader et al., 2001). The fundamental response to increased biomechanical stress observed during hypertension and heart failure, or increased levels of cardiac Ang II is cardiomyocyte and chamber hypertrophy. Several lines of evidence suggest that Ang II can function as a growth factor for cells of the cardiovascular system and can induce cell proliferation and cell differentiation, lead to cardiac hypertrophy and vascular remodeling. Once Ang II binds to the AT_1R, a series of signaling cascades are activated which lead to various effects of Ang II such as contraction, smooth muscle cell growth, hypertrophy, and cell migration, events that contribute to both normal vascular function and disease progression.

In cell cultures Ang II through AT_1R promotes hypertrophy of the embryonic, neonatal and adult cardiomyocytes (Thomas et al., 2002). Overexpression of the AT_1R in the heart of transgenic mice induces a massive cardiac hypertrophy and fibrosis with conduction and rhythm defects and heart failure without hypertension and this phenotype can be reversed by angiotensin receptor blocker (Hein et al., 1997; Paradis et al., 2000). Several studies *in vivo* have suggested that AT_1R is a critical factor in mediating Ang II effects on cardiac hypertrophy. Chronic infusion of subpressor doses of Ang II to rats caused ventricular hypertrophy without changes in blood pressure. In a genetic model of hypertension, normalization of blood pressure by sympatholytic agents or by direct vasodilators did not cause regression of cardiac hypertrophy, whereas treatment with an ACE inhibitor did. AT_1R mediate the Ang II-stimulated collagen synthesis and enhance cardiac remodeling through perivascular and myocardial fibrosis, as well as vascular medial thickening (Lim et al., 2001). The expression of many immediate-early genes (*c-fos, c-jun, junB, Egr-1 and c-myc*) and fetal marker genes of cardiac hypertrophy atrial

natriuretic peptide (ANP), brain natriuretic peptide (BNP), myosin heavy chains (MHC), and skeletal α-actin are stimulated by AT_1R activation (Sadoshima and Izumo, 1993). Ang II, via AT_1R activation, stimulates NADPH oxidase and enhances production of reactive oxygen species (ROS) in the heart, which in turn contributes to cardiac hypertrophy and remodeling. Increase in ROS also leads to inflammation by stimulating nuclear factor-kappaB (NF-κB), and by upregulating adhesion molecules, cytokines, and chemokines (Bader et al., 2001; Griendling et al., 2000b).

Renin Angiotensin System and the Brain. The brain expresses the genes that encode all the components of the RAS including angiotensinogen, renin, Ang II, ACE, AT_1R, AT_2R, and ACE2 (Harmer et al., 2002; Phillips, 1987; Phillips and Sumners, 1998). The brain responds to both circulating and tissue Ang II, and participates in regulation of blood pressure by increasing sympathetic activity, vasopressin release, thirst, and sodium appetite (Phillips, 1987). The components of the RAS have been identified in all brain areas involved in the central regulation of blood pressure, including the subfornical organ (SFO), paraventricular nucleus (PVN), rostral ventrolateral medulla (RVLM), area postrema, and nucleus tractus solitarius, amongst others (Davisson, 2003). Acute central Ang II injections induce a pressor response and an increase in sympathetic nerve activity, while central Ang II blockade attenuates the hypertensive response and decreases sympathetic activity, suggesting the important role of brain in hypertensive response (Davisson et al., 2000; Davisson et al., 1998).

The PVN of the hypothalamus is recognized as a critical (CNS) nervous system center for the coordination of autonomic and neuroendocrine homeostatic responses (Dampney, 1994; Swanson and Sawchenko, 1983). Several studies support the role of the PVN in blood pressure control. Microinjection of Ang II into the PVN of rats increased mean arterial pressure, which

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was attenuated following systemic administration of losartan, an AT_1R antagonist (Bains et al., 1992). We have recently reported that chronic peripheral Ang II infusion results in increased production of proinflammatory cytokines in the PVN of hypothalamus (Kang et al., 2009). Evidence is accumulating for the importance of brain ACE2 in the pathogenesis of neurogenic hypertension as ACE2 may play a protective role in the brain by activating ACE2/Ang (1-7)/Mas receptor axis and balancing the ACE/Ang II/AT_1R axis. Various studies have demonstrated that ROS generation and NF-κB activation by Ang II modulates some of these effects in the CNS, acting in different regions of the brain.

INFLAMMATION AND HYPERTENSION

Pro-inflammatory Cytokines. Recent studies suggest that hypertension is a chronic low grade inflammatory condition and pro-inflammatory cytokines (PIC) such as TNF-α, interleukin (IL)-6, and IL-1β, have emerged as critical players in the development of hypertension and other cardiovascular diseases (Ferreri et al., 1997; Kang et al., 2009; Shi et al., 2010; Sriramula et al., 2008). Ang II, the effector peptide of the RAS, plays a key role in the regulation of the vascular inflammatory response by activating the recruitment of inflammatory cells. In addition, inflammatory cells can produce Ang II, resulting in a local positive feedback response, thereby perpetuating the inflammatory cycle. Ang II is known to regulate the expression of adhesion molecules, chemokines, and cytokines, as well as the growth factors that participate at various points in the inflammatory pathway. For example, Ang II can upregulate TNF-α, IL-1, and IL-6, all of which act to enhance the inflammatory response (Arenas et al., 2004; Ferreri et al., 1997).

TNF-α. TNF-α is a multifunctional cytokine, whivh plays an important role in diverse physiological and pathophysiological processes, such as inflammation, cell survival, growth, differentiation and apoptosis (Mann, 2002; von Haehling et al., 2004). TNF-α is produced by

many different cell types. The main sources in vivo are stimulated monocytes, fibroblasts, and endothelial cells. Macrophages, T- and B-lymphocytes, granulocytes, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cells, cardiomyocytes and keratinocytes also produce TNF-α after appropriate stimulation. TNF-α is central in initiating and sustaining the proinflammatory cytokine cascade and can stimulate the production of other cytokines such as IL-1β and IL-6 (Mann, 2002).

TNF-α Signaling. TNF-α is synthesized as a monomeric type-2 transmembrane protein (tmTNF) that is inserted into the membrane as a homotrimer and cleaved by the matrix metalloproteinase, TNF-α converting enzyme (TACE) to a 51 KDa soluble circulating trimer (solTNF); both tmTNF and solTNF are biologically active. TNF-α binds to two distinct surface receptors, type 1 (p55, TNFR1) and 2 (p75, TNFR2), and induces intracellular signaling cascades, which regulate cell survival, apoptosis, differentiation, proliferation and activation of immune functions (Idriss and Naismith, 2000; MacEwan, 2002). TNFR1 is expressed in most cell types, and can be activated by binding of either solTNF or tmTNF, with a preference for solTNF; whereas TNFR2 is expressed primarily by cells of the immune system, including microglia, and by endothelial cells, and is preferentially activated by tmTNF. The complexity of TNFR1-mediated signaling is what allows many divergent outcomes to occur as a result of TNF-α signaling and contributes to difficulties inherent with and the side effects resulting from broad TNF-α signaling inhibition (McCoy and Tansey, 2008).

Several *in vitro* and *in vivo* studies suggest the existence of cross-talk between the RAS and TNF-α (Arenas et al., 2004; Brasier et al., 1996; Kalra et al., 2002; Sasamura et al., 1997). For instance, Ang II treatment induces the production of TNF-α in cultured cardiomyocytes and fibroblasts (Kalra et al., 2002; Yokoyama et al., 1999). In patients with hypertension or heart

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failure, chronic blockade of AT_1R resulted in a significant decrease in the circulating levels of TNF-α (Cottone et al., 1998; Tsutamoto et al., 2000). A recent study showed that mice treated with etanercept prevented hypertension and blunted the increase in super oxide production in response to Ang II (Guzik et al., 2007). However, the functional importance of TNF-α in Ang II induced responses is not yet clearly defined.

Anti-inflammatory Cytokines. The balance between the PIC and anti-inflammatory cytokines (AIC) is considered an important determinant in the outcome of cardiovascular disease. IL-10 is one of the most important anti-inflammatory cytokines. It was shown to downregulate the production of TNF-α, IL-1, and IL-6 (Bolger et al., 2002; Nishio et al., 1999). Circulating levels of IL-10 were reported to be decreased in patients with hypertension and a decrease in IL-10 and the IL-10/TNF-α ratio correlated with depressed cardiac function (Kaur et al., 2006). It has been shown that Ang II infusion results in decreased expression of IL-10 in the hypothalamic paraventricular nucleus (Kang et al., 2009; Shi et al., 2010).

OXIDATIVE STRESS AND HYPERTENSION

The cellular mechanisms and signaling pathways whereby Ang II mediates its physiological and pathophysiological vascular effects are complex. A growing body of evidence indicates that production of ROS and activation of reduction-oxidation dependent signaling cascades leading to oxidative stress are centrally and critically involved in Ang II-induced cardiovascular events (Griendling et al., 2000b; Touyz, 2000). Oxidative stress is defined as the imbalanced redox state where pro-oxidants overwhelm intrinsic antioxidant systems, resulting in increased production of ROS. Under physiological conditions, ROS are produced in a controlled manner at low concentrations and function as signaling molecules to maintain vascular integrity.

Under pathological conditions, increased ROS production contributes to endothelial dysfunction, increased contractility, vascular smooth muscle cell growth, monocyte invasion, lipid peroxidation, inflammation, and increased deposition of extracellular matrix proteins. All these are important factors in hypertensive vascular damage (Papaharalambus and Griendling, 2007). ROS are produced by all vascular cell types, including endothelial, vascular smooth muscle and adventitial cells, and can be formed by numerous enzymes. Enzymatic sources of ROS in the vascular wall that are involved in hypertension are NADPH oxidase, xanthine oxidase, uncoupling of the mitochondrial respiratory chain, cytochrome p450, and uncoupling of endothelial nitric oxide synthase (eNOS) (Griendling et al., 2000b).

The major ROS are superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH^-$). Superoxide anion can further combine with nitric oxide (NO), forming reactive compounds such as peroxynitrite (ONOO·), and generating nitroso-redox imbalance (Hare and Stamler, 2005). In addition, peroxynitrite oxidizes tetrahydrobiopterin thereby leading to eNOS uncoupling and diminished NO production. These ROS are generated as intermediate products in oxidative phosphorylation reactions and play a role in normal redox control of physiological signaling pathways. They also act as important second messengers and intracellular signaling molecules in cell growth, survival and apoptosis. However, excessive ROS generation leads to oxidative stress, triggers cell dysfunction, lipid peroxidation, and DNA mutagenesis (Murdoch et al., 2006).

The major source of Ang II-stimulated ROS generation in the cardiovascular system is NADPH oxidase. Vascular ROS are produced primarily from NADPH oxidase, a multi-subunit enzyme catalyzing superoxide ($\cdot O_2^-$) production by the 1 electron reduction of oxygen using NADPH as the electron donor: $2O_2 + NADPH \rightarrow 2O_2^- + NADP + H^+$. Vascular NADPH oxidase

comprises at least 4 components: cell membrane associated p22phox and gp91phox (or gp91phox [nox2] homologues, nox1 and nox4), and cytosolic subunits, p47phox, p67phox and Rac1. Activation of NADPH oxidase is regulated by many vasoactive hormones, cytokines, growth factors (platelet-derived growth factor, transforming growth factors) and mechanical stimuli (shear stress and stretch). The best studied pathway of ROS production in vascular cells is that of NADPH oxidase activation by Ang II, which has been shown to involve protein kinase C, phospholipase D, c-Src and receptor tyrosine kinases (Seshiah et al., 2002).

Several studies have established the role of ROS in vascular and cardiac hypertrophy and remodeling that contribute further to the pathogenesis of hypertension. It is well established that there is increased production of superoxide and depletion of nitric oxide in the endothelium of the blood vessels and in the heart of hypertensive animals and this contributes to contractile dysfunction. This depletion in nitric oxide could be due to either a direct decrease in NO (inhibition/depletion of nitric oxide synthase) or due to decreased bioavailability of NO due to its interaction with superoxide to form peroxynitrite. In Ang II-infused animal models and SHR rats, NADPH activity is increased, ROS generation is enhanced; these processes are mediated through AT_1Rs and associated with overexpression of vascular and cardiac NADPH oxidase units (Cifuentes et al., 2000; Heymes et al., 2003; Kakishita et al., 2003). Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats (Vaziri et al., 2000). It has been shown that Ang II-induced superoxide production and hypertension are markedly blunted in mice lacking the p47phox subunit of NADPH oxidase (Landmesser et al., 2002). Treatment with cell permeable SOD mimetic tempol lowers blood pressure in the 1-kidney, 1-clip model of renovascular hypertension (Dobrian et al., 2001) and SHR model of hypertension (Schnackenberg et al., 1998). In *in vitro* studies using cultures of vascular smooth muscle cells,

vascular fibroblasts, endothelial cells, and cardiomyocytes, Ang II stimulation increases both mRNA and protein expression of gp91phox (Griendling et al., 2000a). These studies support a role for NADPH oxidase-derived ROS and increased oxidative stress in the pathogenesis of Ang II-induced cardiovascular disease.

Hypertension is also an inflammatory condition in which various cytokines play a significant role in the progression of vascular and cardiac lesions. Both Ang II and TNF-α were shown to induce activation of NF-κB in a ROS dependent manner, which in turn can increase the production of other proinflammatory cytokines and chemokines (Papaharalambus and Griendling, 2007).

NF-κB AND HYPERTENSION

One of the major mechanisms involved in increased cytokine gene expression and inflammatory process of hypertension is the activation of transcription regulator nuclear factor-κB. NF-κB is a key transcription factor that regulates inflammatory processes, which can be activated by angiotensin II, proinflammatory cytokines, and reactive oxygen species. NF-κB complexes comprise homodimers or heterodimers of the structurally related proteins, NF-κB1 (p50), NF-κB2 (p52), Rel A (p65), c-Rel, and Rel B, which share the amino-terminal Rel-homology domain, with dimerization, nuclear localization and DNA-binding functions (Li and Verma, 2002). p50/p65 heterodimers are predominant in many cell types. NF-κB proteins are present in an inactive form in the cytosol bound to IκB inhibitor proteins, including IκBα, IκBβ, IκB-γ, IκBε and Bcl-3. Activation of NF-κB involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein IκB by specific IκB kinases. The free NF-κB then passes into the nucleus, where it binds to κB sites in the promoter regions of genes for inflammatory proteins such as TNF-α, inducible nitric oxide synthase, and adhesion molecules.

Thus the activation of NF-κB leads to a coordinated increase in the expression of many genes whose products mediate inflammatory and immune responses, ultimately involved in cardiac remodeling, hypertension, and heart failure (Barnes and Karin, 1997).

Recent evidence suggests that activation of NF-κB by Ang II induces gene transcription of proinflammatory cytokines, which leads to further increase in ROS production, fostering a positive feedback mechanism eventually leading to the progression of hypertension (Kang et al., 2009; Mehta and Griendling, 2007; Papaharalambus and Griendling, 2007). Therefore, an understanding of the regulation of NF-κB function and how its activity is integrated with other cell-signaling pathways involving Ang II and TNF-α provides an opportunity for the effective exploitation of these proteins in the treatment of hypertension and other inflammatory cardiovascular diseases.

STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

Although the majority of literature regarding hypertension to date focuses on role of RAS, particularly Ang II, emerging notion implicates inflammatory molecules in the pathogenesis of cardiovascular diseases. Since inflammation is a key component in the pathogenesis of hypertension and other cardiovascular diseases, the interaction between Ang II and TNF-α may play an important role in the modulation of hypertensive response. Hence studying the interaction between the RAS and TNF-α peripherally and centrally in hypertension and the possible roles of oxidative stress and NF-κB in mediating Ang II induced hypertensive response may provide an important clue in our quest in understanding the pathophysiology of hypertension and other cardiovascular diseases. The present work was undertaken in order to gain more insight into the mechanisms of regulation involved in cardiac remodeling and

hypertension through interaction between TNF-α and Ang II. The specific aims of this study were:

1. Determine the involvement of TNF-α in angiotensin II mediated effects on salt appetite, hypertension and cardiac hypertrophy.

2. Determine the specific mechanisms and downstream signaling pathways involved in the interaction between TNF-α and renin angiotensin system.

3. Determine the role of central inhibition of TNF-α in angiotensin II mediated hypertension.

4. Determine the role of ACE2 overexpression in the brain on Ang II-induced hypertensive response.

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CHAPTER 2

INVOLVEMENT OF TNF-α IN ANGIOTENSIN II MEDIATED EFFECTS ON SALT APPETITE, HYPERTENSION AND CARDIAC HYPERTROPHY*

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INTRODUCTION

Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system (RAS), plays a key role in regulation of body fluid homeostasis, the development of hypertension, and the maintenance of cardiovascular function (Brunner, 2001; Ruiz-Ortega et al., 2001). Ang II is widely recognized for its vasoconstrictor effect, thereby regulating vascular tone and systemic blood pressure (Kim and Iwao, 2000) and exerts its actions by binding to G-protein coupled receptors, angiotensin type 1 (AT_1R) and type 2 (AT_2R), located on the plasma membrane of target cells throughout the body(Allen et al., 2000; Murphy et al., 1991). The AT_1R plays a predominant role in the central regulation of arterial blood pressure and cardiovascular remodeling (Allen et al., 2000; Kim and Iwao, 2000).

Ang II has been shown to have both central and peripheral effects. In the peripheral vasculature, it normally acts to raise arterial pressure by AT_1R mediated vasoconstrictor effects. This pressor response of Ang II administration is also known to be partially modulated by the concomitant release of endothelin, prostaglandins, nitric oxide, superoxide and other free radicals from endothelial cells (Barton et al., 1997). Ang II also contributes to cardiac and vascular remodeling through its direct effect on the heart and the blood vessels (Griffin et al., 1991; Ruiz-Ortega et al., 2001; Sadoshima and Izumo, 1993). In addition, Ang II stimulates aldosterone, which acts on the renal distal tubules and collecting ducts to retain sodium and water, thereby raising blood pressure (Corvol and Jeunemaitre, 1997). Centrally, Ang II plays an important role in regulating salt appetite and thirst mediated by AT_1R (Davisson et al., 2000; McKinley et al., 2003; Morris et al., 2002). Apart from these, Ang II also acts as a growth factor and stimulator of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and chemokines (Funakoshi et al., 1999; Kalra et al., 2002; Ruiz-Ortega et al., 2002).

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TNF-α is a multifunctional cytokine that plays an important role in diverse physiological and pathophysiological processes such as inflammation, cell survival, growth, differentiation and apoptosis (Mann, 2002; von Haehling et al., 2004). Since inflammation is a key component in the pathogenesis of hypertension and cardiovascular disease, the interaction between Ang II and TNF-α may play an important role in the modulation of hypertensive response.

Several *in vitro* and *in vivo* studies suggest the existence of cross-talk between the RAS and TNF-α (Arenas et al., 2004; Brasier et al., 1996; Kalra et al., 2002; Sasamura et al., 1997). For instance, Ang II treatment induces the production of TNF-α in cultured cardiomyocytes and fibroblasts (Kalra et al., 2002; Yokoyama et al., 1999). In addition, TNF-α treatment increased AT_1R mRNA levels in neonatal rat cardiac fibroblasts (Gurantz et al., 1999). Administration of the AT_1R antagonist, valsartan, inhibited the expression of TNF-α in a murine model of arterial injury (Wu et al., 2001). In patients with hypertension or heart failure, chronic blockade of AT_1R resulted in a significant decrease in the circulating levels of TNF-α (Cottone et al., 1998; Tsutamoto et al., 2000). More importantly, blockade of TNF-α by etanercept has been shown to prevent renal damage in genetically hypertensive rats, and to lower blood pressure in rats with hypertension induced by Ang II and salt, suggesting a role for TNF-α in blood pressure regulation and renal injury (Elmarakby et al., 2006; Muller et al., 2002). A recent study showed that mice treated with etanercept prevented the hypertension and blunted the increase in super oxide production in response to Ang II (Guzik et al., 2007). However, the functional importance of TNF-α in Ang II induced responses is not yet clearly defined. Therefore, in the present study, we examined the role of TNF-α in the mediation of Ang II induced responses, particularly its effects on salt appetite, thirst, blood pressure and in myocardial cell growth. The effects of chronic administration of Ang II has been evaluated in TNF-α knockout mice and compared with

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those responses in wild type control mice to dissect out the role played by TNF-α in the Ang II induced effects.

METHODS

Experimental Animals. Twelve-week-old male B6129S-*Tnf^{tm1Gkl}*/J TNF-α knockout (TNF-α$^{-/-}$) mice and control B6129SF2/J (WT) mice (Jackson Laboratories) weighing between 25 and 30 grams were used in this study. The mice were housed in a temperature-controlled room (23 $\pm$ 2°C) with a 12:12 hour light-dark cycle from 07:00 to 19:00 in the animal quarters. The studies were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee. They were randomly divided into different groups according to chronic treatment with or without Ang II. Osmotic minipumps were implanted subcutaneously to deliver Ang II (1μg/kg/min; Sigma Chemical) for 14 days. The control animals were implanted with sterile saline pumps. These groups are as follows: 1) WT,- Sham operated control; 2) WT + Ang II, - wild type treated with chronic Ang II; 3) TNF-α$^{-/-}$, - Sham operated control; 4) TNF-α$^{-/-}$ + Ang II, - knockout mice treated with chronic Ang II; and 5) TNF-α$^{-/-}$ + Ang II + TNF-α , - TNF-α replaced knockout mice treated with chronic Ang II. In this group 6 of TNF-α$^{-/-}$ mice, along with Ang II infusion, human recombinant TNF-α (rTNF-α) was given intraperitoneally at a dose of 10 ng/g/day for 14 days. The overall experimental design was presented in figure 2.1. It should be noted here that we have not seen any sign of increasing susceptibility to infection in these TNF-α$^{-/-}$ mice in pre or post-operative periods of surgical intervention for telemetry probes and minipump implantations.

Blood Pressure Measurements. Blood pressure in conscious mice was measured using a radio telemetry system with carotid arterial catheters (Data Sciences Intl, DSI; MN). For the

implantation of the radiotransmitter, mice were anaesthetized with a ketamine-xylazine mixture (90 and 10 mg/kg, ip). A midline skin incision 2cm long from chin to manubrium was performed to isolate the carotid artery. The catheter portion of the telemetric probe (Model TA11PA-C10) was inserted into the ascending aorta through the left carotid artery, and the body of the probe was placed subcutaneously on the right flank. Mice were placed on a 12-hour light/dark cycle and received food and water ad libitum throughout the study. Mice were allowed to recover for 7-10 days before experiments were begun. Data was collected, stored, and analyzed using Dataquest A.R.T. software (DSI). Only animals giving stable records were included in the final analysis.
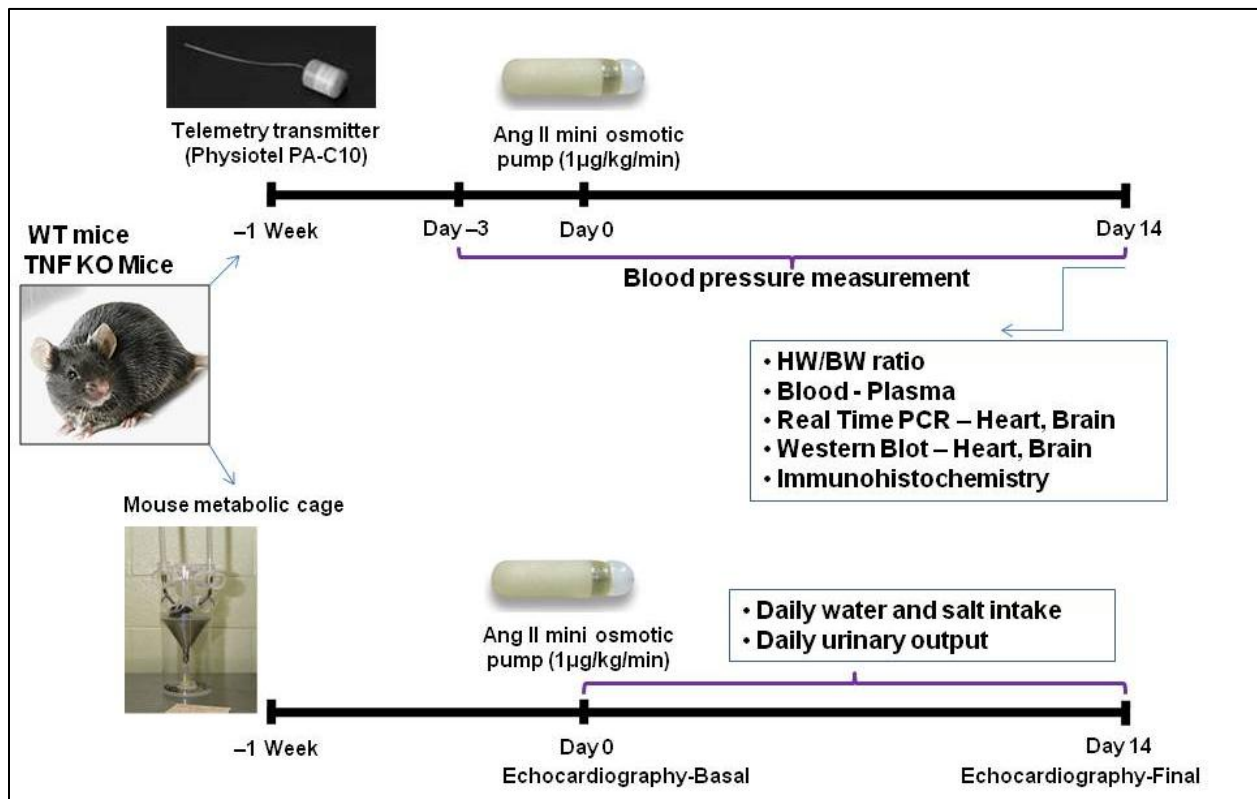
Figure 2.1. Schematic diagram showing the experimental design.

