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Tumor necrosis factor induced oxidative stress in the central nervous system contributes to sympathoexcitation in heart failure

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TUMOR NECROSIS FACTOR INDUCED OXIDATIVE STRESS IN THE CENTRAL NERVOUS SYSTEM CONTRIBUTES TO SYMPATHOEXCITATION IN HEART FAILURE

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ABSTRACT

Despite advanced therapeutic strategies for post-myocardial infarction (MI) patients, many ultimately develop congestive heart failure (CHF), rendering the disease a major cause of death in the United States. MI is associated with an acute increase in sympathetic nervous system activity, becoming persistent in CHF patients. Increased pro-inflammatory cytokines (PICs) following MI are implicated in the pathogenesis of CHF. The increase in tumor necrosis factor (TNF), a primary PIC, correlates closely with heart disease severity. Moreover, central PIC production increases post-MI, and can affect the brain's cardiovascular regulatory regions that control sympathoexcitation. Therefore, understanding how PICs modulate sympathoexcitation is important for development of new therapeutics. Recent studies underscore the importance of central NADPH oxidases in the pathogenesis of hypertension. However, the role of central NADPH oxidase-induced reactive oxygen species (ROS) production in the development of CHF remains limited. In this dissertation, the hypothesis that central PICs induce ROS production and modulate sympathoexcitatory neurons of the paraventricular nucleus (PVN) is explored through an array of selective animal models combined with novel technologies for sympathoexcitation and cardiovascular function assessment. The effect of the TNF blocker, pentoxyfylline, was investigated on the expression of the catalytic subunits of NADPH oxidase (Noxs) in the PVN neurons and on the sympathetic activity in CHF rats. Additionally, effects of TNF inhibition on central nitric oxide were explored, as this ROS restrains sympathoexcitation. More specifically, central TNF was inhibited to understand the interaction between superoxide and nitric oxide in the PVN neurons during CHF. TNF knock-out mice were also used to study the effect of TNF on volume overload associated with CHF. Finally, to understand the role of peripheral TNF on the PVN's sympathoexcitatory neurons, and to exclude the effects of neurohormones in CHF, human recombinant TNF was injected 5-days systemically to achieve the levels observed following MI

in conjunction with ROS and angiotensin II type-1 receptor blockers. These studies provide new evidence that TNF induces oxidative stress in the PVN through an AT1R mediated mechanism in CHF, and offers new insight into the sympathoexcitatory mechanisms in the brain possibly involved in the pathogenesis of CHF.

CHAPTER 1
BACKGROUND

CYTOKINES AND CARDIOVASCULAR DISEASE

Cardiovascular disease has been the leading cause of mortality among Americans over the last few decades. Current epidemiological studies indicate that there were approximately 1.2 million American adults who suffered from at least one type of cardiovascular disease in 2008 and that 1 of every 2.8 deaths in the United States is from a cardiovascular event (Rosamond *et al.*, 2008). Inflammation has become one of the main themes involved in the pathogenesis of cardiovascular diseases, including myocardial infarction, ischemia-reperfusion injury, myocarditis, atherosclerosis and congestive heart failure (Mehra *et al.*, 2005). Following acute myocardial infarction, systemic response is associated with increased expression of inflammatory cytokines, such as tumor necrosis factor-α (TNF), interleukin-1β (IL-1β) and interleukin-6 (IL-6), both in the blood and myocardium (Torre-Amione *et al.*, 1995). According to the 'cytokine hypothesis of heart failure', the progression of heart failure is, at least in part, a result of the direct toxic effects exerted by elevated cytokines (Seta *et al.*, 1996). TNF, IL-1β and IL-6 are pro-inflammatory cytokines that damage myocytes and exert negative inotropic effects on the heart (Shan *et al.*, 1997). IL-10, on the contrary, is an anti-inflammatory cytokine that protects cardiac function via inhibition of TNF and IL-6 production (Wang *et al.*, 1995). The progression of heart failure, thus, is dependent upon the balance between the production of TNF and IL-10 following the initial insult.

Tumor Necrosis Factor in Congestive Heart Failure. TNF, a multifunctional pro-inflammatory cytokine originally identified as a factor associated with the necrosis of tumor tissue triggers other cytokine and chemokine production, induces expression of cell adhesion molecules, and causes cytotoxicity. This pleiotrophic cytokine exists either as a membrane bound pre-protein or, after being cleaved from its pre-protein by metallopreoteinases, as a mature soluble TNF (Gearing *et al.*, 1994). Both, membrane and soluble TNFs can form homotrimers

which are essential for activation of TNF receptors. TNF actions are initiated by binding to a low affinity 55 kD receptor, TNFR1 or a high affinity 75 kD receptor, TNFR2. These receptors, when stimulated by certain factors, are shed into the circulation and become soluble receptors. The soluble form of receptors act as a buffer by binding to the circulating TNF and preventing their toxic effects, but can release the TNF into circulation when exposed to stress inducing stimuli, exasperating the effects of TNF (Feldman *et al.*, 2000).

Both types of TNF receptors are identified in non-failing (Torre-Amione *et al.*, 1995) and failing human myocardium (Torre-Amione *et al.*, 1996). However, normal myocardium does not produce TNF, whereas stressed myocardium expresses significant amounts of TNF. Feldman's laboratory developed a line of mice that harbored a transgene effecting cardiac-specific overexpression of TNF alpha. This mouse line was generated using the cardiac-specific alpha myosin heavy chain (alpha-MHC) promoter producing a phenotype with dilated cardiomyopathy accompanied by increased inflammation (Kubota *et al.*, 1997). These findings were further confirmed by Bryant and colleagues (Bryant *et al.*, 1998), who over-expressed TNF in hearts of transgenic mice, and determined that they expressed a phenotype characterized by systolic dysfunction, cardiac inflammation, ventricular dilatation, congested tissue, and increased mortality. Additionally, either a single bolus, or continuous infusion of TNF, can induce LV dysfunction and remodeling in various animal models (Bozkurt *et al.*, 1998; Bradham *et al.*, 2002; Kimura *et al.*, 2006). These studies suggest that overproduction of TNF by cardiac myocytes is sufficient to cause severe cardiac disease. When the stress-activated cytokines in the myocardium exceed the limit which can be utilized by the local cellular receptors in an autocrine/paracrine capacity, they become blood borne and enter the systemic circulation, causing some of the additional adverse effects associated with cytokines. Levine *et al.*, first recognized in 1990 that circulating levels of TNF were elevated in patients with end-stage heart

failure (Levine *et al.*, 1990). Subsequent studies further suggested that there was a direct relationship between circulating TNF levels and functional heart failure classification (Torre-Amione *et al.*, 1996).

In heart failure, the expression of cytokines parallels the expression of classical neurohormones (Ferrari *et al.*, 1995). However, increased circulating cytokine levels were observed prior to that of neurohormones (El-Menyar, 2008), suggesting them as potential stimulators of the vicious cycle of congestive heart failure.

SYMPATHOEXCITATION AND HEART FAILURE

Unregulated neurohumoral excitation is the hallmark of congestive heart failure (Francis *et al.*, 2001; Zucker *et al.*, 2004; Negrao and Middlekauff, 2008). This increase in neurohormones is initially targeted to improve cardiac function, but persistent elevation results in a generalized increase in sympathoexcitation, enhanced renal sodium and water reabsorption, and decreased renal perfusion resulting in the activation of a number of peptides including those of the renin-angiotensin system (RAS) (McKinley *et al.*, 2001).

Autonomic Regulation of Sympathoexcitation in Heart Failure. Heart failure is unique among conditions activating the immune system, in the sense that the central effects of the circulating cytokines, acting to regulate the peripheral immune response, are superimposed on, or at least coincidental with, the central effects of the RAS in acting to preserve volume and pressure within a compromised cardiovascular system. Changes in venous volumes of less than 1% are signaled to the brain via the afferent venous volume receptors utilizing autonomic nerves and contribute to the pressure and volume adjustments through the neuroendocrine system (Gupta *et al.*, 1966; Coote, 2005). Similarly, the nucleus tractus solitarius (NTS) of the medulla oblongata is the integral site for the afferent vagal fibers and baroreceptor afferents. The volume signal originating in the NTS travels to the hypothalamus to influence the magnocellular

peptidergic neurohypophyseal neurons of the paraventricular nucleus (PVN), which play a key role in neuroendocrine control of fluid balance. In addition, the parvocellular neurons of PVN send axons to sympathetic outflow centers in the rostral ventrolateral medulla (RVLM) and intermediolateral cell column of the spinal cord, which maintains tonic sympathetic vasomotor outflow.

Besides the systemic baroreceptors and chemoreceptors, the subfornical organ (SFO), organum vasculosum lamina terminalis (OVLT), and area postrema (AP) are unique blood-brain-barrier-deficient regions, also known as circumventricular organs (CVOs), that are primary sensors for blood-borne neuropeptides such as cytokines (Ericsson *et al.*, 1995) and AngII (Potts *et al.*, 1999). Neural pathways originating from these sensory CVOs project into an extensive neural network that is responsible for mobilizing the various systems responsible for maintaining homeostasis, i.e. vasopressin release, autonomic responses, and ingestive behaviors (Wallace Lind *et al.*, 1984; Ferguson and Kasting, 1988). SFO sends direct projections to the magnocellular neurosecretory cells of the SON and PVN and the parvocellular neurons of the PVN (Miselis, 1981; Jhamandas *et al.*, 1989). The magnocellular neurons of the SON and PVN are sites for vasopressin and oxytocin synthesis and fibers from these two regions project to the neurohypophysis where the hormones are released into the circulation to exert their peripheral effects. The parvocellular neurons of the PVN send axons to the RVLM and IML regions of the brain that regulate sympathetic outflow and baroreflexes (Dampney, 1994). Thus, this evidence, demonstrating that cytokine and AngII-sensing nerve cell bodies project to magnocellular and parvocellular hypothalamic nuclei, neurohypophyseal tracts, the ventrolateral medulla and brainstem, led to the hypothesis that these neuropeptides in the central nervous system function as modulators in cardiovascular regulatory networks.

Central Pro-inflammatory Cytokines and RAS in Sympathoexcitation. Intravenous LPS, TNF (Ohashi and Saigusa, 1997) and IL-1β (Saindon *et al.*, 2001) have been shown to increase sympathetic discharge. It is interesting to note that in normal animals, acute and chronic injection of TNF increases renal sympathetic activity (RSNA) (Saigusa, 1990) and production of AngII (Bataillard *et al.*, 1992). It has been well established that circulating levels of cytokines and neuropeptides, like AngII, are elevated in CHF. Already, we have reported that treatment of HF rats with a TNF binding agent, or transcription inhibitory factors, attenuates neuro-humoral excitation (Kang *et al.*, 2008). These findings suggest a direct role for TNF in inducing sympathoexcitation.

REACTIVE OXYGEN SPECIES AND HEART FAILURE

Reactive oxygen species (ROS) encompass a variety of diverse chemical species that are considered as toxic by-products of cellular metabolic processes designed to reduce molecular oxygen. ROS includes free radicals such as superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), or hydroxyl radicals (HO•), as well as non free radicals such as H_2O_2, while the antioxidant systems includes host defense enzymes (catalase, superoxide dismutase, glutathione peroxidase, etc.), vitamins and other molecules (thioredoxin/thioredoxin reductase, glutathione/glutathione reductase, etc.) (Lambeth, 2004). Nitric oxide (NO•), and its oxidized form, peroxynitrite (ONOO-), result from a reaction between NO• with either $O_2^{\bullet-}$ or HO•, also represent ROS, but are often referred to as reactive nitrogen species (RNS). At physiological concentrations, ROS have certain desirable effects. They act as second messengers in various cellular functions. Local ROS production also regulates enzymatic function, and plays a role in signal transduction (redox signaling). ROS were also shown to be essential for cell proliferation and growth, stimulate DNA synthesis and induce expression of protooncogenes such as *c-fos*, *c-jun*, and *c-myc* (Rao and Berk, 1992). But at high

concentrations, ROS are capable of inducing oxidation and damage of macromolecules, proteins, lipids, mitochondria and DNA, thereby leading to cell damage, apoptosis and necrosis.

Multiple lines of evidence suggest that ROS contribute significantly to the hypertrophy and remodeling mechanisms in heart failure. ROS production is triggered in cases of repetitive ischemia-reperfusion, increased pro-inflammatory cytokine levels, auto-oxidation of catecholamines, and synthesis of catecholamines. Some important sources of ROS in left ventricular hypertrophy (LVH) and congestive heart failure (CHF) are xanthine oxidase, mitochondria, uncoupled nitric oxide synthases, NADPH oxidases and infiltrating inflammatory cells. Evidence from the past decade shows that ROS derived from NADPH oxidase are involved in redox signaling. Furthermore, increased NADPH oxidase(s) activity and expression were identified in the myocardium of patients with both ischemic and non-ischemic heart failure (Heymes *et al.*, 2003; Maack *et al.*, 2003; Nediani *et al.*, 2007).

NADPH Oxidase Enzyme Complex as a Source of Superoxide. The classical phagocytic NADPH oxidases, first identified in neutrophils, are involved in oxidative burst aiding in host defense against microbes and in phagocytosis (Roos *et al.*, 2003; Lambeth, 2004). These multi-subunit enzymes consist of the membrane-bound flavocytochrome comprising a catalytic Nox subunit and p^{22phox} subunit, and 4 cytosolic regulatory subunits, p^{40phox}, p^{47phox}, p^{67phox}, and the small GTP-binding protein Rac. Electron transfer occurs from NADPH to molecular oxygen at the catalytic site, resulting in the formation of superoxide. In the last decade, several isoforms of functional NADPH oxidases were identified in various non-phagocytic cell types. Some of the agonists/stimuli related to myocardial remodeling and heart failure that activate NADPH oxidases are G-protein coupled receptor agonists including AngII and endothelin (Duerrschmidt *et al.*, 2000; Li and Shah, 2003), cytokines such as TNF (Frey *et al.*, 2002; Li *et al.*, 2002),

growth factors, mechanical forces (Hwang *et al.*, 2003) and hypoxia-reoxygenation (Kim *et al.*, 1998).

There are 5 Nox isoforms (Nox1–5), expressed in a tissue-specific manner. Each NADPH oxidase isoform contains a catalytic Nox subunit (which facilitates electron transfer) and a smaller $p22^{phox}$ subunit that associate to form a heterodimeric cytochrome. This association is important for the stabilization of the enzyme complex, as well as to bind to the other subunits. Some of the isoforms, such as Nox1 and Nox2, require additional protein subunits for activation of the enzyme. Nox2 (known previously as $gp91^{phox}$) is the isoform that comprises the core of the classical phagocytic NADPH oxidase, but is now known to also be expressed in several other cell types, such as cardiomyocytes (Bendall *et al.*, 2002) and fibroblasts (Pagano *et al.*, 1997). Nox2 requires four other subunits for activation, including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small GTP-Rac. The activation of Nox1-containing oxidase is highly similar to that of Nox2, although it is believed to involve homologues of $p67^{phox}$ and $p47^{phox}$, known as NOXA1 and NOXO1, respectively (Lambeth, 2004). On the contrary, the activation of Nox4 is distinct in that it does not appear to require any of the conventional regulatory subunits (Ambasta *et al.*, 2004; Martyn *et al.*, 2006). The mechanisms responsible for regulating Nox4 activity remain poorly understood.

Li *et al.* (2002) reported that the subunit expression of NADPH oxidase and its activity is increased in parallel with the activation of MAPKs. An increase in the expression of the subunits Nox2 and $p22^{phox}$ was also observed after MI both in human (Krijnen *et al.*, 2003) and animal myocardium (Fukui *et al.*, 2001). Nox2 plays an important role in contractile dysfunction, while Nox4 is important in the development of LVH. A small GTP-binding Rac1, which is important in the activation of NADPH oxidase, is involved in ANG II-induced cardiomyocyte hypertrophy (Pracyk *et al.*, 1998). Potential redox sensitive downstream targets include Ras, *c*-Src, MAPKs

(ERK1/2, p38MAPK, JNK), p90SRK, PI3K, Akt, AP-1, NFkB, hypoxia-inducible factor-1 (HIF-1) and others (Cave *et al*., 2005).

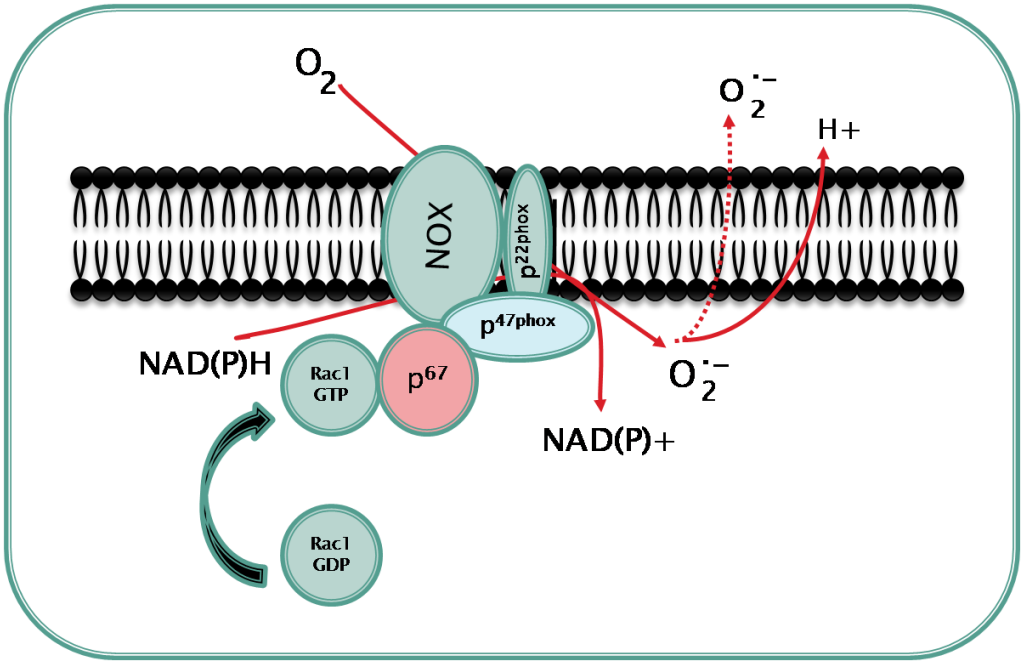

Figure 1. Non-phagocytic NADPH Oxidase may contain Nox1 or Nox4 as homologues for the catalytic subunit gp^{91phox}/Nox2. Superoxide is produced intracellularly and may require the presence of all cytosolic subunits.

Inflammation and NADPH Oxidases in the Central Nervous System. The phagocytic/microglial NADPH oxidase is known to be largely involved in the oxidative damage induced in Alzheimer's disease. The oxidative damage in CNS is generally manifested by lipid peroxidation and formation of protein oxidation products that are toxic to neurons. In Alzheimer's disease, when stimulated by inflammatory stimuli or fibrillar β-amyloid, the NADPH oxidase in the microglia is activated, initiating a series of intracellular signaling cascades responsible for release of ROS and RNS (Akiyama *et al.*, 2000). In addition, the astrocytes, cells that can release pro-inflammatory cytokines TNF and IL-β, and nitric oxide (NO), also produce NADPH oxidase-dependent ROS. Abramov and colleagues, using astrocyte/neuronal co-cultures examined that β-amyloid particles induce oxidative stress in

astrocytes resulting in neuronal cell death. Furthermore, recent studies have implicated neuronal NADPH oxidase in causing neurotoxicity and neuronal cell death. For example, presence of Nox2 in cultures of cortical and sympathetic neurons has been reported and Nox4 expression is also found upregulated in the brain cortex following cerebral ischemia (Vallet *et al.*, 2005). Recent reports also indicate the importance of gp^{91phox}/Nox2 and Nox4 in the regulation of cerebral vascular tone (Chrissobolis and Faraci, 2008).

Accumulating evidence show that schizophrenic patients suffer from diminished antioxidant defenses in the brain. Recently, the role of IL-6 cytokine has been identified in ketamine induced NADPH oxidase activation in the brain, leading to dysfunction of a subset of a GABAergic phenotype of fast-spiking parvalbumin-interneurons, thus causing the pro-psychotic effects of ketamine (Behrens *et al.*, 2008). Although the role of cytokines in the pathophysiology of many inflammatory brain diseases has been well studied, current knowledge on cytokine activated forms of NADPH oxidases expressed in the cardiovascular regulatory centers in the brain in CHF remain limited.

AngII Induced NADPH Oxidase Activation in the Central Nervous System. Recent studies from Griendling's laboratory identified angiotensinII as a potential activator of NADPH oxidase in vascular smooth muscle cells (Lyle and Griendling, 2006; Lee and Griendling, 2008). Subsequently, they, along with others, showed that SOD mimetics dramatically lowered blood pressure in AngII, but not norepinephrine-induced, hypertension (Laursen *et al.*, 1997; Schnackenberg *et al.*, 1998). Studies from Davisson's lab showed that intracerebroventricular injection of AngII modulated blood pressure and drinking behavior are mainly mediated through increased $O_2^{\bullet-}$ produced in the SFO (Zimmerman *et al.*, 2002). Notably, intracerebroventricular injection of adenoviral vectors that expressed Mn-SOD or Cu/Zn-SOD blocked these effects of AngII.

The AngII type-1 receptors (AT-1Rs), the major receptor subtype, is predominantly expressed in central regions that secrete vasopressin and control sympathoexcitation, including the VLM, NTS and PVN (Bunnemann *et al.*, 1992; Johren *et al.*, 1995). In addition, AT-2 receptors expressed in forebrain, brainstem and hypothalamus were also shown to be related to blood pressure regulation (Johren *et al.*, 1997). Intracerebroventricular injection of AngII was shown to increase systemic blood pressure (Davisson *et al.*, 1998; Davisson *et al.*, 2000). Additionally, AngII-induced ROS in the RVLM are shown to modulate sympathetic nerve activity and cardiovascular function in CHF (Mayorov *et al.*, 2004; Gao *et al.*, 2005). AngII also increased the expression of gp^{91phox}/Nox2 and p^{47phox} in the RVLM, suggesting that AngII actions are mediated by NADPH oxidase induced ROS. Blockade of AT-1Rs in the PVN (Han *et al.*, 2007) and RVLM (Mayorov *et al.*, 2004) attenuated the generation of ROS, contributing to decreased sympathetic activity in CHF.

Furthermore, Chan *et al.* showed that AngII-induced superoxide produced via NADPH oxidases increased the phosphorylation of p38 MAPK and/or ERK1/2 in the RVLM, but not JNK. These increases in the RVLM were attenuated by application of a flavin oxidase inhibitor, DPI, antisense oligonucleotides that target p^{22phox} and p^{47phox} mRNA, or tempol. They also observed that DPI, tempol and a p38MAPK inhibitor attenuated the frequency of glutamate-sensitive excitatory postsynaptic currents and the blood pressure increase induced by AngII (Chan *et al.*, 2005).

Additionally, several studies report a cross-talk between pro-inflammatory cytokines and the RAS in both humans and animals. These studies show that treatment with angiotensin II (AngII) resulted in elevation of TNF-α in isolated heart preparations (Frolkis *et al.*, 2001), while pre-treatment with losartan, an AT-1R blocker, attenuated the TNF-α biosynthesis induced by AngII (Gurlek *et al.*, 2001), suggesting that AT-1R expression is closely related to that of TNF-α

in the heart (Tsutamoto *et al.*, 2000; Gurlek *et al.*, 2001). These studies explain an apparent interaction between cytokines and AngII in the periphery. However, the cytokine-AngII interaction in the PVN of CHF animals is currently unexplored.

Nitric Oxide in Sympathetic Regulation of Heart Failure. Functional studies suggest that apart from AngII, nitric oxide (NO) within the PVN and SON, as an atypical neurotransmitter, can elicit multiple actions and play a crucial role in the regulation of sympathetic tone. NO synthase (NOS) positive neurons are detected primarily in the PVN and SON regions. Electrophysiological studies indicated that administration of a NO donor into the PVN siginificantly reduced BP, HR, and RSNA (Horn *et al.*, 1994; Zhang *et al.*, 1997), while the converse has resulted with administration of a NO blocker (Zhang *et al.*, 1997), suggesting the role of NOS positive neurons in the PVN. The message and the number of nNOS positive neurons were shown to be substantially decreased in CHF (Patel *et al.*, 1996; Zhang and Patel, 1998). On the contrary, overexpression of nNOS in the rostral ventrolateral medulla (RVLM) normalized the reduced baroreflexes in CHF, suggesting the role of central NO in baroreflex function (Wang *et al.*, 2003).

More recent evidence shows that ROS decrease NO bioavailability, which is an important factor in the pathology of many disease processes (Cai and Harrison, 2000). All cell types in the brain, in response to inflammation, produce significant amounts of NO via inducible nitric oxide synthase (iNOS) that acts synergistically with superoxide producing NADPH oxidase to kill neurons by formation of OONO-. Peroxynitrite can directly oxidize proteins, lipids and DNA and promote tyrosine nitration and nitrosylation of cysteine, resulting in enzyme and protein dysfunction, eventually leading to neuronal cell death. Formation of OONO- has been attributed in the pathogenesis of many CNS disorders. Moreover, non-cytotoxic concentrations of the

excitatory amino acid, glutamate has been shown to increase production of $O_2^{\bullet-}$ and OONO- via induction of inflammatory mediators (Scott *et al.*, 2007).

STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

Increased circulating levels of cytokines lead to increased production of ROS and AngII, resulting in the progression of heart failure. It has been demonstrated that the PVN and RVLM of the CNS play a key role in the regulation of sympathetic outflow and progression of cardiovascular disease. Peripheral cytokines can act on specialized brain regions known as circumventricular organs, which lack a blood-brain-barrier, altering central cardiovascular outputs including sympathoexcitation, release of vasopressin, and dampening of baroreflexes. In addition, cytokines produced in the PVN and RVLM of the brain may also alter cardiovascular and body fluid homeostasis. In order to better understand the central actions of cytokines in the development of sympathoexcitation and to identify novel, central therapeutic targets of the disease, it is essential to investigate the signaling mechanism(s) of cytokines in the central nervous system.

Recent work in hypertension has identified ROS produced by NADPH oxidase as key signaling intermediates of AngII in the PVN and SON. Furthermore, decreased NO in these key central cardiovascular regulatory centers results in sympathoexcitation. However, the role of NADPH oxidase-derived ROS in cytokine signaling in neurons within the CNS, and the role of centrally produced ROS in cytokine-induced changes in sympathetic outflow and body fluid balance, has not been studied. Therefore, we tested the hypothesis that the $O_2^{\bullet-}$-NO interaction via the activation of AT1Rs are key signaling molecules and mediate the cardiovascular actions of cytokines acting in the central nervous system. Furthermore, we hypothesize that dysregulation of cytokine/AngII/ROS signaling in the brain is involved in the pathogenesis of congestive heart failure.

In order to explore our hypothesis, we performed a series of *in vivo* experiments integrated with cardiovascular physiological, molecular and immunohistochemical techniques.

Aim 1: Determine the role of TNF in mediating oxidative stress in the PVN and inducing sympathoexcitation in congestive heart failure.

Aim 2: Investigate the effects TNF on nitric oxide in the PVN, and its effect on sympathoexcitation in heart failure.

Aim 3: Examine the effects of TNF upon the superoxide-nitric oxide interaction and AT1R expression in the sympathoexcitatory neurons of the PVN, and upon volume overload in congestive heart failure.

Aim 4: Determine the chronic systemic treatment effects of TNF in inducing oxidative and nitrosative stress in the heart and the PVN, and understand the mechanism by which increased TNF in the PVN contributes to increased oxidative stress and sympathoexcitation.

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CHAPTER 2

TNF-α BLOCKADE DECREASES OXIDATIVE STRESS IN THE PARAVENTRICULAR NUCLEUS AND ATTENUATES SYMPATHOEXCITATION IN HEART FAILURE RATS*

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INTRODUCTION

Congestive heart failure is characterized by a generalized state of neurohumoral excitation that contributes to progressive deterioration of cardiac function resulting in the premature death of patients. In the past, most of the treatments were aimed at blocking this exaggerated neurohumoral excitation by using β-adrenergic receptor antagonists, angiotensin converting enzyme inhibitors, and angiotensin receptor blockers in heart failure (HF) patients. These treatments have considerably reduced mortality and morbidity, however the clinical course of CHF is still progressive, hence the need for innovative approaches to therapy.

In addition to neurohormones, the activation of proinflammatory cytokines such as TNF-α is known to play a role in the pathogenesis of cardiovascular disease. These cytokines are increased with the severity of heart disease and are of prognostic significance. Despite the abundant evidence that TNF-α contributes significantly to cardiac dysfunction in heart failure in animal models, the results of two large clinical trials using etanercept, a truncated, soluble TNF receptor antagonist (RENAISSANCE) and infliximab (RECOVER), a TNF-α blocking antibody, were largely negative (Mann *et al.*, 2004). However, Pentoxyfylline (PTX), a phosphodiesterase inhibitor, which also blocks cytokine expression, has been found to be promising in small clinical trials (Sliwa *et al.*, 2002). In addition to elevating intracellular cyclic AMP, PTX increases the production of prostacyclins and vasodilatory eicosanoids (Myers *et al.*, 1994; Schermuly *et al.*, 2001) and depresses the production of TNF-α (Strieter *et al.*, 1988).