Metabolic Cage Study. In another set of experiments, mice were individually housed in specially designed metabolic cages that prevented food and fecal contamination of urine samples. Food and water were available ad libitum. Mice were given both water and salt (1.8% Sodium Chloride) solution in two separate receptacles and were allowed to adapt to the metabolic cages for 7 days. After the acclimatization period, daily water intake, salt intake, and urine volume were measured at baseline and during the 14 day Ang II infusion period. At the end of 14 days, the mice were euthanized and the organs were weighed, and the hypothalamus and left ventricular tissues were collected for mRNA and protein measurements.

Echocardiography. Transthoracic echocardiography was performed on mice under isoflurane anesthesia using a Toshiba Aplio SSH770 (Toshiba Medical, Tustin, CA) fitted with 12MHz transducer at baseline, and after 14 days of Ang II infusion. Left ventricular internal dimensions at end-systole and end-diastole (LVS and LVD), and interventricular septal wall thickness at the end of systole and at the end of diastole (IVSS and IVSD) were measured digitally on the M-mode tracings and averaged from at least 3 cardiac cycles. Left ventricular fractional shortening (%FS) was calculated as [(LVD-LVS)/LVD] x100.

Protein Analysis by Western Blot. The protein expression in the heart and hypothalamus was analyzed by western blot with the use of anti-AT_1R antibody (Santa Cruz). Protein was extracted from heart and hypothalamus tissue with lysis buffer. The total protein concentration in samples was measured by Bio-Rad Dc protein assay. Protein extracts (25µg) were combined with an equal volume of 2X Laemmli loading buffer, boiled for 5 minutes, and separated by electrophoresis on 10% SDS-Polyacrylamide gels. Then proteins were transferred from the gel to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) by electroblotting. Membranes were blocked with 1% casein in PBS-T for 1 hour and then incubated with anti-AT_1R antibody or anti-

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TNFR1 antibody (Santa Cruz) overnight at 4°C. Membranes were washed four times in wash buffer (1X TBS, 0.1% Tween-20), followed by incubation with HRP-labeled anti-rabbit IgG for 1 h at room temperature. Membranes were washed four times with wash buffer at room temperature before the antigen-antibody complexes were detected by an enhanced chemiluminescence kit (ECL Plus, Amersham). Autoradiographs were scanned and analyzed by densitometry using VersaDoc MP 5000 System (Bio-Rad). Protein expression of GAPDH was used to check equal loading. All primary antibodies were used at a dilution of 1:1000 and secondary antibodies were used at a dilution of 1:10,000.

Analysis of mRNA Expression by Real Time RT-PCR. Total RNA was isolated from left ventricular tissue and the hypothalamus using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. The RNA concentration was calculated from the absorbance at 260 nm and RNA quality was assured by 260/280 ratio. The RNA samples were treated with DNaseI (Ambion) to remove any genomic DNA. First strand cDNA was synthesized from 1μg RNA with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real Time PCR amplification reactions were performed with iTaq SYBR Green Super mix with ROX (Bio-Rad) in triplicate using the ABI Prism 7900 Real time PCR machine (Applied Biosystems, Fostercity, CA). The primer sequences were used as follows: ANP forward 5′-TGC CGG TAG AAG ATG AGG TC-3′; ANP reverse 5′-AGC CCT CAG TTT GCT TTT CA-3′; BNP forward 5′-AGG GAG AAC ACG GCA TCA TT-3′; BNP reverse 5′-GAC AGC ACC TTC AGG AGA T-3′; AT$_1$R forward 5′-CTG CGT CTT GTT CTG AGG TG-3′; AT$_1$R reverse 5′-ACT GGT CCT TTG GTC GTG AG-3′; NF-κB p50 subunit forward 5′-CGA GGC AGC ACA TAG ATG AA-3′; NF-κB p50 subunit reverse 5′-AGG TCC TTC CTG CCC ATA AC-3′; TNFR1 forward 5′-AAT ATC CTC GAG GCT CTG AGA-3′; TNFR1 reverse 5′-ATG TAC ACC AAG TTG GTA

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GC-3′; TNFR2 forward 5′-AGC CCA GGG CGG GAT A-3′; TNFR2 reverse 5′-GGT AAT TCT GGG AAG CCG TAA A-3′. Gene expression was measured by ΔΔCT method and was normalized to 18S ribosomal RNA or GAPDH mRNA levels. The data are presented as the fold expression of the gene of interest relative to their control animals.

Statistical Analysis. Results are presented as mean values ± SEM. One-way analysis of variance (ANOVA) was used for comparisons of results from more than two groups, where as Student *t*-test was used to analyze differences between two groups. For repeated measurements analysis a two-way ANOVA followed by Bonferroni post hoc test was used. GraphPad Prism version 4.03 software (GraphPad Software, San Diego, CA) was used for the analysis. Differences were considered significant at a value of $P < 0.05$.

RESULTS

Blood Pressure Measurements. Continuous radio-telemetry recordings of arterial pressure showed that there were no significant differences in baseline blood pressure measurements among the groups (Figure 2.2 A). Ang II infusion for 14 days significantly increased mean arterial pressure in WT mice from 115 ± 1 to 151 ± 3 mm Hg ($P < 0.001$) but not in TNF-$\alpha^{-/-}$ mice (113 ± 2 to 123 ± 3 mm Hg). But when TNF-$\alpha^{-/-}$ mice were given replacement therapy with rTNF-α, Ang II administration caused an increase in mean arterial pressure (109 ± 1 to 153 ± 3; $P < 0.001$) similar to as noted in WT mice.

Metabolic Parameters. At baseline, there were no significant differences in salt and water intakes and urine volume between the WT and TNF-$\alpha^{-/-}$ groups. As illustrated in Figure 2, Ang II infusion for 14 days in WT mice caused significant increases in salt and water intake as well as urine output, the increases of which were seen as early as 5th day of infusion. However, the salt and water intakes, and urine output remained unchanged in the TNF-$\alpha^{-/-}$ mice infused with Ang

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II (Figure 2.2 B, C, and D). Interestingly, the same treatment in the TNF-α^{-/-} mice receiving replacement therapy of rTNF-α resulted in increases in salt intake, water intake, and urine output, noted especially in the 2nd week of Ang II infusion.
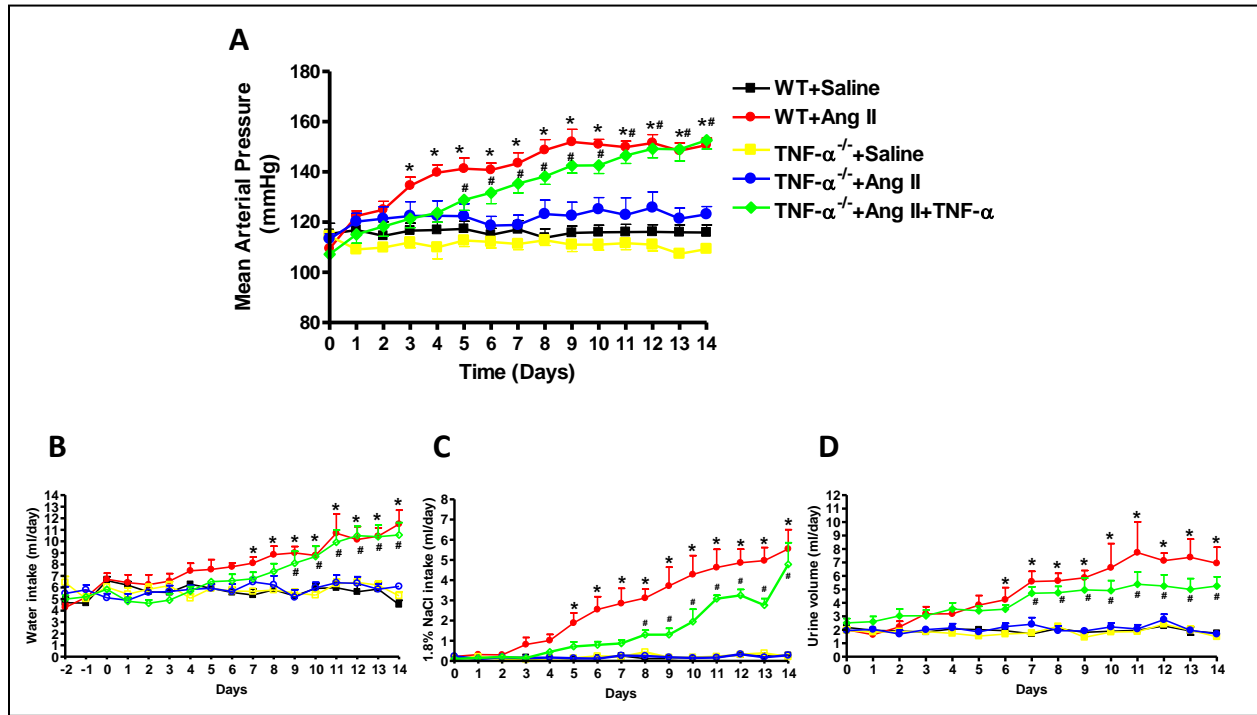
Figure 2.2. Effect of angiotensin II on A). Mean arterial pressure (MAP), B). Water intake, C). Salt (1.8% NaCl) intake, and D). Urine excretion. MAP was measured using radio telemetry for the period of Ang II infusion. Ang II was infused from day 1 to day 14. Values are mean ± SEM (n=6 animals per group). $P < 0.05$ compared with WT group (*) and TNF-α^{-/-} group ([#]).

Echocardiography. Echocardiography was performed to evaluate the effect of Ang II on left ventricular function in these WT and TNF-α^{-/-} mice (Table 2.1). The Ang II infusion significantly increased cardiac wall thickness, end-diastolic and end-systolic dimensions, and decreased fractional shortening in WT mice. Conversely, in TNF-α^{-/-} mice, wall thickness, end-diastolic and end-systolic dimensions, or fractional shortening were not affected by Ang II infusion. These results indicated that cardiac function was well preserved in TNF-α^{-/-} mice.

Table 2.1. Echocardiographic analysis of cardiac hypertrophy and function.

Parameters	WT	WT+Ang II	TNF-α$^{-/-}$	TNF-α$^{-/-}$+Ang II	TNF-α$^{-/-}$+ Ang II+TNF-α
N	6	6	6	6	6
IVSD (mm)	0.45 ± 0.03	0.54 ± 0.02*	0.40 ± 0.03	0.44 ± 0.02	0.67 ± 0.06$^{\#}$
IVSS (mm)	0.60 ± 0.04	0.81 ± 0.08*	0.65 ± 0.03	0.70 ± 0.03	0.87 ± 0.05$^{\#}$
LVD (mm)	3.50 ± 0.10	4.28 ± 0.22*	3.14 ± 0.07	3.17 ± 0.06	3.69 ± 0.07$^{\#}$
LVS (mm)	2.55 ± 0.08	3.03 ± 0.14*	2.22 ± 0.05	2.27 ± 0.06	2.73 ± 0.12$^{\#}$
PWD (mm)	0.50 ± 0.03	0.57 ± 0.02*	0.42 ± 0.05	0.46 ± 0.02	0.60 ± 0.04$^{\#}$
PWS (mm)	0.63 ± 0.07	0.74 ± 0.03*	0.60 ± 0.04	0.67 ± 0.04	0.80 ± 0.10$^{\#}$
%FS (%)	29.18 ± 1.36	23.57 ± 0.62*	27.77 ± 0.90	27.84 ± 1.06	24.40 ± 0.75$^{\#}$
HR (beats/min)	422 ± 12	470 ± 33	455 ± 27	423 ± 7.5	472 ± 20

Values are mean ± SEM. *n,* no. of mice. IVSD and IVSS, interventricular septal thickness at end diastole and end systole, respectively; LVDD and LVDS, left ventricular internal diameter at end diastole and end systole, respectively; PWD and PWS, posterior wall thickness at end diastole and end systole, respectively; HR, heart rate. $P < 0.05$ compared with WT group (*) and TNF-α$^{-/-}$ group ($^{\#}$).

Cardiac Hypertrophy Responses to Ang II Infusion. To evaluate changes in the cardiac weight in these Ang II treated WT and TNF-α$^{-/-}$ mice, the hearts were harvested and weighed at the end of 2 weeks experimental period. The ratio of heart weight to body weight is calculated. Figure 2.3 illustrated these results on cardiac weight. There were no differences in vehicle treated TNF-α$^{-/-}$ and WT mice. In WT mice, Ang II infusion had increased heart weight and also increased the ratio between the heart weight and body weight. In contrast, the TNF-α$^{-/-}$ mice with Ang II infusion did not show any increase in heart weight to body weight ratio (Figure 2.3A). To further verify the attenuated hypertrophic response in TNF-α$^{-/-}$ mice, mRNA levels of atrial natriuretic peptide (ANP) in the left ventricle were measured by RT-PCR. While the infusion of Ang II significantly increased myocardial levels of ANP in WT mice, this up-regulation of ANP was significantly attenuated in TNF-α$^{-/-}$ mice (Figure 2.3B).
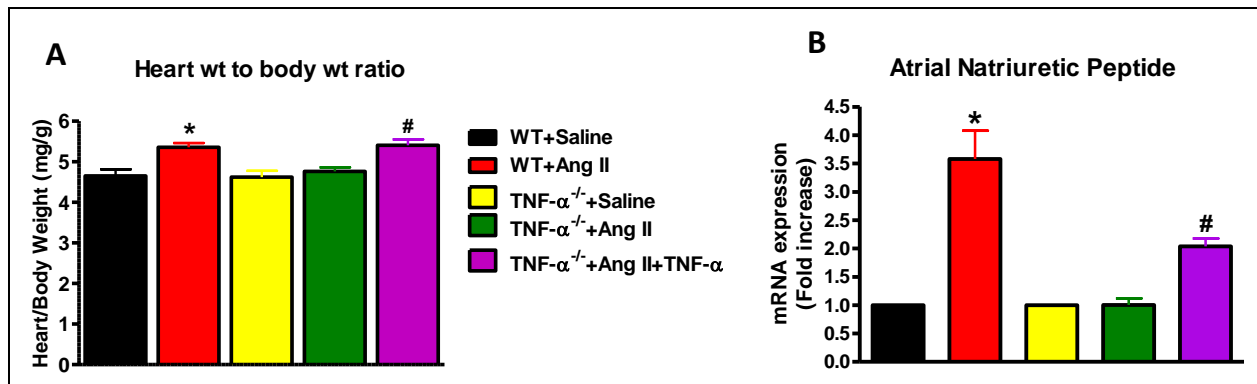
Figure 2.3. Effect of Angiotensin II infusion on cardiac hypertrophy. (A). Heart weight to body weight ratio in wildtype (WT) and TNF knockout (TNF-$\alpha^{-/-}$) mice. (B). mRNA expression of atrial natriuretic peptide in left ventricle. Values are mean ± SEM. $P < 0.05$ compared with WT group (*) and TNF-$\alpha^{-/-}$ group ($^\#$).

Gene Expression Studies. To determine whether the infusion of Ang II alters the expression of AT$_1$R, we examined the mRNA levels of AT$_1$R in the heart and hypothalamus by real time PCR. AT$_1$R mRNA expression was also assessed in the samples collected from WT (n=5-6) and TNF-$\alpha^{-/-}$ mice (n=5-6) prior to Ang II infusion. The basal level of AT$_1$R mRNA expression was not significantly different between the groups. The AT$_1$R mRNA expression in both heart and hypothalamus was significantly increased in Ang II infused WT mice, whereas that in Ang II infused TNF-$\alpha^{-/-}$ mice remained unchanged. These results were confirmed at protein levels by the western blot analysis (Figures 2.4 and 2.5).
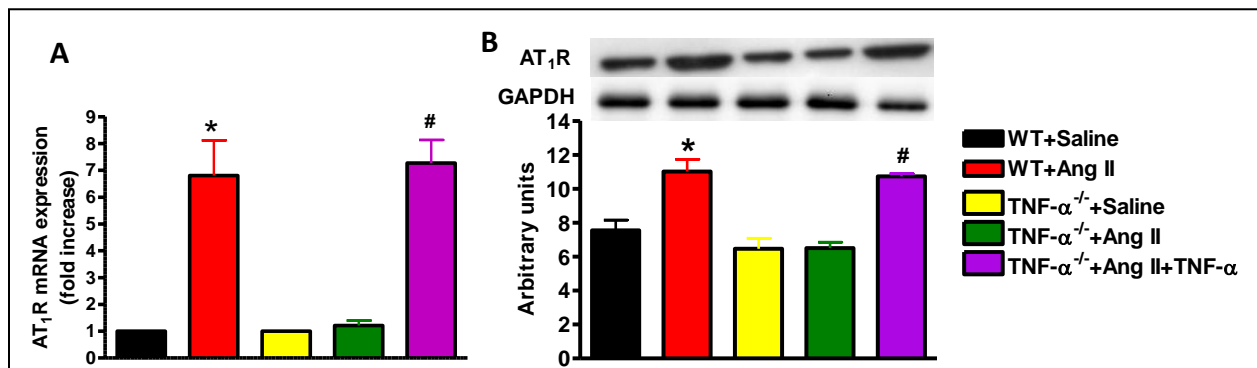
Figure 2.4. Effect of Ang II on (A). mRNA and (B). protein expression of AT$_1$ receptor (AT$_1$R). Representative Western blot and densitometric analysis of AT$_1$R protein in Left ventricle (B). $P < 0.05$ compared with WT group (*) and TNF-$\alpha^{-/-}$ group ($^\#$).
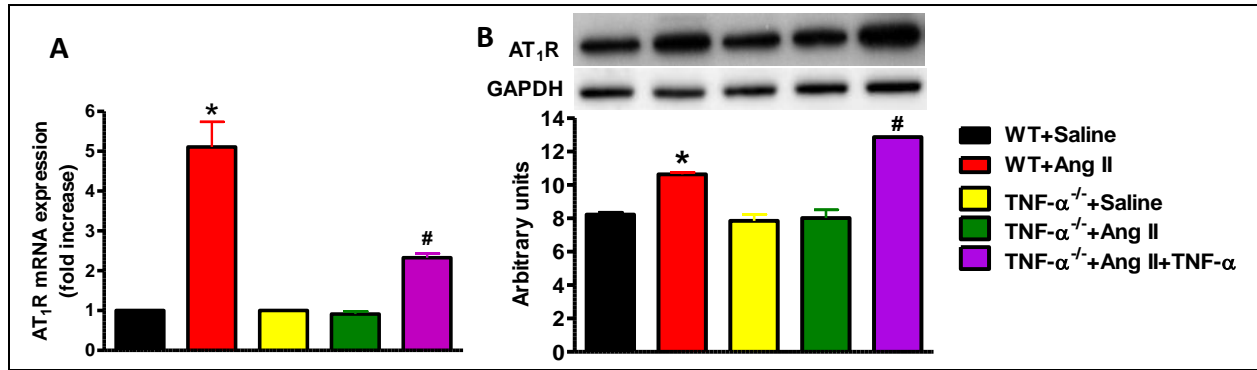
Figure 2.5. Effect of Ang II on (A). mRNA and (B). protein expression of AT_1 receptor (AT_1R). Representative Western blot and densitometric analysis of AT_1R protein in Hypothalamus (B). $P < 0.05$ compared with WT group (*) and TNF-$\alpha^{-/-}$ group ($^\#$).

Because both Ang II and TNF-α have been shown to act through the NF-κB mediated pathways, we analyzed the P50 subunit of NF-κB mRNA expression using real time PCR. Ang II infusion significantly increased the NF-κB mRNA expression in WT mice but not in TNF-$\alpha^{-/-}$ mice (Figure 2.6).
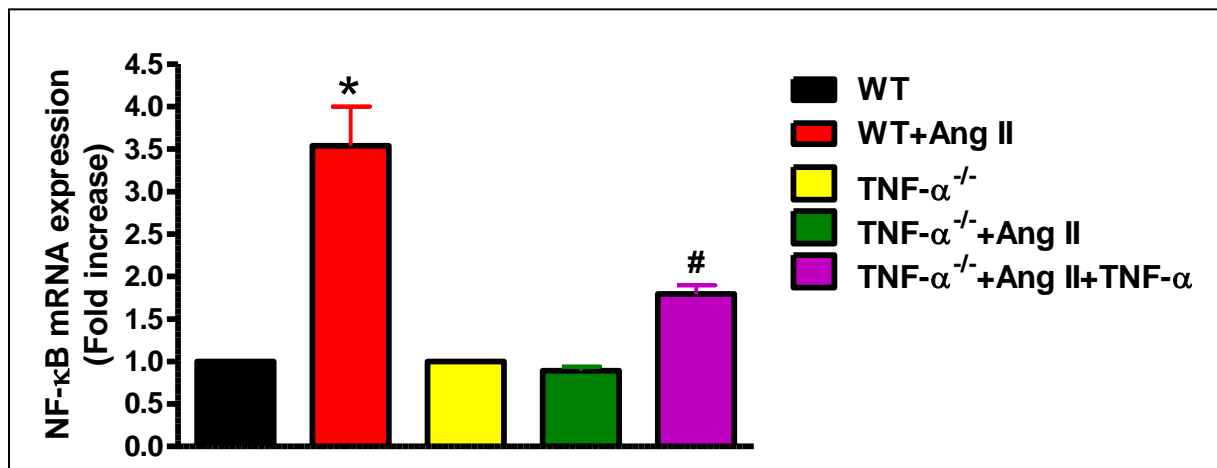
Figure 2.6. Effect of Ang II on mRNA expression of NF-κB in the heart. $P < 0.05$ compared with WT group (*) and TNF-$\alpha^{-/-}$ group ($^\#$).

To determine which TNF receptor is involved in Ang II infusion, the expression of TNF type I (TNFR1) and type II (TNFR2) in the left ventricle was analyzed. After Ang II infusion, there was a significant increase in TNFR1 mRNA and protein expression in the WT but not in the

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TNF-α$^{-/-}$ mice. However, there was no significant difference in the TNFR2 mRNA expression (Figure 2.7).
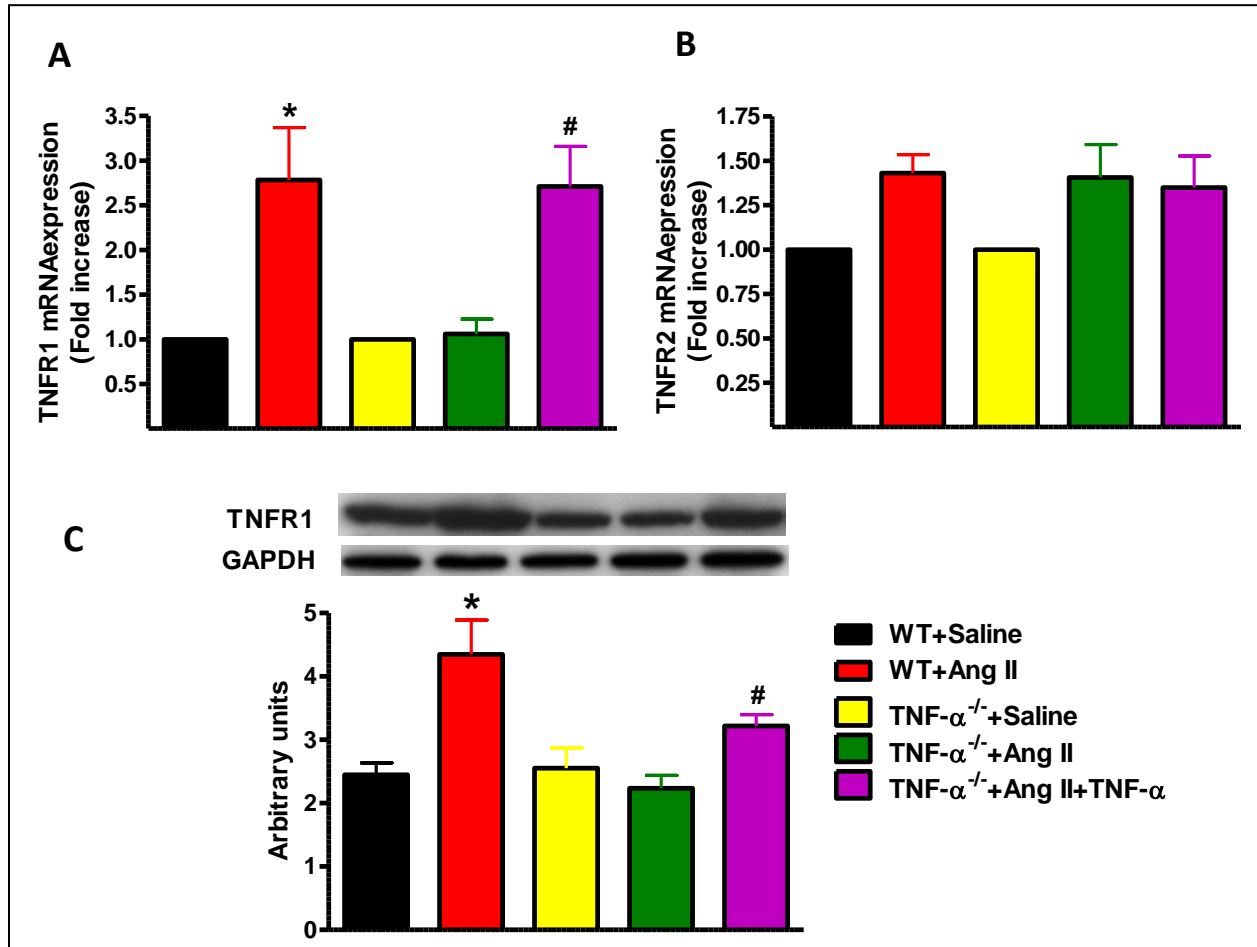
Figure 2.7. Effect of angiotensin II infusion on the expression of TNF receptor type 1 (TNFR1) and type 2 (TNFR2) in the left ventricle. (A). TNFR1 mRNA expression. (B). TNFR2 mRNA expression. (C). Representative western blot and densitometric analysis of TNFR1 protein in Left ventricle. Values are mean ± SEM (n=5-6 animals per group). $P < 0.05$ compared with WT group (*) and TNF-α$^{-/-}$group ($^{\#}$).

DISCUSSION

The present study demonstrated that the responses to chronic Ang II administration on salt and water intake, blood pressure and cardiac function were markedly attenuated in mice lacking the gene for the pro-inflammatory cytokine, TNF-α (TNF-α$^{-/-}$ mice). However, these Ang II responses had been restored in TNF-α$^{-/-}$ mice when these mice were given a replacement

therapy with human recombinant TNF-α. In addition, it was also observed that the mRNA levels of AT_1R, as well as NF-κB mRNA expressions in the heart and hypothalamus, were increased in response to chronic Ang II in WT but not in TNF-$α^{-/-}$ mice. These results suggest that a concomitant generation of TNF-α is involved in the complete expression of Ang II-induced salt appetite, hypertensive, as well as cardiac hypertrophic responses, possibly via the TNF-α induced up-regulation of AT_1R as well as activation of NF-κB activity.

Most of the known physiological actions of Ang II, such as vasoconstriction, increased aldosterone secretion, increased sympathetic nerve activity, and increased water and sodium intake, are mediated by the activation of AT_1R which are widely distributed in all organs, including the liver, adrenal glands, brain, lung, kidney, heart, and blood vessels (Allen et al., 2000; Timmermans et al., 1993). AT_1R in the brain are linked to vasopressor responses, along with regulation of salt appetite, thirst, and modulation of vasopressin release (de Wardener, 2001; Llorens-Cortes and Mendelsohn, 2002; McKinley et al., 2003). Salt appetite and thirst are central nervous system phenomenon. Injection of Ang II into the brain or into the periphery increases salt appetite and thirst in rodents (Davisson et al., 2000; Denton et al., 1990; Wright et al., 1987). Ang II is a relatively large peptide and it does not readily cross the blood brain barrier. The CNS effect of the Ang II could be via the circumventricular organ (CVO) where the blood brain barrier is week or absent (McKinley et al., 2003). These include the organum vasculosum lamina terminalis (OVLT), subfornical organ and area postrema (AP). Ablation of the AP or OVLT regions attenuates the Ang II induced response on salt appetite and thirst (McKinley et al., 2003). Thus, all the components of the RAS system are expressed within the central nervous system, thereby facilitating some of the Ang II induced effect observed centrally. Activation of the RAS and the subsequent increase in the local production of Ang II is one of the main

mechanisms responsible for hypertension and the progression of cardiovascular disease. Ang II has been shown in many reports to increase the expression of various cytokines and chemokines that induce cardiac hypertrophy, inflammation and vascular remodeling that result in the long term regulation of blood pressure (Kim and Iwao, 2000; Ruiz-Ortega et al., 2001). Several studies have shown that blockade of the RAS by ACE inhibitors or by AT_1R blockers attenuate hypertensive response and end organ damage as well as inflammatory markers in many cardiovascular diseases (Devereux et al., 2004; Mathew et al., 2001; Schiffrin, 2002). It can be argued that the attenuated cardiac hypertrophy induced by Ang II in TNF-$\alpha^{-/-}$ mice could be the result of reduced blood pressure response in those animals and thus pose a potential limitation to data interpretation in the present study. Further studies are needed to examine the pressure dependent and independent component of the attenuated hypertrophic response in these TNF-$\alpha^{-/-}$ mice. However, accumulating evidence from clinical and experimental studies indicates that there is a functional crosstalk between Ang II and several pro-inflammatory cytokines, including TNF-α and IL-6, in the regulation of cardiovascular function (Arenas et al., 2004; Brasier et al., 1996; Kalra et al., 2002; Sasamura et al., 1997).

Although there is considerable evidence from previous studies supporting a role for AT_1R activation in Ang II mediated hypertension, there have been very limited studies that examined the functional importance of TNF-α in Ang II induced hypertension and AT_1R expression. The results from our present study demonstrated a more clear assessment of the functional involvement of the pro-inflammatory cytokine, TNF-α, in the chronic Ang II induced effects, particularly on salt appetite, blood pressure and cardiac hypertrophy. However, it may be argued that the component of RAS may be altered which may influence the responsiveness of Ang II in these TNF-$\alpha^{-/-}$ mice. However, this possibility may be unlikely as we have observed that the

basal tissue AT_1R mRNA expression in both heart and hypothalamus was not different between these TNF-$\alpha^{-/-}$ mice and WT mice. Further studies are required to define the various components of RAS in TNF-$\alpha^{-/-}$ mice.

It is well known that Ang II, by its direct effect on the activation of immune cells, induces the production of inflammatory mediators such as TNF-α and contributes to tissue damage in hypertensive response (Funakoshi et al., 1999; Kalra et al., 2002; Ruiz-Ortega et al., 2002). Recently, blockade of TNF-α using etanercept has been shown to prevent renal damage in genetically hypertensive rats and to lower blood pressure in rats with hypertension induced by Ang II and salt, suggesting a role for TNF-α in blood pressure regulation and renal injury (Elmarakby et al., 2006; Muller et al., 2002). Thus, an interaction between Ang II and TNF-α has been suggested to play an important role in hypertensive response (Ferreri et al., 1997; Guzik et al., 2007; Muller et al., 2002). A recent study by Guzik et al. (Guzik et al., 2007) showed that Ang II infusion caused infiltration of T lymphocytes in the aortic adventitia and periaortic fat, increased T lymphocytes production of TNF-α, increased vascular superoxide production, and led to hypertension in mice. Treatment with the TNF-α antagonist etanercept prevented the hypertension and the increase in vascular superoxide caused by Ang II (Guzik et al., 2007). Collectively, these data suggest the notion that chronic Ang II caused infiltration of T lymphocytes in various organ systems including cardiovascular and central nervous system that facilitates the production of TNF-α. This enhancement of TNF-α production resulted in up-regulation of AT_1R to further enhance the direct actions of Ang II in the target organs. Additions to these direct actions of Ang II, there are also effects of TNF-α mediated enhanced oxidative stress induced by activation of NADPH oxidase, possibly via activation of NF-κB activity (Guzik et al., 2007). However, earlier studies had reported differing results regarding the role of

TNF-α in the regulation of blood pressure. It had been shown that TNF-α opposed the vasoconstrictor effects of phenylephrine in rat aortic ring preparations (Hollenberg et al., 1991). Ferreri et al. (Ferreri et al., 1997) demonstrated that the administration of anti-TNF-α antiserum causes additional increase in mean arterial pressure in a model of Ang II induced hypertension, indicating that TNF-α may oppose the pressor actions of Ang II. However, a study by Alexander et al. (Alexander et al., 2002) showed that infusion of TNF-α at a dose of 50 ng/day for 5 days into virgin rats had no significant effect on blood pressure, but it produced a hypertensive response in pregnant animals. A recent study (Guzik et al., 2007) showed that mice treated with etanercept prevented the hypertension and blunted the increase in super oxide production in response to Ang II. These results also suggest that an interaction between TNF-α and other factors, including oxidative stress, is required for full expression of this cytokine induced hypertensive response.

Interaction between the RAS and TNF-α *in vivo* in cardiac hypertrophy was apparent when losartan, an AT_1R blocker, was given to transgenic mice over expressing TNF-α in the heart. Losartan prevented the development of hypertrophy, while vehicle treatment produced a significant increase in the heart weight-to-body weight ratio and LV wall thickness in transgenic mice overexpressing TNF-α (Flesch et al., 2003). Ang II has been shown to induce TNF-α biosynthesis in the heart by activating NF-κB, which in turn induces various pro-inflammatory cytokines and chemokines, including TNF-α (Barnes and Karin, 1997; Kalra et al., 2002). Sustained application of TNF-α induces an increase in AT_1R mRNA levels in cardiac fibroblasts and is dependent on NF-κB activation (Cowling et al., 2002; Gurantz et al., 1999). Ang II, on binding with its receptor, becomes internalized, resulting in the activation of its intracellular signaling mechanism. AT_1R mediated cellular signaling events have been postulated to occur via

Gαq mechanism. Interestingly, one of the downstream signaling mechanisms of Gαq also involves NF-κB activation. Similarly TNF-α production also involves NF-κB translocation into the nucleus resulting in the perpetuation of TNF-α and other proinflammatory cytokines. In support of the present finding, it has been shown that NF-κB inhibition attenuates hypertensive response and end organ damage in spontaneously hypertensive rats (Rodriguez-Iturbe et al., 2005). Clearly, further studies are needed to understand the molecular mechanism involved in the TNF-α and RAS interaction.

The present results may provide a beneficial therapeutic implication of TNF-α blocker in hypertensive patients those are also suffering from arthritis. At this moment, no direct clinical report is available that may indicate that treatment with a TNF-α blocker may cause a decrease in blood pressure in arthritic patients, or have any additive hypotensive effects with the blockers of RAS. Obviously, more comprehensive studies would be required to examine the therapeutic benefit of a TNF-α blocker in the management of hypertension and/or arthritis in patients.

In conclusion, the results from the present study suggest that a concomitant generation of TNF-α is required for the full expression of chronic Ang II-induced effects, such as salt appetite, hypertension, as well as cardiac hypertrophy, possibly via its action in up-regulating AT_1R as well as enhancing NF-κB activity. The findings of the present study emphasize an important mechanistic role of TNF-α in the mediation of hypertensive as well as cardiac hypertrophy responses induced by chronic Ang II administration. These results demonstrate an existence of a cross-talk between the renin-angiotensin system and the pro-inflammatory cytokines in the regulation of cardiovascular and other organ functions. However, the specific mechanisms and the downstream signaling pathways by which these two systems interact with each other are not yet clearly defined. These present findings provide an important clue in our quest in

understanding the pathophysiology of hypertension and other cardiovascular diseases. Thus, it is imperative that further emphasis should be focused on complete elucidation of the interactive role of the renin-angiotensin system and pro-inflammatory cytokines to increase our understanding of cardiovascular diseases that are linked with inflammatory process.

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CHAPTER 3

OXIDATIVE STRESS MEDIATES INTERACTION OF ANGIOTENSIN II AND TNF-α IN ANGIOTENSIN II-INDUCED HYPERTENSION AND CARDIAC HYPERTROPHY

INTRODUCTION

Angiotensin II (Ang II) plays an important role in blood pressure regulation and cardiac hypertrophy. Multiple signaling pathways that regulate Ang II-mediated cardiac hypertrophy and hypertension have been identified; these included activation of protein kinase-C, mitogen activated protein kinases (MAPK), and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Mehta and Griendling, 2007). Ang II, via the type 1 receptors (AT_1R), enhances the production of ROS through stimulation of NADPH oxidase. Increased oxidative stress contributes to endothelial dysfunction and to vascular inflammation by stimulating the redox-sensitive transcription factors such as NF-κB and by up regulating adhesion molecules, cytokines, and chemokines (Papaharalambus and Griendling, 2007).

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine with a wide range of biological effects. It is implicated in the pathophysiology of many cardiovascular diseases, including hypertension (Sriramula et al., 2008; Zhang et al., 2006). TNF-α is central in initiating and sustaining the proinflammatory cytokine cascade and can stimulate the production of other cytokines such as IL-1 and IL-6. TNF-α increases the production of ROS in cultured cardiac myocytes (Nakamura et al., 1998). TNF-α overexpression in transgenic mice leads to adverse cardiac remodeling, characterized by increased total matrix metallo protein (MMP) activity and increased fibrosis (Bryant et al., 1998; Sivasubramanian et al., 2001).

Both Ang II and TNF-α were shown to induce activation of NF-κB in a ROS-dependent manner, which in turn can increase the production of other proinflammatory cytokines and chemokines (Papaharalambus and Griendling, 2007; Sun et al., 2007; Zhang et al., 2006). Several studies have established the role of ROS in the hypertrophy and remodeling of the heart

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and blood vessels, which contribute further to the pathogenesis of hypertension (Touyz, 2003; Xiao et al., 2002). It is well established that there is increased production of superoxide and depletion of nitric oxide (NO) in the endothelium and in the heart of hypertensive animals, and this contributes to contractile dysfunction. This depletion in nitric oxide could be either due to a direct decrease in NO production (inhibition/depletion of nitric oxide synthase [NOS]) or due to decreased bioavailability of NO because of its interaction with superoxide to form peroxynitrite (Griendling et al., 2000b; Touyz, 2003). In Ang II-infused animal models and spontaneously hypertensive rats (SHR), NADPH activity is increased and ROS generation is enhanced. These processes are mediated through AT_1Rs and associated with overexpression of vascular and cardiac NADPH oxidase subunits (Cifuentes et al., 2000; Heymes et al., 2003; Kakishita et al., 2003). Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats (Vaziri et al., 2000). It has been shown that Ang II-induced superoxide production and hypertension are markedly blunted in mice lacking the p47phox subunit of NADPH oxidase (Landmesser et al., 2002). Treatment with the cell permeable superoxide dismutase (SOD) mimetic tempol lowers blood pressure in the 1-kidney, 1-clip model of renovascular hypertension (Dobrian et al., 2001) and in the SHR model of hypertension (Schnackenberg et al., 1998). In studies using cultures of vascular smooth muscle cells, vascular fibroblasts, endothelial cells, and cardiomyocytes, Ang II stimulation increases both mRNA and protein expression of gp91phox (Griendling et al., 2000a). These studies support a role for NADPH oxidase-derived ROS and increased oxidative stress in the pathogenesis of Ang II-induced cardiovascular disease.

We recently demonstrated that TNF-α was involved in Ang II mediated cardiac hypertrophy, hypertension and salt intake (Sriramula et al., 2008). However, the molecular mechanisms involved in these responses have not been elucidated. In the present study, we have

investigated whether or not oxidative stress and NF-κB are mediating the interaction between Ang II and TNF-α.

METHODS

Experimental Animals. Male, 8-10 week-old, wild type (WT) and TNF-α knockout (TNF-α$^{-/-}$) mice underwent implantation of osmotic minipumps, and received either angiotensin II (1µg/kg/min) or vehicle saline for 14 days. These groups are as follows: 1) WT+Saline - control mice with saline pumps; 2) WT+Ang II - wild type treated with chronic Ang II; 3) TNF-α$^{-/-}$ +saline – knockout mice with saline pumps; 4) TNF-α$^{-/-}$+Ang II - knockout mice treated with chronic Ang II. At the end of 14 days, the mice were euthanized. The hypothalamus and left ventricular tissues were collected for mRNA and protein measurements and cardiac blood was collected for plasma. The experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee.

Measurement of Total ROS, Superoxide and Peroxynitrite. Total ROS, superoxide and peroxynitrite were measured in left ventricular tissue using a bench top electron paramagnetic resonance (EPR) spectrophotometer e-scan R (Noxygen science transfer and diagnostics, Elzach, Germany) (Elks et al., 2009). Tissue pieces were incubated at 37°C with CMH (200 µM) for 30 minutes. Aliquots of the incubated probe medium were then taken into 50 µl glass capillary tubes and measured using EPR spectroscopy. ROS released by tissues react with the probe and form stable adducts which can be measured using EPR spectroscopy.

Measurement of Collagen. To determine collagen content, heart sections (10µm thickness) were cut and stained with Picro-Sirius Red. Fibrillar collagen was identified in the Picro-Sirius stained sections by its red appearance. With the use of NIH Image J software, these sections were

analyzed morphometrically. The area of perivascular fibrosis was calculated as the ratio of the fibrosis area surrounding the vessel to the total vessel area.

Western Blot Analysis. The left ventricular tissue was homogenized with RIPA lysis buffer and proteins were separated. The total protein concentration in samples was measured by Bio-Rad Dc protein assay. Equal amounts of protein (30 µg) were separated by SDS-PAGE on 10% (wt/vol) gels and transferred on to PVDF membrane (Immobilon-P, Millipore; Bedford, MA), and blocked with 1% BSA in TBS-T at room temperature for 60 minutes. The membranes were subjected to immunoblot analyses with primary antibody (1:1000 dilution). Immunodetection was accomplished with a HRP conjugated secondary antibody (1:2000 dilution) using an enhanced chemiluminescence kit (Amersham). The data were quantified by the densitometry using NIH image. Protein expression of GAPDH was used as a loading control.

Real Time RT-PCR. Total RNA was isolated from left ventricular tissue with TRI reagent (Sigma), and cDNA was synthesized from 1µg RNA with the iScrpt cDNA synthesis kit (Bio-Rad). Real Time PCR amplification reactions were performed with iQ SYBR Green Super mix with ROX (Bio-Rad) in triplicate using the ABI Prism 7900 Real time PCR machine (Applied Biosystems, Fostercity, CA). The list of primers used was shown in Table 3.1. Data were normalized to GAPDH expression by the $\Delta\Delta C_T$ comparative method.

Measurement of NF-κB Activity. Hearts were harvested from the mice and nuclear extracts were obtained using a commercially available nuclear extraction kit (Active Motif, Carlsbad, CA). Protein concentrations were then quantified using a Bio-Rad protein assay. Equal amounts of protein were utilized in a colorimetric NF-κB assay specific for the activated form of p65 subunit of NF-κB using a commercially available kit (TransAm NF-κB p65; Active Motif).

Table 3.1. List of primers used for the real time RT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
α-MHC	AGCTGACAGGGGCCATCAT	ACATACTCGTTCCCCACCTTC
β-MHC	TTCATCCGAATCCATTTTGGGG	GCATAATCGTAGGGGGTTGTTGG
BNP	AGGGAGAACACGGCATCATT	GACAGCACCTTCAGGAGAT
Collagen-I	GATGGATTCCCGTTCGAGTA	CCTTCTTGAGGTTGCCAGTC
Collagen-III	CCTGGAGCCCCTGGACTAATAG	GCCCATTTGCACCAGGTTCT
CTGF	CAAAGCAGCTGCAAATACCA	GGCCAAATGTGTCTTCCAGT
iNOS	GGCAGAATGAGAAGCTGAGG	GAAGGCGTAGCTGAACAAGG
NOX-2	TGTGGCTGTGATAAGCAGGA	CTTGAGAATGGAGGCAAAGG
p22phox	TGGACGTTTCACACAGTGGT	CAGCCCGGACGTAGTAATTC
p47phox	CCACACCTGCTGGACTTCTT	CTGTTCCCGAACTCTTCTCG
p67phox	GCCACAGTCATGTTCAATGG	GGGCTCTTTTTGCTTGTGAC
TGF-β	CTGCTGCTTTCTCCCTCAAC	GCGAGCCTTAGTTTGGACAG
GAPDH	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT

Statistical Analysis. Data are presented as mean ± SEM. Comparisons of treated versus untreated animal data were made by independent Student's t tests. Between groups comparisons were made by ANOVA, followed by a post-hoc Bonferroni's test. $P < 0.05$ was considered significant.

RESULTS

Cardiac Hypertrophy and Fibrosis. Because both Ang II and TNF-α are involved in cardiac hypertrophy and fibrosis, we investigated the effect of blocking TNF-α on cardiac hypertrophy and fibrosis. Ang II-mediated cardiac hypertrophy is shown to be associated with typical induction of the fetal gene expression. As shown in Figure 3.1, expression levels of brain natriuretic factor and β-myosin heavy chain (MHC) mRNA was significantly increased, and α-MHC mRNA was significantly reduced in WT mice after Ang II infusion. The expression profile of these genes, however, was not significantly altered in Ang II infused TNF-α$^{-/-}$ mice, thus indicating attenuation of the typical Ang II-mediated hypertrophy response in TNF-α$^{-/-}$ mice.
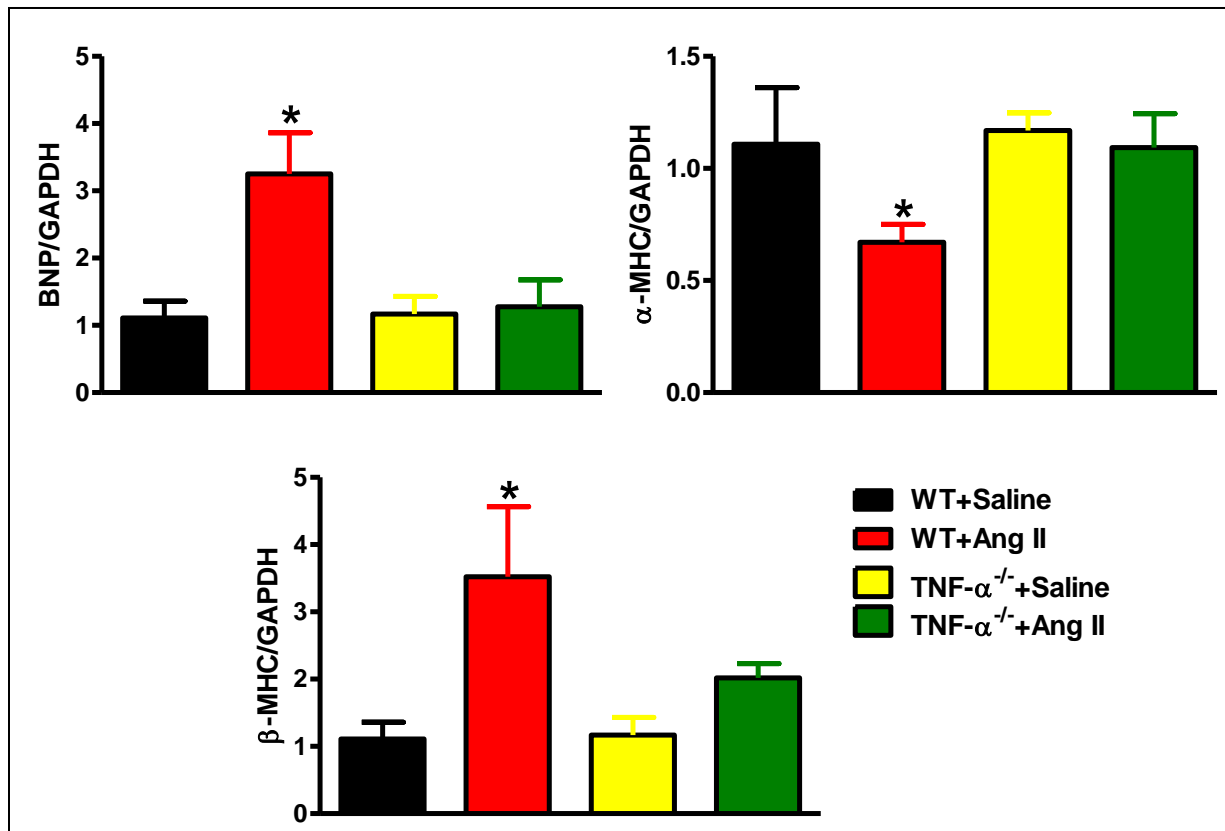
Figure 3.1. Effect of Ang II on mRNA expression of fetal genes in of WT and TNF-α$^{-/-}$ mice. Expression levels of brain natriuretic factor and β-MHC mRNA was increased, and α-MHC mRNA was significantly reduced in WT mice after Ang II infusion, but not in TNF-α$^{-/-}$ mice. Values are mean ± SEM. $P < 0.05$ compared with WT group (*).