The concept of targeting brain production of neurohormones is relatively new and understudied. Recent evidence from our lab suggests that cytokines are not only increased in the circulation and LV tissues, but also in the hypothalamus of CHF rats (Francis *et al.*, 2004). We also showed that cardiac sympathetic afferents contribute to hypothalamic production of cytokines and that the vagal efferents regulate the peripheral production of cytokines, thus

suggesting a nervous system link in the activation of cytokines in CHF (Francis *et al.*, 2004). In the brain, the PVN of the hypothalamus is an important center regulating cardiovascular and fluid homeostasis. Electrophysiological studies show that stimulation of PVN results in increased sympathetic activity(Kannan *et al.*, 1989). PVN neuronal activity is increased in HF rats. Injection of TNF-α into the PVN or rostral ventrolateral medulla (RVLM) increased sympathetic activity suggesting a direct role of TNF-α in sympathetic activity(Zhang *et al.*, 2003). Nevertheless, the mechanisms by which cytokines contribute to the sympathoexcitation in heart failure are not known.

Oxidative stress plays an important role in the progression of CHF. Both *in vitro* and *in vivo* studies have associated TNF-α as an important contributor to oxidative stress, either directly or indirectly, by decreasing coronary or systemic perfusion resulting in cardiac dysfunction. Furthermore, TNF-α modulates the activity and expression of NAD(P)H oxidases (Noxs), a potential source of reactive oxygen species (ROS) in cardiovascular disease. NAD(P)H oxidase is a multisubunit enzyme complex that consists of two membrane subunits, p22phox and Nox2, and four cytoplasmic subunits, p40phox, p47phox, p67phox and Rac1. The catalytic subunit of NAD(P)H oxidases is Nox2, and few isoforms of Nox2, such as Nox1 and Nox4, are found in cardiomyocytes and neurons. The role of TNF-α in eliciting NAD(P)H oxidase subunit expression in CHF is relatively unknown.

Taken together, it is evident that TNF-α contributes significantly to oxidative stress and sympathoexcitation in the pathogenesis of CHF. To understand the mechanistic link between CHF induced production of cytokines, and neurohumoral excitation, we hypothesized that increased levels of TNF-α in CHF may modulates the expression of Nox2 or its isoforms in the PVN and contributes to the exaggerated sympathetic activity. In this study we used PTX to block

the production of cytokines both at the center and the periphery, as it is the only available drug known to cross the blood-brain barrier (BBB).

METHODS

Animals. Adult male Sprague-Dawley rats weighing 350–375 g were used for this study. They were housed in temperature (23 ± 2°C) and light-controlled (lights on between 7 AM and 7 PM) animal quarters and were provided with water and rat chow ad libitum. The experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee.

Experiment Protocol. Rats underwent coronary artery ligation (CAL) to induce HF under ketamine + xylazine anesthesia (90 and 10 mg/kg IP), or Sham operation (Sham), as described previously (Francis *et al.*, 2001). Induction of HF was confirmed by transthoracic echocardiography 24 hours after recovery from surgery and the rats were assigned to different treatment groups. Thereafter, HF or SHAM rats were chronically injected with PTX (30 mg/kg daily IP; in 10% ethanol), or vehicle (10% ethanol alone) for 5 weeks. A second echocardiogram was obtained at the end of the treatment protocol and the rats were sacrificed under isoflurane anesthesia; plasma and other tissues were collected for further analysis.

Echocardiographic Assessment of LV Function. A first echocardiography was performed 24h after coronary artery ligation followed by a second echo at the end of 5week study as described previously (Francis *et al.*, 2004). In brief, transthoracic echocardiography was performed under ketamine anesthesia, using a Toshiba Aplio SSH770 (Toshiba Medical, Tustin, California) fitted with a PST 65A sector scanner (8 MHz probe) which generates two dimensional images at a frame rate ranging from 300-500 frames per second. LV end diastolic volume (LVEDV) and ejection fraction (EF) were computed using the area length method. The portion of the LV that displays akinesis was electronically planimetered and expressed as a percent of the total LV

silhouette to estimate the size of the ischemic zone (% IZ). Only rats with large infarct (% IZ ≥ 45%) were used for the study. LV fractional shortening (FS %) was calculated using the following equation FS % = [(LVDD-LVESD)/LVEDD] x 100. Tei index was determined from Doppler recordings of LV inflow and outflow as described previously (Dujardin *et al.*, 1998). From mitral inflow, isovolumetric relaxation time and isovolumetric contraction time were measured. Ejection time was measured from LV outflow velocity curve recorded from long-axis view. The Tei index was calculated using the equation, Tei index = (isovolumetric relaxation time + isovolumetric contraction time)/ventricular ejection time.

Detection of ROS in the Heart and Brain. ROS were detected using Dihydroethidium (DHE) staining. DHE is a fluorogenic probe, which in presence of ROS is converted to ethidium bromide that intercalates with nuclear DNA, resulting in a punctuate appearance. The rats received an intracardiac injection of DHE at a dose of 80 μg/kg body weight. Hearts and brains were harvested, placed into freezing mold with Tissue-Tek® OCT (Sakura Finetek, Torrance, CA), snap frozen with liquid nitrogen, sectioned on a cryostat and placed on slides. Cryosections (12 μm) were immediately viewed and imaged under epifluorescence with a Zeiss Axiovert 200 microscope using an Ethidium Bromide (EtBr) compatible filter set (Chroma Filters #41006); images were captured with an Olympus Q Capture 5 camera and Q Capture Pro software.

Extraction of PVN by Laser Capture Microscopy (LCM). LCM was conducted in a dehumidified room (humidity ≤35%), and was kept to less than 30 min per slide to reduce the loss in recovery of intact RNA. A 7.5 μm laser spot size was used to capture the PVN, at a power range of 65–80 mW and pulse duration of 550–750 μs. This combination of parameters allowed efficient retrieval of the entire PVN area and a consistent lifting efficiency of >80%. The number of laser ‘shots’ used for each sample was kept constant at 1400. These parameters secured a sufficient and near constant amount of input RNA for comparative real-time RT-PCR analyses

and protein for western blotting. All experiments were performed no less than five times with five different animals.

Measurement of Circulating TNF-α. At the end of the 5-week study, one group of rats was sacrificed by decapitation with guillotine under deep anesthesia by isoflurane and approximately 4 mls of trunk blood was collected in heparinized tubes. Plasma samples obtained by centrifugation of heparinized blood at 4ºC (Beckman-Coulter) at 2500 rpm for 15 min were used for estimation of circulating TNF-α and catecholamines. Circulating levels of TNF-α were quantified using commercially available rat TNF-α ELISA kit (Biosource, Camarillo, CA) as described previously (Tei *et al.*, 1995; Francis *et al.*, 2003).

Estimation of Circulating Catecholamine Levels. Plasma norepinephrine (NE) and epinephrine (EPI) were measured using high performance liquid chromatography (HPLC) as described previously (Francis *et al.*, 2001) with minor modifications in plasma sample preparation**.** Plasma samples were prepared by adding activated alumina, Tris buffer, EDTA and internal standard DHBA, along with 0.5 ml of rat plasma. The samples were centrifuged and supernatant separated and rinsed twice in ultra pure water and filtered through a Millipore filter (Ultrafree MC UFC30GV00, Millipore Corp). Samples were filtered and injected into an Eicom HTEC-500 system fitted with an HPLC-ECD.

Renal Sympathetic Nerve Activity (RSNA). One set of rats were subjected to recording of RSNA. The left kidney was exposed by left retroperitoneal flank incision. The renal sympathetic nerves were identified under a dissecting microscope, isolated free of the surrounding connective tissue, and placed on a pair of platinum recording electrodes. Once an optimal signal-to-noise ratio was achieved, the electrode and the renal nerve were covered with a dentistry impression material (Coltene President). The signal was amplified with a Grass P511 band-pass amplifier with low-frequency cutoff set at 30 Hz and high-frequency cutoff at 3 kHz. The amplified and

filtered signal was channeled to an audio amplifier-loudspeaker (Grass model AM 8 audio monitor) for auditory evaluation, and to a rectifying voltage integrator for quantification (Grass model 7P10). The integrated voltage signals were acquired by a commercially available data aqusition system (Acknowledge for Windows; Biopack, Santa Barbara, CA). Minimum and maximum RSNA was detected using a intravenous bolus administration of phenylephrine (20 g/kg) and sodium nitroprusside (SNP; 100g/kg), respectively. At the end of the experiment the background noise, defined as the signal recorded postmortem, was subtracted from actual RSNA recorded, and subsequently expressed as percent of maximum (in response to SNP).

RNA Isolation and Real-time RT PCR. Total RNA was extracted from the LV and microdissected PVN using TRIzol reagent (Invitrogen), and reverse transcribed using oligo dT and reverse transcriptase. Expression levels of Nox1, Nox2, Nox4, TNF-α, and IL-1β mRNA were determined using specific rat primers shown in table 1. GAPDH was used as housekeeping gene. Real-time RT–PCR (qRT–PCR) was performed in 384 well PCR plates using Bio-Rad PCR Master Mix (The iTaq SYBR^{TM} Green Supermix with ROX) and the ABI Prism 7900 sequence detection system (Applied Biosystems). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles (15 s at 95°C, 1 min, at 60°C). A dissociation step (15 s at 95°C, 15 s at 60°C and 15 s at 95°C) was added to check the melting temperature of specific PCR product.

Table 2.1. Sequence of Primers Used in Real-time RT-PCR.

Primer	Sense	Antisense	Genebank ID
TNF-α	3′-GTCGTAGCAAACCACCAAGC-5′	5′-TGTGGGTGAGGAGCACATAG-3′	D00475
IL-1β	3′-CTGTGACTCGTGGGATGATG-5′	5′-AGACCTGACTTGGCAGAGGA-3′	NM_031512
Nox 1	3′-CCCTGGAACAAGAGATGGAC-5′	5′-AATTGGTCTCCCAAAGGAGGT-3′	NM_053683
Nox 2	3′-CGGAATCCTCTCCTTCCT-5′	5′-GCATTCACACACCACTCCAC-3′	AF298656
Nox 4	3′-TTCTACATGCTGCTGCTGCT-5′	5′-AAAACCCTCCAGGCAAAGAT-3′	AY027527

Western Blot Analysis of Nox Subunits. Protein was extracted from LV samples and laser captured PVN samples in ice-cold buffer (10 mM Tris·HCl pH 7.4, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (Roche). The protein content in the supernatant was determined using a detergent compatible protein assay (Bio-Rad). Protein samples (25µg) were resolved in a 10% SDS-polyacrylamide gel along with a molecular weight marker and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked at RT for 1h in 1% casein in PBS-T. Blots were then incubated overnight at 4°C with the following primary antibodies: Nox1 (1:1000 dilution), and Nox2 (1:1000 dilution) (Santa Cruz Biotechnology). GAPDH (1:1000 dilution) was used as internal control. Bound primary antibodies were detected with a horseradish peroxidase–labeled secondary antibody (1:20,000; 1 hour) and enhanced chemiluminescence (AmerSham). The band intensities were quantified using Kodak ID 3.6 imaging systems and normalized with GAPDH levels.

Localization of TNF-α and Nox Subunits by Immunohistochemistry. Brain and heart tissue were fixed in 4% paraformaldehyde and cut into 8µm thick-sections, then pretreated with 0.3% hydrogen peroxide and 0.1% sodium azide in PBS for 10 min to inhibit endogenous peroxidase activity. These sections were washed twice in PBS and incubated in blocking medium (1% BSA and 10% normal goat serum in PBS) for 10 min. The sections were then treated with respective primary antibodies Nox1 (1:100 dilution), Nox2 (1:100 dilution), Nox4 (1:100 dilution) and TNF-α (1:100 dilution) (Santa Cruz Biotechnology) and incubated overnight at 4°C. The sections were again washed twice in PBS and incubated with secondary antibody, a peroxidase conjugated IgG antibody for 30 min. Bound antibodies were detected with streptavidin-peroxidase complex using 0.2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride in PBS containing 0.003% hydrogen peroxide. Negative control sections were incubated with secondary antibody alone.

Statistical Analysis of Data. All results are expressed as mean ± SEM. For statistical analysis of the data, one-way ANOVA followed by Bonferroni's post hoc test, was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA, to determine differences among groups. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Effect of PTX Treatment on Survival of CHF Rats. A total of 101 rats were subjected to CAL or Sham surgery and subsequently assigned to three sets of animals, one set for immunohistochemistry, the second for mRNA, and the third for western blot analysis. The average survival within 24h of surgery was 80% in the rats undergoing CAL and 100% in the Sham group. Over the five week study, 2 of the CHF rats and 2 of the CHF+PTX rats died prior to the designated time point, while none of the Sham animals died during the investigation. Thus, over the course of the five weeks, PTX treatment had no apparent influence on the survival (Table 2.1).

Effect of PTX on ROS Production in HF. ROS production in the heart and brain was assessed by DHE fluorescence (Fig. 2.1). As indicated by the punctate staining of the nuclei, CHF-induced an increase in ROS production in the heart and brain tissues. In contrast, PTX inhibited CHF-induced ROS production.

Effect of PTX on LV Function. Table 2.1 shows LV function in rats as measured by echocardiography. Compared to Sham animals, CHF rats had reduced LVEF and increased LVEDV and increased volume/mass ratio. There was no significant difference in the HR between the two CHF groups. The average %IZ in both the CHF groups was >56% with a range of 50-58% before and after the treatments. At the end of the five weeks, Tei index was increased

by 59.38% and LVEF further declined by 21.21 % in the CHF group. In contrast, treatment with PTX in the CHF group prevented further decline in LVEF and decreased Tei index.

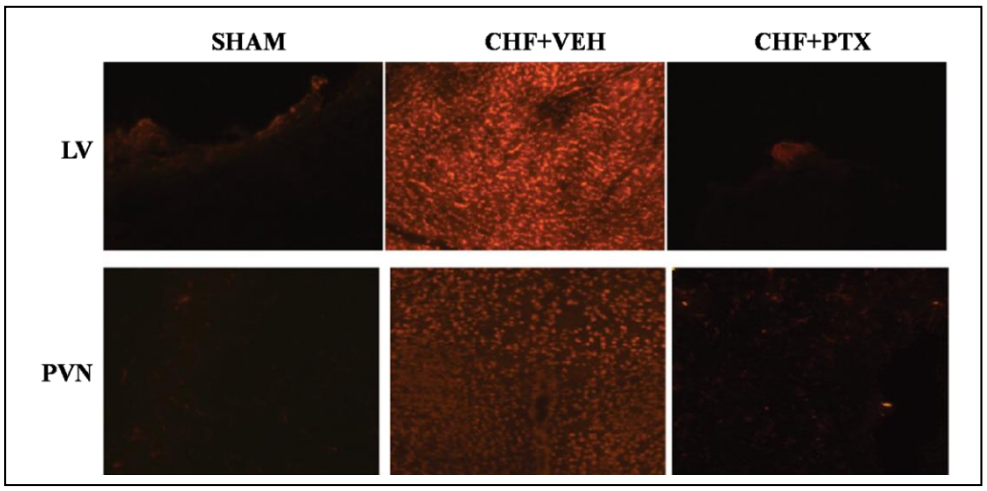

Fig.2.1. Detection of Reactive Oxygen Species (ROS) by dihydroethidium (DHE) staining. Under identical imaging conditions, production of superoxide is elevated significantly in the heart (*top*) and brain (*bottom*) compared with Sham and pentoxifylline (PTX)-treated rats.

Effect of PTX on Cytokine Production in HF. To determine the effect of PTX treatment on CHF induced production of TNF-α in the heart and PVN, we determined mRNA transcript levels by real-time RT-PCR in the LV tissue, and in the PVN isolated using laser capture micro dissection. Circulating levels of TNF-α were also measured by ELISA to estimate the anti-cytokine effects of PTX. As shown in figure 2.2A, the elevated levels of circulating TNF-α found in CHF rats were restored to near normal levels with PTX-treatment. The LV of CHF rats showed increased levels of TNF-α (~3.7 fold increase) when compared with those of PTX-treated rats (~1.7 fold increase), although the levels did not exactly reach the levels of Sham rats (Figs. 2.2B and 2.2C).

To further ascertain the anti-cytokine effects of PTX in CHF rats, the protein levels of TNF-α were assessed in the LV and PVN by immunohistochemistry. Immunohistochemistry revealed that elevated levels of TNF-α in CHF were significantly attenuated by treatment with

PTX (Fig. 2.2D). These results show that the TNF-α expression is significantly inhibited by PTX both in the LV and the PVN of CHF rats.

Table 2.2. Echocardiographic Findings

	Sham	**Sham + PTX**	**MI 24 h**	**CHF 5 wks**	**CHF + PTX 5 wks**
n	18	18	48	23	23
HR	415 ± 12.0	410 ± 5.00	409 ± 12.0	418 ± 6.00	411 ± 10.0
IVSD (mm)	1.52 ± 0.05	1.55 ± 0.04	1.22 ± 0.09	1.14 ± 0.08*	1.53 ± 0.10$^{\#}$
IVSS (mm)	2.80 ± 0.09	2.72 ± 0.08	1.76 ± 0.12*	1.67 ± 0.13*	2.00 ± 0.16*
LVDD (mm)	6.41 ± 0.18	6.33 ± 0.14	7.75 ± 0.40	10.91 ± 0.72*	8.99 ± 0.17*$^{,\#}$
LVDS (mm)	3.03 ± 0.18	3.01 ± 0.06	5.92 ± 0.45*	8.12 ± 0.63*	7.80 ± 0.86*
PWD (mm)	1.67 ± 0.05	1.74 ± 0.08	1.17 ± 0.04*	1.26 ± 0.12*	1.45 ± 0.06
PWS (mm)	2.66 ± 0.10	2.70 ± 0.06	1.87 ± 0.12*	1.85 ± 0.15*	2.17 ± 0.07*
%EF	83.7 ± 0.90	84.6 ± 1.20	34.0 ± 0.20*	26.0 ± 1.20*	33.2 ± 2.40*$^{,\#}$
%FS	52.94 ± 1.96	51.79 ± 1.30	24.45 ± 2.34*	15.42 ± 1.26*	23.42 ± 2.41*
%IZ	0	0	54.9 ± 1.80	58.9 ± 2.60	55.8 ± 3.10
Tei	0.46 ± 0.02	0.44 ± 0.02	0.34 ± 0.03	0.51 ± 0.04	0.32 ± 0.03*$^{,\#}$
LVEDV (µl)	548 ± 56	520.3 ± 48.5	865 ± 40.2*	1015 ± 93.6*	868 ± 103*$^{,\#}$

Values are means ± SE; IVSD, Interventricular septal thickness at end-diastole; IVSS, Interventricular septal thickness at end-systole; LVDD, Left ventricular internal diameter at end-diastole; LVDS, Left ventricular internal diameter at end-systole; PWD, Posterior wall thickness at end-diastole; PWS, Posterior wall thickness at end-systole; %FS, percent fractional shortening
*$P < 0.05$, compared with Sham group
#$P < 0.05$, compared with CHF 5 wks group

Modulation of Expression of Nox1, Nox2 and Nox4 in HF. The mRNA expression of the NAD(P)H oxidase subunits Nox2 and its isoforms (Nox1 and Nox4), were assessed by real-time RT-PCR. CHF induced a ~6, 10 and 4 fold increase in Nox1, Nox2 and Nox4 in the LV (Figs. 2.3A, B and C), respectively. A similar increase was also seen in the PVN of CHF rats (~28, 15 and 6 fold increase, respectively) for Nox1, Nox2 and Nox4 compared to the Sham group (Figs. 2.3D, E and F). PTX treatment in the CHF group significantly reduced the expression of these subunits in both the LV and PVN.

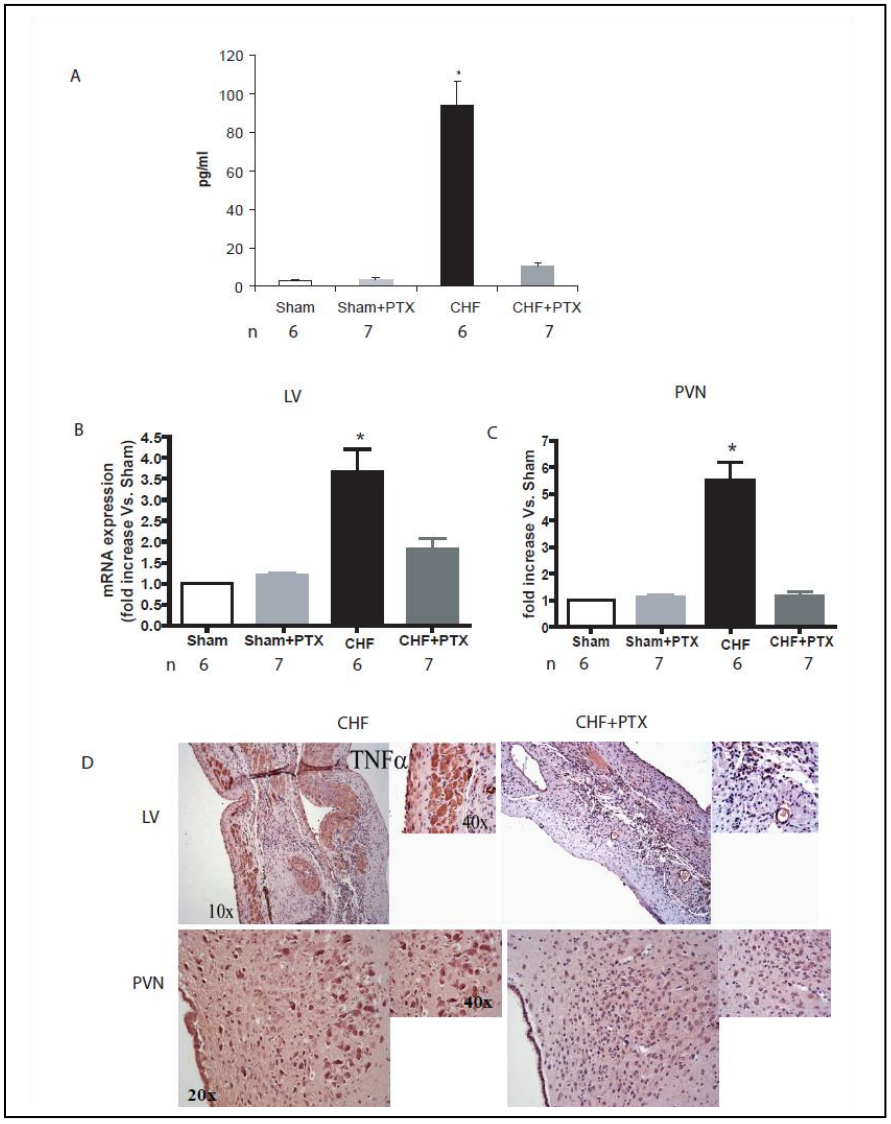

Fig.2.2. Tumor Necrosis Factor (TNF)-α Levels. *A*: PTX inhibited congestive heart failure (CHF)-induced production of circulating TNF-α (**P*<0.05). *B* and *C*: CHFinduced mRNA expression of TNF- α inhibited by PTX in the left ventricle (LV; *B*) and the paraventricular nucleus (PVN; *C*). *D*: Immunohistochemistry photomicrographs of TNF-α in the LV and PVN.

Protein expression for Nox1, Nox2 and Nox4 was measured in the LV and PVN by Western blot analyses. Unfortunately, we did not get a good signal on the Western blot with the commercially available Nox4 antibody. As shown in figures 2.4A and 2.5A, CHF rats treated with PTX exhibited a decrease in the protein expression of Nox1and Nox2 in the LV and PVN (Figs. 2.4B and 2.5B).

Immunohistochemistry also revealed an increased expression of Nox1, Nox2 and Nox4 in the LV of CHF rats as compared to Sham and those treated with PTX (Figs. 2.4C, 2.5C and 2.6, respectively). In the PVN, Nox1 was predominantly detected in the magnocellular neurons of CHF rats (Fig. 2.4C). Nox2 and Nox4 expression was increased both in the magnocellular and parvocellular neurons of the PVN of CHF rats. Treatment with PTX reversed all these changes in the PVN of CHF rats (Figs. 2.5C and 2.6).

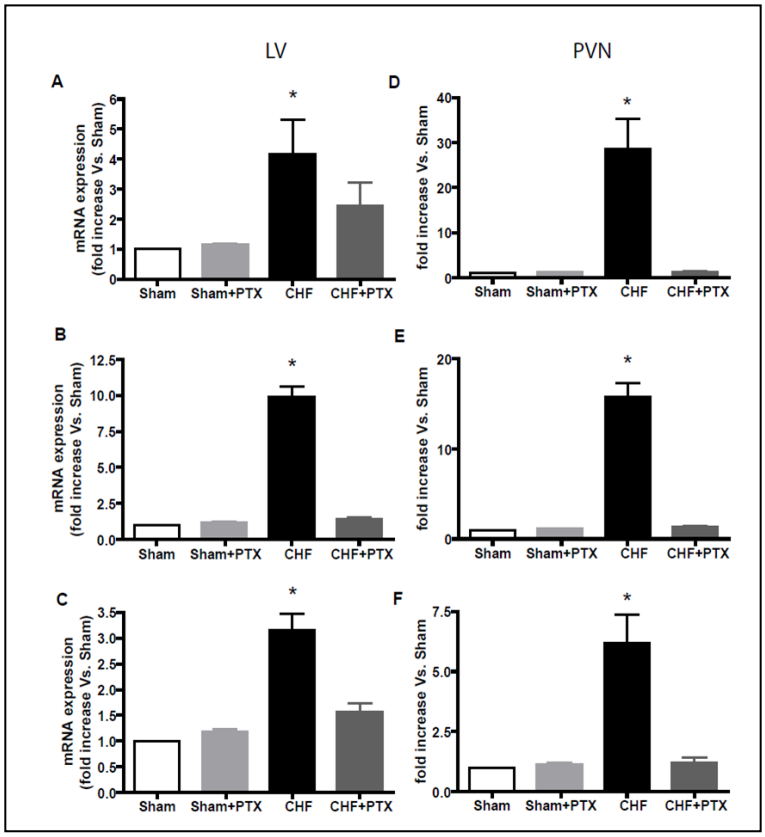

Fig.2.3. Effect of PTX Treatment on mRNA Levels of NAD(P)H Oxidase Subunits [Nox1 (*A*), Nox2 (*B*), and Nox4 (*C*)] in the LV and PVN [Nox1 (*D*), Nox2 (*E*), and Nox4 (*F*)] of Experimental Rats. Levels of Nox1, Nox2, and Nox4 were elevated in the LV and PVN of CHF rats compared with the Sham and PTX-treated rats. *P <0.05.

Effect of Blocking Cytokines on Sympathetic Activity. Plasma epinephrine and norepinephrine levels were significantly increased (995.5±89 and 576±56 pg/ml, respectively; p<0.05) in the CHF rats. Following inhibition of cytokines with PTX, these catecholamine levels were decreased significantly (347±42 and 192.9±29 pg/ml, respectively; p<0.05). These levels

showed a significant difference when compared to those of Sham rats (225±30 and 193±40 pg/ml, respectively; $p<0.05$) (Fig. 2.7A).

Figure 2.7B shows a raw tracing from a vehicle treated CHF rat and PTX treated CHF rat. Figure 2.7C shows RSNA as a percent of maximum response to the hypotension induced by intravenous injection of SNP. RSNA response to hypotension was maximum in CHF rats treated with vehicle when compared to that of PTX-treated CHF rats. There was no difference in the RSNA activity in vehicle and PTX treated Sham rats in response to hypotension.

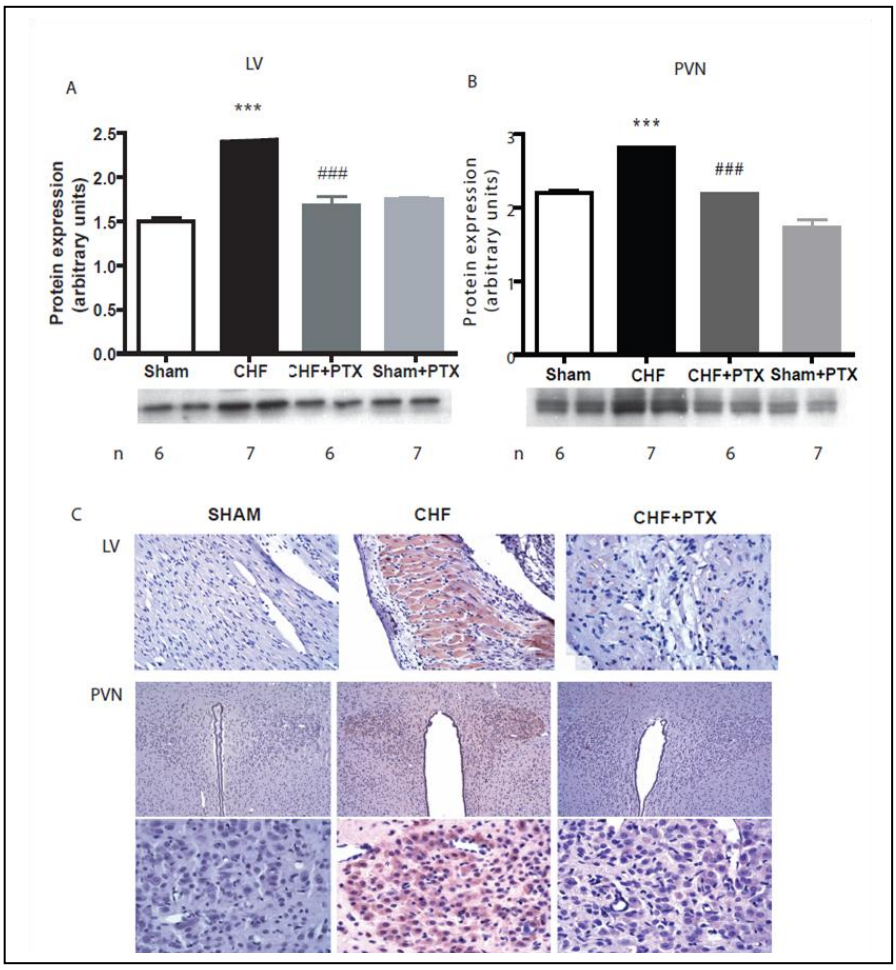

Fig.2.4. Effect of PTX Treatment on Protein Expression of Nox1 Subunit. Representative Western blot and densitometric analysis of Nox1 in the LV (*A*) and PVN (*B*). ***$P<0.001$ vs. Sham; $^{###}P<0.001$ vs. CHF. Immunohistochemical localization of Nox1 in the LV and PVN of CHF rats (C).

DISCUSSION

The major findings of this study are 1) The expression of the NAD(P)H oxidase subunits, Nox1, Nox2 and Nox4 were elevated in the LV and the PVN of CHF rats, 2) administration of PTX, a blocker of proinflammatory cytokine production, normalized the enhanced NAD(P)H oxidase subunit and TNF-α expression, both in the PVN and the LV, and 3) PTX also decreased the circulating catecholamine levels and RSNA, indicators of sympathetic activity, in CHF rats. These findings suggest that the cytokines play a role in inducing oxidative stress in the PVN, thereby contributing to the increased sympathetic activity in CHF rats.

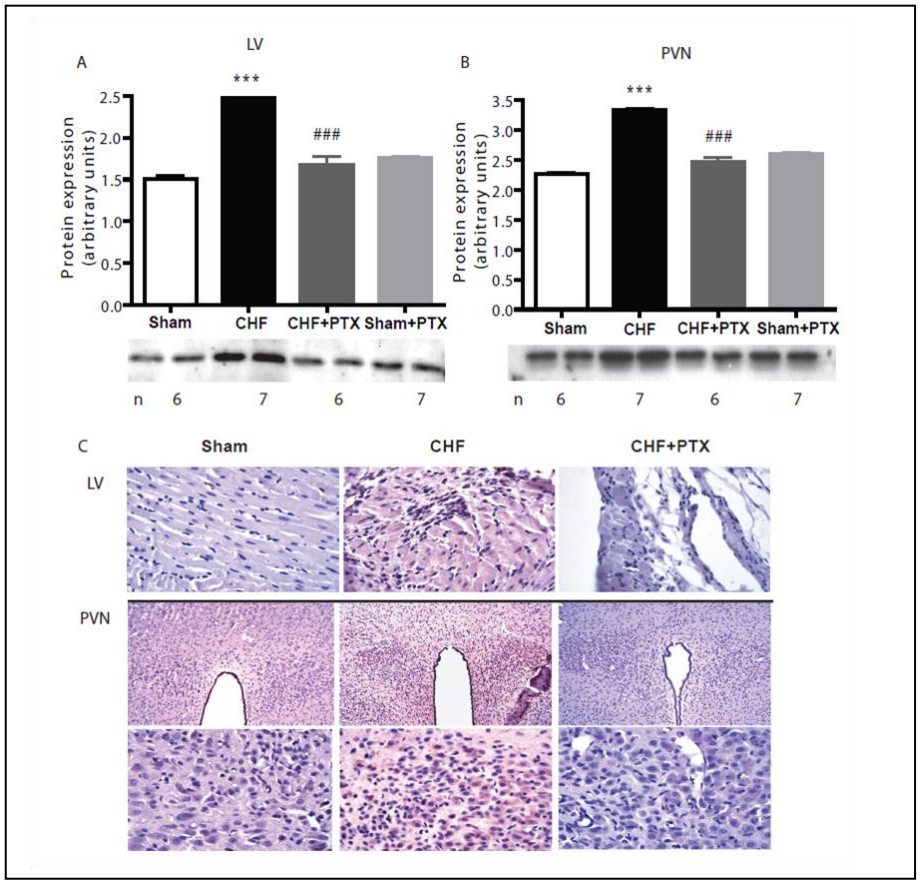

Fig.2.5. Nox2 Protein Expression. Representative blot and densitometric analysis of LV (*A*) and PVN (*B*). ***P <0.001 vs. Sham; ###P< 0.001 vs. CHF. *C*: photomicrographs showing expression of Nox2 in the LV and PVN of CHF rats. PTX attenuated Nox2 subunit expression both in the LV and PVN of CHF rats. The sections shown are representative of results from 3 different experiments. Magnification is X100 in *top* and X400 in *bottom*.

Pentoxifylline and LV Function. Cytokines are produced locally in the myocardium in response to stimuli such as hemodynamic pressure overload and myocardial ischemia or infarction (Torre-Amione *et al.,* 1996; Baumgarten *et al.,* 2002). Several clinical studies have shown that PTX decreases proinflammatory cytokine production and improves LV function in CHF patients (Sliwa *et al.,* 1998; Skudicky *et al.,* 2001; Sliwa *et al.,* 2002; Bahrmann *et al.,* 2004; Sliwa *et al.,* 2004). In this study also, we show that treatment with PTX in CHF rats resulted in a significant improvement in LVEF and prevented an increase in the Tei index, an indicator of systolic dysfunction. The Tei index is a combination of contraction and relaxation time intervals constituting an overall index of LV function as assessed by Doppler echocardiography. The Tei index has been shown to have prognostic importance in dilated (Dujardin *et al.,* 1998) and restrictive cardiomyopathies (Harjai *et al.,* 2002), and also in acute myocardial infarction (Moller *et al.,* 2001). The Tei index has a narrow range in normal subjects and seems to progressively increase with deterioration of LV function (Dujardin *et al.,* 1998). TNF-α exerts a negative inotropic effect on the contractility of the ischemic heart in hamsters, dogs, and humans (Finkel *et al.,* 1992) by interfering with calcium homeostasis, and thus interfering with contraction-excitation coupling (Meldrum *et al.,* 1998). PTX has been demonstrated to exert protective effects on ischemic myocardium by reducing TNF-α production (Zhang *et al.,* 2005). In the present study, we saw a deterioration of LV function in the vehicle treated CHF rats as indicated by decreased EF and increased Tei index. We also observed an increase in LVDD and LVEDV, which indicate a decrease in LV contractile function. Treatment with PTX improved LV contractile function in our study that could be due to a combined reduction in cytokines and oxidative stress. This is supported by our observation that PTX treatment decreased ROS production. It has been shown that the surviving myocytes in the infarcted region are potential sources of cytokines, which may contribute to the progression of

CHF (Irwin *et al.*, 1999). This study further shows that PTX, by virtue of its anti-cytokine activity, exerts its role in improving the systolic function in CHF, which is comparable to other studies (Irwin *et al.*, 1999; Skudicky *et al.*, 2001; Gurantz *et al.*, 2005).

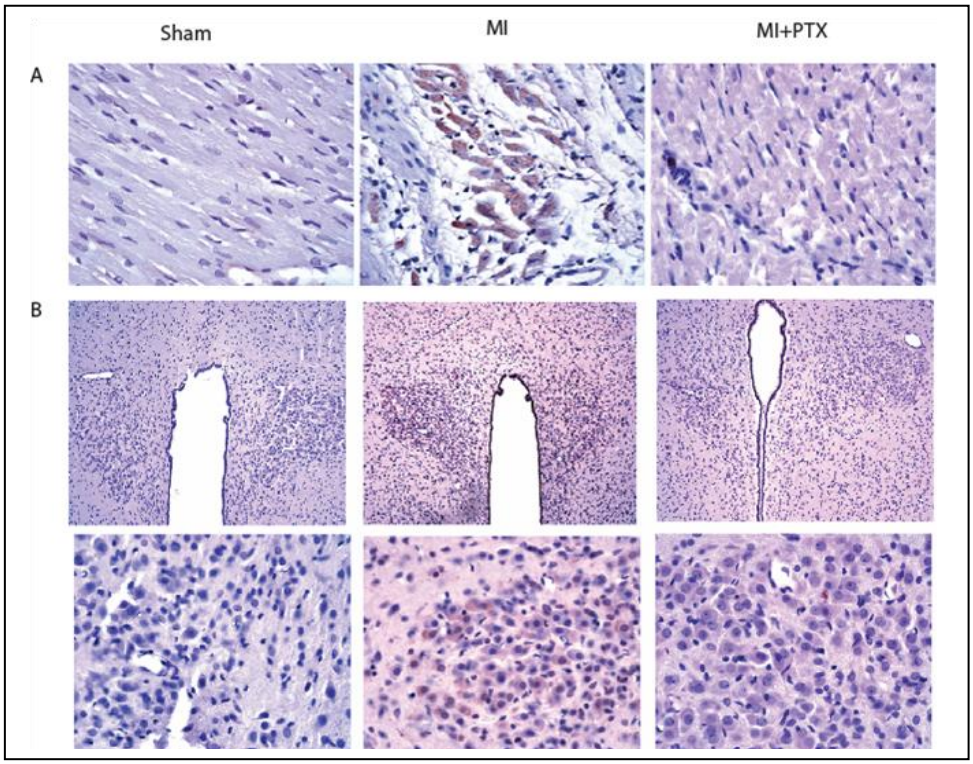

Fig. 2.6. Photomicrographs Showing Expression of Nox4 in the LV (*top*) and PVN (*bottom*). CHF increased the expression of Nox4 both in the LV and PVN of heart failure rats, whereas PTX treatment attenuated this increased expression.

Cytokines and CNS in Congestive Heart Failure. When the stress-activated cytokines in the myocardium exceed the limit to be utilized by the local cellular receptors in autocrine/paracrine functions, they become blood-borne and enter the systemic circulation, causing some of the adverse effects associated with cytokines. One critical function of blood-borne cytokines is to stimulate self-production via a feed-forward mechanism. These blood borne cytokines are large molecules and do not readily cross the BBB. However, they can enter the brain through a saturable transport mechanism, or a passive transport mechanism via the circumventricular organ, where the BBB is either weak or absent. Our previous studies showed that cardiac

sympathetic afferents are a potential mechanism for the induction of hypothalamic cytokines in CHF (Francis *et al.*, 2004). Thus, the circulatory cytokines could induce inflammatory and sympathoexcitaory mediators in the brain that have been shown to be antagonized by a mineralocorticoid receptor blocker (Kang *et al.*, 2006). The deleterious role of these stress-activated cytokines in the progression of heart disease could be due to the direct toxic effects exerted by these cytokines on heart and circulation.

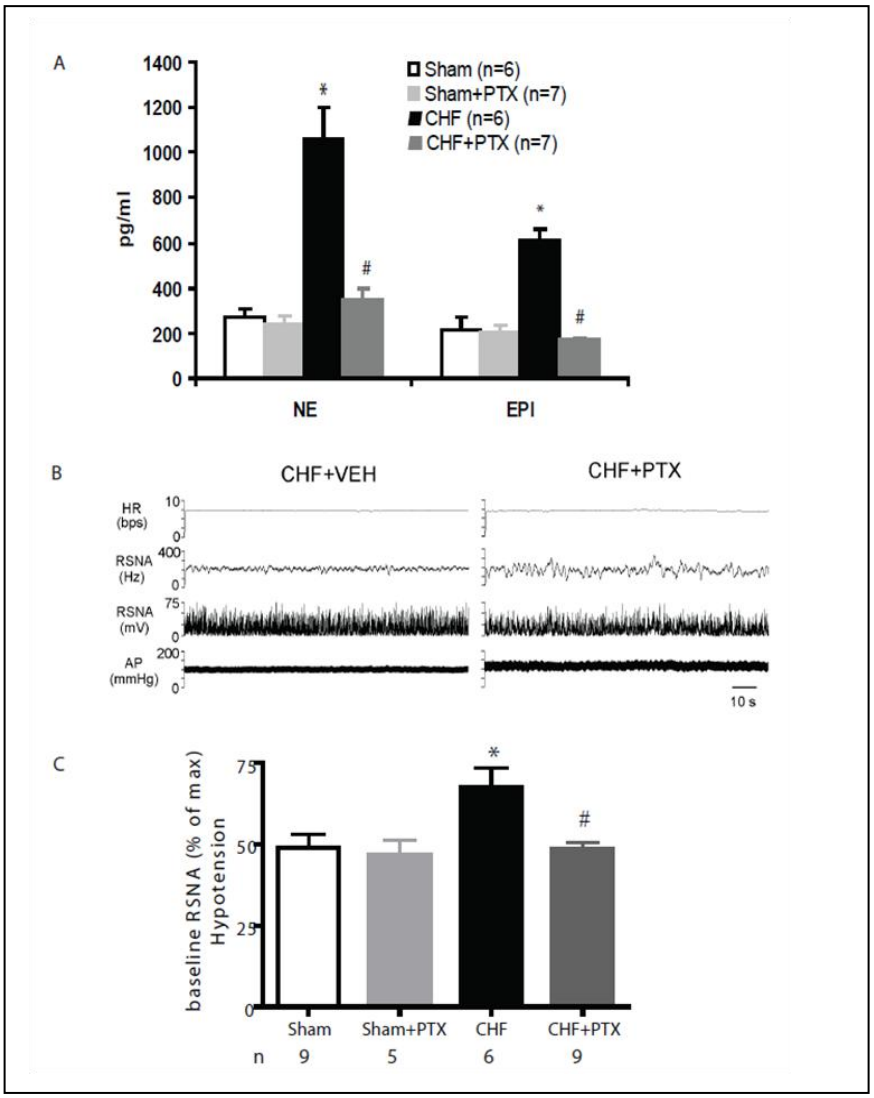

Fig. 2.7. Sympathetic Activity. *A*: circulating norepinephrine (NE) and epinephrine (Epi) levels were significantly higher in CHF rats, whereas they were lower in those treated with PTX. **P*<0.05 compared with Sham. *B*: and *C*: raw tracing showing renal sympathetic nerve activity (RSNA; *B*) and integrated RSNA (*C*) of CHF rats was higher in the vehicle-treated rats compared with the PTXtreated rats. **P*<0.05 vs. Sham; $^{\#}$*P*<0.05 vs. CHF.

The PVN of the hypothalamus is one of the five major regions in the brain controlling sympathetic outflow. It regulates sympathetic activity via its inputs from the nucleus tractus solitarius (NTS) (Swanson and Sawchenko, 1983) and efferent projections to the RVLM and intermediolateral column (IML) of the spinal cord (Pyner and Coote, 2000). The PVN is composed of two kinds of neurons (Swanson and Sawchenko, 1983); the larger magnocellular neurons projecting into the pituitary are responsible for humoral regulation of fluid balance, whereas the smaller parvocellular neurons projecting to other sites in the CNS are involved in mediating sympathetic activity (Patel, 2000).

It has also been shown that many putative mediators, like prostaglandins, facilitate the transport of cytokines into the brain. These cytokines then stimulate the microglial cells in the brain to produce even more cytokines. We have also shown that injection of prostaglandin-E2 increased sympathetic activity in HF rats (Zhang *et al.,* 2003). In this study, we show cardiosympathoexcitatory neurons of the PVN as yet another potential source of cytokines in CHF. Moreover, the enhanced sympathetic activity in HF rats was decreased in the present study concurrent with the administration of PTX as evidenced by attenuated circulating catecholamine levels and RSNA. To observe the effects of central administration of PTX on RSNA, we administered PTX (10µg/kg body wt.) chronically for 5 weeks using osmotic minipumps in a group of rats (n=8) through intracerebroventricular (ICV) cannulation in CHF rats. No significant difference in RSNA or other parameters was observed in the rats treated with PTX either peripherally or centrally (data not shown). This shows that intraperitonial administration of PTX exerts both central and peripheral effects. Though the antagonism of TNF-α was not successful in larger clinical trials (possibly due to the fact that the Etanercept could bind to only the circulating and not the membrane-bound tissue TNF-α) (Kelly and Smith, 1997), a small clinical trial using PTX was successful in improving cardiac outcomes (Sliwa *et al.*, 2002). Our

results in the present study strongly suggest that using PTX decreases the tissue-bound TNF-α in the PVN and LV.

Oxidative Stress and CHF. Apart from cytokines, ROS have also been implicated in the pathogenesis of CHF. The NAD(P)H oxidases are one of the major sources of superoxide in the heart. This multisubunit enzyme complex consists of two membrane subunits, p22phox and Nox2, and four cytoplasmic subunits, p40phox, p47phox, p67phox and Rac1. Nox2 is the catalytic subunit of NAD(P)H oxidase and it has other isoforms, namely Nox1 and Nox4, that substitute Nox2 in cardiomyocytes and neurons.

NAD(P)H oxidases activated by Angiotensin-II, cytokines (eg.,TNF-α), endothelin-1 and mechanical forces are shown to play an important role in cardiovascular dysfunction (Griendling *et al.,* 2000). Nox1 has been reported to be upregulated in TNF-α induced oxidative stress in coronary arteries of hyperhomocysteinemic rats (Ungvari *et al.,* 2003), while Nox4 is upregulated in aortic smooth muscle cells treated with TNF-α (Moe *et al.*, 2006). Nox2 is demonstrated to be elevated in human CHF (Heymes *et al.,* 2003) and this was also confirmed in cardiomyocytes (Krijnen *et al.,* 2003). In this study we show that Nox2 and its homologues, Nox1 and Nox4, are upregulated in the LV and PVN of CHF rats. These results are also positively correlated to the ROS production, as indicated by the DHE staining (Fig. 1). Inhibition of TNF-α production by PTX has reversed these changes, suggesting a role for cytokines in redox-sensitive signaling in CHF.

PVN and supraoptic nucleus are most strongly implicated in the re-dox mechanisms in the post-MI neurodysregulation (Lindley *et al.,* 2004). In our study, we too show that there is increased ROS in the PVN, more specifically we show that ROS producing machinery is increased in the PVN of CHF rats. Among the Nox's shown, Nox1 is predominantly seen in the magnocellular neurons of the PVN. Thus, it is possible that Nox1 could play a role in regulating

neurohumoral mechanism contributing to CHF. Interestingly, Nox4 expression is increased both in the magnocelluar and parvocellular neurons of the PVN. Nox2 is also increased in the magnocellular and parvocellular neurons of PVN in CHF rats. These finding suggests that expression of different Nox2 homologues in these cell bodies has a significant role in the pathogenesis of CHF and raises the possibility that Nox2 and Nox4 overexpression in the parvocellular neurons contribute significantly to neurohumoral excitation in CHF. Further studies are needed to identify the role of these homologues in sympathoexcitation.

Recent studies showed that microinjection of a NAD(P)H oxidase inhibitor into the PVN reduced the cardiac sympathetic afferent reflexes elevated by central ANG II or the epicardial application of bradykinins (Zhang *et al.*, 2006). Coupled with our findings that a cytokine blocker, PTX, inhibits CHF-induced Nox expression in the PVN and LV, it suggests that oxidant signaling via cytokines in the PVN may be involved in the reduction of catecholamine levels, and RSNA in CHF. It is plausible that one might extrapolate that the effects of PTX observed in the PVN could be due to its other varied effects. However, from the precise anti-cytokine and anti-oxidant effects of PTX observed in this study, it is convincing to conclude that decreased cytokines resulted in the reduction of NAD(P)H oxidase subunits in the PVN, and thus, sympathoexcitaion in CHF.

In conclusion, this study clearly demonstrates that the CHF induced an increase in TNF-α in the PVN and in the LV contributes to the increased expression of NAD(P)H oxidase subunits, Nox1, Nox2 and Nox4. Treatment with PTX, an inhibitor of cytokine production, attenuated the mRNA and protein expression of these subunits and superoxide production, and also decreased circulating levels of catecholamines in CHF rats. Our results suggest that cytokine-induced oxidative stress in the central nervous system and in the periphery contribute to the pathophysiology of CHF. Furthermore, these studies demonstrate that, for the first time, both

cytokines and NAD(P)H oxidase in the PVN contribute to increased plasma catecholamine and exaggerated neurohumoral excitation in CHF.

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CHAPTER 3

CYTOKINE BLOCKADE ATTENUATES SYMPATHOEXCITATION IN HEART FAILURE: CROSS-TALK BETWEEN nNOS, AT-1R AND CYTOKINES IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS*

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INTRODUCTION

Sympathetic hyperactivity is a striking feature of the syndrome of congestive heart failure (CHF). Initially after myocardial injury, there is increased sympathetic activity even before the onset of overt heart failure (Francis *et al.*, 1990). When this sympathetic hyperactivity fails to restore the functioning of the injured myocardium, it results in generalized sympathoexcitation leading to increased vasoconstriction and ventricular remodelling.

Regulation of sympathetic activity is a complex process that involves the activation of several neurohormones, including those of the renin angiotensin system (RAS). Blockade of the AT-1R in the paraventricular nucleus (PVN) restores sympathetic activity in CHF (Zhang *et al.*, 2002). In a previous study we showed that elevated TNF-α and IL-1β in the PVN of rats with acute myocardial infarction (MI) is mediated through cardiac sympathetic afferents (Francis *et al.*, 2004). Furthermore, we demonstrated that elevated cytokines in the PVN induce production of reactive oxygen species (ROS) (Guggilam *et al.*, 2007). ROS, produced in the neurons of the brain, in turn, are cytotoxic, further perpetuating sympathoexcitatory effects (Dawson *et al.*, 1996). Studies from this and other labs have shown that pentoxifylline (PTX), a cytokine synthesis blocker, reduced the central and peripheral production of cytokines and attenuated the production of ROS, renal sympathetic nerve activity (RSNA), as well as plasma norepinephrine levels, an indirect measure of sympathetic activity in CHF rats (Francis *et al.*, 2004; Kang *et al.*, 2006; Guggilam *et al.*, 2007). Additionally, several studies report a cross-talk between pro-inflammatory cytokines and the RAS in both humans and animals. These studies show that treatment with angiotensin II (AngII) resulted in elevation of TNF-α in isolated heart preparations (Frolkis *et al.*, 2001), while pre-treatment with losartan, an AT-1R blocker, attenuated the TNF-α biosynthesis induced by AngII (Gurlek *et al.*, 2001), suggesting that AT-1R expression is closely related to that of TNF-α in the heart (Tsutamoto *et al.*, 2000; Gurlek *et*

al., 2001). These studies explain an apparent interaction between cytokines and AngII in the periphery. However, the cytokine-AngII interaction in the PVN of CHF animals is currently unexplored.

Studies by Patel *et al.* showed that rats with CHF had a decreased message for neuronal nitric oxide synthase (nNOS), and consequently, decreased nitric oxide (NO) in the PVN (Patel *et al.*, 1996; Zhang *et al.*, 1998), indicating loss of regulation of sympathetic tone. Furthermore, AngII-induced sympathetic hyperactivity disrupts the antagonistic mechanism of NO, while increasing superoxide production. This emphasizes the cross-talk between ROS and RAS mechanisms (Rajagopalan *et al.*, 1996). However, it is unknown whether increased cytokines interact with AT-1Rs within the PVN to modulate nNOS and contribute to sympathetic hyperactivity in CHF rats.

In the current study, we examined the hypothesis that increased cytokines in the PVN up-regulate AT-1R expression and deplete nNOS, contributing to exaggerated sympathetic activity in CHF rats. We used PTX to block cytokine synthesis in CHF rats, as this phosphodiesterase inhibitor has been documented to cross the blood-brain barrier (BBB) rapidly and efficiently after systemic administration (Watkins *et al.*, 2003), and inhibit production of TNF-α and IL-1β (Yoshikawa *et al.*, 1999).

METHODS

Studies were performed in male Sprague-Dawley rats weighing 250–300 g. To study the effect of PTX on survival of CHF rats, the study was conducted in two phases. In phase I, rats were pre-treated with PTX (30 mg/kg IP), or its vehicle (10% ethanol, IP), 24 h prior to induction of CHF or Sham surgery, to study the effects on survival of pre-treatment with a cytokine synthesis blocker. In Phase II, rats were subjected to CHF or Sham surgery and subsequently treated with PTX (30 mg/kg IP, daily) or vehicle for a period of 5-weeks. No

significant difference was observed in the molecular and biochemical parameters analyzed between PTX pre-treated and post-treated CHF groups. Therefore, the results from both of these groups were combined and presented as CHF+PTX.

All procedures on animals in this study were approved by the Louisiana State University Institutional Animal Care and Use Committee, and were in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

CHF Model. Heart failure was induced under ketamine+xylazine (90 and 10 mg/kg IP) anaesthesia by coronary artery ligation. In brief, the trachea was intubated, and the rat was placed on an Anesthesia Work Station (Hallowell EMC). A left thoracotomy was performed, the heart was exteriorized and the left anterior descending coronary artery was ligated. Sham-operated rats underwent thoracotomy and manipulation of the heart, except for the ligation of the coronary artery. All rats received analgesics (Buprex, 1 ml/kg SC) following the surgery.

Assessment of LV Function

Echocardiography: Echocardiography was performed 24h after coronary artery ligation or Sham surgery under ketamine (25 mg/kg IP) sedation. Infarct size was estimated by planimetric measurement of the percentage of the LV that demonstrated systolic akinesis. Rats with infarct size $\geq$50% were selected and thereafter treated with PTX or vehicle for 5-weeks. At the end of the 5-week study, a second echocardiographic assessment was performed. Percent ischaemic zone (%IZ), LV ejection fraction (EF), LV end-diastolic volume (LVEDV), and LV end-diastolic volume-to-mass ratio, all indexes of severity of CHF, were determined from short- and long-axis images of the left ventricle (LV). LV mass and volume were calculated using the area length method. After completion of two-dimensional imaging, pulse-wave Doppler interrogation of mitral inflow was performed to determine heart rate (HR). Cardiac output (CO) was calculated as

the product of HR and stroke volume (SV). From mitral inflow, isovolumetric relaxation time and isovolumetric contraction time were measured. The Tei index was calculated as described previously (Guggilam *et al.*, 2007).

Morphological Parameters: At the end of the study, the rats were sacrificed under deep anaesthesia using a carbon dioxide chamber. Lung and left and right ventricular masses were recorded, and the respective indices were obtained by dividing with body mass.

Survival. To calculate the survival in pre- and post-PTX treated groups, rats were monitored for mortality following induction of MI.

Measurement of Circulating TNF-α. Circulating levels of TNF-α were quantified in the plasma samples using a commercially available rat TNF-α ELISA kit (Biosource, Camarillo, CA) as described previously (Tei *et al.*, 1995; Francis *et al.*, 2003).

Estimation of Circulating Catecholamine Levels. Plasma norepinephrine (NE) and epinephrine (EPI) were measured in plasma samples using an Eicom HTEC-500 system fitted with an HPLC-ECD using HPLC-EC as described previously (Guggilam *et al.*, 2007).

Measurement of Renal Sympathetic Nerve Activity (RSNA). RSNA was measured in Sham and CHF rats anesthetized with pentobarbital, as described previously (Guggilam *et al.*, 2007). Following equilibration, the maximum change (increase) in RSNA in response to an intravenous bolus injection of sodium nitroprusside (SNP; 100 μg/kg) was measured in each animal. The raw nerve activity, integrated nerve activity, mean arterial pressure (MAP), and HR were recorded on a Biopac Acknowledge system. At the end of the experiment, the background noise, defined as the signal recorded post-mortem, was measured and subtracted from the actual RSNA recorded, and subsequently expressed as percent change in RSNA from baseline (in response to SNP).

Extraction of PVN by Laser Capture Microscopy (LCM). PVN was captured from frozen brain sections by LCM, as described previously (Guggilam *et al.*, 2007). All the parameters were

set constant to attain a relatively equal amount of input RNA for comparative real-time RT-PCR analyses and protein for western blotting.

RNA Isolation and Real-time RT PCR. Total RNA was extracted from the tissues as described previously (Guggilam *et al.*, 2007). Real-time RT–PCR (qRT–PCR) was performed in 384-well PCR plates, using Bio-Rad PCR Master Mix (The iTaq SYBR^{TM} Green Supermix with ROX) to study the expression levels of TNF-α (X 66539), IL-1β (NM 031512), iNOS (NM 012611), nNOS (NM 052799), AT-1R (NM 031009), and 18S (NR 003278) as the housekeeping gene using the ABI Prism 7900 sequence detection system (Applied Biosystems).

NADPH Diaphorase Staining for nNOS. At the end of week 5, the rats for NADPH diaphorase staining were anaesthetized and perfused transcardially with heparinized saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS, pH 7.4). The brains were removed and post-fixed at 4°C for 4 h in 4% paraformaldehyde solution and then placed in 20% sucrose at 4°C for 24h. Brains were blocked in the coronal plane, and 30-µm-thick sections were cut with a cryostat. The sections were collected in 0.1 M PBS, containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium and 1.0 mg/ml β-NADPH, and were then placed in an incubator at 37°C for 1 hr. After incubation, the sections were rinsed in PBS (pH 7.4) and mounted on glass slides. NADPH-diaphorase positive neurons in the PVN of three adjacent sections at the same coronal level were counted as described by Zheng *et al* (Zheng *et al.*, 2005).