To determine whether profibrotic gene expression which leads to fibrosis in the heart was altered in TNF-α$^{-/-}$ mice, we analyzed cardiac expression of connective tissue growth factor (CTGF), which was shown to be involved in cardiac fibrosis (Chen et al., 2000). Infusion of Ang II increased CTGF mRNA expression in WT mice. This increase in Ang II-induced CTGF expression was markedly attenuated in TNF-α$^{-/-}$ mice. The critical markers for fibrosis such as TGF-β, collagen I and collagen III in hearts of WT mice were also significantly increased by Ang II (Figure 3.2). However, these changes were significantly less in TNF-α$^{-/-}$ mice compared with WT mice.
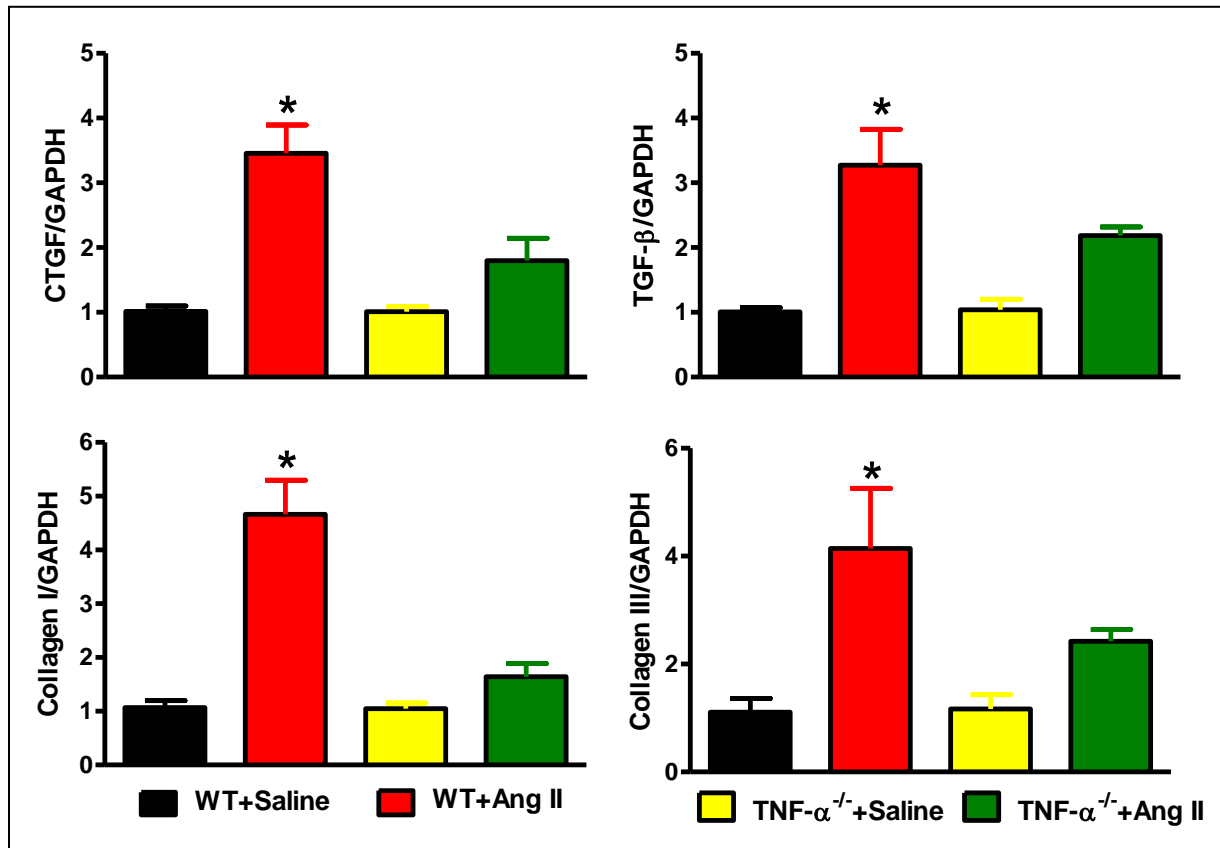
Figure 3.2. Effect of Ang II on mRNA expression of profibrotic genes in WT and TNF-α$^{-/-}$ mice. Ang II infusion increased CTGF, TGF-β, collagen I and collagen III mRNA expression in the hearts of WT mice but not in TNF-α$^{-/-}$ mice. Values are mean ± SEM. *P* < 0.05 compared with WT group (*).

Because Ang II-induced cardiac hypertrophy and remodeling is shown to be involved fibrosis and collagen deposition, we investigated the effect of Ang II on interstitial and perivascular fibrosis in the heart sections using Picro-Sirius Red staining. Chronic Ang II infusion for 14 days showed a marked increase in collagen deposition in hearts of WT mice as indicated by red staining. In contrast, no significant change in interstitial fibrosis and perivascular fibrosis were observed in Ang II infused TNF-α$^{-/-}$ mice (Figure 3.3). These data indicate that the cardiac fibrosis response of Ang II is, in part, contributed by the presence of TNF-α. Taken together these results suggest that TNF-α$^{-/-}$ mice had an attenuated cardiac remodeling response to the Ang II infusion.
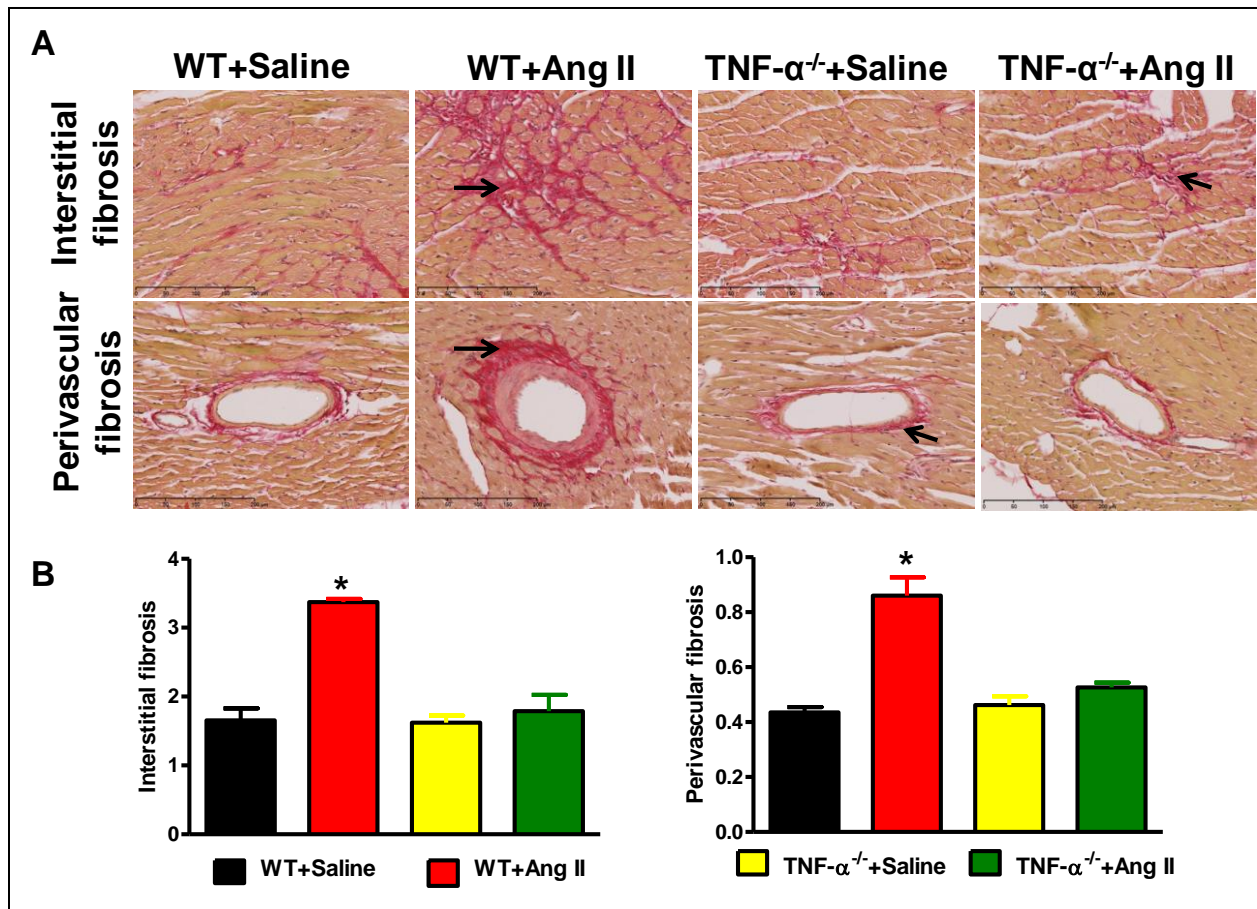
Figure 3.3. Effect of Ang II on fibrosis in WT and TNF-$\alpha^{-/-}$ mice. (A) The heart sections were stained with Picro-Sirius Red. The red stained collagen deposition is marked by arrows. Representative photo images are shown. (B) The quantitative data showed that Ang II infusion increased interstitial fibrosis and perivascular fibrosis in the hearts of WT mice but not in TNF-$\alpha^{-/-}$ mice. Values are mean ± SEM. $P < 0.05$ compared with WT group (*).

Cardiac Oxidative Stress. ROS have been shown to act as important signaling molecules in the cardiovascular system and activate many signaling pathways mediated by Ang II. To evaluate the effect of Ang II on ROS formation in the left ventricle, we measured and quantified total ROS, superoxide and peroxynitrite production in the heart tissue by electron paramagnetic resonance (Figure 3.4). Ang II infusion significantly increased the total ROS, superoxide and peroxynitrite production in WT mice compared with saline infused control mice leading to increased oxidative stress. These changes were attenuated in TNF-$\alpha^{-/-}$ mice infused with Ang II and suggested an attenuated oxidative stress response.

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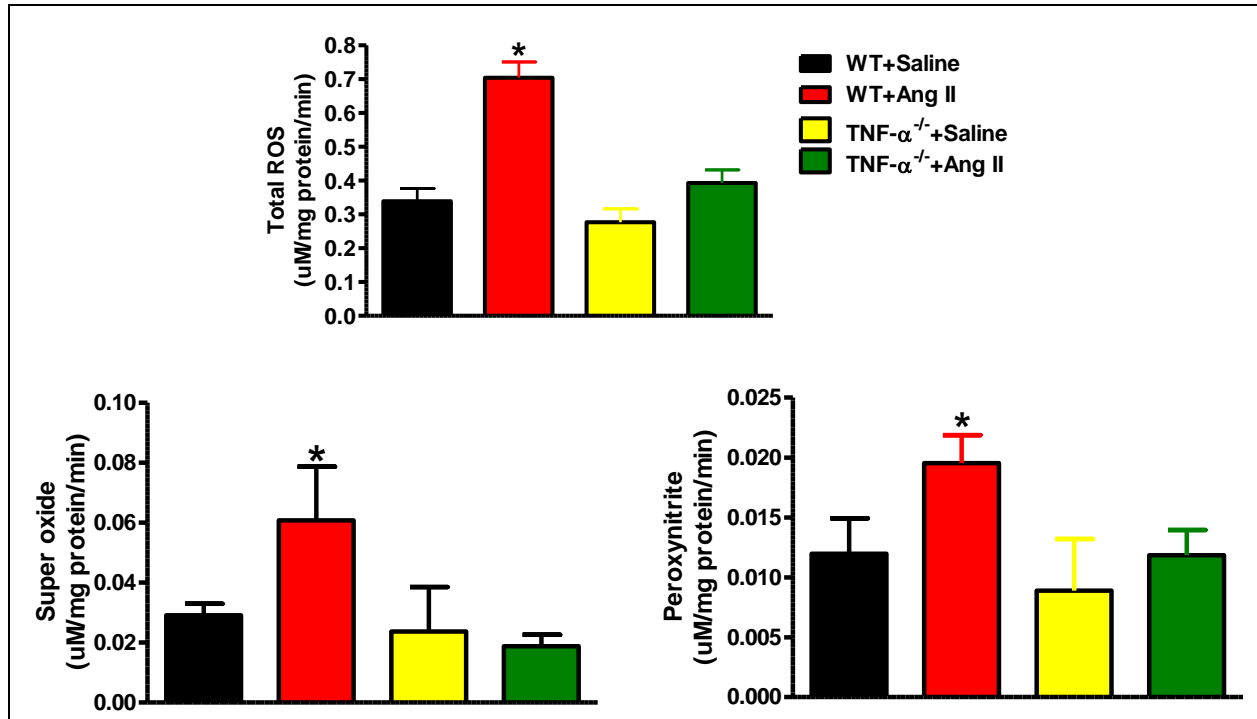
Figure 3.4. Effect of Ang II cardiac oxidative stress in WT and TNF-$\alpha^{-/-}$ mice. Ang II infusion increased total ROS, superoxide and peroxynitrite production in the hearts of WT mice but not in TNF-$\alpha^{-/-}$ mice. Values are mean ± SEM. $P < 0.05$ compared with WT group (*).

Activation of NADPH Oxidase. Because Ang II is a profound stimulator of NADPH oxidase, we investigated the cardiac expression of the NADPH oxidase subunits Nox2, p22phox, p47phox and p67phox. We observed that TNF-$\alpha^{-/-}$ mice had no changes in myocardial expression of NADPH oxidase enzyme subunits when compared with WT control mice. Chronic Ang II infusion caused a significant increase in the mRNA expression of NADPH oxidase subunits in WT mice. However, these changes in mRNA were attenuated in Ang II infused TNF-$\alpha^{-/-}$ mice (Figure 3.5). Also, we have confirmed these mRNA changes in NADPH oxidase enzyme subunits with protein expression measured by western blot analysis (Figure 3.6).

Expression of iNOS. Previous studies suggest that hypertensive animals show significantly higher mRNA and protein expression of myocardial inducible NO synthase (iNOS), so in our study we measured iNOS expression in the heart. Ang II infusion significantly increased the

mRNA and protein expression of iNOS in the cardiac tissue of WT mice. However, TNF-$\alpha^{-/-}$ mice showed an attenuated iNOS expression to Ang II infusion (Figure 3.7).

Activation of NF-κB. Both Ang II and TNF-α can induce oxidative stress, which in turn, activates transcription factors such as NF-κB. In our study, the activity of p65 NF-κB in nuclear extracts of the cardiac tissue was increased in Ang II infused WT mice compared to saline infused WT mice. However, Ang II infused TNF-$\alpha^{-/-}$ mice showed a decreased activity of p65 NF-κB in the heart (Figure 3.8).
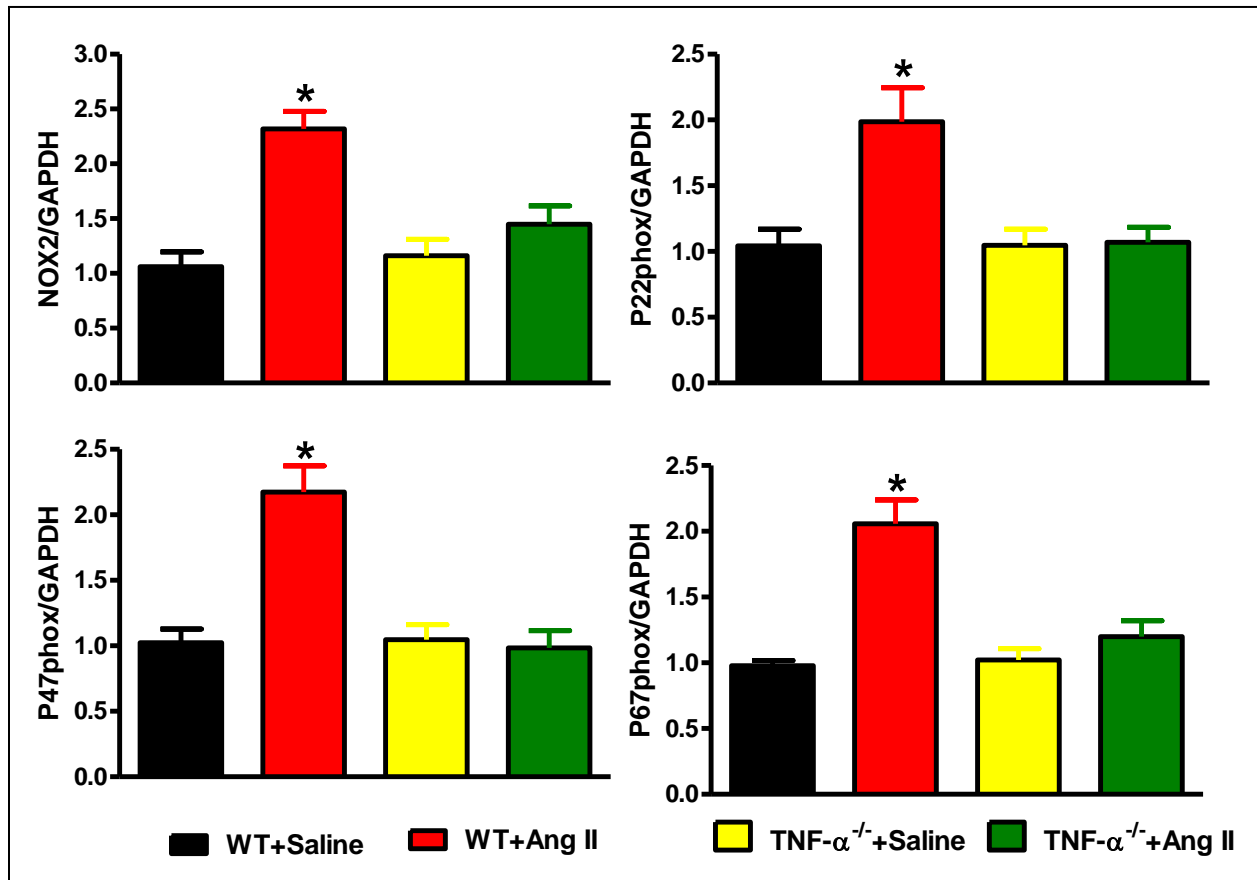
Figure 3.5. Effect of Ang II on mRNA expression of NADPH oxidase subunits in WT and TNF-$\alpha^{-/-}$ mice. Ang II infusion increased NOX-2, p22phox, p47phox and p67phox mRNA expression in the hearts of WT mice but not in TNF-$\alpha^{-/-}$ mice. Values are mean ± SEM. $P < 0.05$ compared with WT group (*).
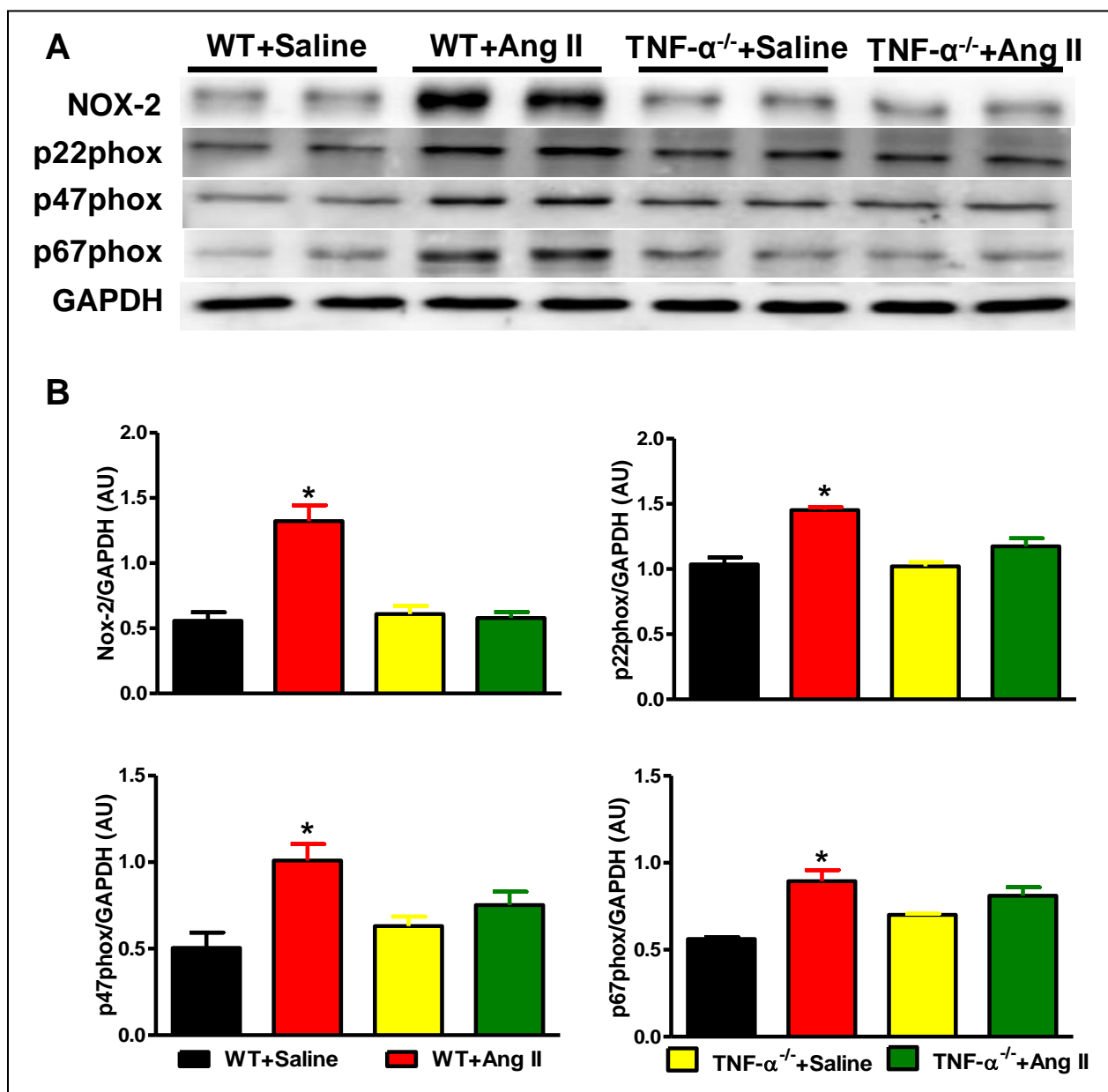
Figure 3.6. Effect of Ang II on protein expression of NADPH oxidase subunits in WT and TNF-$\alpha^{-/-}$ mice. (A) Representative western blots showing levels of NADPH subunits in heart tissue. (B) Densitometric analysis of western blots (n=6), AU-arbitrary units. Ang II infusion increased NOX-2, p22phox, p47phox and p67phox protein expression in the hearts of WT mice but not in TNF-$\alpha^{-/-}$ mice. Values are mean ± SEM. $P < 0.05$ compared with WT group (*).
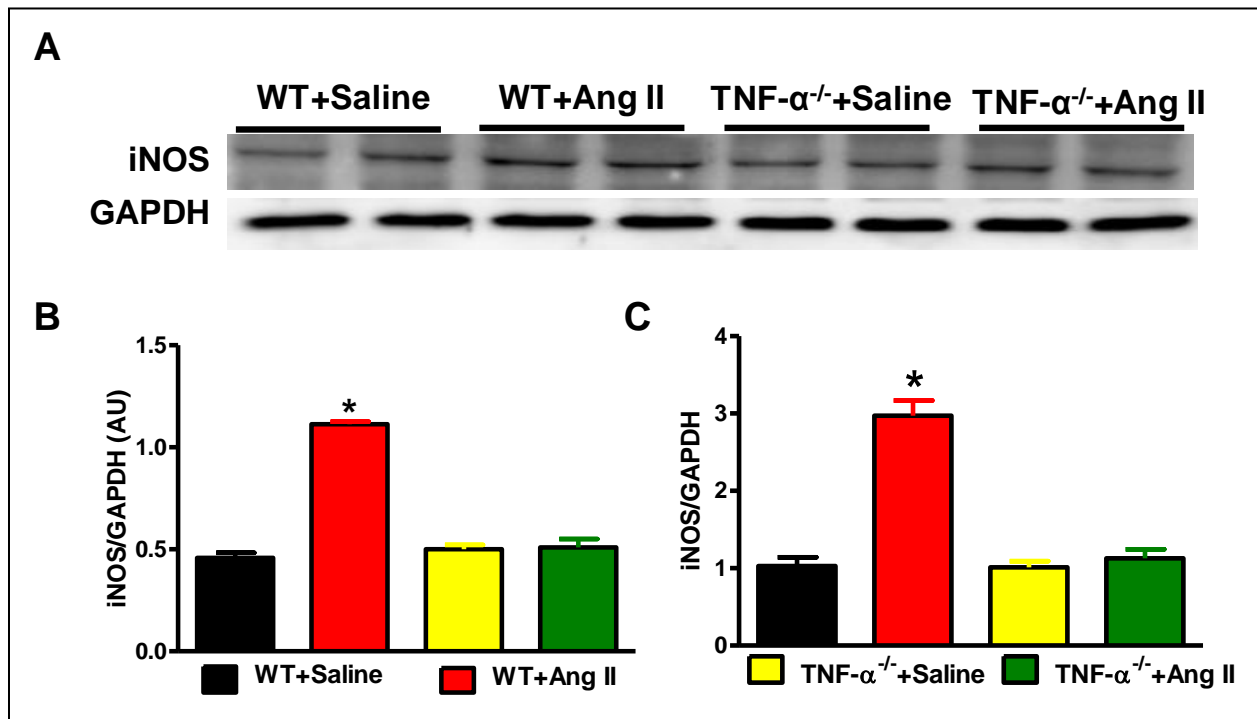
Figure 3.7. Effect of Ang II on protein and mRNA expression of iNOS in WT and TNF-$\alpha^{-/-}$ mice. (A) Representative western blot (B) Densitometric analysis of western blot. (C) mRNA expression of iNOS in the heart. Both protein and mRNA expression levels of iNOS are increased by Ang II infusion in the hearts of WT mice but not in TNF-$\alpha^{-/-}$ mice. Values are mean $\pm$ SEM. $P < 0.05$ compared with WT group (*).
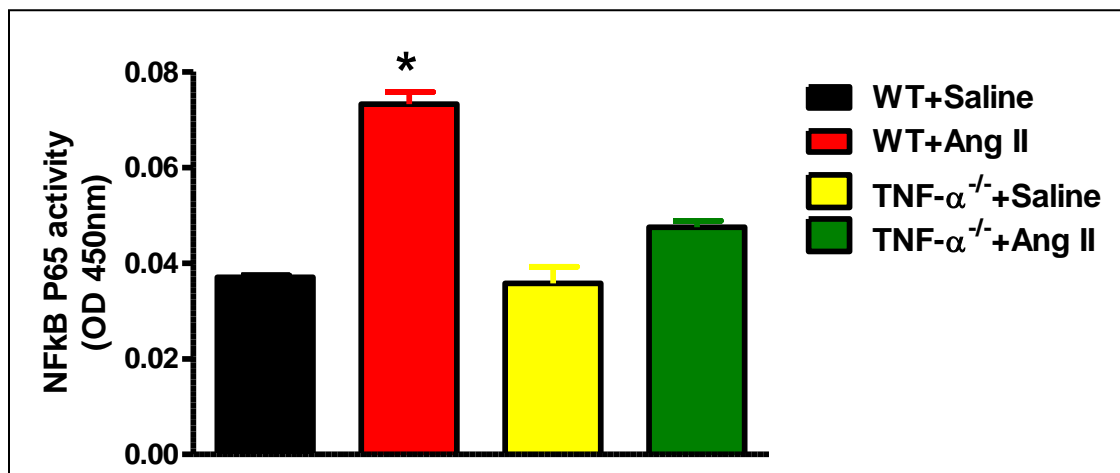
Figure 3.8. Effect of Ang II on activity of NF-κB in WT and TNF-$\alpha^{-/-}$ mice. Ang II infusion increased NF-κB p65 activity in the hearts of WT mice but not in TNF-$\alpha^{-/-}$ mice. Values are mean $\pm$ SEM. $P < 0.05$ compared with WT group (*).

DISCUSSION

Previously we have shown that TNF-α is involved in Ang II-induced cardiac hypertrophic and hypertensive response (Sriramula et al., 2008). In this study we show that attenuation of Ang II mediated hypertension and cardiac hypertrophy by TNF-α deletion involves decreased oxidative stress and superoxide production.

A marked attenuation of Ang II-induced cardiac hypertrophy and fibrosis was demonstrated in TNF-α$^{-/-}$ mice by measuring various hypertrophy markers, including profibrotic genes, and interstitial and perivascular fibrosis in the cardiac tissue. It has been previously shown that TNF-α can induce myocardial fibrosis by inhibiting collagen phagocytosis in the heart (Chou et al., 1996), and also by upregulating Ang II receptors on cardiac fibroblasts leading to enhancement of the effects of Ang II (Gurantz et al., 1999). Also, in our study, enhanced TGF-β and CTGF expression was observed in Ang II-infused WT mice but not in TNF-α$^{-/-}$ mice. CTGF is well known for its role as a downstream mediator of the chronic fibrotic effects of TGF-β, which, when activated stimulates fibroblasts to differentiate into myofibroblasts, the key cells for collagen synthesis in cardiac remodeling (Chen et al., 2000; Villarreal and Dillmann, 1992). These results suggest that the effects of Ang II on cardiac hypertrophy and fibrosis are mediated by a concomitant increase in TNF-α. Consistent with this finding, a recent report showed that locally generated TNF-α is involved in cardiac remodeling by stimulating cardiac hypertrophy and fibrogenic response in pressure overloaded heart through modulation of CTGF and TGF-β expression, ultimately leading to ventricular dysfunction (Sun et al., 2007).

Oxidative stress and increased ROS have been implicated in hypertension in human studies of essential hypertension and experimental models of hypertension (Griendling et al.,

2000b; Nakazono et al., 1991; Suzuki et al., 1998). Many studies have shown that Ang II causes ROS generation by activating NADPH oxidase enzyme via AT_1R activation, which, in turn, activate redox sensitive transcription factor such as NF-κB (Touyz, 2003). Ang II-mediated superoxide generation requires functionally active NADPH subunits Nox-2, p22phox, p47phox, and p67phox because downregulation or absence of these subunits results in attenuated or abrogated Ang II-induced cell growth, contraction, and inflammation (Brandes et al., 2002; Xiao et al., 2002), whereas upregulation has shown to be associated with enhanced effects (Lassegue et al., 2001). Also TNF-α can induce oxidative stress by activating NADPH oxidase and decreasing the release of NO (Frey et al., 2002; Li et al., 2002; Zhang et al., 2006). Therefore, in the present study, we measured cardiac oxidative stress and activation of NADPH oxidase subunits. The TNF-$α^{-/-}$ mice infused with Ang II did not show any significant changes in total ROS generation, superoxide and peroxynitrite production, and NADPH subunit activation suggesting an attenuated oxidative response. This result is in agreement with those of previous studies showing that blockade of TNF-α can decrease the production of ROS and attenuate the Ang II induced hypertension (Guzik et al., 2007).

TNF-α is known to affect eNOS expression by affecting the half life of its mRNA (Bitar et al., 2005). TNF-α also inhibited NO-mediated, endothelium dependent-vasorelaxation in coronary arterioles (Zhang et al., 2006). Furthermore, increased iNOS was shown to be associated with hypertension (Chou et al., 1998). Excessive NO production by iNOS result in peroxynitrite anion formation, protein tyrosine nitration, hydroxyl radical generation thereby causing oxidative and nitrosative stress leading to hypertension (Espey et al., 2002; Modlinger et al., 2004). It has been shown that in SHR rats, even though excess NO is produced by iNOS (Hong et al., 2000), reduction in NO bioavailability, which is, in part, due to Ang II-mediated

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increase in superoxide production and impaired superoxide scavenger activity is observed (Adler and Huang, 2004). In our study, mRNA and protein expression of myocardial iNOS were markedly increased by Ang II infusion in WT mice. However, Ang II infusion did not increase the iNOS expression in TNF-$\alpha^{-/-}$ mice. In addition Ang II significantly increased peroxynitrite production in WT mice but not in TNF-$\alpha^{-/-}$ mice. These results suggest that despite reduced iNOS mediated NO production due to Ang II infusion in TNF-$\alpha^{-/-}$ mice, the concomitant decrease in superoxide production and peroxynitrite production lead to increased NO bioavailability and an attenuated hypertensive response.

Recent evidence also suggests that one of the most important downstream signaling molecules common for both TNF-α and Ang II is the transcription factor NF-κB. NF-κB is not only involved in the activation of proinflammatory cytokines, but also in the induction of oxidative and nitrosative stress (Papaharalambus and Griendling, 2007). We have previously shown that the increase in Ang II-mediated NF-κB p50 mRNA is attenuated in TNF-$\alpha^{-/-}$ mice (Sriramula et al., 2008). In the present study, we documented that TNF-$\alpha^{-/-}$ mice infused with Ang II showed an attenuated NF-κB p65 activity in the cardiac tissue.

In conclusion, by using TNF-$\alpha^{-/-}$ mice, we have demonstrated that TNF-α is involved in Ang II-mediated cell signaling, which leads to cardiac hypertrophy, fibrosis and hypertensive response. Improved cardiac function, as indicated by decreased cardiac hypertrophy and fibrosis, blunted NADPH oxidase expression, and decreased ROS formation in Ang II-infused TNF-$\alpha^{-/-}$ mice, suggests that TNF-α is mediating these Ang II-induced effects. Taken together, these data suggest that Ang II-induced cardiac hypertrophy (i.e., myocyte hypertrophy and interstitial fibrosis) and hypertension are dependent on the presence of NADPH oxidase, increased oxidative stress, and activation of NF-κB, and requires concomitant generation of TNF-α. More

importantly, this study provides the evidence that the attenuation of Ang II-induced hypertensive and hypertrophic response by blockade of TNF-α is mediated by decreased oxidative stress (decreased total ROS, superoxide, and peroxynitrite), increased NO bioavailability, and downregulation of NF-κB activity.

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CHAPTER 4

INHIBITION OF TUMOR NECROSIS FACTOR-α IN THE BRAIN ATTENUATES ANGIOTENSIN II-INDUCED HYPERTENSION

INTRODUCTION

Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system (RAS), exerts diverse physiological actions in both the peripheral and central neural systems. Essential components of the RAS, i.e., renin, angiotensinogen, angiotensin converting enzyme, angiotensin converting enzyme 2, angiotensin type 1 (AT_1R) and type 2 (AT_2R) receptors , as well as various aminopeptidases, are synthesized within the brain suggesting the existence of an intrinsic brain RAS (Bader and Ganten, 2002; Sakai and Sigmund, 2005; Veerasingham and Raizada, 2003). Recent evidence suggests that hyperactivity of the brain RAS may play a critical role in the development and maintenance of hypertension. Ang II, acting through AT_1R, plays a prominant role in the central regulation of blood pressure by activating the sympathetic nervous system, regulating fluid balance and the secretion of aldosterone (Veerasingham and Raizada, 2003). The pressor response to infusion of chronic low-dose Ang II in animals has been shown, at least in part, to be sympathetically driven (Bruner and Fink, 1986; Gorbea-Oppliger and Fink, 1994). Previous studies suggest that systemically delivered Ang II likely acts upon the circumventricular organs, where the blood brain barrier is weak or absent, and subsequently activates hypothalamic and brain stem sites such as the paraventricular nucleus (PVN) and ventrolateral medulla, contributing to sympathoexcitation and hypertensive response (Collister and Hendel, 2003; Fink et al., 1987; Simpson, 1981).

Experimental evidence indicates that the hypothalamic PVN is an important center for integrating neural signals of the pressor response to Ang II and modulates sympathetic vasomotor tone (Martin et al., 1991; Zhu et al., 2004). Recent findings from our lab and others suggest that RAS, in addition to inducing neurohumoral excitation, also increases the production of proinflammatory cytokines (PIC), such as tumor necrosis factor-alpha (TNF-α), and decreases

the expression of anti-inflammatory cytokines, like IL-10, in the brain's cardiovascular regulatory centers (Kang et al., 2009; Shi et al., 2010). A persistent increase in peripheral Ang II levels is proposed to initiate a cascade of signaling events involving reactive oxygen species (ROS) in central cardioregulatory regions leading to increased sympathetic activity, hypertension and end organ damage. These observations coupled with the emerging role of cytokines in hypertension led us to hypothesize that the central effects of Ang II are, at least in part, mediated by the activation of TNF-α within the cardiovascular relevant brain areas, and ultimately lead to an enhanced hypertensive response.

In the present study, we investigated the effect of central TNF-α inhibition using etanercept (ETN), a soluble TNF-α receptor fusion protein, in Ang II-induced hypertensive response. In addition, we investigated the downstream signaling pathways involved in interaction of TNF-α and Ang II.

METHODS

Animals. Male Sprague-Dawley rats (8-10 weeks old) were used in this study. Animals were housed in a temperature-controlled room ($25 \pm 1^{o}C$) and maintained on a 12:12 hour light: dark cycle with free access to water and food. All animal and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University and complied with NIH guidelines.

Experimental Protocol. The rats were implanted with radio telemetry transmitters to measure blood pressure, and subjected to intracerebroventricular (ICV) infusion of etanercept (10μg/kg/day) or artificial cerebrospinal fluid (aCSF), with and without subcutaneous infusion of Ang II (200 ng/kg/min) for 4 weeks. Osmotic minipumps (Alzet, model 2004) were filled with

Ang II dissolved in 0.9% saline or saline alone, and were implanted subcutaneously in the retroscapular area. The rats were divided into 3 groups: 1) Control group - saline minipumps + ICV aCSF, 2) ETN group - saline minipumps + ICV ETN, 3) Ang II group - Ang II minipump + ICV aCSF, 3) Ang II+ETN group - Ang II minipump + ICV etanercept. At the end of the study, rats were euthanized; the brain and hearts were removed and stored for further analysis.

Blood Pressure Measurement. Blood pressure was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN). Rats were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (ip) and placed dorsally on a heated surgical table. An incision was made on the ventral surface of the left leg, and the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and a small clamp was used to temporarily interrupt the blood flow. The catheter tip was introduced through a small incision in the femoral artery, advanced ~6 cm into the abdominal aorta such that the tip was distal to the origin of the renal arteries, and secured into place. The body of the transmitter was placed into the abdominal cavity and secured to the abdominal wall. The abdominal musculature was sutured and the skin layer was closed following implantation. Rats received benzathine penicillin (30000 U, im) and buprenorphine (0.1 mg/kg, sc) immediately following surgery and 12 h postoperatively and allowed to recover for seven days.

ICV Cannula Implantation. Following the transmitter recovery period, the rats were implanted with ICV cannula for infusion of ETN or aCSF (Francis et al., 2003). The rats were anaesthetized and the head was positioned in a Kopf stereotaxic apparatus. An ICV cannula was implanted into the right lateral cerebroventricle (1.3 mm caudal to bregma, 1.5 mm lateral to the midline, and 3.5 mm ventral to the dura) according to Paxinos and Watson, and fixed to the

cranium using small screws and dental cement. A 4-week osmotic minipump was implanted subcutaneously and connected to the infusion cannula via the catheter tube to deliver ETN or aCSF into the brain.

Real Time RT-PCR. PVN punches were made from frozen brain sections using a Stoelting brain punch (Stoelting). Total RNA was isolated from PVN tissue using RNeasy plus micro kit (Qiagen) and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Real Time PCR amplification reactions were performed with iQ SYBR Green Super mix with ROX (Bio-Rad) using the ABI Prism 7900 Real time PCR machine (Applied Biosystems). List of primers used was provided in Table 4.1. Data were normalized to GAPDH expression by the $\Delta\Delta C_T$ comparative method.

Table 4.1. List of primers used for real time RT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
ANP	ATCACCAAGGGCTTCTTCCT	CCTCATCTTCTACCGGCATC
AT₁R	CAAAAGGAGATGGGAGGTCA	TGACAAGCAGTTTGGCTTTG
IL-1β	GCAATGGTCGGGACATAGTT	AGACCTGACTTGGCAGAGGA
IL-6	CACAAGTCCGGAGAGGAGAC	ACAGTGCATCATCGCTGTTC
IL-10	CTAACGGGAGCAACTCCTTG	ATCATGGAAGGAGCAACCTG
MCP-1	GTGCTGACCCCAATAAGGAA	TGAGGTGGTTGTGGAAAAGA
nNOS	AGGTCAAGAACTGGGAGACG	GAAGAGCTGGTCCTTTGTGC
NOX-2	CGGAATCTCCTCTCCTTCCT	GCATTCACACACCACTCCAC
TNF-α	GTCGTAGCAAACCACCAAGC	TGTGGGTGAGGAGCACATAG
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

Immunohistochemical Analysis. Rats (n=4 in each group) were transcardially perfused with 200 ml of ice-cold PBS (7.4 pH; 0.1M) followed immediately by 200 ml of 4% paraformaldehyde in PBS. The brains were removed, postfixed in 4% paraformaldehyde solution for 2 hours, and transferred to a phosphate buffer containing 20% sucrose (pH 7.4) and stored overnight. For immunostaining, 10 µm coronal sections from paraffin embedded brains were

collected on slides. First the sections were incubated with 0.3% H_2O_2 in methanol for 10 minutes. For antigen retrieval, citrate buffer with microwave heating technique is used. Then the sections were incubated with 1.5% goat or rabbit serum in PBS containing 0.3% Triton X100 for 30 minutes. The sections were incubated with primary antibodies overnight at 4°C followed by incubation with biotinylated goat-anti rabbit or rabbit-anti goat secondary antibodies for 60 minutes, and stained with Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. Each step was followed by washing the sections with PBS containing 0.3% Triton X100. Sections incubated without primary antibody were used as negative controls.

Measurement of Plasma IL-10. At the end of the study, blood was collected in chilled EDTA tubes, plasma was separated and stored at -80°C until assayed. Circulating levels of IL-10 were quantified in the plasma using a commercially available rat IL-10 ELISA kit (Invitrogen) according to manufacturer's instructions.

Statistical Analysis. Data were analyzed, when appropriate, by Student's t test, repeated measures ANOVA, or 1-way ANOVA followed by Newman-Keuls correction for multiple comparisons between means. Statistical comparisons were performed using Prism5 (GraphPad Software). Values are expressed as mean ± SEM, with $P < 0.05$ considered significant.

RESULTS

Effect of Ang II Infusion on the Mean Arterial Pressure. To assess the effect of central blockade of TNF-α on Ang II-induced hypertensive response, mean arterial pressure (MAP) was measured using a radio telemetry system. Figure 4.1 shows the effect of ICV treatment with ETN on MAP. Chronic Ang II infusion for 28 days significantly increased the mean arterial pressure

(MAP) in rats when compared with control rats (106 ± 4 mmHg in control versus 154 ± 3 mmHg in Ang II). In contrast, ICV treatment with etanercept attenuated the increase in MAP induced by Ang II infusion (127 ± 4 mmHg in control versus 154 ± 3 mmHg in Ang II). Furthermore, ETN treatment alone had no effect on MAP.
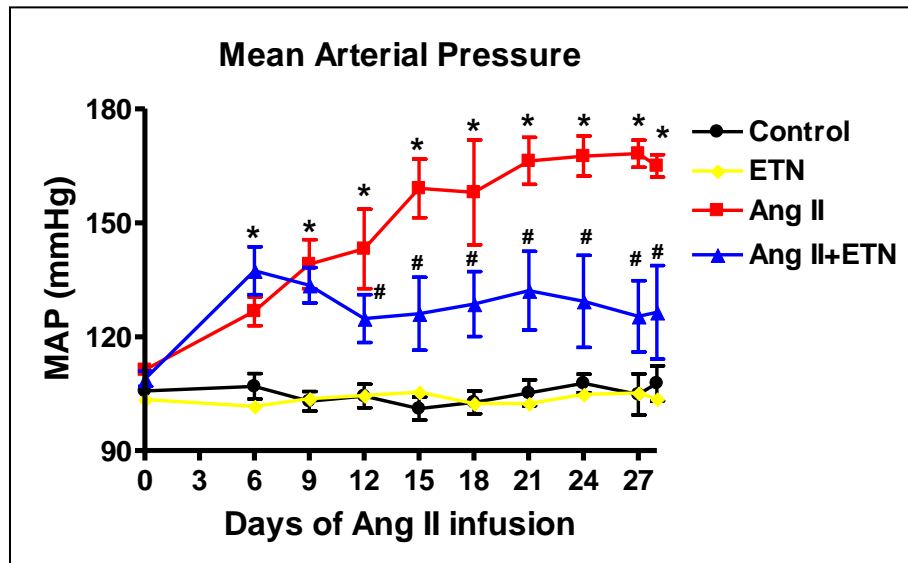
Figure 4.1. Effect of ICV treatment with etanercept on mean arterial pressure (MAP). Ang II infusion resulted in significant increase in MAP whereas treatment with etanercept significantly decreased the MAP. *$p<0.05$ Control vs. Ang II; #$p<0.05$ Ang II vs. Ang II+ETN.

Effect of Ang II Infusion on the Expression of PIC in the PVN. To determine the effect of Ang II on the production of PIC and chemokines, we examined the mRNA expression in the PVN by real time RT-PCR. Figure 4.2 shows that Ang II infusion induced an increase in mRNA of the PIC TNF-α, IL-6 and IL-1β, and the chemokine MCP-1 in the PVN. This trend was reversed in the PVN of Ang II-infused rats that were treated with ETN.

Effect of Ang II Infusion on the Expression of PIC in the Heart. We also measured the expression of PIC in the heart by real time RT-PCR. Figure 4.3 shows that Ang II infusion caused increase in mRNA expression of TNF-α, IL-6 and IL-1β, and chemokine MCP-1 in the heart, which was decreased by central treatment with ETN.

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Figure 4.2. Effect of ICV treatment with etanercept on PVN expression of proinflammatory cytokines. Ang II infusion significantly increased the mRNA expression of TNF-α, IL-6, IL-1β and MCP-1 in the PVN. ICV treatment with etanercept attenuated these mRNA changes. n=5-9 per group. *$p<0.05$ Control vs. Ang II; #$p<0.05$ Ang II vs. Ang II+ETN.