Immunohistochemistry. Immunohistochemistry was performed in formalin fixed sections of the brain as described previously (Guggilam *et al.*, 2007). The primary antibodies against nNOS and 3-nitrotyrosine (3-NT) (Santa Cruz, CA) (1:100) were incubated overnight at 4°C. The sections were then washed twice in PBS, and incubated with a peroxidase conjugated 2° IgG antibody for 30 minutes. Bound antibodies were detected with a streptavidin-peroxidase complex using 3, 3'-diaminobenzidine tetrahydrochloride in PBS containing 0.003% hydrogen peroxide.

Western Blot Analysis. Frozen LV, PVN and hypothalamus proteins were prepared and fractionated in 7.5-10% polyacrylamide gel and transferred to an Immobilon membrane, as described previously (Guggilam *et al.*, 2007). Due to limitation in the quantity of PVN, we used the hypothalamus for protein analysis of AT-1R. The membranes were blocked in 1% Casein in Tris-buffered solution containing 0.1% (v/v) Tween-20 for 1 h and then incubated overnight with antibodies (1:1000) against TNF-α, AT-1R, or nNOS (Santa Cruz, CA), followed by a peroxidase-conjugated goat anti-mouse IgG antibody (1:10,000). The signal was detected using an enhanced chemiluminescence immunoblotting detection system and the net intensity was determined and expressed in relative arbitrary units by normalizing the protein intensity to that of anti-GAPDH antibody.

Statistical Analysis of Data. All results are expressed as mean ± SEM. For statistical analysis of the data, student's *t* test, or one-way ANOVA followed by Bonferroni's post hoc test were used. Survival among treated and untreated groups was analyzed using the Kaplan-Meier analysis of survival followed by the Log-rank test (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA). Values of $p<0.05$ were considered significant.

RESULTS

Effect of PTX Treatment on the Survival of CHF Rats. As shown in table 3.1, pre-treatment with PTX significantly improved 24h survival after induction of CHF as compared to the post-treatment group. None of the Sham animals died during the entire study protocol.

Effect of Blocking Cytokines on Sympathetic Activity. Plasma norepinephrine (NE) and epinephrine (EPI) levels were significantly increased in the CHF rats as compared to those of Sham (Fig. 3.1A). Following PTX treatment, these catecholamine levels were decreased significantly. However, the NE and EPI levels in the Sham rats treated with PTX were not different from those of Sham operated rats.

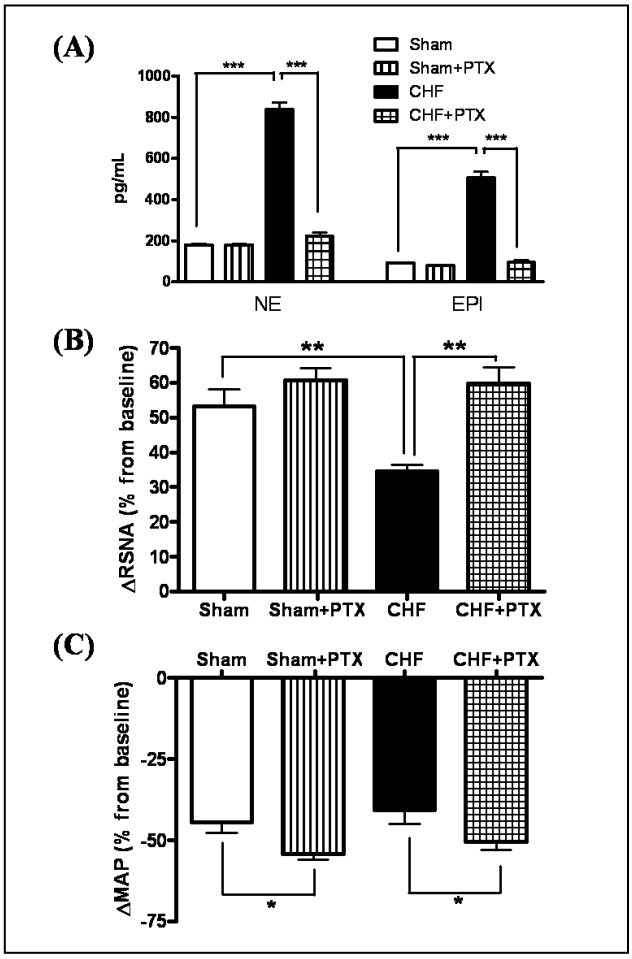

Fig.3.1. Assessment of Sympathetic Activity. (A) Norepinephrine (NE) and epinephrine (EPI) levels in the plasma, samples were analysed using HPLC-ECD. ***p<0.001 (n=5 per group). (B) Percent change in RSNA from baseline in rats in response to SNP IV bolus. (C) Percent change in MAP from baseline in response to IV SNP bolus. Note that although IV SNP produced a similar decrease in MAP between vehicle-treated Sham and CHF rats (respective baseline values; 127.2 ±5.5 mmHg and 124.2 ± 4.1 mmHg), the magnitude increase in RSNA to IV SNP was blunted in the CHF rats when compared to Sham rats. As compared to respective vehicle treated groups, IV SNP produced a greater decrease in MAP in PTX-Sham and PTX-CHF animals (respective baseline values; 112.0 ± 6.7 mmHg and 127.0 ± 4.1 mmHg, respectively). In CHF rats, PTX treatment also augmented the baroreflex control of RSNA in response to IV SNP. *p<0.05; **p<0.01 (n for Sham=9; Sham+PTX=5; CHF=6; CHF+PTX=11)

As shown in figures 3.1B and 3.1C, in Sham rats, IV SNP produced a marked decrease in MAP and a concurrent increase in RSNA. The hypotensive and sympathoexcitatory responses to IV SNP were further augmented in Sham rats treated with PTX. In CHF rats, IV SNP also decreased MAP to a level similar to that observed in Sham rats; however, the magnitude increase

in RSNA evoked by SNP at this dose was significantly blunted. Of merit, IP PTX treatment in CHF rats not only enhanced the hypotensive response to IV SNP, but also augmented/restored the renal sympathoexcitatory response to this hypotensive stimulus.

Effect of PTX Treatment on Cardiac Function and Morphology in HF. Compared to the Sham rats, CHF rats demonstrated increased LVEDV, LVEDP, LV mass, mass/volume ratio and Tei index, and a decreased SV, CO and EF. However, treatment with PTX attenuated only Tei index. The carotid artery was catheterized to measure MAP and pulse pressure (PP). There were no differences in the baseline HR, MAP and PP between Sham and CHF groups (Table 3.1).

Compared with Sham rats, CHF rats had increased right ventricle mass index and lung mass index, which were decreased with PTX treatment, indicating less pulmonary vascular congestion and hypertrophy. No significant difference was observed in the left ventricular mass index.

Effect of PTX Treatment on Cytokine Expression Levels. As shown in figure 3.2, plasma TNF-α and IL-1β levels at 5 weeks remained at, or around, baseline levels during the entire study in the Sham groups. In contrast, levels significantly elevated in the CHF group were attenuated by PTX treatment.

At 5 weeks, the mRNA expression of TNF-α (Fig. 3.3A) and IL-1β (Fig. 3.3B) was significantly increased both in the LV and PVN of CHF rats. PTX treatment significantly decreased these increased levels. PTX treatment in the Sham group had no effect on the myocardial or PVN cytokine mRNA expression. Figure 3.3C illustrates that the elevated TNF-α protein levels in the LV and PVN, as analyzed by Western blot, were significantly attenuated by PTX treatment.

Table 3.1: Echocardiographic and Cardiac Morphological Parameters

	Sham	Sham+PTX	CHF	CHF+PTX
Survival				
PTX pre-treatment				
n	7	7	14	15
24h survival	7 (100%)	7 (100%)	10 (71.4%)*	14 (93.4%)*,#
5-week survival	7 (100%)	7 (100%)	9 (64.8%)*	12 (80%)*,#
PTX post-treatment				
n	7	7	10	10
24h survival	7 (100%)	7 (100%)	7 (70%)	7 (70%)
5-week survival	7 (100%)	7 (100%)	7 (70%)	7 (70%)
Echocardiography				
n	7	7	10	10
HR (bpm)	415.2 ± 17.19	410.0 ± 4.88	407.0 ± 14.09	410.7 ± 16.42
MAP (mmHg)	109±6	108±7	104±6	102±5
PP (mmHg)	39±4	37±3	26±4*	27±2*
LVEDP (mmHg)	4.19±0.08	4.52±0.35	24.38±1.23*	14.17±2.7*,#
LVEDV (μl)	548.4 ± 88.56	517.3 ± 56.87	759.1 ± 24.3*	738.7 ± 52.49*
Mass (mg)	763.4 ± 58.23	807.8 ± 33.77	939.3 ± 28.76*	872.0 ± 46.0
Vol/Mass (μl/mg)	0.62 ± 0.09	0.52 ± 0.07	0.82 ± 0.03*	0.86 ± 0.08*
SV (μl)	473.2 ± 77.15	320.6 ± 41.5	261.7 ± 15.59*	250.6 ± 31.2*
CO (ml/min)	192.6 ± 22.0	157.3 ± 16.9	106.5 ± 7.7*	114.6 ± 16.3*
EF (%)	0.84 ± 0.01	0.82 ± 0.01	0.35 ± 0.02*	0.36 ± 0.03*
IZ (%)	0	0	54.42 ± 1.25*	56.26 ± 0.89*
Tei index	0.39 ± 0.01	0.40 ± 0.01	0.65 ± 0.02*	0.49 ± 0.02*,#
Morphology				
Bodyweight (g)	360±15	359±23	355±12	353±18
LVM (mg)	0.87±0.03	0.88±0.05	1.00±0.08	0.95±0.07
LVMI (mg/g)	2.42±0.09	2.45±0.21	2.81±0.17	2.73±0.19
RVM (mg)	0.23±0.02	0.24±0.03	0.46±0.04*	0.35±0.04*,#
RVMI (mg/g)	0.64±0.09	0.67±0.07	1.31±0.15*	0.99±0.11*,#
LM (g)	1.49±0.064	1.52±0.09	4.53±0.31*	3.17±0.19*,#
LMI (mg/g)	4.15±0.13	4.23±0.10	12.78±0.84*	8.99±0.39*,#

Data are mean ± SEM. HR, heart rate; MAP, Mean arterial pressure; PP, Pulse pressure; LVEDV and LVEDP, left ventricular end-diastolic volume and pressure, respectively; SV, Stroke volume; CO, cardiac output; EF%, percent ejection fraction; IZ%, percent ischaemic zone; LVM and RVM, left and right ventricular mass, respectively; LVMI and RVMI, left and right ventricular mass index, respectively; LM, lung mass; LMI, lung mass index. Mass Index= mass of the organ/body weight. *p<0.05 vs. Sham; #p<0.05 vs. CHF rats

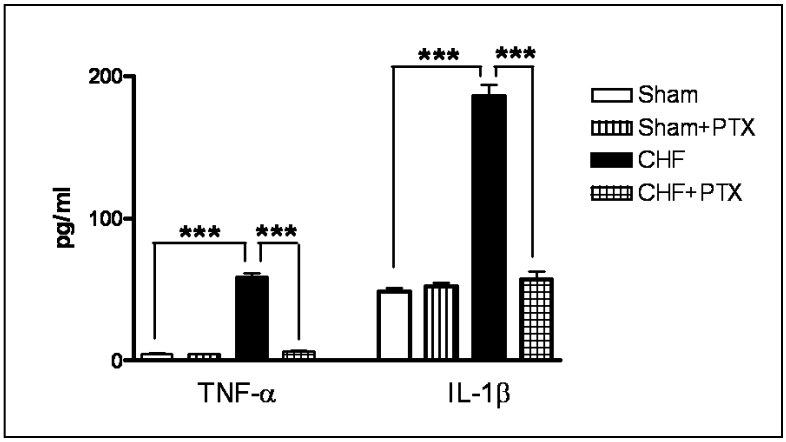

Fig.3.2. Plasma TNF-α and IL-1β levels from Sham and CHF rats Treated with Vehicle or PTX. Data are mean ± SEM. ***p<0.001. For each group n=6.

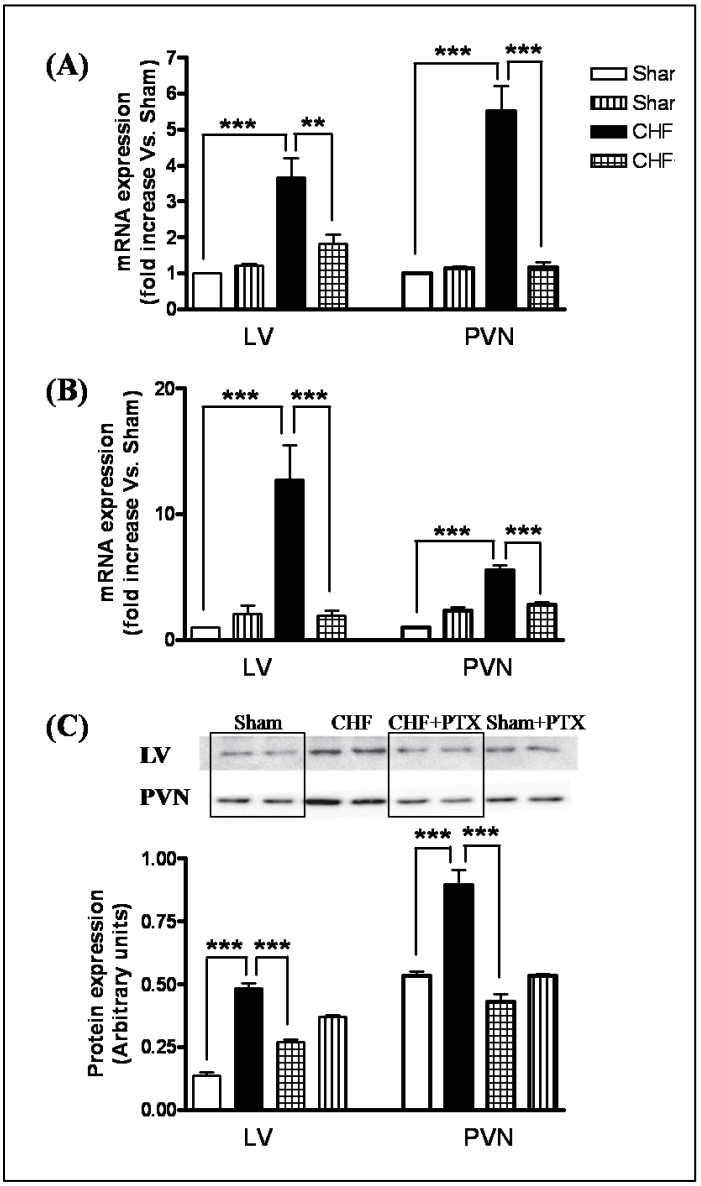

Fig.3.3. Expression of Cytokines in the LV and the PVN. (A) mRNA expression of TNF-α in the LV and the PVN as estimated by real-time RT-PCR. Values are mean ± SEM. (n=6 per group) (B) mRNA expression of IL-1β in the LV and the PVN. Values are mean ± SEM. (n=6 per group) (C) Western blot analysis of anti-TNF-α antibody in the LV and PVN. Bar graphs are mean ± SEM values of band intensities representing four independent experiments. **p<0.01; ***p<0.001

Effect of PTX Treatment on AT-1R Expression. The AT-1R expression was increased significantly in the LV and PVN of CHF rats compared to that of Sham rats, while treatment with PTX restored these levels to normal (Fig. 3.4A). Similarly, the elevated protein levels of AT-1R in the LV and the hypothalamus were restored to normal in PTX treated CHF rats (Fig. 3.4B).

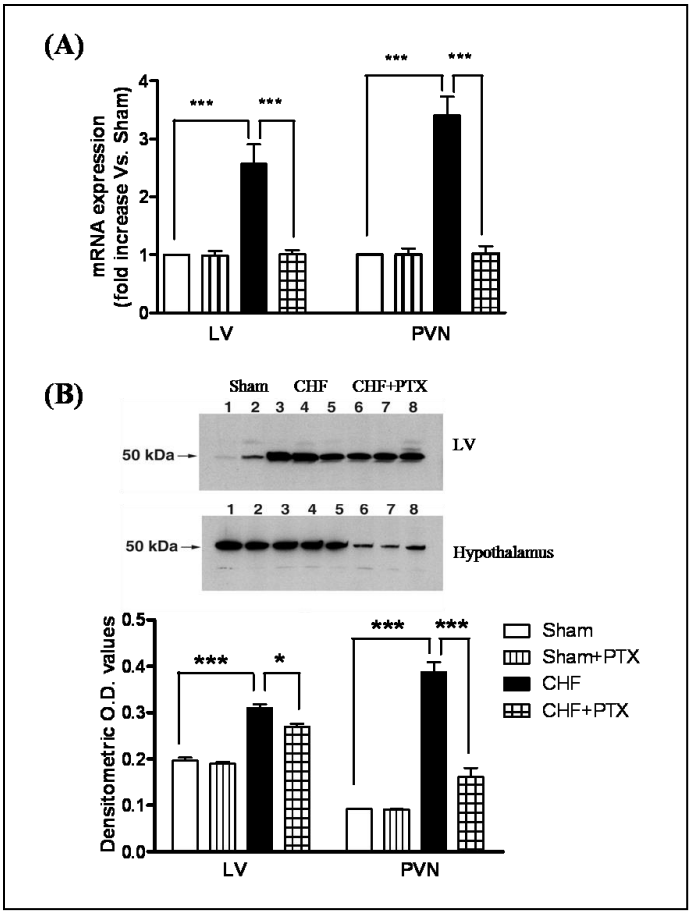

Fig.3.4. AT-1R Levels in the LV and the PVN. (A) AT-1R mRNA expression in the LV and the PVN. ***p<0.001. (n=6 per group) (B) Western blot analysis of anti-AT-1R antibody in the LV and the hypothalamus. Bar graphs are mean ± SEM values of band intensities representing four independent experiments. *p<0.05; ***p<0.001

Effect of PTX Treatment on NOS Levels. The mRNA expression of iNOS was significantly increased, while that of nNOS was significantly reduced in the PVN of CHF rats as compared to those of Sham rats. PTX normalized the expression of iNOS and nNOS within the PVN of CHF rats. No significant change in the nNOS expression was noticed in the PTX treated Sham rats (Fig. 3.5).

Figure 3.6A illustrates the PVN stained positive for NADPH-diaphorase activity. The number of NOS-positive cells in the PVN of CHF rats was significantly less than that of the Sham group. In CHF rats, treatment with PTX restored the number of nNOS-positive neurons in the PVN to a level similar to that observed in the Sham group (Fig. 3.6B). Comparably, the protein expression of nNOS, as determined by Western blot, showed a significant decrease in CHF rats compared to both Sham rats and PTX treated CHF rats. PTX treated Sham rats showed nNOS levels comparable to that of the vehicle treated Sham rats (Fig. 3.6C). Immunohistochemical staining for nNOS protein showed that compared to Sham rats and PTX treated CHF rats, a significant decrease in the number of neurons positively stained for nNOS was observed in CHF rats (Fig. 3.6D).

Effect of Blockade of Cytokines on 3-nitrotyrosine (3-NT) Staining. Diffuse positive immunostaining for 3-NT, an indicator of peroxynitrite, was observed in the surviving cardiomyocytes of the peri-infarct region of the LV of CHF rats (Fig. 3.7A). The PVN of CHF rats also demonstrated an increased staining for 3-NT (Fig. 3.7B and 3.7C). Treatment with PTX significantly reversed these changes both in the LV and in the PVN of CHF rats.

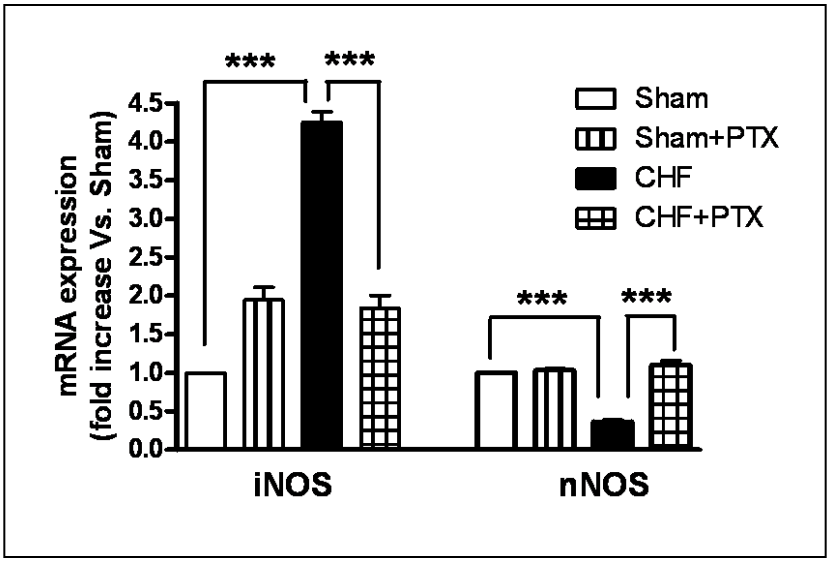

Fig.3.5. mRNA Expression of iNOS and nNOS in the PVN. Values are means ± SEM. (n=6 per group). ****p*<0.001

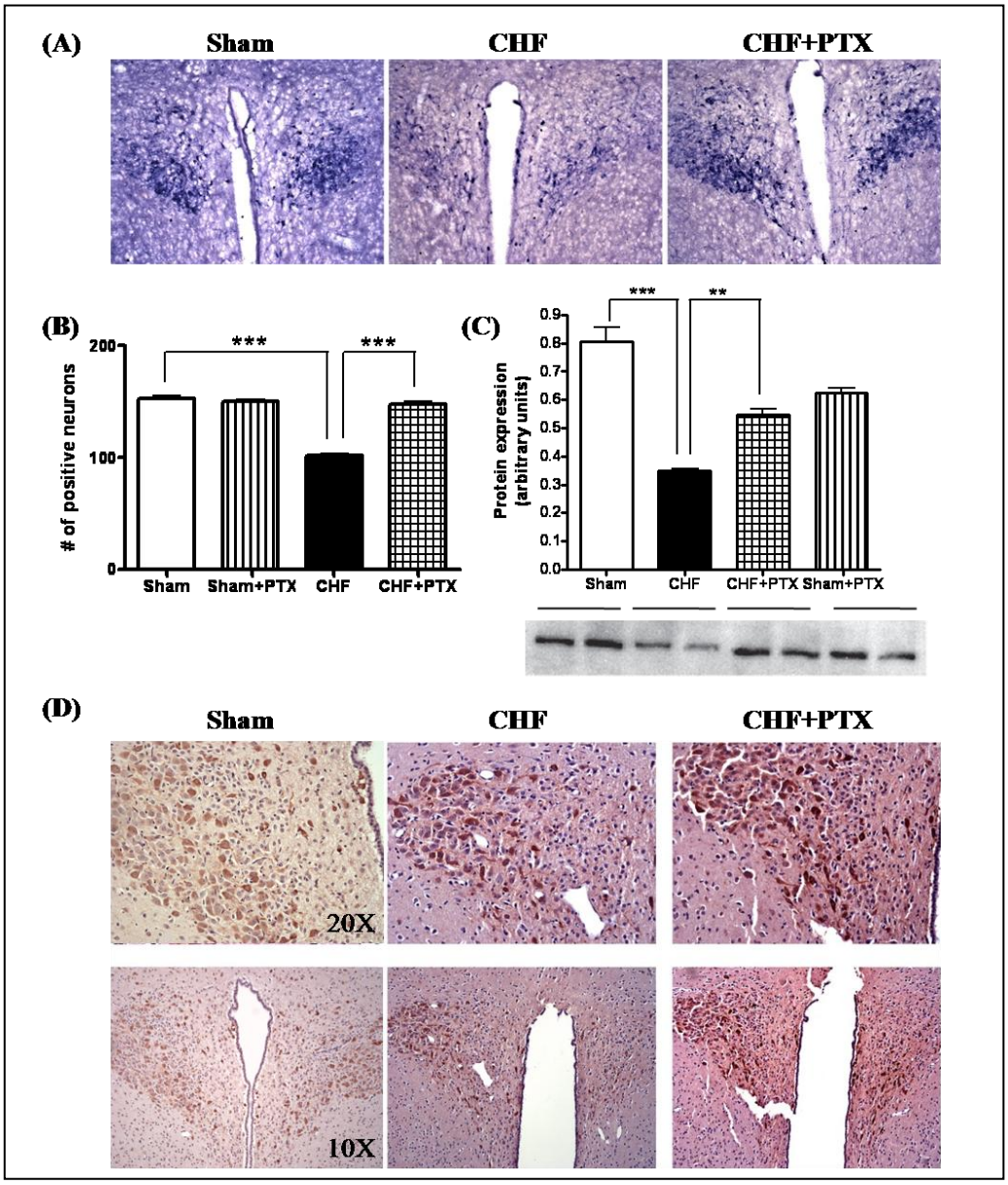

Fig.3.6. Protein Expression of nNOS in the PVN. (A) nNOS activity detected by NADPH diaphorase staining in the PVN of Sham, PTX treated and untreated CHF rats. (Magnification shown 200X). (B) Quantification of nNOS positive neurons from the NADPH diaphorase staining. Data are mean ± SEM. (n=10 per group). (C) Western blot analysis of anti-nNOS antibody in the PVN. Bar graphs are mean ± SEM values of band intensities representing four independent experiments. $^{**}p<0.01$; $^{***}p<0.001$ (D) Immunostaining of the PVN for anti-nNOS antibody (Magnification in the upper panel=400X; lower panel=200X).

DISCUSSION

The novel findings of the present study are 1) Pre-treatment with a cytokine blocker improved survival and LV function in CHF rats; 2) CHF is associated with an increase in TNF-α and IL-β, and a depletion of nNOS, within the PVN. Our molecular and biochemical findings

indicate that blockade of cytokine production by treatment with PTX restored the nNOS levels in the PVN and reduced sympathoexcitation in CHF; 3) AT-1 receptor levels were significantly elevated in the PVN of CHF rats, but not in those treated with PTX, suggesting a cross-talk between cytokines and the RAS in the PVN of HF rats.

Taken together, these results suggest that cytokines contribute to deleterious cardiac effects and decreases in NO bioavailability in the PVN, contributing to enhanced sympathoexcitation and poor survival rate, which is likely mediated via the AT-1R, ultimately suggesting a cross-talk between cytokines and RAS.

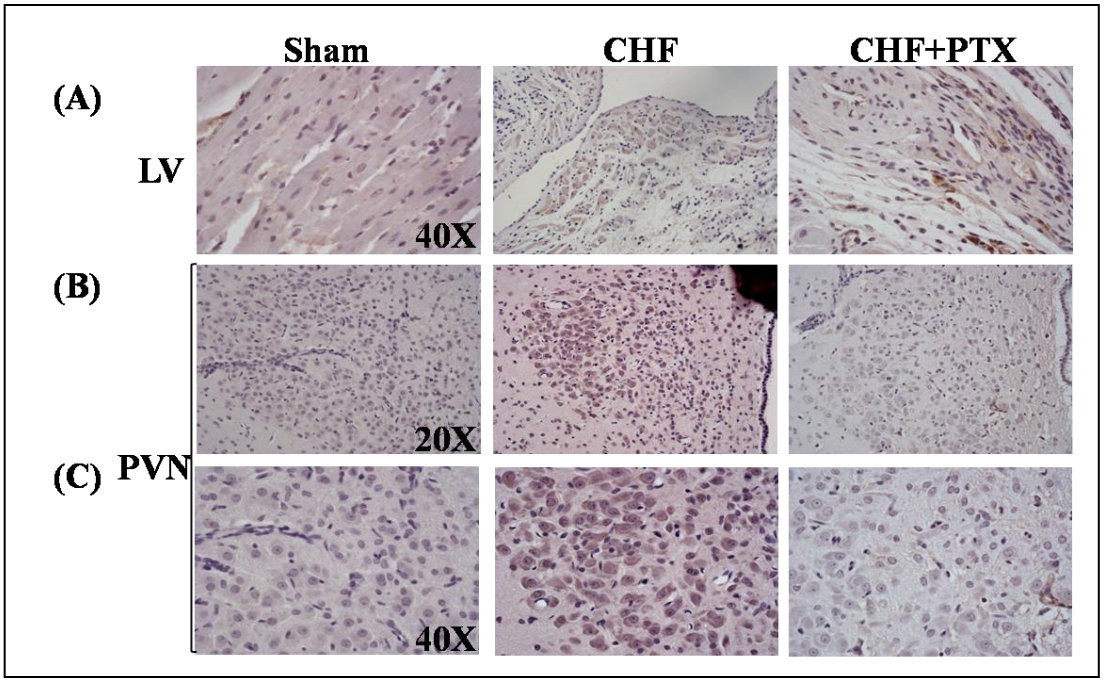

Fig.3.7. Micrograph Showing Immunostaining for Anti-3-Nitrotyrosine in (A) the LV and (B, C) the PVN. Note the evident increase in anti-3-NT signalling in the PVN of vehicle treated CHF rats compared to those of Sham and PTX treated CHF rats.

Increased sympathetic activity (Aronson *et al.*, 2002) and inflammatory cytokines (Kowalewski *et al.*, 2002) result in potentially serious ventricular arrhythmias, the main cause of mortality in CHF. Our results demonstrate that the increased LVEDP, lung weight and right ventricle mass in CHF were significantly lowered after PTX, indicating a selective effect on LV

diastolic function which is comparable with decreased LVEDV. The increased Tei index in CHF was also significantly decreased by PTX. These results indicate an improved LV diastolic and systolic function. In addition, compared to the Sham group, CHF rats demonstrated a blunted sympathoexcitatory response to IV SNP. This finding is consistent with the notion that in CHF, there is an impaired baroreflex control of RSNA (Wang *et al.*, 2003; Francis *et al.*, 2004). Of interest, IP PTX treatment in CHF rats enhanced not only the hypotensive response to IV SNP, but also the magnitude of renal sympathoexcitation to this stimulus. These findings suggest that in CHF, PTX treatment improved the baroreflex mechanisms that influence central sympathoexcitatory outflow to the kidneys. Recent evidence also suggests that blocking the production of cytokines in CHF rats, decreases sympathetic activity (Guggilam *et al.*, 2007; Yu *et al.*, 2007). These adverse effects, including LV dysfunction and remodelling resulting in the progression of HF, might be due to a sustained increase in TNF-α (Mann, 2002; Anker *et al.*, 2004). Thus, treatment with PTX improved cytokine-induced diastolic and systolic dysfunction and reduced sympathetic hyperactivity resulting in improved LV function.

Nevertheless, more complex mechanisms are associated with increased sympathetic activity in CHF than TNF-α alone. Recent studies underscore the importance of the interaction between cytokines and the RAS in cardiac remodelling and increased sympathetic activity in the progression of CHF. AngII and TNF-α can potentiate the effects of each other, resulting in a vicious cycle towards CHF(Tsutamoto *et al.*, 2000; Frolkis *et al.*, 2001; Gurlek *et al.*, 2001). The brain RAS also plays an important role in sympathetic hyperactivity and cardiac remodelling in CHF (Zucker *et al.*, 2004). In the present study, treatment with PTX significantly decreased the elevated expression of AT-1R in the heart and the PVN, improved LV function, and decreased plasma catecholamines alongside a decrease in cytokine levels. These results further reinforce the cross-talk between cytokines and AngII in the PVN in CHF.

Besides its interaction with cytokines, AngII also interacts with NO in the PVN of CHF animals. A reduction in NO in CHF may mediate an amplification of the AngII signal to further increase sympathetic activity (Liu *et al.*, 1998). Positive nNOS neurons of the PVN are important in regulating central sympathetic outflow (Zhang *et al.*, 2001) and increased sympathetic activity in CHF is attributed, at least in part, by decreased nNOS neurons in the PVN (Zhang *et al.*, 1998). The blunted baroreflex in CHF also causes central AngII to augment sympathetic activity in CHF (Barron *et al.*, 1989). Apart from the intrinsic AngII of the brain, circulating AngII molecules can cross the blood brain barrier (BBB) at the circumventricular organs (CVOs), which express AT-1Rs and project multiple neurons into the PVN (McKinley *et al.*, 1998). Upon entry into the brain, these AngII molecules can potentiate the AT-1R as well as TNF-α expression. In addition, MI-activated cytokines within the myocardium that exceeds the limit for utilization by the local cellular receptors in autocrine/paracrine functions become blood-borne and enter the systemic circulation. These cytokines enter the brain through a saturable transport mechanism or a passive transport mechanism via the CVOs, further potentiating TNF-α and AngII, while attenuating nNOS expression. Interestingly, results from the current study also demonstrate that IP PTX treatment normalized the increased levels of AT-1R and decreased levels of nNOS in the PVN of CHF rats, while improving the baroreflex control of RSNA to a hypotensive stimulus. This clearly corroborates our hypothesis that a cross-talk exists between cytokines, AngII and NO within the PVN, contributing to sympathoexcitation in CHF rats.

We also recently reported that increased oxidative stress in the PVN by cytokines is one possible reason for increased sympathetic activity in CHF rats (Guggilam *et al.*, 2007). ROS-induced cytotoxicity in the RVLM was shown to result in sympathoexcitation (Dawson *et al.*, 1996). AngII also exerts a positive feed-forward mechanism in the production of AngII and superoxide, which are further sympathoexcitatory (Rajagopalan *et al.*, 1996). The decreased

nNOS expression, and the increased iNOS expression and formation of peroxynitrite in the PVN, explain NO dysregulation in CHF rats. In the present study, treatment of CHF rats with PTX decreased iNOS expression and prevented the formation of peroxynitrite, thereby reducing the exaggerated sympathetic activity in CHF.

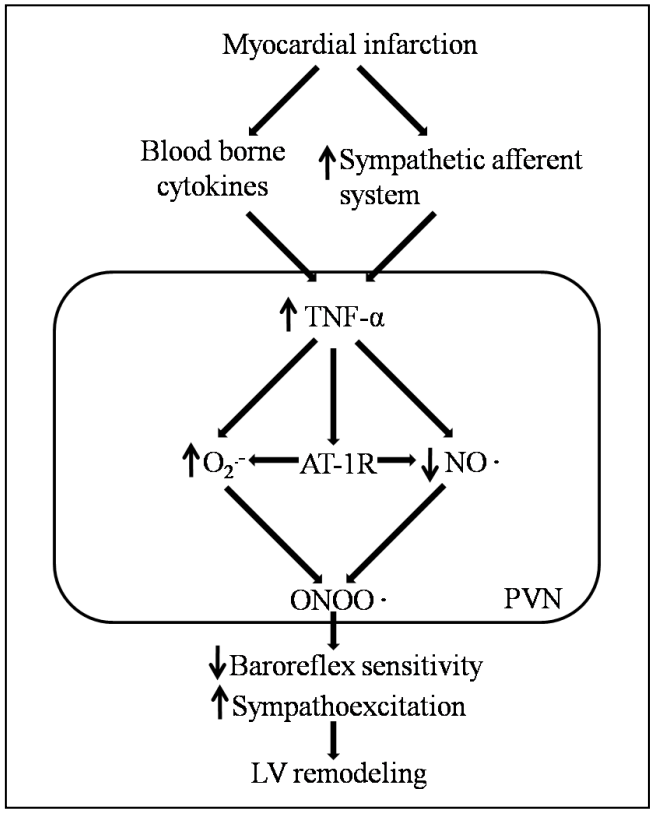

Fig 3.8. Mode of Action of TNF-α in Modulating ROS Production in the PVN and Contributing to Sympathoexcitation. The MI-activated cytokines in the myocardium that become blood-borne, as well as the sympathetic afferent activation, potentiate the expression of TNF-α in the PVN that in turn up-regulates AT-1R expression in the PVN. TNF-α and AngII feed forward each others' effects in the PVN, and together contribute to increased superoxide and decreased bioavailability of NO via peroxynitrite (ONOO·) formation, thus contributing to impaired baroreflex sensitivity and increased sympathoexcitation, ultimately resulting in LV dysfunction.

In addition to the blood-borne cytokines crossing the BBB, cardiac sympathetic afferents also activate hypothalamic synthesis of cytokines in rats (Francis *et al.*, 2004). Similarly, the elevated AT-1R expression in the heart and PVN suggests that both peripheral and central AngII could possibly play a role in depleting nNOS positive neurons in the PVN, possibly via sympathetic afferents. Restoration of nNOS levels by treatment with a cytokine blocker indicates

a cross-talk between cytokines, AngII and NO. This ultimately implies that cytokines might decrease NO either directly, or indirectly, via a pathway involving the AT-1R, the action of which results in sympathetic hyperactivity in CHF (Figure 3.8).

There are a few limitations to this study. In addition to blocking cytokines PTX has a positive inotropic effect on the heart; it is not known at this point whether the effects exerted by PTX are by virtue of its phosphodiesterase inhibitory activity. Nevertheless, we chose this drug due to its effect as a general cytokine blocker. The concept we are introducing in this study is that there is an interaction between cytokines, RAS and nitric oxide in the sympathoexcitatory process observed in CHF. Clearly, further studies are required to specifically delineate the mechanism by which the RAS and NO system interact within the neurons of the PVN in the presence or absence of cytokines.

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CHAPTER 4

TNF CONTRIBUTES TO SYMPATHOEXCITATION IN HEART FAILURE THROUGH MODULATION OF SUPEROXIDE AND NITRIC OXIDE IN THE CENTRAL NERVOUS SYSTEM

INTRODUCTION

Recent studies underscore the importance of central nervous system mechanisms in the regulation of blood pressure and cardiovascular homeostasis. It is well known that increased sympathoexcitation after myocardial infarction (MI) is the major cause of progression of this disease. Elevated tumor necrosis factor (TNF), along with other circulating hormones such as angiotensin II (AngII), in heart failure are typically transported into the brain via the circumventricular organs (CVOs), areas that lack a well formed blood brain barrier (BBB). Once through the BBB, AngII acts in the paraventricular nucleus (PVN) of the hypothalamus to regulate thirst, salt appetite and sympathetic nerve activity, primarily mediated through reactive oxygen species (ROS) production (Zimmerman *et al.*, 2002; Zimmerman *et al.*, 2004; Campese *et al.*, 2005). Our previous study showed that in congestive heart failure (CHF), the increased TNF is related to increased NADPH oxidase subunit expression, the primary source of superoxide anions ($O_2^{\bullet-}$), within the PVN (Guggilam *et al.*, 2007). Moreover, blockade of $O_2^{\bullet-}$ in the PVN completely abolished the increased sympathetic activity associated with CHF (Han *et al.*, 2007). AngII-induced ROS in the rostral ventrolateral medulla (RVLM), an important autonomic regulatory center, also plays a key role in the modulation of sympathetic nerve activity and cardiovascular function (Mayorov *et al.*, 2004; Gao *et al.*, 2005). Blockade of AngII type-1 receptors (AT1Rs) in the PVN (Han *et al.*, 2007) and RVLM attenuated generation of ROS, contributing to decreased sympathetic activity in CHF. In addition, systemic AT1R blockade can decrease the production of TNF, thus limiting cardiac remodeling and dysfunction. However, the importance of central TNF in increased sympathoexcitation is not completely understood.

Recently we reported that the decreased neuronal nitric oxide synthase (nNOS), a primary source of nitric oxide (NO) in the PVN, is attenuated in CHF animals treated with

pentoxifylline, a TNF inhibitor (Guggilam *et al.*, 2008). It is well known that decreased NO production in CHF is associated with increased sympathoexcitation. AngII plays a role in the modulation of NO, which acts, in part, in the production of ROS. Intracerebroventricular (ICV) administration of AngII can decrease nNOS, with its concurrent increase in ROS, in the PVN, ultimately leading to increased sympathetic activity (Campese *et al.*, 2002; Campese *et al.*, 2005). However, after myocardial infarction (MI), elevation of TNF has been observed even before that of neurohormones, including AngII. Therefore, it is possible that the increased TNF observed following MI can trigger the production of other peptides that lead to neurohumoral excitation. To date, the importance of central TNF in increased sympathoexcitation is not completely understood.

We, therefore, hypothesized that TNF induces $O_2^{\bullet-}$ and modulates NO in the PVN and RVLM, possibly through AT1R activation, and contributes to sympathoexcitation in CHF. To explore our hypothesis, we used two approaches: 1) chronic central blockade of TNF with etanercept (ETN, a human recombinant TNF receptor fusion protein that competitively binds with TNF, thereby preventing the binding of TNF to its receptor) in heart failure mice; 2) whole body TNF gene knockout (TNF KO) mouse model to study the role played by TNF in body fluid homeostasis and sympathoexcitation in heart failure. We also explored the interaction between $O_2^{\bullet-}$ and NO in the PVN and RVLM and its contribution to sympathoexcitation in heart failure. The results of this study provide insight into the mechanisms that induce sympathoexcitation and disease progression in the failing heart.

METHODS

Mice. Male TNF KO (B6;129S-*Tnf*^{tm1Gkl}/) and wild-type (WT) mice (Jackson Laboratory, ME) of 12-16 weeks of age with similar B6129SF2/J genetic background were used in the present study. Mice were housed in a light (12 h light-dark cycle) and temperature-controlled room and

standard chow and water were provided *ad libitum*. All surgical procedures in animals were performed at Louisiana State University School of Veterinary Medicine and were carried out in accordance with the regulations of the Louisiana State University Animal Care and Use Committee and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Drugs. Etanercept (Enbrel) was purchased from Amgen and Wyeth Pharmaceuticals (Collegeville, PA). The drug was dissolved in artificial cerebrospinal fluid (aCSF) for ICV infusion. The dose used in this study was optimized by preliminary experiments conducted in our lab.

Experimental Protocol and Surgical Procedures. To explore our hypothesis, the study was conducted in two protocols. In protocol I, we studied the effect of central blockade of cytokines on sympathoexcitation. In this protocol, WT mice had a cannula implanted into their right lateral cerebral ventricle, using the following stereotaxic coordinates: 0.3mm posterior to bregma, 1.0mm lateral from midline and 2.8mm from the surface of the skull. After one week of recovery, they underwent either coronary artery ligation to induce MI or sham surgery. While still under anesthesia, an 28-day osmotic minipump (Alzet) was implanted subcutaneously on the back of the neck. The minipump was connected to the cannula in the lateral ventricle for continuous infusion (0.11μl/hr) of ETN (5μg/hr) or vehicle (VEH; aCSF) over a 4-week treatment interval.

In protocol II, we used TNF KO mice, along with WT mice, to study the effect of TNF gene ablation on sodium and fluid retention and subsequent sympathoexcitation. In this phase, mice were acclimated in custom designed metabolic cages for one week. The mice then underwent coronary artery ligation and thereafter were maintained in metabolic cages with free

access to food, water and 1.8% NaCl solution. Water and salt intake were measured and 24 h-urine collections were obtained for the duration of the study.

At the end of the study, mice from both protocols were anesthetized and sacrificed by decapitation. Blood samples were collected for plasma norepinephrine (NE) measurement. Hypothalamus and brainstem samples were collected for gene expression studies. Mice that were used for ESR studies were injected with heparin (100U/25 g body wt.) and then perfused transcardially with ice-cold buffer, and these brain regions were collected and processed for ROS measurement. For immunofluorescence studies, anesthetized mice were perfused transcardially with ice-cold heparinized saline, followed by formalin. The tissues were stored in formalin until further processed.

Surgical Procedures

ICV Cannula: Mice were anesthetized with ketamine/xylazine (80 mg/kg ketamine+10 mg/kg xylazine, I/P). Following placement into the stereotaxic apparatus (Kopf instruments, CA), an incision was made through the skin, fascia was removed and the skull was exposed. After the skull was leveled in the rostral/caudal direction, a small, stainless steel screw was inserted into the skull and the cannula was inserted through a drilled hole at the position previously mentioned. Dental acrylic was used to fix the cannula in place and the skin was sutured over the hardened dental acrylic cap. Mice were treated with analgesics and allowed to recover for 1 week before induction of myocardial infarction.

Experimental Myocardial Infarction (MI): Mice were premedicated with atropine sulfate (0.04 mg/kg SC) and anesthetized 5 min later with 2% v/v isoflurane/oxygen. Mice were placed on a heating pad in supine position and the trachea was intubated using a 24-guage intravenous catheter with a blunt end. Anesthesia was maintained by supplementing oxygen (1.8 L/min) and 0.7%-2.0% isoflurane at a rate of 105/min and with a tidal volume of 2.1-2.5 ml using a rodent

ventilator (Harvard Apparatus, Inc., MA). A left lateral thorocotomy was performed by blunt incision in the fourth intercostal space, the left anterior descending (LAD) coronary artery was ligated using a prolene monofilament with tapered needle (Ethicon USP 7-0, Johnson & Johnson Co., NJ) 2-3 mm from the tip of the left auricle with the aid of a dissecting microscope (Zeiss) to induce MI. After coronary artery ligation (CAL), the chest was closed, and the mouse was allowed to recover. In sham-operated animals, the suture was placed under the LAD artery and removed without ligation of the vessel. Operated mice were monitored and studied for a period of 5-weeks following surgery.

Osmotic Mini-pump Implantation: Immediately following MI, while still under anesthesia, a subcutaneous incision was made upon the back of the mouse, a pocket was made between the skin and muscle and washed with sterile saline. Single 28-day osmotic mini-pumps with polyvinyl tubing (0.027"I.D. x 0.045"O.D.) attached to the pump injector were implanted in each mouse. The tubing was inserted under the skin and connected to the cannula in the lateral ventricle and fixed into place with dental acrylic cement. The skin incision was closed and the mice were treated with analgesics.

Metabolic Studies. To study relative fluid and sodium balances among different treatment groups, we performed metabolic studies by placing animals in custom-designed individual metabolic cages with free access to food, water and 1.8% NaCl solution, in a temperature-controlled environment and a 12/12 h light–dark cycle. Three days after acclimation, water and salt solution intake were measured and 24 h-urine collections were obtained for the duration of the study. Sodium intake (mEq) was calculated as salt solution intake multiplied with salt content/equivalent weight of sodium. All sodium chloride is expressed as sodium. Urine samples were collected under saturated oil in graduated conical tubes to avoid evaporative losses and their volumes recorded. All aliquots of urine were immediately frozen at -20°C until urinary

sodium (U_{Na}) was determined by a Nova electrolyte 16+ analyzer (Nova Biomedical, Waltham, MA). Total 24 h U_{Na} excretion was calculated by multiplying the measured concentration with the daily urine volume.

Echocardiography. Cardiac function and development of CHF were analyzed by echocardiography 24 h and 4- weeks after induction of MI. Echocardiography was performed in mice anesthetized with 1.5% isoflurane/oxygen with Toshiba Aplio SSH770 system (Toshiba Medical Systems, CA) fitted with a PLT 1202 linear transducer (12 or 14 MHz), which generates two-dimensional images at frame rates ranging from 300 to 500 frames per second. Left ventricular (LV) wall thickness, LV end-diastolic dimension (LVD), LV end-systolic dimension (LVS), LV end-systolic posterior wall thickness (PWS), and LV end-diastolic posterior wall thickness (PWD) were measured using two-dimensional short-axis imaging. LV percent fractional shortening (%FS) was calculated as: %FS = (LVD – LVS)/LVD x100%. The portion of the LV that displays akinesis was electronically planimetered and expressed as a percent of the total LV silhouette to estimate the size of the infarct. Only the mice that had an infarct size of 40-50% were used for the study.

Semi-quantitative Real-time RT-PCR. RNA was isolated from hypothalami and brainstems with TRIzol (Invitrogen, CA), treated with DNAase, and reverse transcribed using random primers reverse transcriptase. Gene transcripts were determined by quantitative real-time polymerase chain reaction using SYBR-Green master mix (Applied Biosystems, CA) on an Applied Biosystems 7900. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to 18S gene. The level of change was expressed as fold change versus respective sham values.

Electron Spin Resonance (ESR) Studies. One of the most sensitive and definitive methods of measurement of superoxide production is electron spin resonance (ESR). In this study, we

utilized an established technique for superoxide anion ($O_2^{\bullet-}$) and $OONO^{\bullet}$ measurement in the hypothalamus/brainstem using ESR and the spin trap. Two different spin probes were used for ESR studies. 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH) was used to measure $O_2^{\bullet-}$ levels, and 1-hydroxy-3-carboxypyrrolidine (CPH) was used to measure peroxynitrite ($OONO^{\bullet}$) levels. All ESR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super-high quality factor (Q) microwave cavity (Bruker Company, Germany).

Sample Preparation for ESR Studies*:* The dissected hypothalamus/brainstem from each animal was placed into a 24-well plate containing Kreb's HEPES buffer (KHB) (20mM, pH 7.4) Tissue pieces were then washed twice with the same buffer to remove any trace contamination. Samples were then incubated at 37^{o}C with specific spin probes for 30 minutes.

Total Tissue $O_2^{\bullet-}$ Production: Tissue pieces were incubated at 37^{o}C with CMH (200 μM) for 30 minutes. Aliquots of the incubated probe media were then taken in 50 μl glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) for determination of total ROS production, under the following ESR settings: field sweep 50 G; microwave frequency 9.78 GHz; microwave power 20 mW; modulation amplitude 2 G; conversion time 327 ms; time constant 655 ms; receiver gain 1 x 10^5. For superoxide production, samples were pre-incubated at 37^{o}C with PEG-SOD (50 U/ml) for 30 minutes, then CMH (200uM) for an additional 30 minutes. Aliquots of the incubated probe media were taken in 50 μl glass capillary tubes for determination of total superoxide production. Addition of PEG-SOD to CMH allowed competitive inhibition of the $O_2^{\bullet-}$-CMH oxidation reaction by the quenching of $O_2^{\bullet-}$ radicals. Since it is cell permeable, PEG-SOD can competitively inhibit the CMH-$O_2^{\bullet-}$ interaction both intracellularly and extracellularly, thus allowing accurate measurement of total tissue $O_2^{\bullet-}$ production. To determine actual total tissue superoxide production, the values obtained from

incubation with PEG-SOD and CMH were subtracted from the values obtained from incubation with CMH only.

Tissue $OONO^{\bullet}$ Production: Tissue pieces were incubated at 37°C CPH (500 μM) and the reaction was initiated by adding cysteine (100uM) for 30 minutes. The reaction of $OONO^{\bullet}$ with the thiols of free cysteine is the first direct reaction of $OONO^{\bullet}$, and cysteine is the amino acid that reacts the fastest with peroxynitrite. Aliquots of the incubated probe media were then taken in 50 μl glass capillary tubes for determination of $OONO^{\bullet}$ production following the parameters previously outlined.

Double-labeling Immunofluorescence. Fluorescent immunohistochemistry was performed as described previously (Khaleduzzaman *et al.*, 2007) with minor modifications. The anesthetized mice were perfused transcardially with heparinized saline followed by 10% neutral buffered formalin. Brains were then collected and stored in 4% paraformaldehyde until analyzed. The brains were embedded in paraffin and 10 μm thick sections were cut on Superfrost plus slides (Fischer scientific) and incubated overnight at 56˚C. The slides were then deparaffinized in xylene, and rehydrated in descending grades of ethanol. Antigen retrieval was performed by incubating slides in Reveal decloaker, pH 6.0, for 30 min at 120°C in a decloaking chamber (Biocare Medical, Concord, CA) at 17 to 19 lb/in^2 and cooled to 90°C. Following equilibration to RT, slides were incubated in 0.2% fish skin gelatin (FSG) (Sigma-Aldrich, St. Louis, MO) in PBS for 10 min. This solution was also used to wash slides between all incubations. Tissues were blocked in a humidity chamber for a minimum of 30 min with blocking solution consisting of 2% donkey serum (Sigma), 1% bovine serum albumin (BSA) (Sigma), 0.05% FSG, 0.1% Triton X-100 (Sigma), and 0.05% Tween 20 (Bio-Rad) in PBS. To identify neuronal cells, the slides were incubated overnight at 4°C with a 1/100 dilution of mouse polyclonal anti-neuronal nuclear protein (NeuN) (Molecular Probes). Slides were then incubated with 1/300 dilution of

biotinylated mouse anti-goat immunoglobulin G (IgG) or goat anti-rabbit IgG (Molecular Probes) for 30 min at RT and then incubated with 1/500 dilution of streptavidin conjugated to Alexa Fluor 594 (Invitrogen).

For dual staining, slides were incubated in blocking solution for 30 min, incubated with rabbit polyclonal anti-nNOS or anti-3-nitrotyrosine (3-NT) or an antibody that recognizes c-Fos, FosB, Fra-1, or Fra-2 (c-Fos K25, Santa Cruz) in 0.2% FSG in PBS at 4°C overnight. Slides were then incubated with 1/300 dilution of biotinylated goat anti-rabbit IgG (Molecular Probes) for 30 min at RT and then incubated with 1/500 dilution of streptavidin conjugated to Alexa Fluor 488 for 30 min at RT. Slides were mounted with ProLong Gold antifade reagent (Molecular Probes) and allowed to set for overnight at 4°C. The lack of nonspecific staining was confirmed by using no primary antibody controls (data not shown).

Data Analyses. All data illustrated are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, CA, www.graphpad.com. Log-rank (Mantel-Cox) test was used to compare the survival rates between groups. Student's t-test was used to compare the fold changes in mRNA levels between WT+MI and TNF KO+MI groups. One-way ANOVA was used to observe the differences among groups, and two-way ANOVA or ANOVA with repeated measures was used comparison of metabolic parameters, followed by Bonferroni's correction. Spearman's correlation was used to measure the strength of the relationship between 24-h sodium excretion and calculated daily sodium intake. Survival rates were compared using log rank test. In all cases, $p<0.05$ was considered statistically significant.

RESULTS

Improved Survival in TNF KO Mice After MI. In protocol I, within the first one week, 33.3% mortality was observed in MI+VEH mice, while the mortality rate was reduced to 10% in mice

treated centrally with TNF blocker (MI+ETN mice) (Figure 4.1A). In protocol II, within 24 h after CAL surgery, <5% mortality was observed in both TNF KO and WT sham mice. At the end of the study, the mortality was 20.1% in WT+MI mice while the mortality was 6.7% TNF KO+MI mice. Most deaths occurred within the first 10 days and were a result of cardiac rupture as determined by findings at autopsy. However, compared to the Sham mice, significantly more WT+MI mice (p=0.0054) died during the study than the TNF KO+MI mice (p=0.15) (Figure 4.1B).

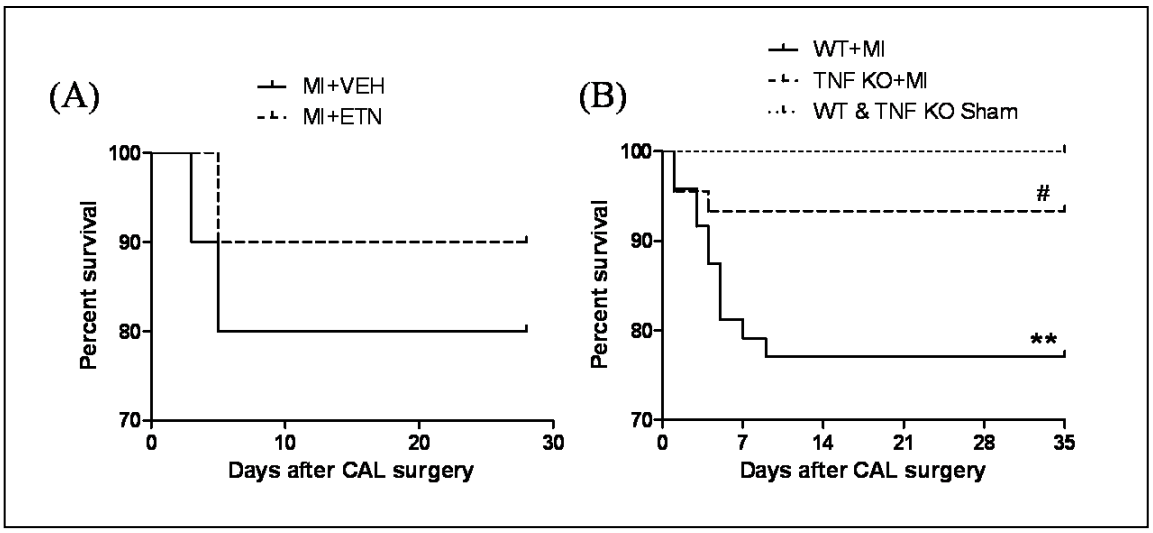

Figure 4.1 Kaplan–Meier Survival Curve in WT and TNF KO Mice at the End of the Study. (A) Survival rate was improved in CHF mice treated with ETN compared to those treated with VEH. (B) The WT mice with CHF exhibited a significant (**, $p < 0.01$) decrease in survival compared with sham-operated WT and TNF KO mice 4wks after MI. TNF KO mice demonstrated significantly ($^{\#}$, $p<0.05$) greater survival compared with WT 4wks after MI.

Decreased Fluid and Sodium Retention in TNF KO Mice. Figure 4.2 shows the effect of TNF on fluid intake, urine output (UO), sodium intake and sodium excretion (U_{Na}) in WT and KO mice. Water intake was significantly decreased, while salt or sodium intake was dramatically increased by day 2 and day 3 in WT+MI mice. After day 3, water intake remained constant in all the experimental groups, while sodium intake by hypertonic saline drinking remained elevated in WT+MI mice throughout the study, but not in the Sham or TNF KO+MI mice. UO and U_{Na} were

significantly decreased in WT+MI mice. On the contrary, the UO and U_{Na} tended to decrease till day 3 but later returned to be in line with those of sham-operated WT and TNF KO mice. In this study, dietary sodium intake was not considered as there was no significant difference in food intake among different groups.

In sham-operated WT and TNF KO mice, the correlation coefficient between daily sodium intake and U_{Na} excretion was 0.26 (p=0.24) and -0.24 (p=0.27), respectively. However, the corresponding correlation coefficients for WT and TNF KO heart failure mice were -0.82 (p<0.0001) and 0.66 (p<0.01), respectively.