Effect of Ang II Infusion on the Expression of IL-10. We also examined the effect of TNF-α inhibition on the expression of anti-inflammatory cytokine IL-10. Chronic subcutaneous Ang II infusion resulted in significant decrease in PVN mRNA expression of IL-10 and plasma levels of IL-10. However, Ang II infusion and simultaneous inhibition of TNF-α in the brain using ETN, increased PVN mRNA expression of IL-10 and plasma levels of IL-10 (Figure 4.4).
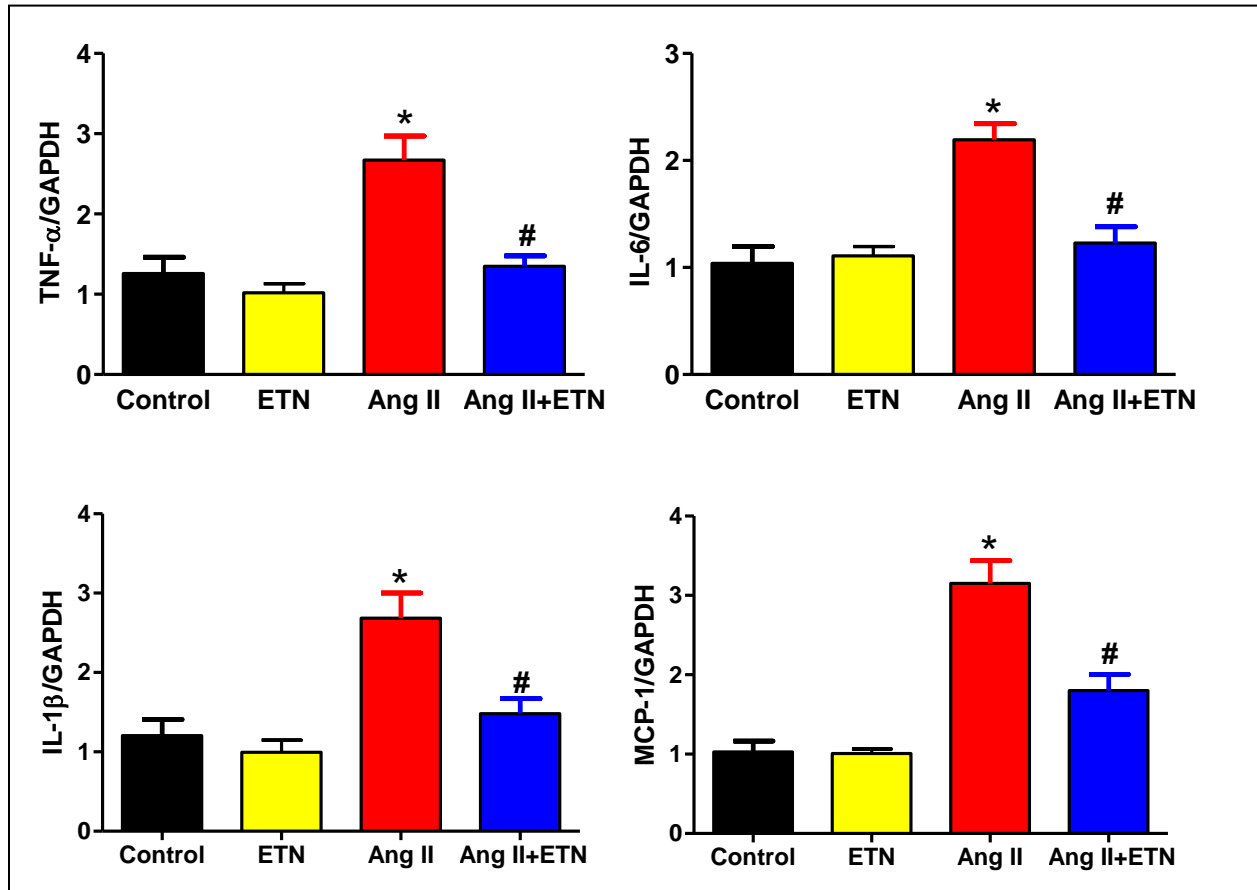
Figure 4.3. Effect of ICV treatment with etanercept on cardiac expression of proinflammatory cytokines. Ang II infusion significantly increased the cardiac mRNA expression of TNF-α, IL-6, IL-1β and MCP-1. ICV treatment with etanercept attenuated these mRNA changes. n=5-9 per group. *$p<0.05$ Control vs. Ang II; #$p<0.05$ Ang II vs. Ang II+ETN.

Effect of Ang II Infusion on the Cardiac Hypertrophy. To evaluate Ang II-induced changes in the cardiac hypertrophy in these rats, the hearts were harvested and weighed at the end of experimental period. The ratio of heart/body weight was calculated as an indicator of cardiac hypertrophy. Also mRNA expression of a molecular marker of cardiac hypertrophy, atrial natriuretic peptide (ANP), was measured in cardiac tissue using real time RT-PCR. Chronic Ang II infusion lead to cardiac hypertrophy as indicated by increased heart/body ratio and increased mRNA expression of ANP in the heart (Figure 4.5). ICV treatment with ETN inhibited Ang II mediated cardiac hypertrophy as indicated by decreased heart/body weight ratio and decreased

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mRNA expression of ANP in the heart suggesting a beneficial effect of brain TNF-α blockade in
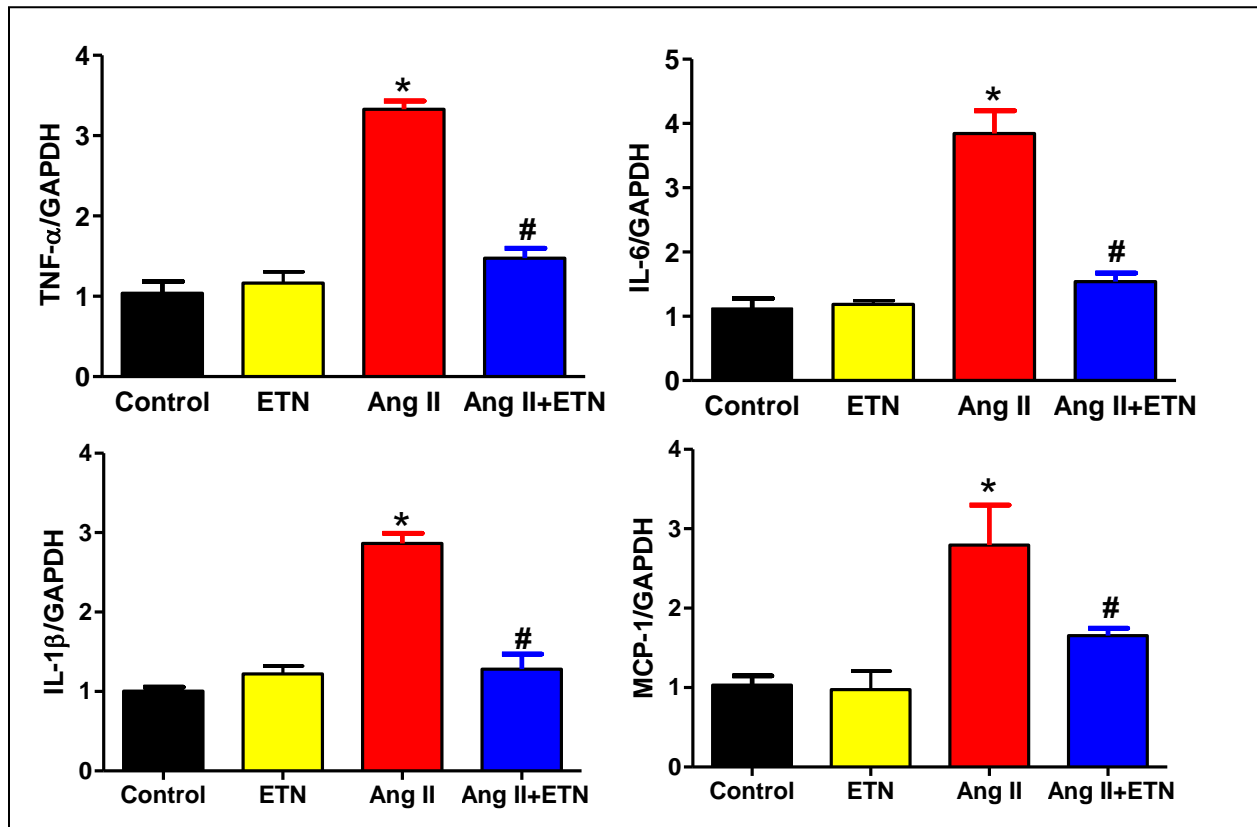
on cardiac hypertrophy.
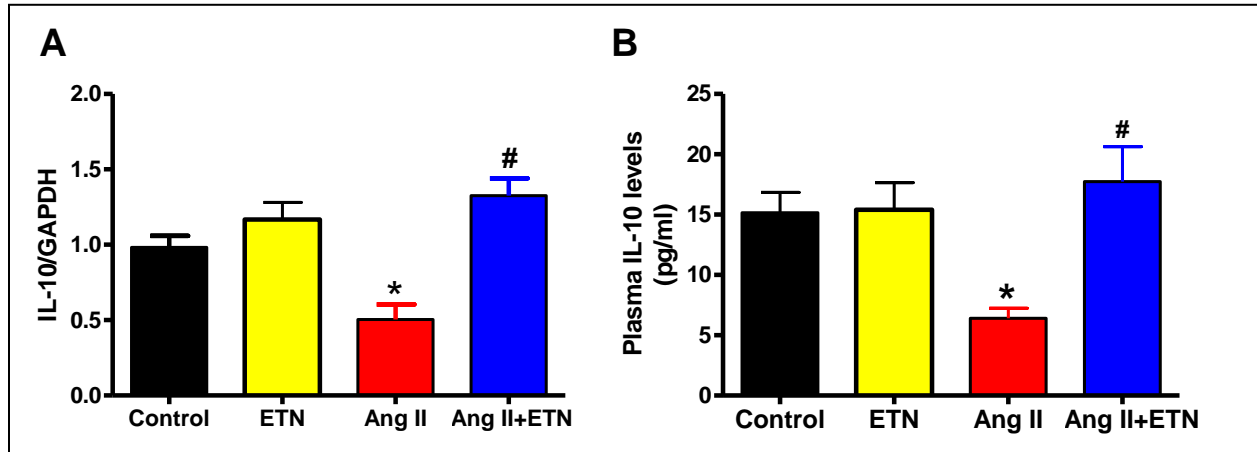
Figure 4.4. Effect of ICV treatment with etanercept on expression of anti-inflammatory cytokine IL-10. Ang II infusion significantly decreased the PVN mRNA expression of IL-10 (A) and plasma IL-10 levels (B). ICV treatment with etanercept resulted in increased expression of IL-10. n=5-9 per group. *$p<0.05$ Control vs. Ang II; #$p<0.05$ Ang II vs. Ang II+ETN.

Figure 4.5. Effect of ICV treatment with etanercept on cardiac hypertrophy. Ang II induced cardiac hypertrophy as indicated by heart weight to body weight ratio and molecular marker atrial natriuretic peptide expression, is attenuated by central inhibition of TNF-α using etanercept. *$p<0.05$ Control vs. Ang II; #$p<0.05$ Ang II vs. Ang II+ETN.

Effect of Ang II Infusion on the Expression of AT_1R. Both Ang II and TNF-α have been shown to modulate AT_1R expression. To determine whether the infusion of Ang II alters the expression of AT_1R in the PVN, we examined the mRNA levels of AT_1R by real time RT-PCR and the protein expression using immunohistochemistry. The AT_1R mRNA expression in the PVN was significantly increased in Ang II infused rats compared with control rats, whereas, in Ang II infused rats treated with ETN, it remained unchanged (Figure. 4.6A). These results were confirmed at the protein level in the PVN by immunohistochemical analysis (Figure. 4.6B).

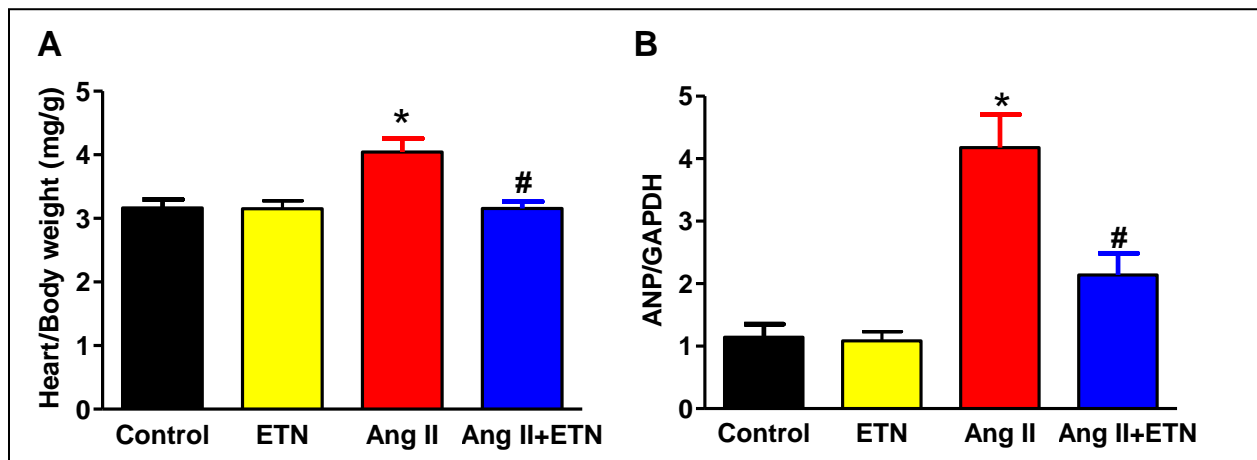**Figure 4.6.** Effect of ICV treatment with etanercept on AT_1 receptor expression in the PVN. Ang II infusion significantly increased the mRNA (A) and protein (B) expression of AT_1 receptor. Increased expression of AT_1R is shown by arrows. ICV treatment with etanercept attenuated these mRNA and protein expression. *$p<0.05$ Control vs. Ang II; #$p<0.05$ Ang II vs. Ang II+ETN.

Effect of Ang II Infusion on the Expression of NOX-2 and nNOS. Because both Ang II and TNF-α have been shown to act through the oxidative stress mediated pathways, we analyzed the NOX-2 (previously gp91phox) subunit of NADPH and nNOS mRNA expression in the PVN using real time PCR. Ang II infusion significantly increased NOX-2 and decreased nNOS mRNA expression in Ang II infused rats but these changes were reversed in Ang II infused rats treated with ETN (Figure 4.7).
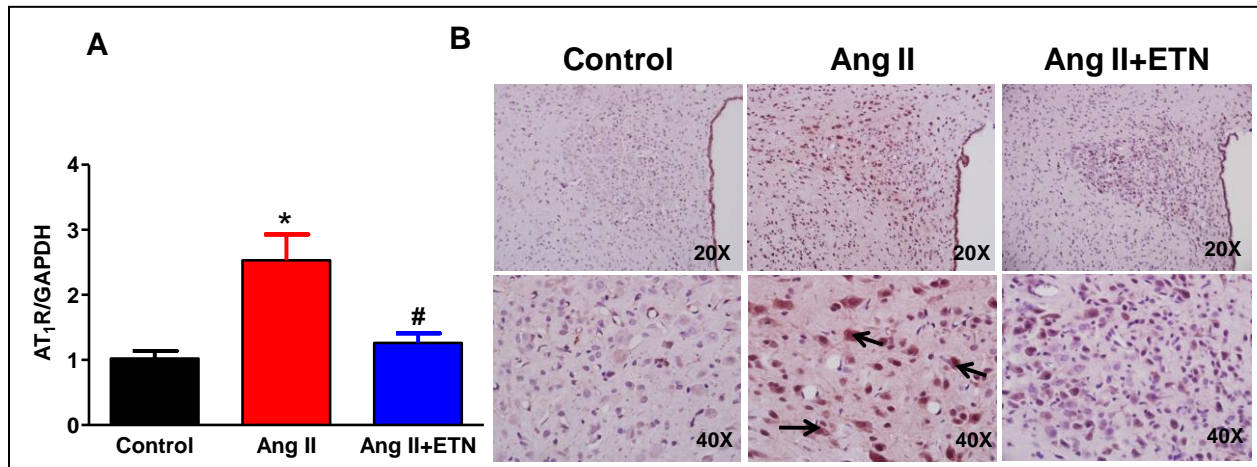
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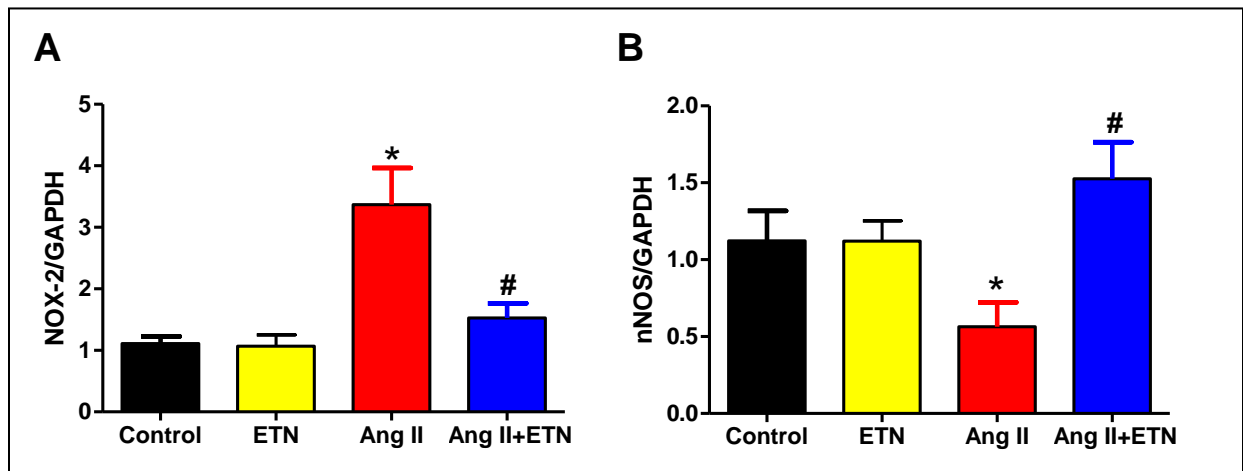
Figure 4.7. Effect of ICV treatment with etanercept on NOX-2 and nNOS expression in the PVN. Ang II infusion significantly increased the mRNA expression of NOX-2 and decreased the expression of nNOS. ICV treatment with etanercept reversed these mRNA changes in the PVN. *$p < 0.05$ Control vs. Ang II; #$p < 0.05$ Ang II vs. Ang II+ETN.

DISCUSSION

In the present study, our data demonstrate that Ang II-induced hypertension is attenuated by treatment with the TNF-α blocker etanercept. Central TNF-α inhibition with etanercept treatment also reduced the expression of PIC, reduced cardiac hypertrophy, decreased the expression of NOX-2 and AT$_1$R, and increased nNOS and IL-10 expression.

Recent evidence suggests that hypertension is an inflammatory condition where various PIC such as TNF-α, IL-6 and IL-1β, both centrally and peripherally, have been shown to play an important role in the pathogenesis of hypertension (Chae et al., 2001; Lu et al., 2009; Shi et al., 2010; Sriramula et al., 2008; Sun et al., 2004; Veerasingham and Raizada, 2003). We have recently reported that chronic peripheral Ang II infusion results in increased production of PIC in the PVN (Kang et al., 2009). More importantly, blockade of TNF-α by etanercept has been shown to prevent renal damage in genetically hypertensive rats and to lower blood pressure in

rats with hypertension induced by Ang II and salt, suggesting a role for TNF-α in blood pressure regulation and renal injury (Elmarakby et al., 2006; Muller et al., 2002). Etanercept is a soluble recombinant fusion protein consisting of an extracellular ligand-binding domain of TNF receptor 2. Etanercept inhibits TNF-α by binding to the TNF receptor and acting as a competitive inhibitor of TNF-α (Goffe and Cather, 2003). A recent study also showed that mice treated with etanercept prevented hypertension and blunted the increase in super oxide production in response to Ang II (Guzik et al., 2007). Our present observations complement these findings and show that central blockade of TNF-α in the brain using etanercept protects rats against Ang II-dependent hypertension and cardiac hypertrophy.

Ang II can act as a potent proinflammatory agent and stimulate the production of chemokines such as MCP-1, and cytokines, such as TNF-α, IL-6 and IL-1β, and downregulates the anti-inflammatory cytokine IL-10 (Shi et al., 2010). TNF-α is considered as one of the initiators of the cytokine cascade, which can induce production of other cytokines and chemokines (Zhang et al., 2006a; Zhang et al., 2006b). A recent study demonstrated that Ang II-induced hypertension involves activation of microglia and increased expression of PIC within the PVN (Shi et al., 2010). In our study, TNF-α blockade with etanercept decreased expression of other cytokines such as IL-6, IL-1β, and chemokine MCP-1, and upregulated the expression of IL-10, supporting the hypothesis that PIC are involved in Ang II-induced hypertensive response.

Excessive ROS production in the brain cardiovascular regulatory centers contribute to the neuropathogenesis of hypertension by enhancing sympathetic nervous system activity (Paravicini and Touyz, 2006). It has been shown that NADPH oxidase is the main source of Ang II-induced ROS in neurons (Paravicini and Touyz, 2006). Treatment with the cell permeable superoxide dismutase (SOD) mimetic tempol inhibits Ang II-mediated superoxide production and

hypertension (Nishiyama et al., 2001). Also TNF-α can induce activation of NADPH oxidase leading to oxidative stress and decreasing the bioavailability of NO (Zhang et al., 2006b). Our present study provides further support for these observations by showing that central TNF-α blockade with etanercept reduced the expression of NOX-2, the catalytic subunit of the NADPH oxidase enzyme, and increased the expression of nNOS in the PVN.

In many animal models of hypertension, the expression of AT_1R is upregulated in the central cardiovascular regulatory centers including the PVN (Veerasingham and Raizada, 2003). In the brain AT_1R mediates the central effects of Ang II, including vasopressin release, water and salt intakes, and increased sympathetic drive, contributing to the development of hypertension (Paul et al., 2006; von Bohlen und Halbach and Albrecht, 2006). Both *in vitro* and *in vivo* studies have demonstrated the existence of cross-talk between Ang II and TNF-α (Arenas et al., 2004; Sasamura et al., 1997). Also, we have shown that attenuation of Ang II-induced hypertension in TNF-α knockout mice involves decreased expression of AT_1R (Sriramula et al., 2008). In the present study, treatment with etanercept resulted in reduction of Ang II-induced AT_1 receptor upregulation in the PVN. These results suggest that the AT_1R is involved in the beneficial effects of TNF-α inhibition.

In summary, chronic Ang II infusion resulted in cardiac hypertrophy and hypertension, increased AT_1R and PIC expression, and decreased IL-10 expression in the PVN. Ang II infusion also resulted in increased expression of NOX-2 and decreased expression of nNOS in the PVN. Central blockade of TNF-α with etanercept resulted in attenuation of hypertension, cardiac hypertrophy and PIC expression along with increased IL-10 expression. The beneficial effects of central TNF-α blockade in Ang II-induced hypertensive responses are mediated by AT_1R downregulation and decreased oxidative stress in the PVN. Our findings provide further

evidence for the involvement of the brain RAS and its interaction with TNF-α in neurogenic

hypertension and may be beneficial towards further development of hypertensive therapies.

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CHAPTER 5

BILATERAL ACE2 OVEREXPRESSION IN THE PARAVENTRICULAR NUCLEUS ATTENUATES ANGIOTENSIN II-INDUCED HYPERTENSION

INTRODUCTION

The renin angiotensin system (RAS) plays an important role in the regulation of blood pressure and volume homeostasis through its effects on vasoconstriction, cardiac remodeling, sympathetic outflow and vasopressin synthesis and release (Brunner, 2001). Angiotensin II (Ang II), the physiologically active effector peptide of the classical RAS, exerts its various actions contributing to blood pressure regulation mainly via interaction with the angiotensin II type-1 receptor (AT_1R) (Allen et al., 2000). In addition to the classic endocrine system, the discovery of RAS components in various tissues throughout the body led to the concept of local or tissue RAS, which results in the local synthesis, release, and action of angiotensin peptides (Lavoie and Sigmund, 2003). The components of the RAS have been identified in all brain areas involved in the central regulation of blood pressure, including subfornical organ (SFO), paraventricular nucleus (PVN), rostral ventrolateral medulla (RVLM), area postrema, nucleus tractus solitarius, and others (Davisson, 2003). Moreover, a chronic increase in circulating Ang II by peripheral infusion can increase neuronal activity in these brain areas and are implicated in autonomic and cardiovascular regulation, leading to sympathetic hyperactivity and neurogenic hypertension.

The PVN of the hypothalamus is recognized as a critical central nervous system center for the coordination of autonomic and neuroendocrine homeostatic responses (Dampney, 1994; Swanson and Sawchenko, 1983). The PVN contains a complex profile of excitatory and inhibitory neurotransmitters, and receives inputs from a wide variety of sources, including peripheral receptors, higher brain centers such as the cortex and amygdala, and the circumventricular organs (Swanson and Sawchenko, 1983; Tribollet and Dreifuss, 1981).