Improved Cardiac Function in TNF KO Mice After MI. Echocardiography revealed an improvement in the %FS after 4wk ETN treatment, while no improvement in %FS was observed in VEH-treated heart failure mice (Table 4.1). CHF was associated with marked decrease in percent fractional shortening at 4-weeks in WT+MI mice compared to that of TNF KO+MI mice (Table 4.2). However, no significant difference was observed in LV dimensions over the 4wk period, which might be due to the large infarct size in these mice.

Heart and lung weights were measured at the end of the 4-week study (Figure 4.3). Compared to the WT+Sham mice, the wet lung weight ratios, but not the heart weight ratios, were significantly higher in the WT+MI mice, suggesting development of pulmonary congestion. We did not observe any difference among their heart weight ratios possibly because of the infarct size. On the contrary, lung weights in TNF KO+MI mice were significantly lower than those in the WT +MI mice with similar infarct size, indicating decreased pulmonary congestion with TNF ablation.

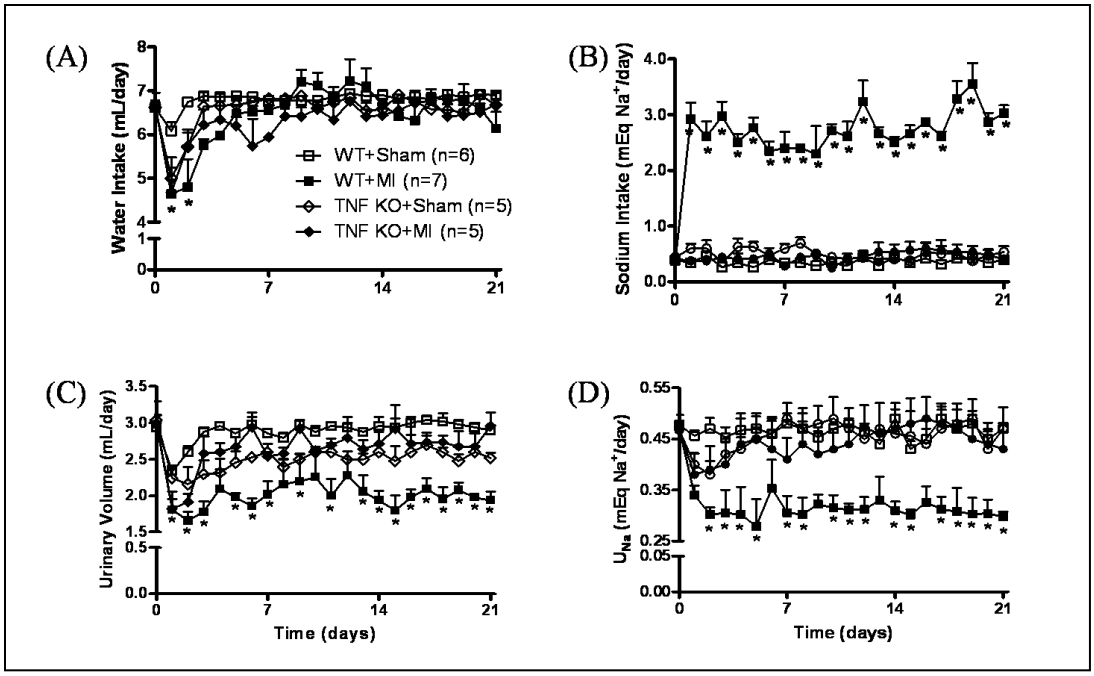

Figure 4.2 Metabolic Parameters. Daily water intake (A), sodium intake (B), urine output (C), and sodium excretion (U_{Na}) (D) of WT and TNF KO mice during the first 3wks after MI or sham surgery. Data are presented as means±SEM.

Table 4.1: Echocardiographic Findings in Mice from Protocol I

	Sham		MI+VEH		MI+ETN	
	24h	4wk	24h	4wk	24h	4wk
IVSS	1.33±0.02	1.41±0.09	1.18±0.11	1.13±0.14	1.25±0.24	1.18±0.13
IVSD	0.84±0.03	0.88±0.05	0.67±0.04	0.74±0.04	0.69±0.04	0.63±0.06
LVS	3.04±0.16	3.21±0.28	2.61±0.42	3.21±0.30	2.95±0.19	3.27±0.92
LVD	4.40±0.11	4.50±0.15	4.03±0.38	4.76±0.32	4.03±0.22	4.75±0.57
PWS	1.33±0.07	1.05±0.06	1.28±0.07	1.21±0.10	1.20±0.06	1.16±0.10
PWD	0.84±0.07	0.82±0.04	0.80±0.07	0.74±0.05	0.78±0.05	0.86±0.29
%FS	32.76±2.83	33.58±0.94	36.37±2.18	27.13±1.20**	34.89±1.17	44.23±3.80*

Data are mean±SEM. IVD and IVS, left ventricular internal diameter at end-systole and end-diastole, respectively; LVD and LVS, left ventricular septal thickness at end-diastole and end-systole, respectively; PWD and PWS, posterior wall thickness at end-diastole and end-systole, respectively; %FS, fractional shortening. (*, $p<0.05$; **, $p<0.01$)

Table 4.2: Echocardiographic Findings in Mice from Protocol II

	WT		TNF KO	
	MI 24h	MI 5wk	MI 24h	MI 5wk
IVSS	1.01±0.08	1.26±0.15	1.03±0.08	1.02±0.06
IVSD	0.69±0.02	0.78±0.07	0.61±0.03	0.69±0.04
LVS	3.17±0.04	2.99±0.18	3.11±0.26	2.95±0.18
LVD	4.21±0.07	4.41±0.08	4.16±0.23	3.96±0.20
PWS	1.04±0.07	1.21±0.08	1.05±0.06	1.08±0.05
PWD	0.68±0.05	0.83±0.08	0.66±0.04	0.70±0.03
%FS	34.60±1.46	25.23±0.79*	25.89±2.48	25.59±1.92

Data are mean±SEM. IVD and IVS, left ventricular internal diameter at end-systole and end-diastole, respectively; LVD and LVS, left ventricular septal thickness at end-diastole and end-systole, respectively; PWD and PWS, posterior wall thickness at end-diastole and end-systole, respectively; %FS, fractional shortening. (*, $p<0.05$; **, $p<0.01$)

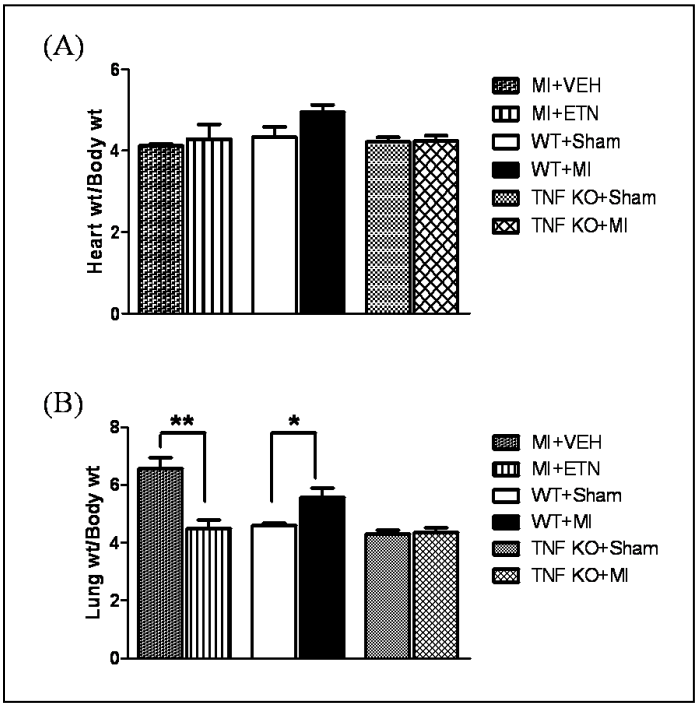

Figure 4.3 (A) Heart weight/body weight ratio of WT, ETN-treated and TNF KO mice 4wks after sham or CAL. (B) Lung weight/body weight ratio of WT, ETN-treated and TNF KO mice 4wks after sham or CAL. WT+CHF mice exhibited significantly greater fluid accumulation in the lungs as compared with TNF KO+CHF.
(*, $p<0.05$; **, $p<0.01$)

Altered Cytokine Expression in the Hypothalamus and Brain Stem in CHF. The increases in transcript levels of TNF, IL-1β and IL-6 were significantly higher in the hypothalamus and brainstem of MI+VEH compared to MI+ETN mice indicating that blocking TNF centrally results in the decreased production of other pro-inflammatory cytokines in these cardiovascular regulatory centers. Compared to the TNF KO+MI mice, these levels were higher in WT+MI mice. On the contrary, WT+MI mice demonstrated a decrease in IL-10, an anti-inflammatory cytokine, and this was restored in MI+ETN and TNF KO+MI mice (Table 4.3).

Altered Expression of $O_2^{\bullet-}$ and NO Sources in the Hypothalamus and Brain Stem in CHF. The expression of Nox2 and Nox4, homologues of the major catalytic subunit of NADPH oxidase, were up-regulated both in the hypothalamus and brainstem regions of MI+VEH compared to the ETN-treated mice. These levels were also higher in WT+MI mice compared to the TNF KO+MI mice (Table 4.3). These findings are consistent with the $O_2^{\bullet-}$ production observed in the PVN and VLM regions (Figure 4.4).

The expression of nNOS was decreased, while that of inducible NOS (iNOS) was increased in the hypothalamus and brainstem of MI+VEH mice as well as WT+MI mice compared to that of ETN-treated or sham-operated/TNF KO+MI mice, respectively (Table 4.3).

Immunofluorescence studies demonstrated a significant decrease in the number of nNOS-positive neurons in the VLM of WT+MI mice as compared to both of the sham-operated groups. Neurons were identified by co-staining with the neuronal marker NeuN. In the VLM of TNF KO+MI mice, the number of nNOS-positive neurons was not different from that of TNF+Sham mice (Figure 4.5). We observed a similar pattern of nNOS expression in the PVN (Data not shown).

Table 4.3. Gene Expression of Cytokines, Nitric Oxide Synthases, Nox Homologues, and AngII Receptors in the Hypothalamus and Brainstem of WT and TNF KO Mice at 4wks following MI.

Category	Transcript	Hypothalamus			Brainstem		
		MI+VEH	MI+ETN	TNF KO+MI	MI+VEH	MI+ETN	TNF KO+CHF
Cytokines	TNF	2.38±0.09	0.40±0.34*	0.88±0.06*	2.01±0.17	0.81±0.85*	1.15±0.09
	IL-1β	3.73±0.06	1.30±1.72*	1.52±0.43*	2.81±0.36	1.96±1.00	1.39±0.29*
	IL-6	1.64±0.11	1.08±0.38	0.96±0.08	1.38±0.14	1.02±0.31	1.11±0.18
	IL-10	1.11±0.08	4.31±1.96*	3.13±0.66*	0.53±0.03	2.21±1.71*	1.01±0.13*
Nitric oxide synthases	nNOS	0.59±0.09	1.27±0.22	1.84±0.11*	0.69±0.09	1.26±0.42*	1.66±0.36*
	eNOS	0.90±0.10	1.20±0.05*	1.94±0.25*	1.07±0.07	1.26±1.13	1.76±0.20
	iNOS	2.57±0.12	0.98±0.06*	0.75±0.05*	0.90±0.19	1.24±0.16	0.63±0.06
Nox homologues	Nox2	3.08±0.38	1.44±1.00*	1.21±0.24*	1.96±0.25	1.88±0.23	0.80±0.17*
	Nox4	1.58±0.15	0.99±0.08*	0.84±0.12**	1.28±0.11	1.03±0.07	0.78±0.04**
AngII receptors	AT1R	1.96±0.19	0.92±0.12*	0.64±0.09**	1.79±0.03	1.04±0.16	0.76±0.03**
	AT2R	0.86±0.09	1.56±0.04*	1.97±0.26*	0.81±0.09	1.28±1.17	1.42±0.07**

Values expressed are means±SEM of fold change ($2^{-\Delta\Delta Ct}$) versus respective Sham group. (*, $p<0.05$; **, $p<0.01$)

TNF Plays a Role in the Interaction between $O_2^{\bullet-}$ and NO in CHF. To assess the interaction between $O_2^{\bullet-}$ and NO in the PVN and VLM regions, we used immunofluorescence co-staining with 3-NT, a product of $OONO^{\bullet}$, and nNOS. The percent nNOS-positive cells that stained for 3-NT were comparatively higher in the PVN after 4-weeks of MI in both WT and TNF KO mice compared to their respective sham-operated groups. However, the number of 3-NT-positive neurons was significantly higher in the WT+MI mice compared to the TNF KO+MI (Figure 4.6). Similar effects with regard to nNOS and 3-NT expression were seen in the VLM region of WT and TNF KO mice with/without CHF (Data not shown).

We further confirmed the formation of $OONO^{\bullet}$ in PVN and VLM by ESR. CHF resulted in increased $OONO^{\bullet}$ levels in these brain regions in ICV ETN-treated mice compared to that of VEH-treated mice (Figure 4.5). In addition, significantly higher $OONO^{\bullet}$ production was observed in WT mice compared to TNF KO heart failure mice (Figure 4.5). Although $O_2^{\bullet-}$ and $OONO^{\bullet}$ are produced in other brain regions, only the PVN and VLM showed significant differences in CHF and sham mice. Therefore, quantitative analyses presented are focused on these regions.

TNF Alters Angiotensin Receptor Expression in the Hypothalamus and Brainstem in CHF. We observed a decrease in AT1R expression in the hypothalamus and brainstem of heart failure mice treated ICV with ETN compared to those of VEH-treated mice (Table 4.3). CHF also induced a significant increase in the AT1R expression in the hypothalamus and brainstem of WT mice over TNF KO mice (Table 4.3). On the contrary, CHF induced a marked decrease in the expression of AT2R in these brain regions in WT mice compared to TNF KO mice (Table 4.3). No difference was observed in the expression of these receptors in sham-operated mice.

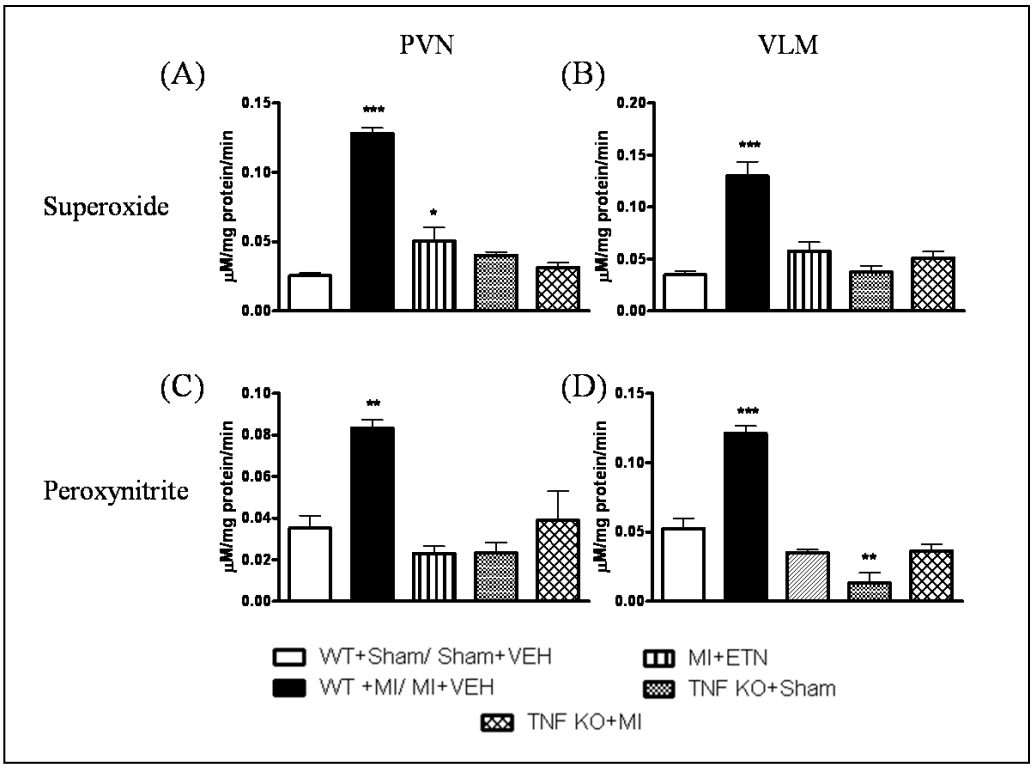

Figure 4.4. (A) Superoxide anion and (B) peroxynitrite production in the PVN of mice 4wks after MI. (C) Superoxide anion and (D) peroxynitrite production in the VLM of mice 4wks after MI. (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Neuronal and Sympathoexcitation Attenuated with TNF Ablation in CHF. Chronic neuronal activation in the PVN and VLM was studied by using an antibody that detects all members of the Fos family; c-Fos, FosB, Fra-1 and Fra-2. As shown in representative photomicrographs in figure 4.7A, CHF induced a markedly greater number of Fos-positive neurons in WT mice compared to the TNF KO mice. It should be noted that in sham-operated mice, the number of Fos-positive neurons were not different from each other (Figure 4.7B).

As shown in figure 4.7C, the plasma NE levels, an indirect indicator for sympathoexcitation, were significantly elevated in MI+VEH and WT+MI mice. However, in ETN-treated, sham-operated as well as TNF KO+MI mice, this increase was attenuated.

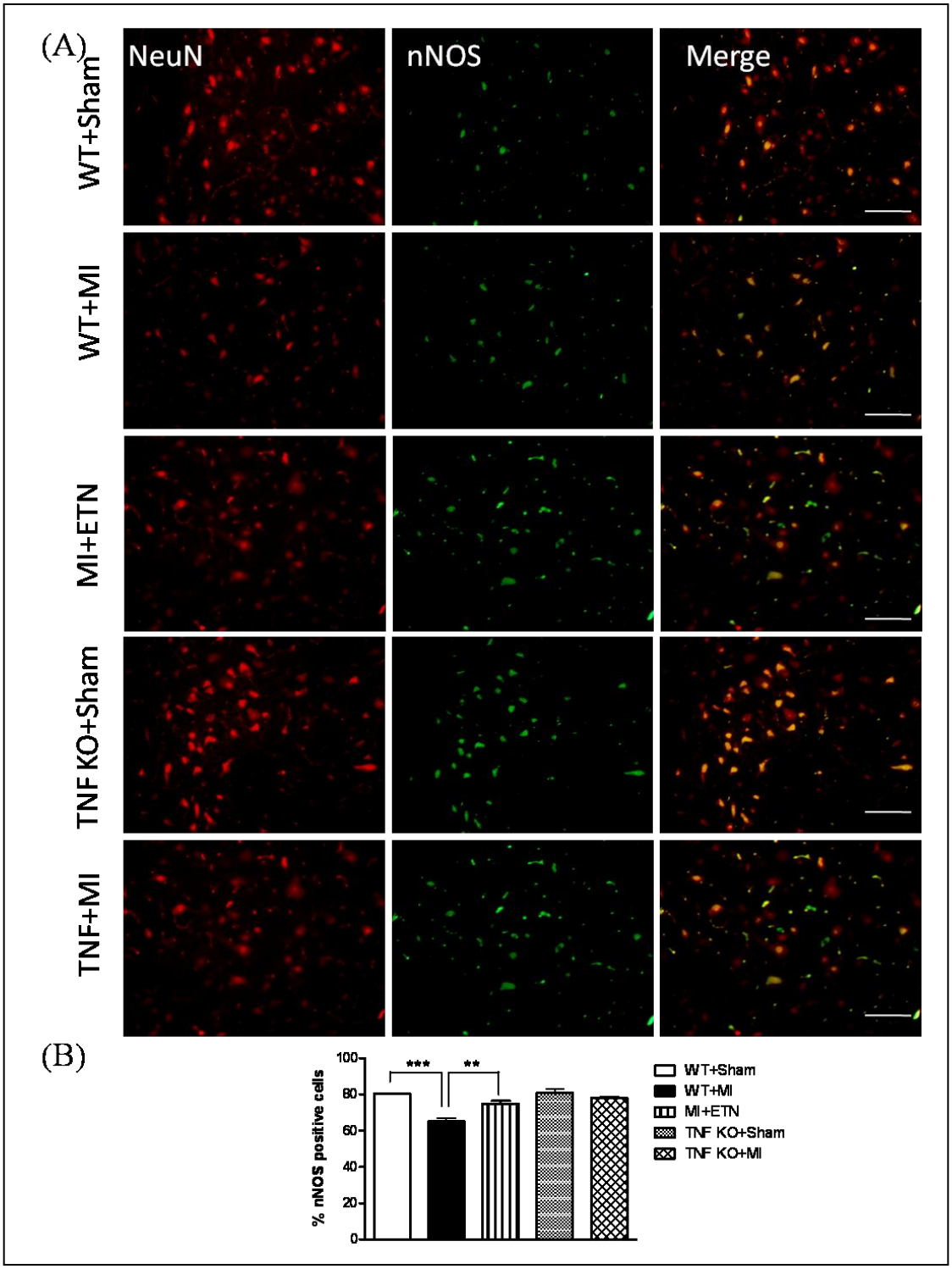

Figure 4.5. (A) Double-immunofluorescence histochemistry for NeuN, a marker for neuronal nuclei (left panel), nNOS (center panel), and their overlay (right panel) in the VLM. NeuN and nNOS were labeled with AlexaFluor 594 and AlexaFluor 488, respectively. Scale bar: 50 μm. (B) Total number of $nNOS^+$ cells are lower in WT+CHF group (***, $p<0.001$) compared with WT+Sham. There is no difference in the $nNOS^+$ neurons in TNF KO mice 4wks after MI.

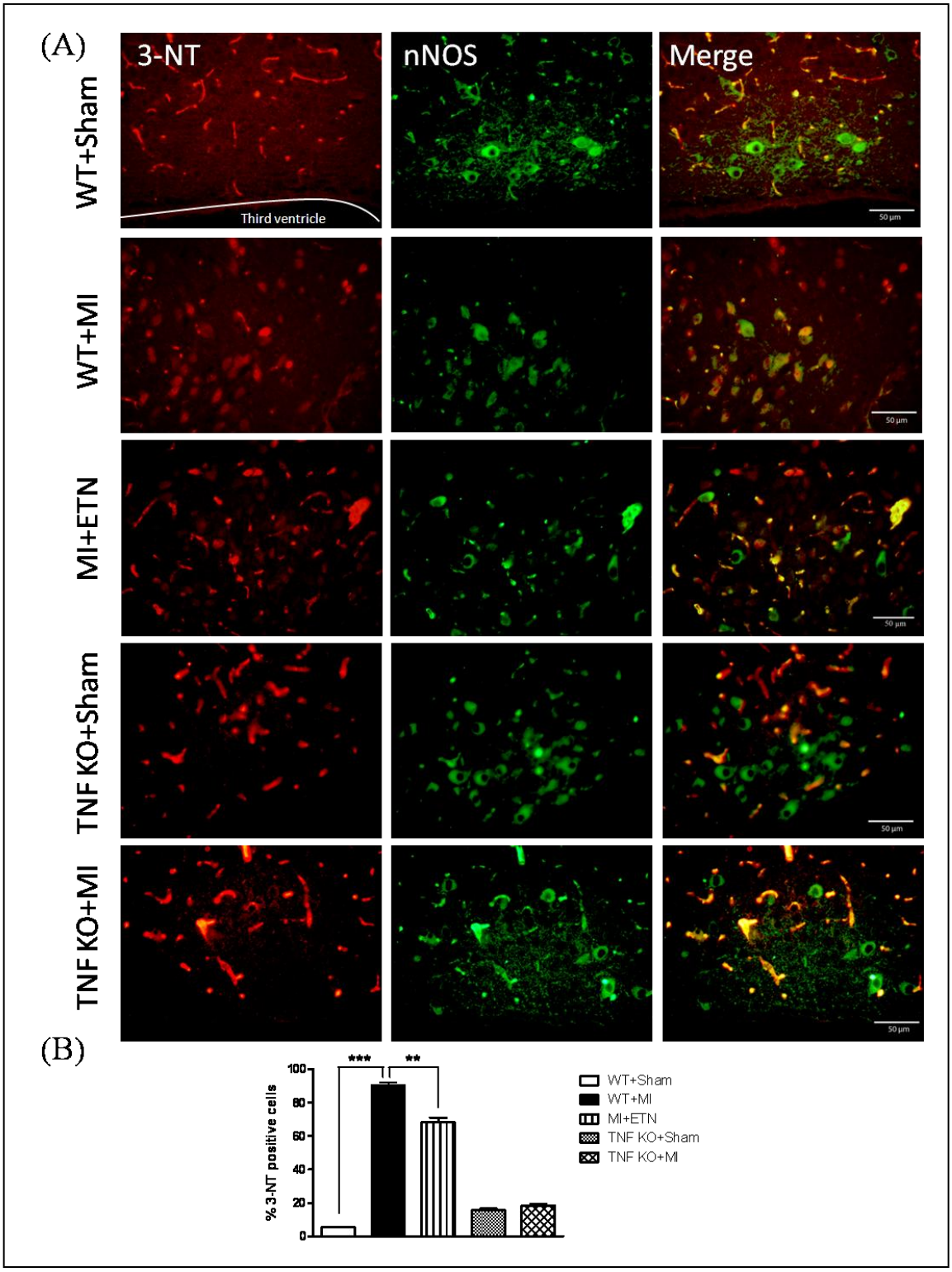

Figure 4.6. (A) Double-immunofluorescence histochemistry for 3-NT (left panel), nNOS (center panel), and their overlapping (right panel) in the PVN. 3-NT and nNOS were labeled with AlexaFluor 594 and AlexaFluor 488, respectively. Scale bar: 50 μm. (B) Total number of 3-NT$^+$ cells are higher in WT+CHF group (***, p<0.001) compared with WT+Sham. There is no difference in the 3-NT$^+$ neurons in TNF KO 4wks after MI compared with sham mice.

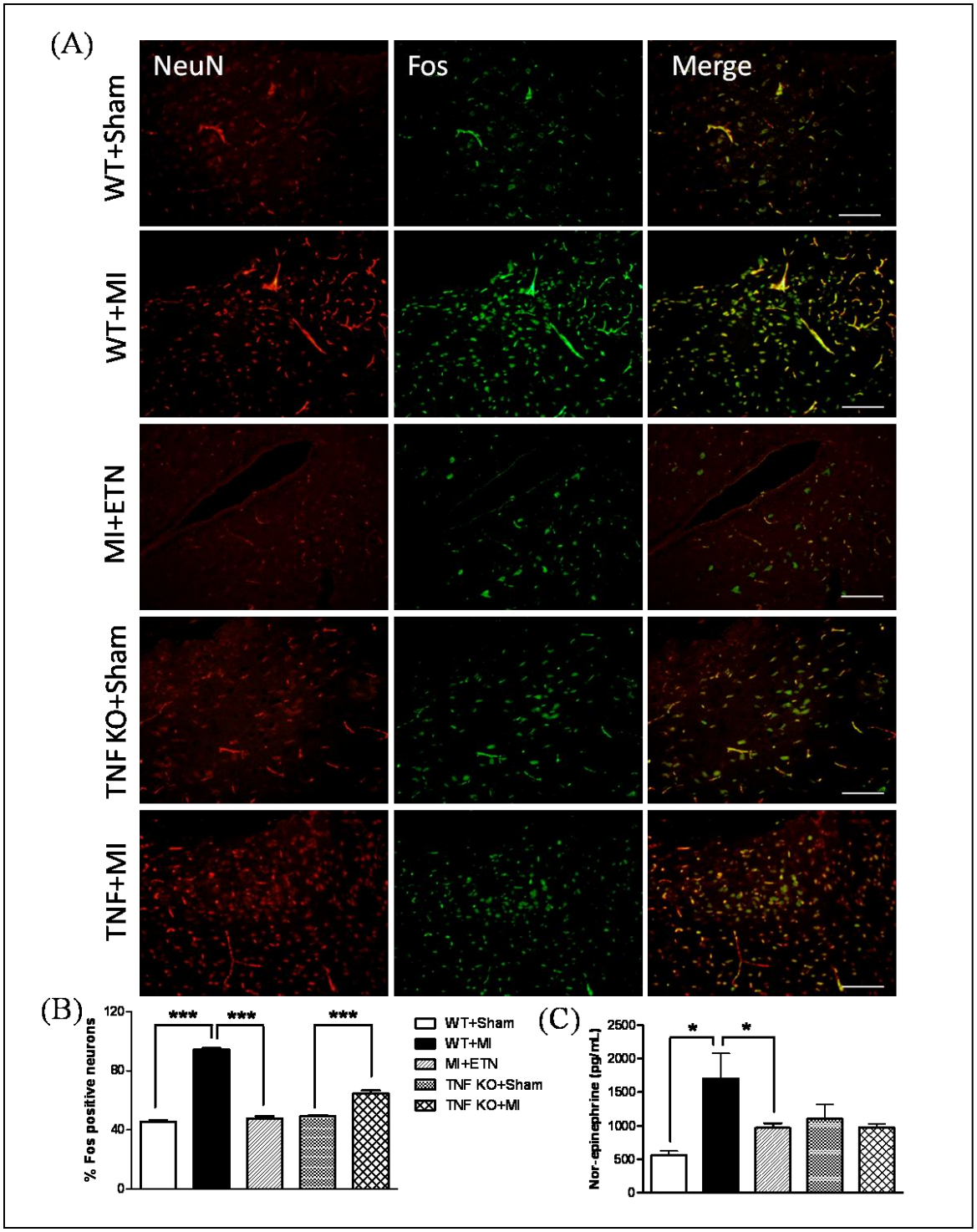

Figure 4.7. (A) Double-immunofluorescence histochemistry for NeuN (left panel), Fos (center panel), and their overlapping (right panel) in the PVN. NeuN and Fos were labeled with AlexaFluor 594 and AlexaFluor 488, respectively. Scale bar: 50 μm. (B) Total number of Fos^+ cells are higher in WT+CHF group (***, $p<0.001$) compared with WT+Sham. There is no difference in the Fos^+ neurons in TNF KO mice 4wks after MI compared to sham mice. (C) Plasma NE levels were significantly higher in WT mice compared with TNF KO mice 4wks after MI.

DISCUSSION

The present study highlights several important findings: 1) central administration of an anti-TNF agent reduces NADPH oxidase subunit expression, ROS production and decreases sympathoexcitation in the presympathetic regions of the brain; 2) increased sodium and fluid retention observed in CHF are partly dependent upon elevated cytokine levels; 3) central TNF blockade as well as knocking out the TNF gene elevates the reduced nNOS expression in the PVN and VLM in CHF; and 4) blocking TNF reduces $OONO^{\bullet}$ formation and reduces neuronal excitation in CHF. These findings support our hypothesis that the resultant sympathetic hyperactivity in CHF is due to TNF-induced oxidative stress in the sympathoexcitatory neurons of the PVN and VLM.

Our previous studies indicate that systemic administration of anti-cytokine agents can lower brain pro-inflammatory cytokine synthesis, NADPH oxidase subunit expression and superoxide production in CHF (Guggilam *et al.*, 2007). The present study, demonstrating that central cytokine inhibition reduces ROS production in the PVN and VLM regions resulting in reduced neurohumoral excitation, strongly indicates that central TNF levels are intrinsically involved in NADPH oxidase activation, ROS production and sympathoexcitation seen in CHF. A recent study showed that subchronic ICV infusion of AngII induced $O_2^{\bullet-}$ production in the RVLM is associated with elevated basal RSNA and impaired arterial baroreflex function (Gao *et al.*, 2005). Moreover, bilateral microinjections of tempol or tiron into the RVLM attenuated the pressor, sympathetic, and tachycardic responses to microinjection of AngII into the RVLM (Mayorov *et al.*, 2004). These studies show that ROS in the RVLM play an important role in the modulation of sympathetic activity and cardiovascular function by central AngII (Gao *et al.*, 2005). Interestingly, the $O_2^{\bullet-}$ source in all the above cases appears to be from NADPH oxidase. Consistent with these findings, in the present study, the expression of the catalytic subunits of

NADPH oxidase, Nox2 or Nox4, were significantly elevated in CHF in the hypothalamus and brainstem of mice treated ICV with the TNF antagonist ETN. The attenuated $O_2^{\bullet -}$ levels in CHF in the PVN and VLM of ETN-treated mice clearly delineates the role of TNF in CHF-induced ROS production. These findings were further confirmed in a TNF gene knockout model.

Another important function of AngII in the hypothalamus is the activation of arginine vasopressin in contributing to the CHF syndrome through increased thirst and sodium appetite (McKinley *et al.*, 2001). In addition, the drinking behavior of mice in response to AngII infusion was shown to be mediated via NADPH oxidase induced superoxide production (Zimmerman *et al.*, 2002; Zimmerman *et al.*, 2004). Our metabolic studies also show that in WT+MI mice, there is a significant negative correlation (r=-0.82) between sodium intake and urinary sodium excretion, indicating an even higher increase in sodium and water retention as compared to TNF KO+MI mice. Conversely, we found a significant positive correlation between sodium intake and urinary sodium (r=0.66) in TNF KO+MI. The 24-h urine volumes were also lower in the WT+MI mice compared to TNF KO+MI mice.

Most of the effects of the RAS, including fluid balance and endocrine secretion, are mediated by AT1Rs (Unger *et al.*, 1988). In this study, the increased fluid intake was accompanied by a decreased urine output, resulting in fluid retention in CHF. These changes were associated with an increased AT1R and a decreased AT2R expression within the hypothalamus and brainstem. Moreover, the lung/body weight ratios were higher in WT+MI mice. These changes were significantly attenuated in TNF KO mice. The elevated superoxide levels observed in CHF in the PVN and VLM regions of WT, but not TNF KO mice, therefore, indicate that TNF contributes to unusual drinking behavior, sodium and fluid retention, all of which can possibly be mediated through AngII mechanisms.

There is enough evidence to show that high levels of AngII in the CNS of CHF animals enhance sympatho-excitation through AT-1R activation (Sasaki *et al.*, 1990; Veerasingham *et al.*, 1997; Li *et al.*, 2006). In the past, Kumagai and Reid (Kumagai *et al.*, 1994) showed that systemic administration of the AT-1R antagonist losartan inhibited the pressor and renal sympathoexcitatory responses to carotid occlusion, demonstrating the role of AngII in carotid occlusion induced sympathoexcitation. Additionally, ICV administration of AngII resulted in BP, RSNA and NE level increases (Campese *et al.*, 2002). AngII induced increases in BP, RSNA, and NE are mediated through up-regulation of AT-1R and downregulation of nNOS, the primary source of NO in CNS neurons (Campese *et al.*, 2005), but the precise mechanism by which AngII induces sympathoexcitation is still not completely clear. NO is a well known sympatho-inhibitory neurotransmitter in the CNS and administration of NO inhibitors ICV resulted in elevated arterial pressure and sympathetic outflow (Sakuma *et al.*, 1992; Zanzinger *et al.*, 1995). Inhibition of NO prior to ICV infusion of AngII attenuated the increase in BP and RSNA. In addition, blockade of AT-1Rs concurrent with infusion of a NO donor lowered the basal RSNA in CHF rabbits, suggesting an important correlation between AngII and NO (Liu *et al.*, 1999; Zucker *et al.*, 2004). Besides AngII, elevated TNF levels in the PVN can also evoke sympathoexcitation (Kang *et al.*, 2006; Guggilam *et al.*, 2007). Previous studies underscore the importance of the interaction between cytokines and the RAS in increased sympathetic activity in CHF. However, elevated cytokine levels were detected even before neurohumoral activation in early HF (Torre-Amione *et al.*, 1996), indicating the important role of cytokines in the progression of CHF. The attenuated NE levels and AT-1R expression in the hypothalamus and brainstem in the WT mice compared to the ETN-treated mice and TNF KO+MI mice, in our study, suggest the major intermediary role of TNF in sympathoexcitation.

The expression of nNOS was significantly decreased in the PVN and RVLM of WT+MI mice as compared to ETN-treated or TNF KO+MI mice, suggesting that TNF has a significant role in altering NO-induced sympatho-inhibitory effects. Alternatively, CHF induced an increased expression of iNOS in the PVN and RVLM with the concurrent increase in $O_2^{\bullet-}$, resulting in an increased formation of $OONO^{\bullet}$ in these brain regions of WT+MI mice, thereby negating the NO effect on reducing sympathoexcitation. A previous study by Zanzinger *et al.* showed that $O_2^{\bullet-}$ and NO interact in the RVLM resulting in formation of $OONO^{\bullet}$, leading to damage of these neurons. They also reported that microinjection of $OONO^{\bullet}$ into the RVLM caused dose-dependent transient excitatory responses (Zanzinger, 2002). However, results from the present study indicate that in CHF, TNF contributes significantly to the interaction of $O_2^{\bullet-}$ and NO in the PVN and RVLM. It is also possible that the NO produced from eNOS and iNOS, as well as NO from other regions of the brain, diffuse into the PVN and VLM contributing towards the formation of $OONO^{\bullet}$, as indicated by our ESR and immunofluorescence studies. The reduced nNOS expression, and the increased ROS produced in the PVN and RVLM (Zanzinger, 2002), decrease the bioavailability of NO contributing to sympathoexcitation.

ROS act as key modulators of increased neuronal activity in the PVN and supraoptic nucleus (SON) of CHF animals (Lindley *et al.*, 2004). The expression of FosB, Fra1, and Fra2 are well recognized to remain elevated under conditions of chronic neuronal stimulation, such as the CHF (Vahid-Ansari *et al.*, 1998). The present study shows that CHF induces Fos expression in the PVN and RVLM of WT mice. Interestingly, the increased Fos expression was attenuated in TNF KO+MI mice, again suggesting the role of TNF in neuronal excitation. These results were comparable to previous studies where the increased numbers of Fos-positive neurons were detected in the PVN and SON at both 2 and 4 weeks after MI (Lindley *et al.*, 2004). In addition, elevated AngII levels in CHF were shown to induce Fos activation in the PVN and SON

(Lindley *et al.*, 2004). These increased Fos levels were inhibited significantly by a ROS scavenger, indicating the importance of ROS in sympathoexcitation (Lindley *et al.*, 2004). Also, the c-Jun/JNK/c-fos pathway is involved in the up-regulation of AT-1R expression by specific transcription factors (Chan *et al.*, 2002). High levels of AngII and AT-1Rs alter potassium channel proteins and decreased the overall K^+ current (Gelband *et al.*, 1997), resulting in sympathoexcitation which is the hallmark in the progression of CHF (Zucker, 2006). Taken together, these results raise the intriguing possibility that TNF induces Fos expression via modulation of ROS in the PVN and RVLM through an AT-1R mediated mechanism.

In addition, at 4 weeks, ETN-treated mice and TNF KO mice had better-preserved cardiac function as indicated by improvement in %FS compared to their VEH-treated and WT counterparts. Pulmonary congestion, as indicated by increased wet lung weight, was reduced in TNF KO mice compared to WT mice 4 weeks after MI. However, ETN-treatment did not reduce the pulmonary congestion in CHF. This might be due to the central administration of ETN as well as the large infarct size. ETN is a soluble fusion protein and cannot cross the blood-brain barrier. Therefore, most of the effects of ETN are due to inhibition of cytokines in the brain and are limited to the CNS. Nonetheless, ETN reduced the sympathoexcitation as shown by decreased NE levels induced by MI.

In conclusion, this study demonstrates that in CHF, TNF induces interaction of $O_2^{\bullet-}$ and NO in the PVN and RVLM, key autonomic regulators of cardiovascular function, through an AT-1R mediated mechanism, resulting ultimately in sympathoexcitation. This study also suggests a biologically important cross-talk between the RAS and pro-inflammatory cytokines in the PVN and RVLM. Functionally, this cross-talk leads to an uncontrolled ROS production that in turn can act as second messengers for further amplification of TNF and/or AngII activation,

resulting in increased neuronal firing in the PVN and RVLM and contributing to

sympathoexcitation and disease progression in the failing heart.

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CHAPTER 5

SYSTEMIC TNF TREATMENT INCREASES SYMPATHOEXCITATION THROUGH OXIDATIVE STRESS AND AT1 RECEPTORS IN THE PARAVENTRICULAR NUCLEUS

INTRODUCTION

Congestive heart failure is mainly characterized by an increase in neurohormones and pro-inflammatory cytokines. Therefore, most of the treatment strategies targeted to improve heart failure include β-blockers, ACE inhibitors and aldosterone antagonists. Following the demonstration by Levine *et al*. that circulating tumor necrosis factor-alpha (TNF) levels are increased in patients with severe heart failure (Levine *et al.*, 1990), subsequent investigations have focused on TNF mechanisms in the pathogenesis of CHF. Overexpression of TNF, by itself, can mimic the phenotype of heart failure and, thus, appears to be a valid therapeutic target for heart failure therapy. Although anti-TNF treatment strategies were successful in smaller short-term studies, larger long-term studies (RENAISSANCE, RECOVER, RENEWAL, ATTACH) were not successful for of various unknown reasons. Nonetheless, most interventions to improve heart failure were accompanied by a decrease in circulating TNF (i.e., milrinone and some phosphodiesterase inhibitors) (Molnar-Kimber *et al.*, 1993).

Experimental findings from Mann's lab revealed that in rats, chronic TNF infusion results in left ventricular contractile dysfunction and dilatation (Bozkurt *et al.*, 1998). TNF acts on cardiac myocytes to increase tetrahydrobiopterin synthesis, a co-factor necessary for the expression of inducible nitric oxide synthase (iNOS) (Nakayama *et al.*, 1994), thus, increasing nitric oxide (NO) production. This increased NO in the heart can decrease the contractility of cardiac myocytes (Finkel *et al.*, 1992) leading to cytotoxicity and ultimately apoptosis (Pinsky *et al.*, 1995). It is well documented that TNF also exerts its toxic effects on heart by altering antioxidant status, thereby, inducing oxidative stress. NADPH oxidase is a multimeric enzyme and is responsible for the reduction of oxygen and electron transport, and constitutes a major source of superoxide production both at cardiac myocytes and vascular smooth muscle level. We have recently demonstrated that chronic TNF infusion induces myocardial toxicity via

mitochondrial dysfunction (Mariappan *et al.*, 2007). In our CHF studies, using TNF blockers, we also showed that TNF is involved in increased NADPH oxidase subunit expression both in the heart and cardiovascular regulatory centers of the brain (Guggilam *et al.*, 2007). However, CHF is a complex phenomenon and further studies are required to delineate the central effects induced by systemic TNF.

Altered fluid homeostasis and increased sympathoexcitation are other important aspects in patients with CHF. Our previous studies have shown that pro-inflammatory cytokine (PIC) levels were increased in the hypothalamic paraventricular nucleus (PVN) within 30 min after myocardial infarction (Francis *et al.*, 2004). Furthermore, we showed that this increase in PICs in the PVN is simply not an epiphenomenon, but are involved in ROS production in the PVN and contribute to sympathoexcitation in CHF. Recent studies also show that angiotensin infusion into the brain can also stimulate ROS production and lead to neuronal excitation. However, in CHF, several neurohumoral systems are activated that might together contribute to the increased ROS production in the PVN and in sympathoexcitation. We predict that the increased systemic TNF can alone stimulate and act on the cardiovascular regulatory center, PVN and contribute to sympathoexcitation and volume overload. Thus, we conducted the present study by treating rats systemically with TNF and to study the effects on the autonomic regulatory neurons of the PVN. This study helps to delineate the relationship between neurohormonal/cytokine activation and sympathoexcitation that is otherwise observed in a heart failure setting. Along with TNF, we also used ROS and AT1R blockers systemically to investigate the effector molecules induced by TNF and the signaling mechanisms.

METHODS

Chronic TNF Infusion Model. Male Sprague-Dawley rats weighing between 275-325 gm were used. After baseline echocardiography, the rats were randomly allocated to different treatments

for 5 days: human recombinant TNF (10 μg/kg, IP) + vehicle or pentoxyfylline (PTX, a cytokine synthesis inhibitor; 30 mg/kg, IP) or Etanercept (ETN, a TNF receptor fusion protein; 1 mg/kg, SC) or tempol (TEM, a superoxide dismutase mimetic; 200 nmol/kg, PO) or Losartan (LOS, an AT1R blocker; 0.2 mg/kg, IP). The study conforms to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). All the experimental procedures were approved by the Louisiana State University Animal Care and Use Committee.

Assessment of Cardiac Structure and Function. Cardiac function was assessed by echocardiography at baseline (day 0) and day 5 of the experiment. Echocardiography was performed in rats anesthetized with 1.5% isoflurane/oxygen with Toshiba Aplio SSH770 system (Toshiba Medical Systems, CA) fitted with a PST 65A sector scanner (8 MHz), which generates two-dimensional images at frame rates ranging from 300 to 500 frames per second. IVSTd and IVSTs, inter-ventricular septal thickness at end-diastoleand end-systole, respectively; LVIDd and LVIDs, left ventricular internal diameter at end-diastole and end-systole, respectively; LVPWTd and LVPWTs, left ventricular posterior wall thickness at end-diastole and end-systole, respectively were measured using two-dimensional short-axis imaging. LV percent fractional shortening (%FS) was calculated as: %FS = (LVIDd – LVIDS)/LVIDd x100%.

After the experiment, the hearts were excised, and left ventricles were dissected and snap frozen in liquid nitrogen and stored at –80°C until further analyzed.

Plasma TNF Levels. At the end of the 5-day study, one group of rats was sacrificed by decapitation with guillotine under deep anesthesia by isoflurane and approximately 4 mls of trunk blood was collected in heparinized tubes. Plasma samples obtained by centrifugation of heparinized blood at 4ºC (Beckman-Coulter) at 2500 rpm for 15 min were used for estimation of circulating TNF-α and catecholamines. Circulating levels of TNF-α were quantified using

commercially available rat TNF-α ELISA kit (Biosource, Camarillo, CA) as described previously (Tei *et al.*, 1995; Francis *et al.*, 2003).

Plasma Catecholamines. Plasma norepinephrine (NE) and epinephrine (EPI) were measured using high performance liquid chromatography (HPLC)**.** Plasma samples were prepared by adding activated alumina, Tris buffer, EDTA and internal standard DHBA, along with 0.5 ml of rat plasma. The samples were centrifuged and supernatant separated and rinsed twice in ultra pure water and filtered through a Millipore filter (Ultrafree MC UFC30GV00, Millipore Corp). Samples were filtered and injected into an Eicom HTEC-500 system fitted with an HPLC-ECD.

Extraction of PVN by Laser Capture Microscopy (LCM). LCM was conducted in a dehumidified room (humidity ≤35%), and was kept to less than 30 min per slide to reduce the loss in recovery of intact RNA. A 7.5 μm laser spot size was used to capture the PVN, at a power range of 65–80 mW and pulse duration of 550–750 μs. This combination of parameters allowed efficient retrieval of the entire PVN area and a consistent lifting efficiency of >80%. The number of laser 'shots' used for each sample was kept constant at 1400. These parameters secured a sufficient and near constant amount of input RNA for comparative real-time RT-PCR analyses and protein for western blotting. All experiments were performed no less than five times with five different animals.

Electron Paramagnetic Spin Resonance (ESR) Studies. Superoxide and peroxynitrite production in the PVN punches were measured using spin-traps and a BenchTop ESR-spectrophotometer e-scan (Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany). The intensity of ESR spectra was quantified after subtraction of the ESR signal of probe without tissue sample.

Real-time RT PCR. RNA was isolated from PVN with TRIzol (Invitrogen, CA), treated with DNAase, and reverse transcribed using random primers reverse transcriptase. Gene transcripts

were determined by quantitative real-time polymerase chain reaction using SYBR-Green master mix (Applied Biosystems, CA) on an Applied Biosystems 7900. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH gene. The level of change was expressed as fold of respective control values.

Immunohistochemical and Immuonofluorescence Studies. The brain samples were embedded in optimum cutting temperature medium, and 5 µm-thick transverse sections were obtained and mounted on Superfrost slides. A general avidin-biotin-peroxidase complex (ABC) procedure was used to identify Nox1, Nox4, nNOS and 3-nitrotyrosine (3-NT) (Santa Cruz Biotechnology) neurons. For immunofluorescence studies, formalin-fixed sections were incubated overnight at 4˚C with the primary anti-rabbit c-Fos (Santa Cruz) or anti-rabbit tyrosine hydroxylase (TH) (AbCam) followed by Cy-3-labeled secondary immunoglobulin (IgG) (Molecular Probes). The slides were washed and mounted with ProLong Gold anti-fade reagent (Molecular Probes) for fluorescent microscopy.

For each animal, positive neurons within the borders of PVN bilaterally were counted manually in two representative 40-µm transverse sections at about –1.80 mm from bregma, and an average value was reported. NIH ImageJ software was used to confirm the manual cell counts in the PVN.

Statistics. All data illustrated are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, CA, www.graphpad.com. One-way ANOVA was used to observe the differences among groups followed by Bonferroni's correction. In all cases, $p<0.05$ was considered statistically significant.

RESULTS

TNF Induces Cardiac Dysfunction. At day 0, there were no significant differences in the cardiac parameters between different treatment groups (data not shown). At the end of 5 days,

TNF treatment resulted in an increased IVSTd and LVPWTd associated with a decreased %FS and increased Tei index as compared to control rats (Table 5.1). These changes indicate that chronic TNF treatment decreased the cardiac pumping performance. However, treatment with TNF blockers, PTX and ETN, superoxide scavenger, TEM, and AT1R blocker, LOS resulted in normalizing the changes induced by TNF.

TNF Induced TNF Expression. Systemic TNF treatment induced an increase in TNF transcripts both in the LV and the PVN (Table 5.2). In addition, the circulating TNF levels were also significantly increased by systemic TNF treatment (Figure 5.1). However, PTX, TEM and LOS treatment attenuated this increase induced by TNF. ETN was not able to restore the tissue levels to normal.

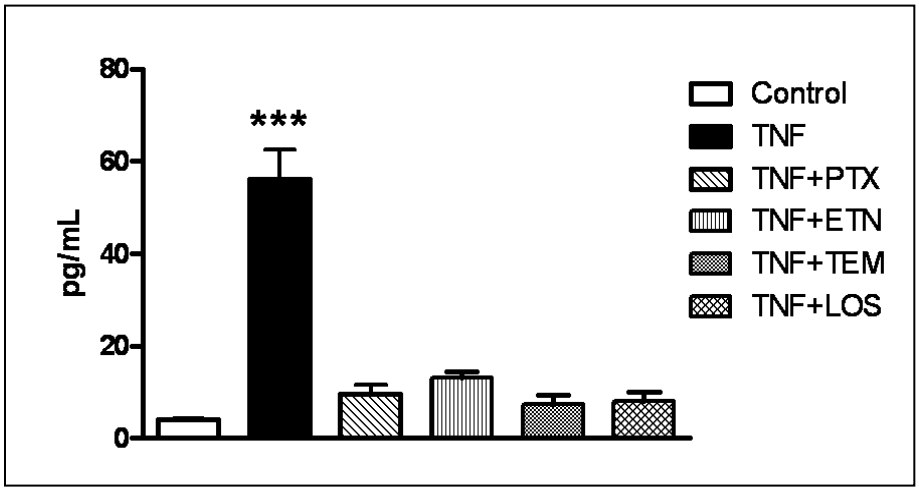

Fig.5.1. Plasma TNF Levels. Data are mean ± SEM. ***p<0.001.

TNF Induces Sympathoexcitation. Figure 5.2 illustrates photomicrograph of c-Fos staining in the PVN neurons. Systemic TNF treatment induced c-Fos expression in the PVN neurons suggesting neuronal excitation. Decreased staining of neurons for c-Fos in the PVN neurons in

rats treated with TEM and LOS suggest that this effect of TNF is mediated through ROS and AT1R mediated mechanisms.

Table 5.1. Echocardiographic Findings.

	Control	**TNF**	**TNF+PTX**	**TNF+ETN**	**TNF+TEM**	**TNF+LOS**
n	20	20	18	18	14	14
Left ventricle						
IVSTd (mm)	1.36 ± 0.02	1.59 ± 0.05*	1.33 ± 0.03	1.41 ± 0.03	1.37 ± 0.05	1.34 ± 0.05
IVSTs (mm)	2.26 ± 0.03	2.48 ± 0.12	2.43 ± 0.08	2.25 ± 0.03	2.50 ± 0.06	2.05 ± 0.05
LVIDd (mm)	6.57 ± 0.08	7.10 ± 0.21	6.98 ± 0.11	7.28± 0.13	7.31 ± 0.13	7.15 ± 0.14
LVIDs (mm)	5.27 ± 0.10	5.69 ± 0.18*	5.03 ± 0.05	4.78 ± 0.15	5.06 ± 0.13	5.16 ± 0.18
LVPWTd (mm)	1.37 ± 0.11	1.57 ± 0.07	1.39 ± 0.03	1.38 ± 0.32	1.38 ± 0.03	1.34 ± 0.31
LVPWTs (mm)	2.25 ± 0.13	2.15 ± 0.06	2.24 ± 0.03	2.15 ± 0.11	2.25 ± 0.16	2.11 ± 0.44
%FS	37.2 ± 0.94	31.1 ± 0.97*	34.2 ± 0.67	32.6 ± 0.23	35.7 ± 1.45	34.2 ± 1.22
Tei	0.29 ± 0.03	0.38 ± 0.11*	0.25 ± 0.01	0.32 ± 0.01	0.30 ± 0.02	0.31 ± 0.04
HR	327 ± 7.46	366 ± 10.1*	334 ± 4.06	337 ± 4.68	329 ± 3.78	337 ± 7.09
MAP	107 ± 6.32	109 ± 5.65	103 ± 5.13	108 ± 4.55	103 ± 7.20	105 ± 6.06

Data are mean±SEM. IVSTd and IVSTs, inter-ventricular septal thickness at end-systole and end-diastole, respectively; LVIDd and LVIDs, left ventricular internal diameter at end-diastole and end-systole, respectively; LVPWTd and LVPWTs, left ventricular posterior wall thickness at end-diastole and end-systole, respectively; %FS, fractional shortening; HR, heart rate; MAP, Mean arterial pressure. (*, $p<0.05$)

Figure 5.3 shows that plasma norepinephrine and epinephrine levels were also increased significantly in rats treated with TNF compared to those treated simultaneously with TNF blockers, ROS inhibitor and AT1R blocker implying that TNF induced sympathoexcitation is via ROS or AT1R or both.

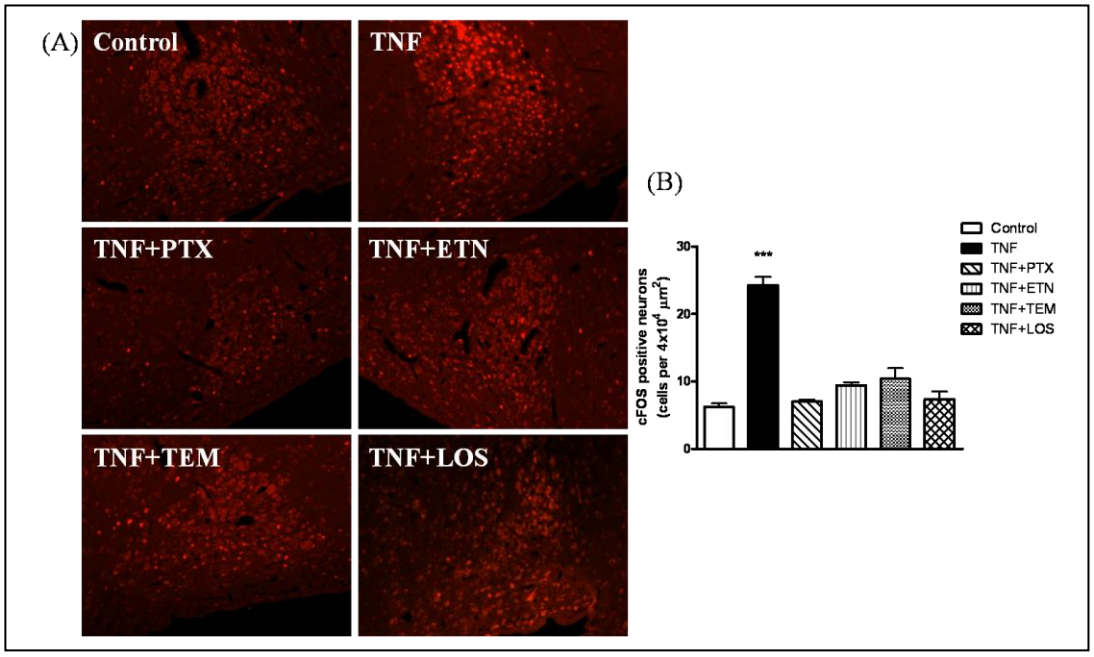

Fig.5.2. Micrograph showing immunostaining for anti-c-FOS in the PVN (A). The bar graph shows the quantification of c-FOS positive neurons in the PVN (B). Note the evident increase in anti-c-FOS in the PVN of TNF treated rats compared to those of control and PTX, ETN, TEM and LOS treated rats.

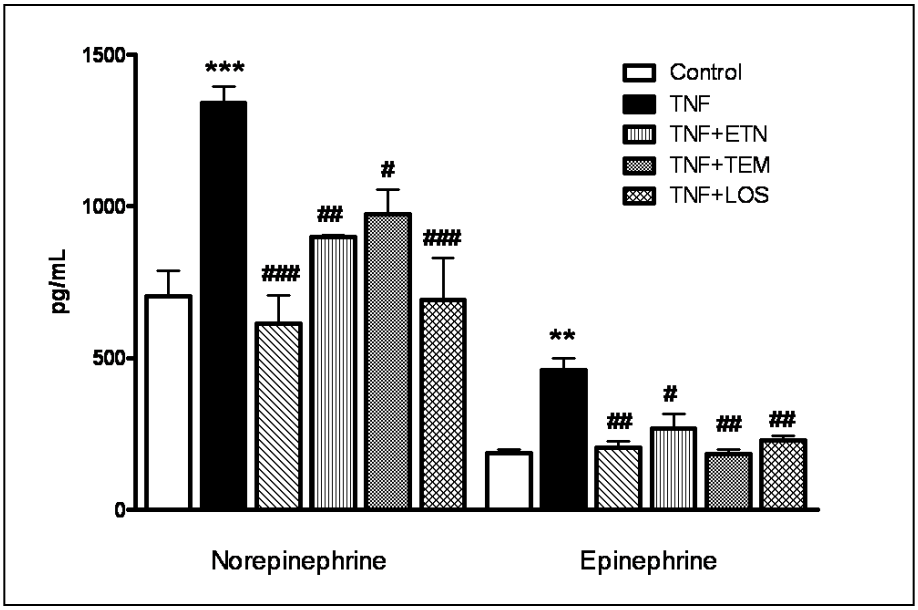

Fig.5.3. Assessment of Sympathetic Activity. Norepinephrine and epinephrine levels in the plasma samples analysed using HPLC-ECD. Data are mean ± SEM. ***p<0.001, **p<0.01versus control; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 versus TNF group.