Several studies support the role of the PVN in blood pressure control. Microinjection of Ang II into the PVN of rats increased mean arterial pressure, which was attenuated following

systemic administration of losartan, an AT_1R antagonist (Bains et al., 1992). It has also been shown that electrolytic or chemical ablation of the PVN attenuated the development of high blood pressure in spontaneously hypertensive (SH) rats, DOCA-salt hypertensive rats and Dahl salt-sensitive rats (Allen, 2002; Ito et al., 2003; Nakata et al., 1989). Previous studies have shown that activation of the PVN resulted in sympathoexcitation and increased blood pressure; both were mediated by AT_1R (Martin et al., 1991; Tagawa and Dampney, 1999). Recent evidence also suggests that hypertension is a inflammatory condition where various proinflammatory cytokines (PIC) such as TNF-α, IL-6 and IL-1β, both centrally and in the periphery, have been shown to play an important role in the pathogenesis of hypertension (Chae et al., 2001; Lu et al., 2009; Shi et al., 2010; Sriramula et al., 2008; Sun et al., 2004). We have recently reported that chronic peripheral Ang II infusion results in increased production of PIC in the PVN (Kang et al., 2009).

Recent studies on the RAS have found new components and have proposed new signaling pathways, such as the ACE2-Ang-(1-7)-Mas receptor pathway, in addition to the classical ACE-Ang II-AT_1R pathway. ACE2 is considered as a critical enzyme of the RAS cascade that is potentially important in counterbalancing the vasoconstrictor and proliferative effects of Ang II with the vasodilatory and anti-proliferative effects of Ang-(1-7) and other related peptides (Ferrario, 1998; Huentelman et al., 2005). Several recent studies showed that alterations of the ACE2 gene or its expression are implicated in cardiovascular disease. Targeted disruption of ACE2 in mice resulted in severely defective cardiac contractility with elevated cardiac Ang II levels (Crackower et al., 2002). Also, ACE2 overexpression by systemic lentiviral delivery prevents cardiac hypertrophy in an Ang II infusion rat model of hypertension (Huentelman et al., 2005), and exerts protective effects on high blood pressure and cardiac pathophysiology induced by hypertension in SH rats (Diez-Freire et al., 2006). Furthermore,

lentiviral mediated overexpression of ACE2 in the RVLM decreases high blood pressure in SH rats (Yamazato et al., 2007). Recently, it has been shown that brain-targeted overexpression of an adenovirus coding for human ACE2 in the SFO reduces the acute Ang II-mediated pressor and drinking responses (Feng et al., 2008).

Based on these observations, we hypothesized that ACE2 overexpression within the PVN will have beneficial effects in counteracting the Ang II-induced hypertensive response. To test this hypothesis rats were injected bilaterally into the PVN with an adenovirus coding for human ACE2 (AdACE2) or its control (AdeGFP). They were than tested for the Ang II-mediated hypertensive response. Our observations demonstrate that overexpression of ACE2 in the PVN attenuated the Ang II-induced inflammatory and hypertensive responses.

METHODS

Animals. Male Sprague-Dawley rats (12 weeks old) were used in this study. Animals were housed in a temperature-controlled room (25 ± 1°C) and maintained on a12:12 hour light: dark cycle with free access to water and food. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University and complied with NIH guidelines.

Experimental Design. The rats were anaesthetized and implanted with radio-telemetry transmitters. After 7 days recovery from the surgery, AdACE2 or AdeGFP virus were injected bilaterally intra-PVN ($2X10^6$ plaque-forming units [pfu], 200nL) using a pressure injector, as described previously (Feng et al., 2008). Osmotic minipumps (Alzet, model 2002) with an infusion rate of 0.5μl/h for 14 days were filled with Ang II (Bachem, 200 ng/kg/min) dissolved in 0.9% saline or saline alone, and were implanted subcutaneously in the retroscapular area. The rats were divided into 3 groups: 1) Control group - Saline minipump + intra PVN AdeGFP, 2)

AdACE2 group - Saline minipump + intra PVN Ad-ACE2, 3) Ang II group – Ang II minipump + intra PVN AdeGFP, 4) Ang II+AdACE2 group - Ang II minipump + intra PVN Ad-ACE2. Rats were euthanized after 14 days and brains were collected for further analysis.

Blood Pressure Measurement. Blood pressure was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN). Rats were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (ip) and placed dorsally on a heated surgical table. An incision was made on the ventral surface of the left leg, and the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and a small clamp was used to temporarily interrupt the blood flow. The catheter tip was introduced through a small incision in the femoral artery, advanced ∼6 cm into the abdominal aorta such that the tip was distal to the origin of the renal arteries, and secured into place. The body of the transmitter was placed into the abdominal cavity and sutured to the abdominal wall. The abdominal musculature was sutured, and the skin layer was closed. Rats received benzathine penicillin (30000 U, im) and buprenorphine (0.1 mg/kg, sc) immediately following surgery and 12 h postoperatively. The rats were allowed to recover for 7 days after the surgery.

Bilateral Intra-PVN Injections. Rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (ip). The rats were placed in a stereotaxic instrument (Kopf instruments; Tujunga, CA) and the skull was exposed through an incision on the midline of the scalp. After bregma was identified, the coordinates for the PVN were determined from the Paxinos and Watson (2007) rat atlas, which is 1.8 mm posterior, 0.4 mm lateral to the bregma, and 7.9 mm ventral to the zero level. AdACE2 or AdeGFP virus were injected bilaterally into the PVN ($2X10^6$ plaque-forming units [pfu], 200nL) using a pressure injector.

Real Time RT-PCR. The PVN punches were made from frozen brain sections using a Stoelting brain punch (Stoelting). Total RNA was isolated from PVN tissue using RNeasy plus micro kit (Qiagen), and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). Real Time PCR amplification reactions were performed with iQ SYBR Green Super mix with ROX (Bio-Rad) using the ABI Prism 7900 Real time PCR machine (Applied Biosystems). The list of primers used for real time RT-PCR was provided in Table 5.1. Data were normalized to GAPDH expression by the $\Delta\Delta C_T$ comparative method. The data are presented as the fold expression of the gene of interest relative to their control animals.

Table 5.1. List of primers used for real time RT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
ACE	TTGACGTGAGCAACTTCC AG	CAGATCAGGCTCCAGTGACA
ACE2	ACCCTTCTTACATCAGCCCTACTG	TGTCCAAAACCTACCCCACATAT
AT_1R	CAAAAGGAGATGGGAGGTCA	TGACAAGCAGTTTGGCTTTG
AT_2R	TGCTGTTGTGTTGGCATTCA	ATCCAAGAAGGTCAGAACATGGA
IL-1β	GCAATGGTCGGGACATAGTT	AGACCTGACTTGGCAGAGGA
IL-6	CACAAGTCCGGAGAGGAGAC	ACAGTGCATCATCGCTGTTC
Mas	CACTGGCCCTCCTGATGAA	GGATGCCAGAATTGAACACAGA
MCP-1	GTGCTGACCCCAATAAGGAA	TGAGGTGGTTGTGGAAAAGA
TNF-α	GTCGTAGCAAACCACCAAGC	TGTGGGTGAGGAGCACATAG
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

Western Blot Analysis. Western blot analysis was performed according to standard protocols. The PVN tissue was homogenized with RIPA lysis buffer. The total protein concentration in samples was measured by Bio-Rad Dc protein assay. Equal amounts of protein (5 µg) were separated by SDS-PAGE on 10% (wt/vol) gels and transferred on to PVDF membrane (Immobilon-P, Millipore), and blocked with 1% BSA in TBS-T at room temperature for 60 min. The membranes were subjected to immunoblot analyses with anti-AT_1R, anti-AT_2R, anti-ACE2, anti-Mas, and anti-GAPDH antibodies (1:200 dilutions). Immunodetection was accomplished

with a horseradish peroxidase-labeled anti-rabbit or anti-goat secondary antibody (1:2000 dilution) using an enhanced chemiluminescence kit (Amersham). The data were quantified by densitometry using Chemidoc XRS system and Quantity-One software (Bio-Rad) and were normalized to GAPDH.

Immunohistochemical Analysis. Rats (n=4 in each group) were transcardially perfused with 200 ml of ice-cold PBS (7.4 pH; 0.1M) followed immediately by 200 ml of 4% paraformaldehyde in PBS. The brains were removed, post fixed in 4% paraformaldehyde solution for 2 hours, and transferred to a phosphate buffer containing 20% sucrose (pH 7.4) and stored overnight. Frozen brain tissues were sectioned in the coronal plane (10 µm) and sections without immunostaining were used to examine GFP expression. For immunostaining, 10 µm coronal sections from paraffin embedded brains were collected on slides. First the sections were incubated with 0.3% H_2O_2 in methanol for 10 minutes. For antigen retrieval, citrate buffer with microwave heating technique was used. Then the sections were incubated with 1.5% goat or rabbit serum in PBS containing 0.3% Triton X100 for 30 minutes. The sections were incubated with primary antibodies overnight at 4°C followed by incubation with biotinylated goat-anti rabbit or rabbit-anti goat secondary antibodies for 60 minutes, and stained with Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. Each step was followed by washing the sections with PBS containing 0.3% Triton X100. Sections incubated without primary antibody were used as negative controls.

Statistical Analysis. Data are presented as mean±SEM. Data were analyzed, when appropriate, by Student's *t* test, repeated measures ANOVA, or 1-way ANOVA followed by Newman-Keuls correction for multiple comparisons between means. Statistical comparisons were performed

using GraphPad Prism 5 for windows (GraphPad Software). Differences were considered statistically significant at a value of $P < 0.05$.

RESULTS

AdACE2 Gene Transfer Increases the Expression of ACE2 in the PVN. To determine the role of ACE2 in the central regulation of blood pressure, we used an adenovirus encoding human ACE2 gene to overexpress ACE2 in the PVN (Figure 5.1A), an important cardiovascular regulatory center of the brain. Figure 5.1B shows the localization of ACE2 gene expression in the PVN as indicated by enhanced green fluorescence protein expression. Figure 5.1C shows increased ACE2 immunoreactivity in the PVN region which received AdACE2 injection compared with the AdeGFP receiving PVN region. Similarly, hACE2 protein expression was detected only in the PVN of AdACE2 injected rats (Figure 5.1D and 5.1E). These results demonstrated that ACE2 expression was increased in the PVN following AdACE2 microinjection.

ACE2 Overexpression and Ang II-induced Blood Pressure Response. To assess the effect of overexpression of ACE2 in the PVN on the Ang II-induced hypertensive response, mean arterial pressure (MAP) was measured using a radiotelemetry system. Figure 5.2 shows the effect of bilateral ACE2 overexpression on MAP. Bilateral PVN microinjection of AdACE2 virus alone did not show any significant change in MAP compared with control rats. Chronic Ang II infusion for 14 days significantly increased the MAP in rats that had received bilateral PVN microinjection of AdeGFP virus (102.3±1.6 mmHg in AdeGFP versus 163.9±3.0 mmHg in Ang II+ AdeGFP). In contrast, Ang II infusion did not produce the same level of increase in MAP in rats that had received bilateral PVN microinjection of AdACE2 virus (134.6±5.9 mmHg in Ang II+AdACE2 versus 163.9±3.0 mmHg in Ang II+ AdeGFP).
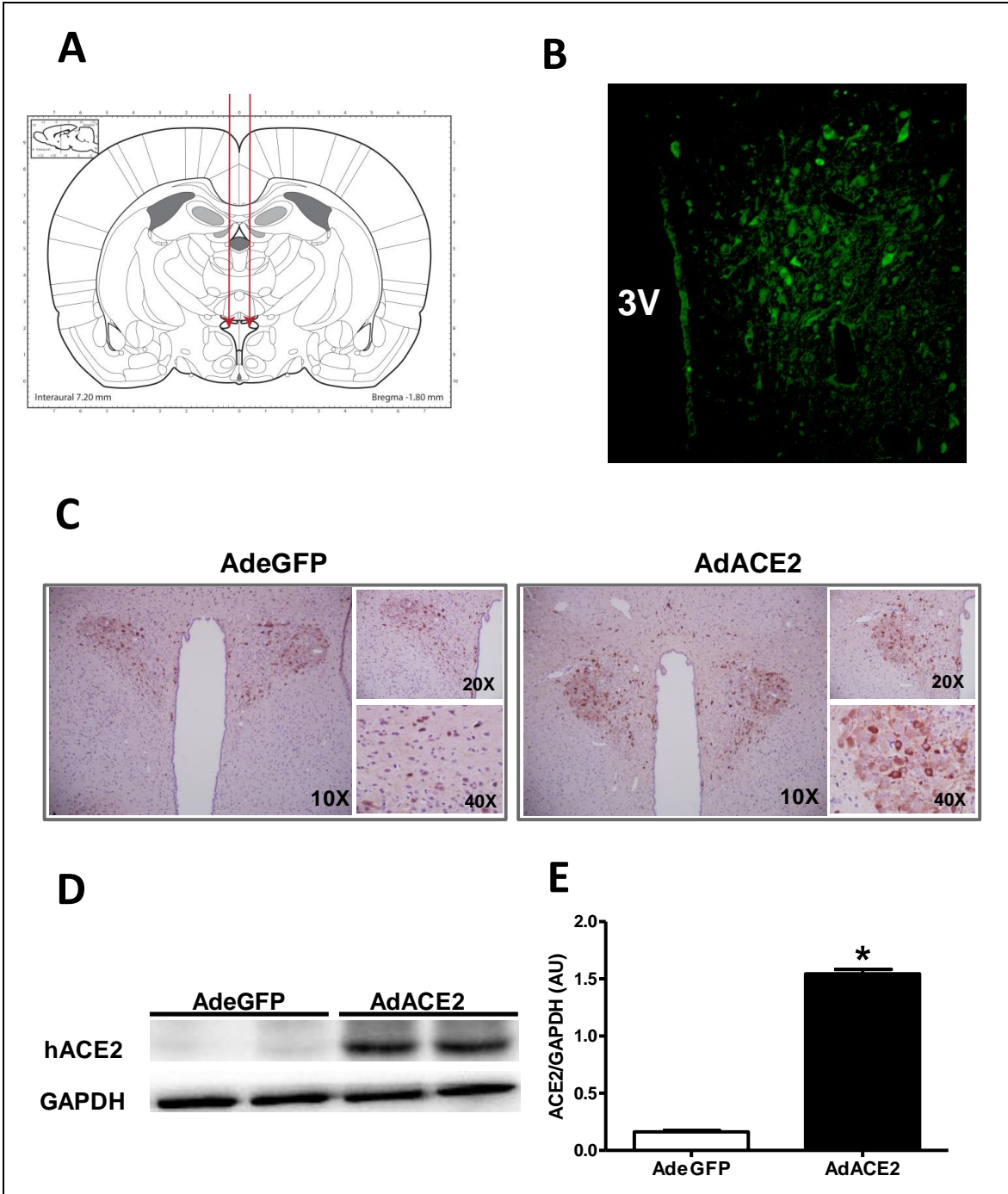
Figure 5.1. Overexpression of ACE2 in the PVN. (A) Schematic showing delivery of Adenovirus to the PVN. (B) Increased ACE2 immunofluorescence within the PVN after AdACE2 injection (C) ACE2 immunoreactivity was increased in the PVN following AdACE2 injection when compared with AdeGFP injection. (D) Western blot and (E) densitometric analysis showing a significant increase in hACE2 protein expression in the PVN compared to control AdeGFP. *, $p<0.05$ compared with AdeGFP.

Figure 5.2. Overexpression of ACE2 in the PVN reduces Ang II pressor response. ACE2 overexpression within the PVN did not change the mean arterial pressure compared with AdeGFP injected control rats. Ang II infusion significantly increased mean arterial pressure compared with AdeGFP injected control rats. B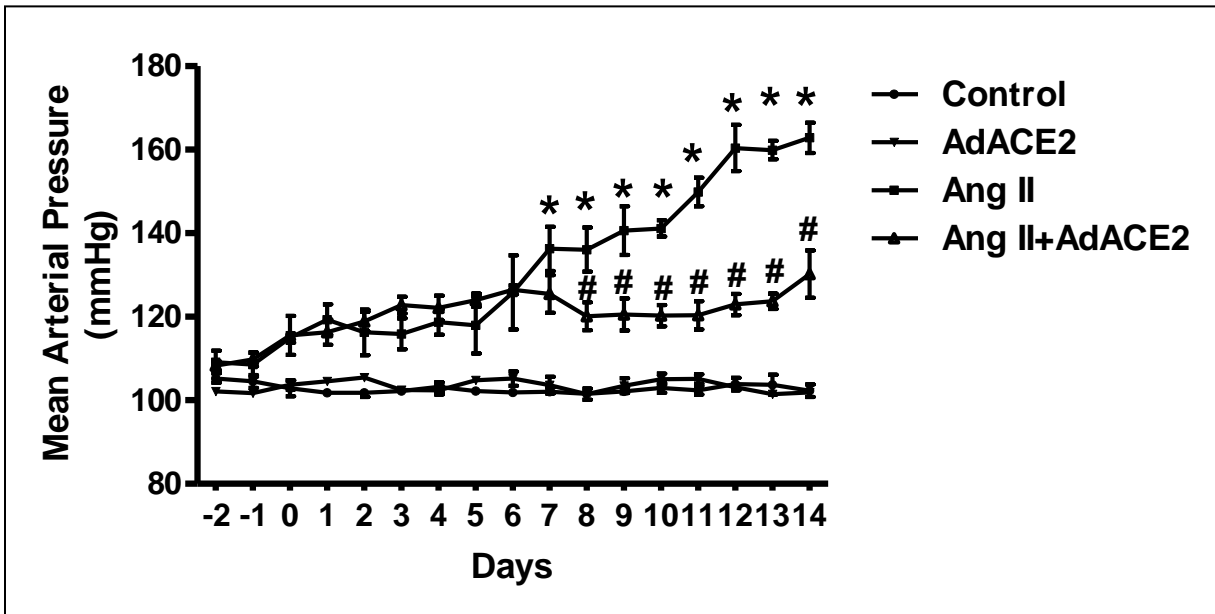ilateral ACE2 overexpression in the PVN significantly reduced the Ang II mediated hypertensive response. n=5-6/group; *, $p<0.05$ compared with control; #, $p<0.05$ compared with Ang II.

ACE2 Overexpression and Expression of the RAS Components. To study the effect of ACE2 overexpression on the other components of RAS, we measured the expression levels of ACE, ACE2, AT_1R, AT_2R and Mas receptor by real time PCR, western blot, and immunohistochemistry. The data indicate that Ang II infusion significantly increased the mRNA expression of ACE and AT_1R, and decreased ACE2, AT_2R, and Mas mRNA expression in the PVN when compared with control rats. Bilateral overexpression of ACE2 in the PVN reversed these gene expression changes (Figure 5.3). These results were further confirmed by western blot and immunohistochemical analysis for AT_1R protein levels in the PVN (Figure 5.4).
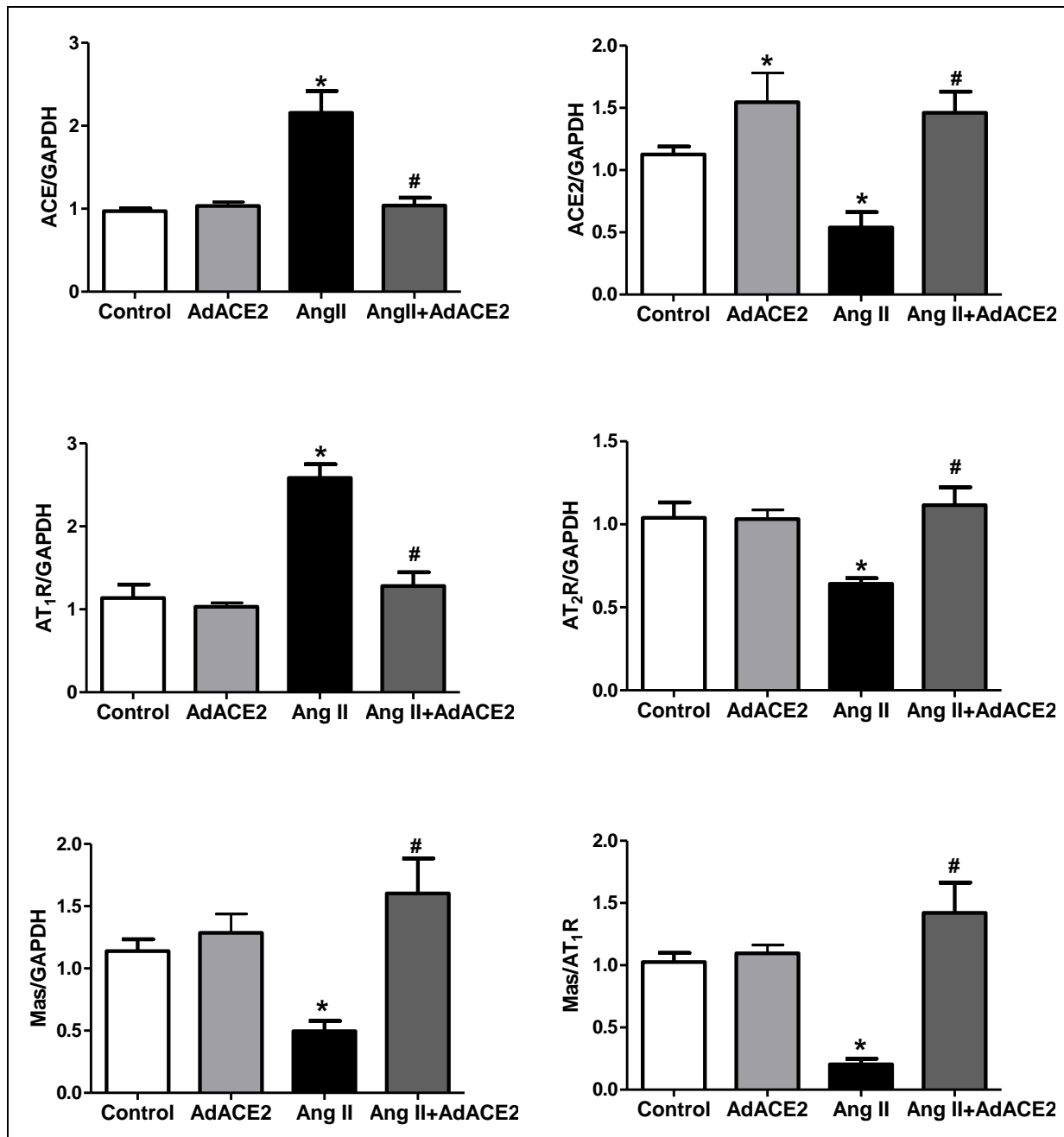
Figure 5.3. Effect of overexpression of ACE2 on the expression of components of RAS in the PVN. Ang II infusion significantly increased the mRNA expression of ACE and AT_1R, and decreased ACE2, AT_2R, Mas, and Mas/AT_1 mRNA expression in the PVN when compared to control rats. Bilateral overexpression of ACE2 in the PVN reversed these gene expression changes. n=9/group; *, $p<0.05$ compared with control; #, $p<0.05$ compared with Ang II.

Figure 5.4. Overexpression of ACE2 downregulates AT_1R expression in the PVN. Typical western blot (A), and quantitative data (B) showing a reduction in AT_1R expression in PVN following ACE2 overexpression. (C) This result was further confirmed by immunohistochemistry against AT1 receptor. *, $p<0.05$ compared with control; #, $p<0.05$ compared with Ang II.

Ang II infusion significantly increased the protein expression of ACE and AT_1R, and decreased ACE2, AT_2R, and Mas protein expression in the PVN when compared with control rats. Bilateral overexpression of A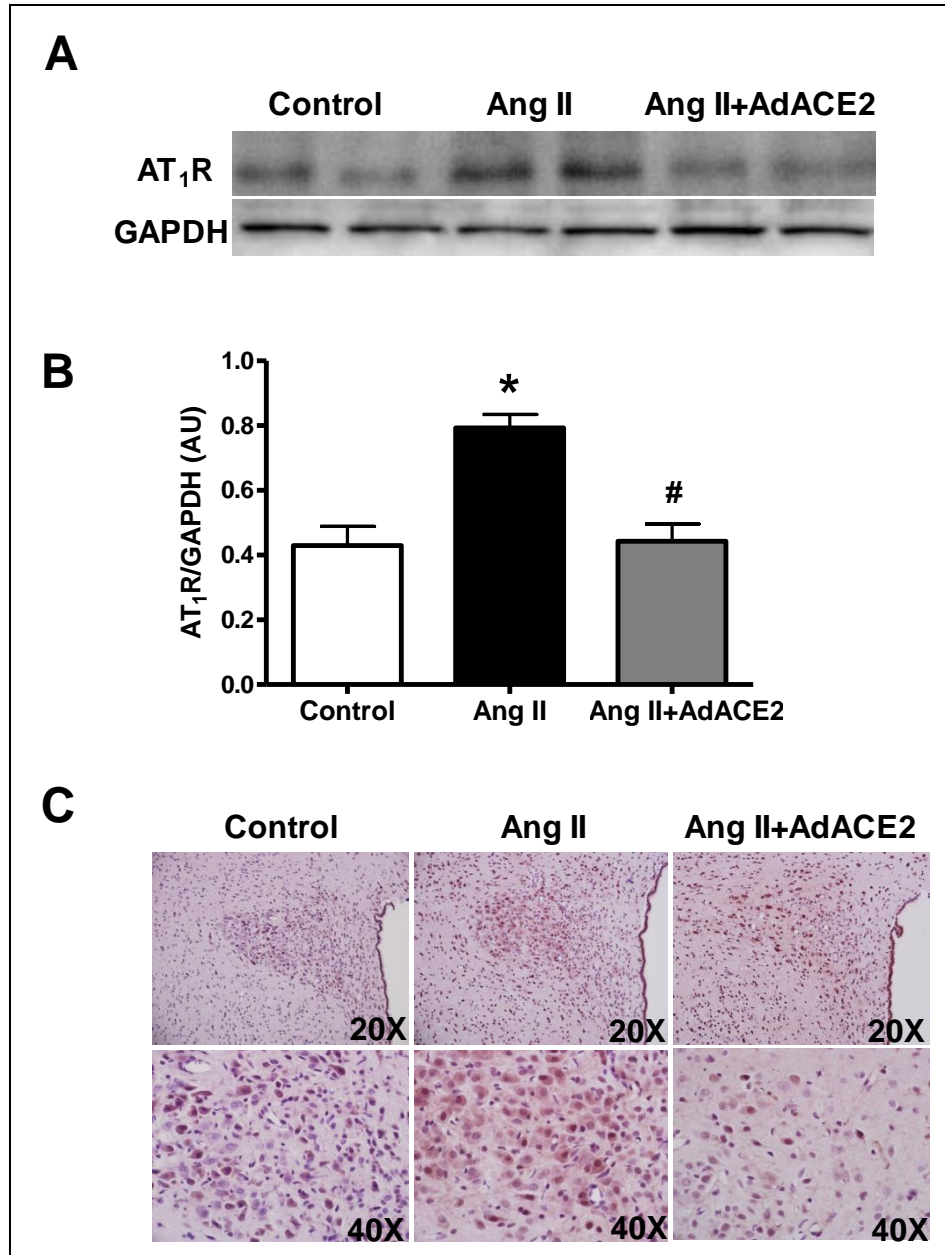CE2 in the PVN reversed these Ang II-induced protein expression changes. Similarly, Ang II infusion significantly increased the protein expression of ACE and AT_1R, and decreased ACE2, AT_2R, and Mas protein expression in the PVN when compared with control rats. Bilateral overexpression of ACE2 in the PVN reversed these protein expression changes (Figure 5.5).
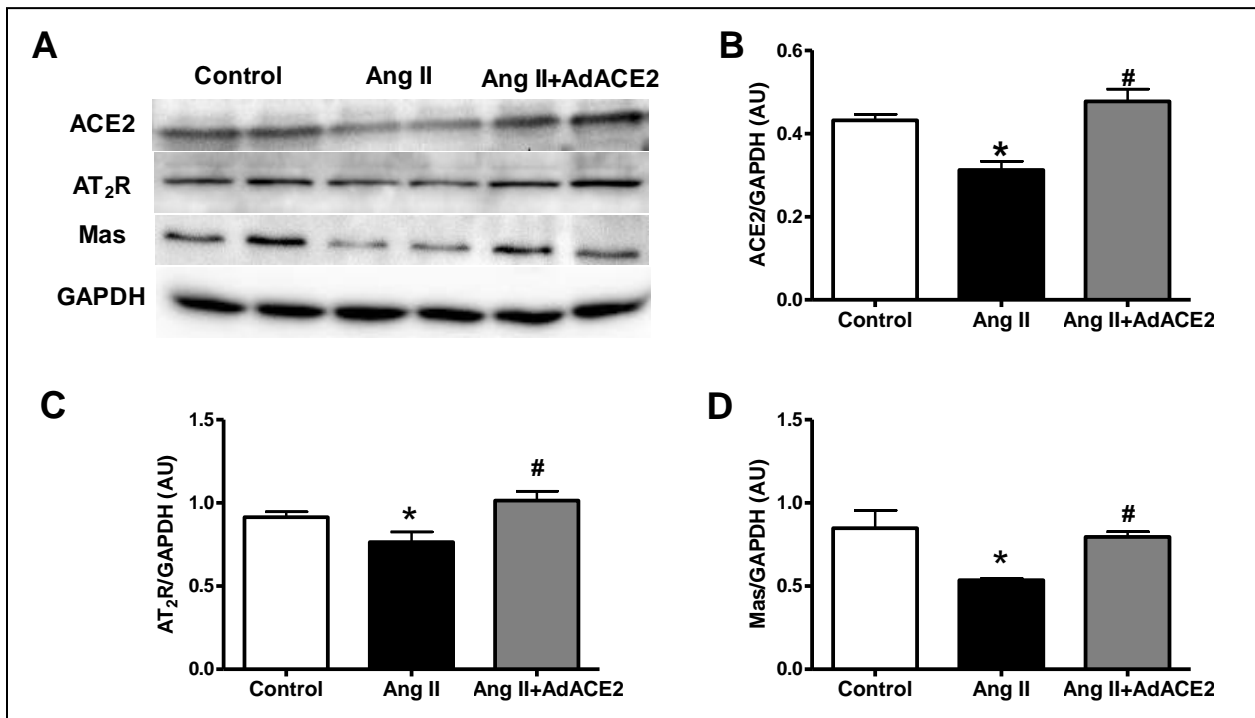
Figure 5.5. Effect of ACE2 overexpression on ACE2, AT_2R and Mas protein expression in the PVN. (A) Representative western blot images. Densitometric analysis of protein expression of (B) ACE2 (C) AT_2R and (D) Mas. Ang II reduced the protein expression of ACE2, AT_2R and Mas expression in the PVN. ACE2 overexpression reversed these changes in the PVN. n=6/group; *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with Ang II.

ACE2 Overexpression and Expression of the Proinflammatory Cytokines. To study the effect of ACE2 overexpression on the PICs production in the PVN, we measured the expression levels of PICs by real time PCR. Ang II infusion significantly increased the mRNA expression of

proinflammatory cytokines TNF-α, IL-1β, and IL-6, and the chemokine MCP-1 in the PVN when compared with control rats. Bilateral overexpression of ACE2 in the PVN attenuated these changes in mRNA expression (Figure 5.6).
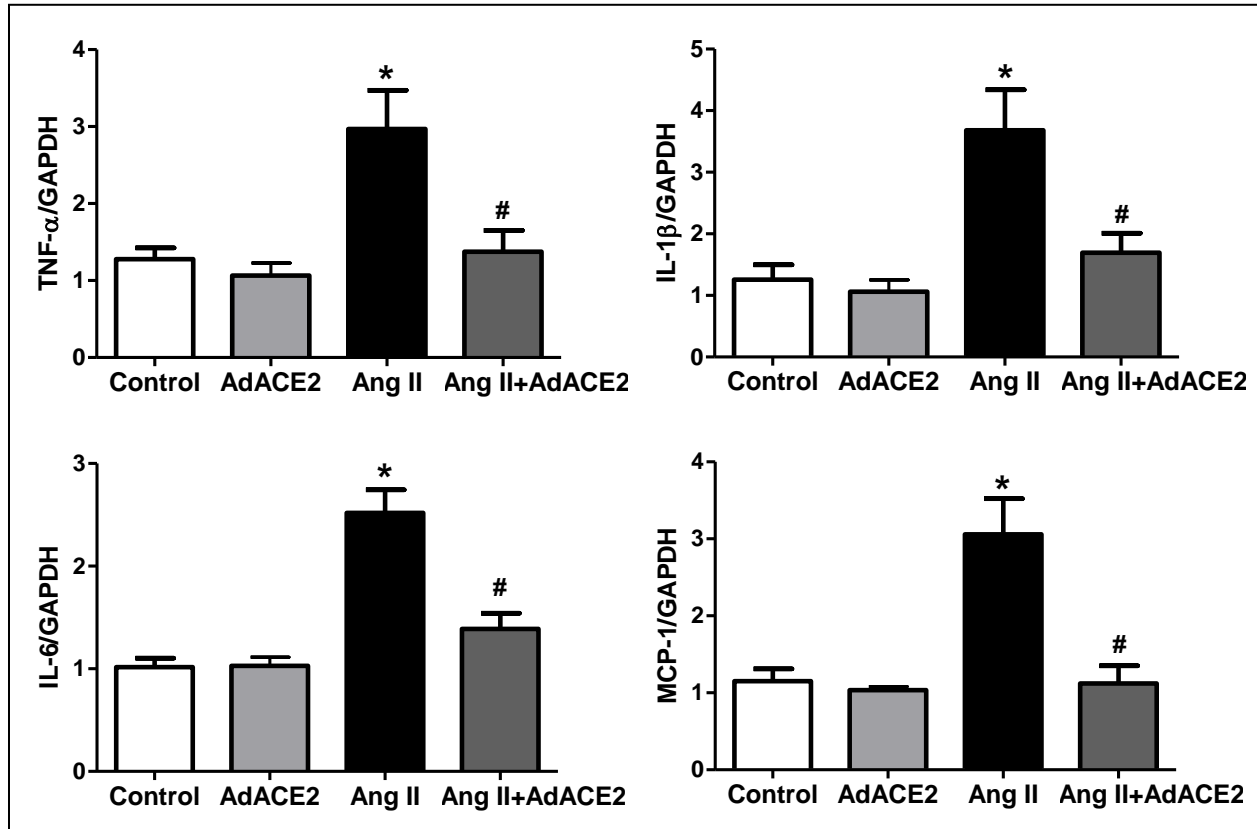

Figure 5.6. Overexpression of ACE2 downregulates proinflammatory cytokine and chemokine expression in the PVN. ACE2 overexpression significantly reduced the Ang II-induced increases in TNFα, IL-1β, Il-6 and MCP-1 expression in the PVN. n=6-9/group; *, $p<0.05$ compared with control; #, $p<0.05$ compared with Ang II.

DISCUSSION

In this study, we investigated the effects of bilateral overexpression of ACE2 in the hypothalamic PVN of the brain on Ang II-induced hypertensive response. The salient findings of this study are as follows: 1) Chronic Ang II infusion significantly increased MAP and reduced the mRNA and protein expression of ACE2 in the PVN; 2) Ang II infusion significantly increased the expression of PIC in the PVN, this was attenuated by ACE2 overexpression; and 3)

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ACE2 overexpression resulted in a down-regulation of AT_1R expression in the PVN, thus preventing the Ang II mediated pressor response. These findings indicate that the Ang II-induced hypertensive effects are attenuated via bilateral PVN ACE2 overexpression. This demonstrates the importance of the PVN as a central cardiovascular regulatory region, as well as the implications an imbalanced RAS can have on hypertensive drive.

In the present study, we found that overexpression of ACE2 in the PVN attenuated the Ang II mediated pressor response, which might be attributed to downregulation of AT_1R expression in the PVN. This supports the notion that the PVN plays an important role in mediating the cardiovascular response elicited by stimulation of the angiotensin system. Previous studies examining the anti-hypertensive effects of ACE2 over-expression during chronic hypertensive response in SH rats (Diez-Freire et al., 2006) and Ang II infused mice (Feng et al., 2008) are consistent with the current results. However, this study not only strengthens the notion that the PVN plays a central role in blood pressure regulation, but that ACE2 overexpression attenuates both the systemic hypertensive response and the associated inflammatory response seen in the PVN during the hypertensive state.

Previous studies showed down-regulation of ACE2 in the heart, kidney, and brain of several hypertensive animal models as well as in patient populations (Xia and Lazartigues, 2008). This ACE2 down-regulation was attributed to AT_1R activation. Furthermore, it has been shown that when ACE2 is over-expressed in the SFO of mice, AT_1R expression was ultimately decreased (Feng et al., 2008). A recent study showed that ACE2 overexpression in the brain resulted in AT_1R downregulation in Syn-hACE2 transgenic mice, leading to reduced Ang II signaling (Feng et al., 2010). These findings suggest an important interplay between ACE2 and AT_1R activity in the hypertensive response. Furthermore, this corroborates with the present

study, where chronic Ang II infusion resulted in both decreased mRNA and protein expression of ACE2 and an increased expression of AT_1R in the PVN and that overexpression of ACE2 reversed these changes. These results further confirm that the AT_1R in the PVN mediate the central inhibitory effects on ACE2 in Ang II-mediated hypertension, as suggested previously (Xia et al., 2009).

Recent evidence show that hypertension is a chronic low grade inflammatory condition where various PIC such as TNF-α, IL-6, and IL-1β have been shown to play an important role in the pathogenesis of hypertension (Chae et al., 2001; Lu et al., 2009; Sriramula et al., 2008; Sun et al., 2004). A recent study showed that microinjection of IL-1β into the PVN increases blood pressure which is mediated by AT_1R and also, microinjection of a subpressor dose of IL-1β into PVN enhances its sensitivity to the central pressor response of Ang II (Lu et al., 2009). We have also previously demonstrated that chronic Ang II infusion increases PIC expression in the PVN (Kang et al., 2009). It has been shown that Ang II-induced increases in cellular adhesion molecules, VCAM-1 and MCP-1, were attenuated in endothelial cells, which had adenovirus-mediated ACE2 over-expression (Lovren et al., 2008). The current study shows that Ang II infusion significantly increased the mRNA expression of the PIC, TNF-α, IL-1β, and IL-6, and the chemokine MCP-1 in the PVN. This increase was attenuated by bilateral ACE2 overexpression in the PVN. These findings suggest that the beneficial effects of ACE2 overexpression are at least in part mediated by decreased expression of PICs in the PVN.

As evidenced extensively over recent years, overactivity of the RAS has been implicated in the development and maintenance of several cardiovascular diseases, including hypertension, and participation of the brain RAS in the pathophysiology of hypertension is now well established (Davisson, 2003; Veerasingham and Raizada, 2003). Levels of Ang II and AT_1R in

the PVN are increased in many animal models of hypertension (Veerasingham and Raizada, 2003). Furthermore, Ang II upregulates ACE and down regulates ACE2 in patients with hypertension (Koka et al., 2008). Previous studies have shown that the Mas and AT_2R oppose the actions of the AT_1R in the brain and periphery (Ferrario, 2006; Widdop et al., 2002). Thus, it is the imbalance between the hypertensive (ACE, Ang II and AT_1R) and anti-hypertensive components (ACE2, Ang1-7, Mas, and AT_2R) of the RAS that results in hypertension. In our study, the use of ACE2 overexpression aided in determining the relevance of this imbalance in perpetuating the hypertensive state. ACE2 over-expression caused a decreased expression of ACE and AT_1R, and an increased expression of ACE2, Mas, and AT_2R, resulting in an increased Mas/AT_1 receptor ratio and therefore counter balancing the Ang II-induced hypertensive response.

In summary, Ang II infusion resulted in a significant increase in MAP with increased AT_1R and ACE gene expression, and decreased ACE2 and Mas gene expression in the PVN. Ang II infusion also increased the expression of PIC TNF-α, IL-1β and IL-6 in the PVN. Bilateral microinjection of AdACE2 into the PVN attenuated the Ang II-induced hypertensive response and reversed these gene expression changes. Our findings, together with previous reports suggest that attenuation of proinflammatory cytokines in combination with the shift of RAS towards the vasoprotective axis may be responsible for the overall beneficial effects of ACE2 overexpression on Ang II-induced hypertensive response.

The present study indicates that bilateral overexpression of ACE2 in the hypothalamic PVN of the brain attenuated Ang II-mediated pressor response. The antihypertensive effects of ACE2 overexpression are likely to result from the net effect of shifting RAS balance, toward decreased expression of ACE and AT_1R, and increased expression of ACE2, Mas, and AT_2R,

and resulting in attenuation of proinflammatory cytokines. This study also demonstrates the importance of the PVN as an important central cardiovascular regulatory region in controlling neurogenic hypertension.

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CHAPTER 6

CONCLUDING REMARKS

OVERALL SUMMARY OF FINDINGS

Hypertension is the single most important predisposing factor for the development of pathological cardiovascular events. Untreated hypertension can lead to coronary heart disease, congestive heart failure, renal failure and stroke in patients. The pathogenesis of essential hypertension is multifactorial and highly complex with multiple signaling pathways. Recent studies suggest that hypertension is a low-grade inflammatory condition induced by interaction of the renin angiotensin system (RAS) with various pro-inflammatory cytokines (PIC), such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6. Several *in vitro* and *in vivo* studies suggest the existence of cross-talk between the RAS and TNF-α. However, the functional importance of the RAS effector peptide, Angiotensin II (Ang II), and its interaction with TNF-α in inducing hypertensive response is not yet clearly defined. Therefore, elucidating the precise signaling mechanisms of TNF-α interaction with local and central RAS is critical for the development of therapeutic treatments targeted to control hypertension.

In chapter 2, we demonstrated that the responses to chronic Ang II administration on salt and water intake, blood pressure and cardiac function were markedly attenuated in mice lacking the gene for the pro-inflammatory cytokine TNF-α (TNF-α$^{-/-}$). However, these Ang II responses were restored in TNF-α$^{-/-}$ mice when undergoing replacement therapy with human recombinant TNF-α. In addition, it was also observed that the mRNA levels of angiotensin II type 1 (AT$_1$R), and nuclear factor-kappaB (NF-κB) mRNA expressions in the heart and hypothalamus were increased in response to chronic Ang II in wildtype (WT) but not in TNF-α$^{-/-}$ mice. These results suggest that a concomitant generation of TNF-α is involved in the complete expression of Ang II-induced salt appetite, hypertensive and cardiac hypertrophic responses, possibly via the TNF-α induced up-regulation of AT$_1$R and NF-κB activity.

In chapter 3, we examined the possible roles of reactive oxygen species (ROS) and NF-κB as signaling molecules involved in the interaction of Ang II and TNF-α in mediating cardiac hypertrophy and hypertensive response. Our results showed that chronic infusion of Ang II for 14 days induced hypertension and cardiac hypertrophy along with increased superoxide production, increased oxidative stress and activation of the transcription factor NF-κB in WT mice, but not in TNF-α$^{-/-}$ mice. These results suggest that TNF-α mediates the Ang II-induced effects via increased oxidative stress and the activation of NF-κB.

In chapter 4, we presented the effect of centrally blocking TNF-α via with etanercept intracerebroventricular infusion in Ang II-treated rats. Etanercept inhibits TNF-α by binding to the TNF receptor 2 and acting as a competitive inhibitor of TNF-α. Our results demonstrated that peripheral Ang II infusion increased blood pressure, induced cardiac hypertrophy, and increased PIC and decreased IL-10, an anti-inflammatory cytokine, in the heart and paraventricular nucleus (PVN). It also increased the expression of NOX-2 and decreased the expression of neuronal nitric oxide synthase (nNOS), leading to oxidative stress. Treatment with etanercept in the brain attenuated the blood pressure response and decreased the expression of PIC, reduced oxidative stress, and increased the expression of the anti-inflammatory cytokine IL-10.

In chapter 5, our results indicate that bilateral overexpression of angiotensin converting enzyme 2 (ACE2), a newly discovered component of the RAS, in the hypothalamic PVN of the brain in rats attenuated the Ang II-mediated blood pressure response. We also showed that the antihypertensive effects of ACE2 overexpression are likely to result from the net effect of shifting the RAS balance toward decreased expression of angiotensin converting enzyme (ACE) and AT$_1$R and increased expression of ACE2, Mas, and AT$_2$R, resulting in attenuation of PIC,

especially TNF-α. This study also demonstrates the importance of the PVN as an important central cardiovascular regulatory region in controlling neurogenic hypertension.

SIGNIFICANCE OF RESEARCH AND FUTURE DIRECTIONS

To date, most of the successful approaches used to treat hypertension have targeted its peripheral causes, especially RAS component blockade. These include ACE inhibitors to prevent the production of Ang II, AT_1R blockers to block the effects of high circulating Ang II, and calcium channel blockers to control blood pressure. These therapies have clearly reduced the morbidity and mortality in hypertensive patients; however, the long term prognosis in patients with hypertension remains poor, and new therapeutic approaches are needed. Despite the enormous amount of research carried out in past decades to understand the pathogenesis of hypertension, the molecular mechanisms underlying hypertension are still relatively unknown.

The complexity of signaling mechanisms involved in the pathogenesis of hypertension is such that selective, mechanistically based antihypertensive treatment is rarely possible in hypertensive patient. Recent evidence also suggests that hypertension is a inflammatory condition where various PIC such as TNF-α, IL-6 and IL-1β, both centrally and in the periphery, have been shown to play an important role in the pathogenesis of hypertension (Chae et al., 2001; Lu et al., 2009; Shi et al., 2010; Sriramula et al., 2008; Sun et al., 2004). Despite the abundant evidence indicating that TNF-α contributes significantly to cardiac dysfunction in animal models, the results of two large clinical trials using etanercept, a soluble TNF-α receptor antagonist (RENAISSANCE), and infliximab (ATTACH), a TNF-α blocking antibody, were largely negative (Anker and Coats, 2002; Mann et al., 2004). However, previous anti-TNF-α therapies have proven effective in the treatment of inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (D'Haens et al., 1999; Elliott et al., 1994; Moreland et

al., 1997). Clearly further studies are required to understand all the possible mechanisms involved in the pathogenesis of hypertension, especially the role of TNF-α.

Recent studies showing the existence of a local RAS, especially in the heart and brain, and identification of new components of the RAS have offered new insights into the pathogenesis of hypertension. Studying the interactive roles of the RAS, PIC, oxidative stress, and NF-κB, both peripherally and centrally, may increase our understanding of cardiovascular diseases that are linked with inflammatory conditions such as hypertension. Our studies suggest that TNF-α interacts with the RAS through increased ROS and superoxide production, and through the transcription factor NF-κB. In addition, we also showed that TNF-α blockade within the brain is a possible treatment option for hypertensive condition. Furthermore, our results showed that the antihypertensive effects of ACE2 overexpression are likely to result from the net effect of shifting the RAS balance toward vasodilatory axis, resulting in attenuation of proinflammatory cytokines. These findings may lead to novel therapies directed towards the treatment of hypertension.

Although we believe that our studies have made significant contributions in identifying novel pathways through which the RAS and TNF-α interact in the heart and brain and contribute to the pathogenesis of hypertension, further studies are required to understand the molecular mechanisms involved in this interaction. We have shown in our study that in Ang II-induced hypertension TNF-α receptor 1 (TNFR1) is expression is increased. Future studies are needed to understand the role of both the TNFR1 and TNFR2 receptors of TNF-α in the pathogenesis of Ang II-induced hypertensive response. Also, our studies show that blocking TNF-α reversed the Ang II-induced decrease in anti inflammatory cytokine IL-10. Further studies are needed to examine the possible cross-talk between IL-10 and TNF-α. These studies could explore how

oxidative stress, superoxide, and transcription factor NF-κB are involved in TNF-α signaling. In summary, a better understanding of TNF-α interaction with the RAS, may help us define better interventions aimed at the treatment of hypertension.

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APPENDIX

LETTER OF PERMISSION

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Srinivas Sriramula was born in Vempet, Hyderabad, Andhra Pradesh, India. He received his Bachelor in Veterinary Science and Animal Husbandry (B.V.Sc. & A.H.) degree in 2001 and Master of Veterinary Science degree in animal genetics and breeding (M.V.Sc.) in 2004 from the college of veterinary sciences, Acharya N.G. Ranga Agricultural University, Rajendranagar, Hyderabad, Andhra Pradesh, India. In January 2006, he joined in the Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, to pursue his doctoral degree in Veterinary Medical Sciences with a specialization in cardiovascular pathophysiology. He will receive his Doctor of Philosophy degree in the summer 2010.