Systemic TNF-treatment caused an increase in the protein (Figure 5.4) and gene expression of TH and AVP (Figures 5.5A and 5.5B), the rate limiting enzyme in catecholamine sysnthesis and AVP in the PVN. TNF blocker, ROS inhibitor and AT1R blocker significantly decreased the TNF-induced TH and AVP expression in the PVN.

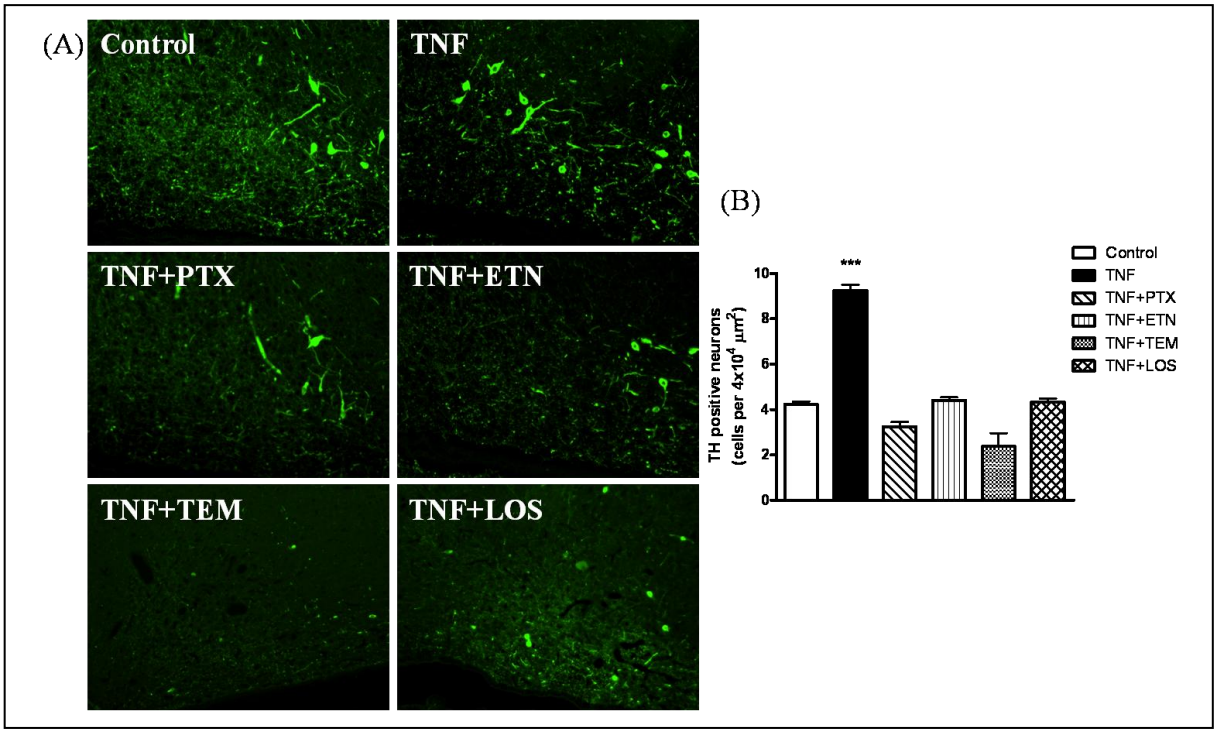

Fig.5.4. Photomicrograph showing immunostaining for anti-TH in the PVN (A). The bar graph shows the quantification of TH positive neurons in the PVN (B). Note the evident increase in anti-TH expression in the parvocellular neurons of the PVN of TNF treated rats compared to those of control and PTX, ETN, TEM and LOS treated rats.

TNF Increases Nox Subunit Expression and Superoxide Production. In accordance with our previous study (Mariappan *et al.*, 2007), and in this study as well, low dose TNF induced the expression of the major catalytic subunit of NADPH oxidase, Nox2 and its homologues, Nox1 and Nox4 in the LV and also in the PVN (Table 5.2). Figure 5.6A shows the protein expression of Nox1, Nox and Nox4 in the PVN. The quantification of positively stained neurons is shown in Figure 5.6B. Systemic TNF treatment resulted in a significant elevation of neurons positively

stained for Nox1, Nox2 and Nox4 indicating an increased production of superoxide in the PVN in these rats. We further confirmed our findings by measuring direct superoxide production in the PVN punches of the rats. In accordance with the Nox subunit expression, superoxide production was increased in the PVN of TNF-treated rats (Figure 5.6C). Treatment with PTX, ETN, TEM and LOS reduced Nox subunit expression and superoxide production induced by TNF.

TNF Reduces nNOS Expression. Previous studies showed that in CHF, nNOS expression is depleted in the PVN. We have also recently showed that the depleted nNOS levels in CHF are restored by treatment with systemic TNF blocker, PTX (Guggilam *et al.*, 2008). Interestingly, in this study, systemic TNF treatment decreased both protein (Figures 5.7A and 5.7B) and mRNA (Figure 5.7C) expression of nNOS in the PVN. These changes were reversed by treatment with TNF blockers, PTX and ETN. ROS scavenger, TEM, and AT1R blocker, LOS.

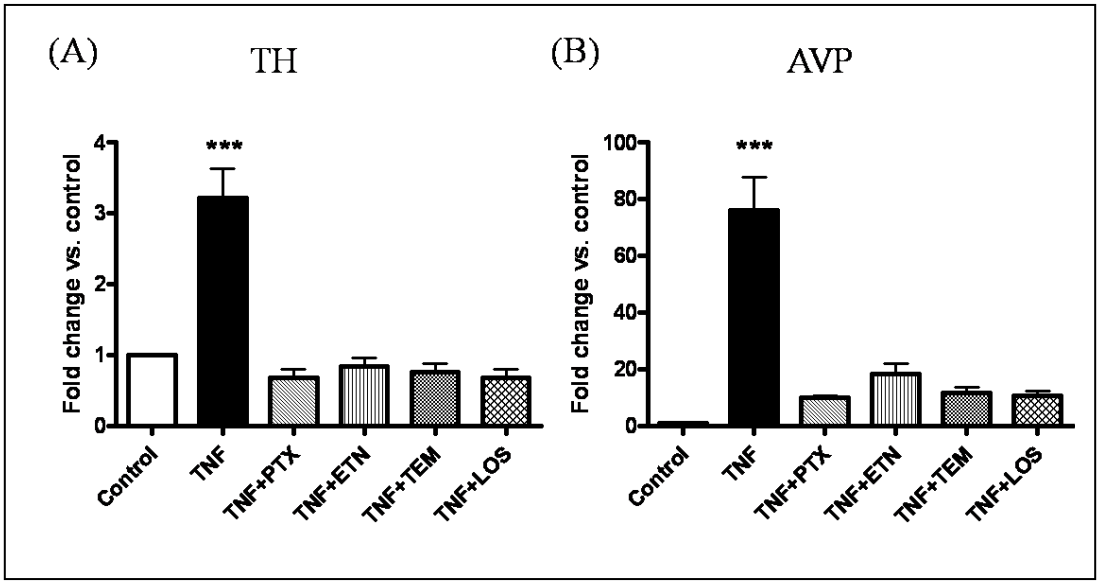

Fig.5.5. Gene Expression of TH and AVP in the PVN. Data are mean ± SEM and are expressed as fold change versus control group data. ****p*<0.001 versus control group.

Table 5.2. Gene Expression in the PVN.

	TNF	TNF+PTX	TNF+ETN	TNF+TEM	TNF+LOS
n	20	18	18	14	14
Left ventricle					
TNF	4.78 ± 1.13	2.57 ± 1.08	3.17 ± 1.27	1.18 ± 6.00	2.91 ± 10.0
NOX1	3.60 ± 1.93	2.35 ± 0.73	1.17 ± 0.09	1.14 ± 0.08*	1.53 ± 0.10$^{\#}$
NOX2	2.60 ± 0.17	1.23 ± 0.07	1.17± 0.14*	1.67 ± 0.13*	2.00 ± 0.16*
NOX4	3.37 ± 0.53	1.67 ± 0.16	1.85 ± 0.09	1.91 ± 0.72*	1.99 ± 0.17*
eNOS	3.05 ± 0.17	1.32 ± 0.07	1.17 ± 0.19*	1.12 ± 0.63*	1.80 ± 0.86*
iNOS	2.45 ± 0.23	0.95 ± 0.09	0.85 ± 0.11*	1.06 ± 0.12*	0.73 ± 0.06
AT1R	2.87 ± 0.23	1.25 ± 0.06	1.85 ± 0.07*	1.09 ± 0.12*	1.33 ± 0.16
Paraventricular nucleus					
TNF	3.67 ± 0.45	1.12 ± 0.38	2.18 ± 0.59	1.57 ± 0.08	1.12 ± 1.20
NOX1	2.91 ± 2.06	0.87 ± 0.08	1.18 ±0.07	1.35 ± 0.13	1.17 ± 0.11
NOX2	2.98 ± 1.30	2.07 ± 0.43	1.19 ±0.19	1.23 ± 0.02	1.37± 0.14*
NOX4	3.93 ± 0.79	1.57 ± 0.14	1.28 ± 0.09	1.65 ± 0.12	1.84 ± 0.19
eNOS	4.20 ± 0.99	0.98 ± 0.06*	0.77 ± 0.22*	1.67 ± 0.40*	0.46 ± 0.09*
iNOS	2.79 ± 0.63	1.33 ± 0.22*	0.71 ± 0.10*	1.10 ± 0.20*	0.52 ± 0.06*
AT1R	3.21 ± 0.28	1.02 ± 0.24*	0.96 ± 0.14*	1.23 ± 0.24*	0.97 ± 0.32*

Values expressed are means±SEM of fold change ($2^{-\Delta\Delta Ct}$) versus control group. (*p<0.05)

TNF Increases Peroxynitrite Formation. In cardiac myocytes, TNF is shown to increase superoxide and NO production eventually resulting in the formation of peroxynitrite which is cytotoxic to the cells. Moreover, increased peroxynitrite formation in certain brain regions has been shown to induce neuronal cell toxicity ultimately leading to sympathoexcitation. We therefore, stained the PVN with 3-nitrotyrosine, a footprint for peroxynitrite formation (Figure 5.8A). Figure 5.8B shows the quantification of 3-NT positive neurons. We observed a significant increase in 3-NT positive neurons in TNF-treated rats while PTX, ETN, TEM and LOS

decreased the TNF-induced peroxynitrite formation in the PVN. These findings were further confirmed by ESR measurements of peroxynitrite production in the PVN punches.

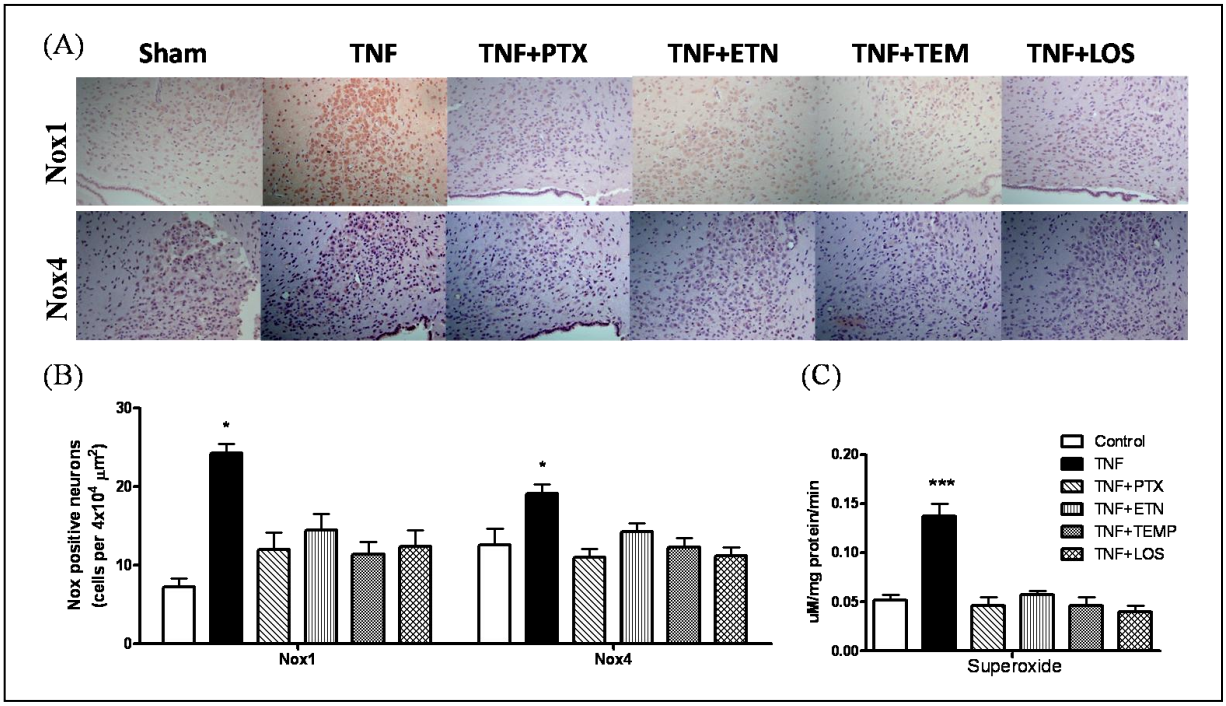

Fig.5.6. (A) Photomicrograph showing immunostaining for anti-Nox1and anti-Nox4 in the PVN. (B) The bar graph shows the quantification of immuno positive neurons in the PVN. Note the evident increased expression of Nox subunits in the neurons of the PVN of TNF treated rats compared to the other study groups. (C) The bar graph represents the superoxide production as measured by ESR spectrometer. Note the increased production of superoxide in the PVN punches of rats treated with TNF alone.

DISCUSSION

Our results show that systemic injections of TNF in rats resulted in a marked increase in the neuronal expression of Fos protein in the parvocellular neuronal division of the PVN which is known to regulate the hypothalamic-pituatary-adrenal (HPA) axis. Concomitantly, an increase in tyrosine hydroxylase (TH)-immunoreactivity and AVP gene expression was observed in this region indicating activation of the nor-adrenergic hypothalamic neurons, following administration of TNF. These results were associated with augmented plasma catecholamine

levels. Furthermore, the expression of nNOS was decreased while that of eNOS, iNOS, AT1R and 3-NT was increased in the PVN. TNF treatment resulted in increased LV posterior wall thickness and Tei index, accompanied by a decrease in %FS. These results support the hypothesis that systemic TNF stimulates sympathetic nervous system, reduces NO bioavailability and an AT1R mechanism may be involved in these responses.

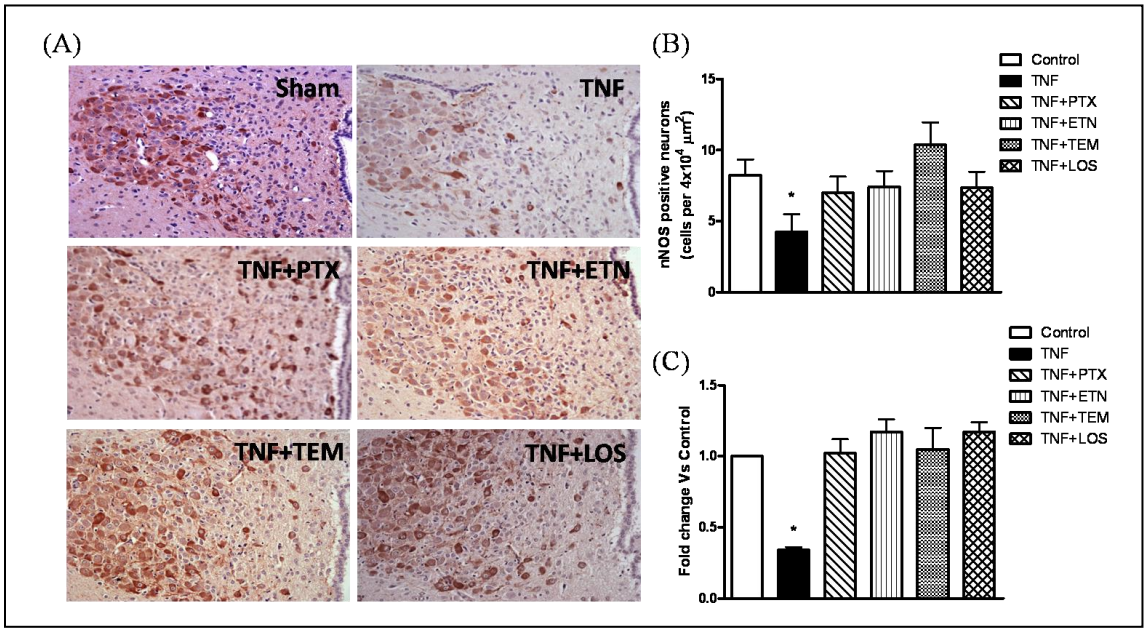

Fig.5.7. (A) Photomicrograph showing immunostaining for anti-nNOS in the PVN. (B) The bar graph shows the quantification of nNOS positive neurons in the PVN. Note the decreased nNOS stained neurons in the PVN of TNF treated rats compared to the other study groups. (C) The bar graph represents the nNOS gene expression in the PVN. Note that the gene expression of nNOS is also decreased in the PVN of rats treated with TNF alone.

Increasing evidence indicates that pro-inflammatory cytokines can engage central nervous system responses by altering neuroendocrine secretion (Beishuizen *et al.*, 2003). Circumventricular organs (CVOs), highly vascular regions that lack blood brain barrier, permit cytokine interaction with brain neurons. In addition, CVOs have blood capillaries with greater permeability and are potential sites of cytokine entry into the brain. These CVOs send efferent fibers to both magnocellular and parvocellular regions of the PVN mediating their influences on

neurohypophysial hormone secretion. A recent study showed that peripheral injection of lipopolysaccharide (LPS) induced Fos-like immunoreactivity in the CVOs, PVN and SON regions (Carnio *et al.*, 2006). The induction of c-Fos, an immediate early gene has been widely used as an indicator of cellular activity in the CNS (Morgan *et al.*, 1991). Present data also demonstrate that Fos, TH and AVP expression are induced in the pPVN by systemic TNF treatment. These activated centers in the brain can send signals to the neuroendocrine organs through release of intermediates that include catecholamines and NO (Carnio *et al.*, 2006). Systemic TNF treatment was also accompanied by an increase in plasma catecholamines, similar to the effect of LPS, indicating the activation of hypothalamic neurosecretory neurons that regulate HPA axis activity.

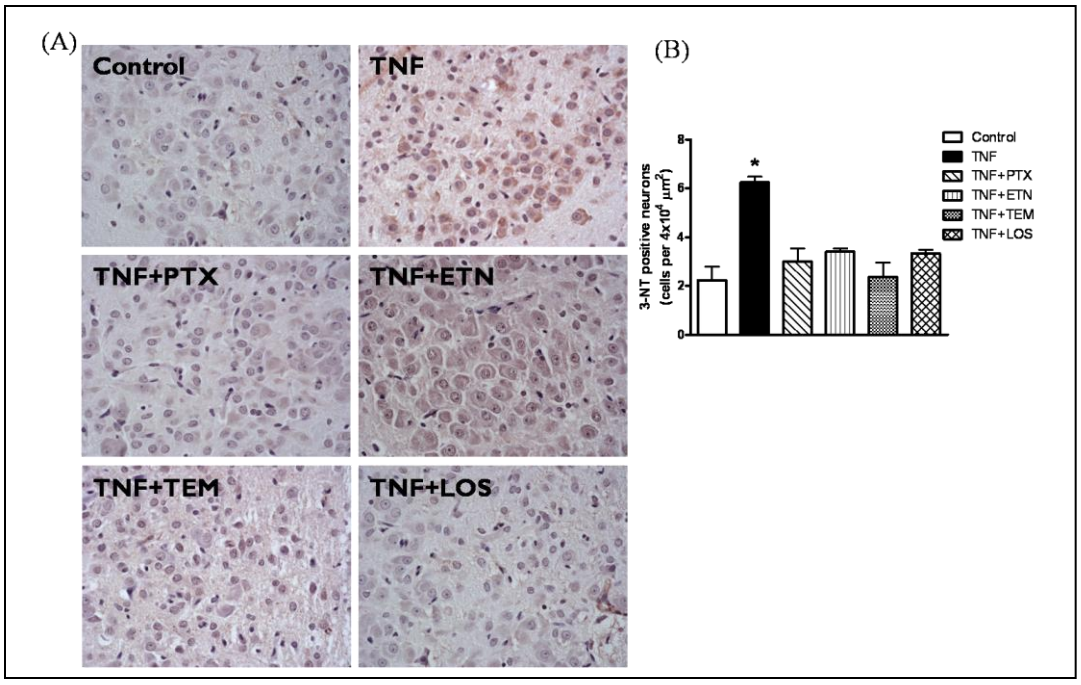

Fig.5.8. (A) Photomicrograph showing immunostaining for anti-3-NT in the PVN. (B) The bar graph shows the quantification of 3-NT positive neurons in the PVN. Note the increased number of 3-NT, a footprint for peroxynitrite formation in tissues, positive neurons in the PVN of TNF treated rats compared to the other study groups.

Growing evidence suggests that NO in the brain acts to restrain the stimulation of HPA axis induced by IL-1β, vasopressin and inflammation, resulting in the suppression of anti-inflammatory corticosteroids release from the adrenals (Rivier *et al.*, 1994). Systemic LPS administration increases iNOS expression not only in the glia cells and astrocytes but also in the neurons. This increase in iNOS in the PVN was shown to be associated with an increased expression for corticotrophin releasing factor (CRF) (Wong *et al.*, 1996). It is now well known that NO has inhibitory effects on the magnocellular secretory neurons of the PVN where oxytocin as well as vasopressin are synthesized. In vitro studies demonstrated that, application of NO donor inhibits the magnocellular secretory neurons of the PVN and SON while that of a NO scavenger enhanced neuronal activity (Kadekaro, 2004). Administration of LPS in rats pre-treated with a NOS inhibitor augmented the release of oxytocin and vasopressin (Rivier, 2003). In the present study, a decrease in nNOS was observed with TNF treatment, while the gene expression of iNOS and eNOS were increased. These changes were associated with a tremendous increase in the expression of AVP in the PVN accompanied by an increase in Fos-immunoreactivity. These results suggest that the bioavailability of NO is reduced in these neurons. The NO produced by iNOS and eNOS could be involved in scavenging the increased superoxide produced in the PVN and this is further confirmed by 3-NT-immunoreactivity, footprint for peroxynitrite formation, observed in the PVN neurons. In addition, noradrenaline can stimulate mRNA expression of AVP in the PVN (Vacher *et al.*, 2002), thus the released catecholamines in response to TNF injections can further potentiate the AVP expression in the PVN. Thus TNF might play a pivotal role in the feed forward relationship between sympathoexcitation and volume overload in stress-induced conditions such as CHF.

However, in CHF, apart from cytokines, elevated AngII also activates the PVN directly via the receptors in the CVOs (Sawchenko *et al.*, 2000). Moreover, in CHF, there is impairment

of baroreflexes, and atrial receptor triggered inhibition of sympathoexcitatory and AVP releasing neurons in the PVN. The increased AngII, acts through AT1Rs and excites PVN neurons that activate sympathoexcitatory outputs to the kidney (Coote *et al.*, 1998). The effects of AT1R are, at least in part, mediated through superoxide production (Lindley *et al.*, 2004). We have shown in our previous study that a cross-talk exists between TNF, ROS and AT1R in the PVN of CHF rats (Guggilam *et al.*, 2008). Here, we validate our results by demonstrating that systemic TNF can induce sympathoexcitation via the activation of HPA axis. Inhibition of this activation by losartan further confirms that the effects of TNF are mediated through AT1R. In this study too, TNF induced activation of the NADPH oxidase subunits, Nox1and Nox4, and was inhibited by tempol co-treatment, demonstrating that the actions of AT1 and TNF are mediated via superoxide production.

Our present results show that systemic TNF can stimulate the activity of sympathetic neurons of the PVN as illustrated by an increase in heart rate. However, at this particular dose, the MAP was not altered by TNF. The TNF-stimulated AVP expression in the PVN suggests its presumptive role in the prompting of ACTH secretion and the modulation of cardiac frequency. In addition, the increase in IVSTd and LVPWTd associated with a decrease in Tei index indicate that TNF induced diastolic dysfunction in these rats. Alternatively, TNF blockers, ROS quencher and AT1R blocker significantly altered these changes induced by TNF. Taken together, our results suggest a putative involvement of AT1 and ROS in the triggering of immediate early genes and gene expression of iNOS, AVP and TH, is an adaptive response to TNF injection in the PVN and in the regulation of sympathoexcitaion in response to stress conditions like CHF.

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CHAPTER 6

CONCLUDING REMARKS

OVERALL SUMMARY OF FINDINGS

Dysregulation of sympathetic activity following MI can exhaust cardiac performance and eventually lead to CHF. Pro-inflammatory cytokines (PICs) play a critical role in the pathogenesis of CHF where increased PICs following MI can gain entry into the brain through the CVOs, or via activation of prostaglandin receptors and thereby affect the CNS. The increase in PIC production in the brain, or activation of the HPA by peripheral afferents, may lead to activation of AngII and ROS, both of which may lead to TNF-dependent ROS signaling in the CNS and the development of cardiovascular diseases, including CHF. Therefore, elucidating the precise signaling mechanisms of TNF in the CNS is critical for the development of therapeutic treatments targeted to central PIC signaling. Through a series of rat and mouse studies, we explored the role of increased TNF in the CNS following MI.

In chapter 2, we demonstrated that in CHF, in addition to plasma TNF, TNF expression is also increased locally in tissues, particularly in the heart and the PVN of the hypothalamus in the brain. These results also demonstrate that this increased TNF is associated with increased expression of the catalytic subunit of the multimeric enzyme NADPH oxidase, Nox2/gp^{91phox}, and its homologues, Nox1 and Nox4 in the LV and the PVN. Furthermore, decreased DHE fluorescence in rats treated with the cytokine blocker, PTX, further confirmed that TNF is involved in the stimulation of superoxide production in the PVN. Taken together, results from this study indicate that the elevated expression of TNF in the PVN directly, or through induction of superoxide production, can increase sympathoexcitation.

In chapter 3, we presented the effects of increased TNF in CHF on the free radical nitric oxide, known to restrain the excitatory neurons of the PVN. At the same time, excessive NO production in the brain can exert toxic effects on neurons via the formation of the peroxynitrite radical. Our results demonstrate that the increased TNF following MI decreased nNOS

expression. The increased iNOS expression observed suggests that the iNOS-derived NO might be involved in the formation of peroxynitrite radicals contributing to the symathoexcitation in CHF. We also showed that the increased AT1R expression in the PVN in CHF is PTX inhibitable. Results from this study clarify the involvement of TNF in decreasing bioavailability of NO in the PVN, possibly through an AT1R mediated mechanism.

In chapter 4, we examined the sympathoexcitatory effects of TNF by 1) treating the CHF mice intracerebroventricularly with Etanercept, a TNF fusion protein that prevents the binding of TNF to its receptors and 2) also by using a TNF knockout mouse model. We found an increase in pro-inflammatory cytokine (TNF, IL-1β and IL-6) expression in the PVN and VLM regions of the brain in CHF mice. Alternatively, the expression of an anti-inflammatory cytokine, IL-10, was decreased in CHF. We also found that superoxide and peroxynitrite production measured in these brain regions by ESR, adopting specific spin probes, was increased in CHF. Immunofluorescence and gene expression studies further indicated that nNOS expression is decreased while that of peroxynitrite and Fos were increased in the PVN and VLM. All these changes were accompanied by decreased % fractional shortening in CHF mice. Interestingly, all these changes were attenuated in TNF KO mice and mice treated ICV with ETN, confirming the sympathoexcitatory effects of TNF. We also demonstrate in this study that the AngII-induced salt appetite and fluid accumulation in CHF are, in part, mediated through TNF.

In chapter 5, we challenged our findings from the previous chapters by treating the rats systemically with TNF to delineate the effects of TNF by excluding the effects of neurohormones that are activated in CHF, contributing to sympathoexcitation. TNF injections resulted in an increase in plasma TNF levels and TNF expression in organs, including heart and autonomic regulatory centers of the brain. In addition, systemic TNF increased c-Fos expression, an immediate-early gene, indicating the TNF-induced neuronal excitation is via the activation of

the HPA axis. Inhibition of these effects by losartan, an AT1R blocker indicates that these effects of TNF are mediated through AngII. Systemic TNF also increased the expression of TH in the PVN, which is a rate limiting enzyme for the synthesis of NE. Furthermore, TNF increased the AVP expression, suggesting its role in fluid retention and volume overload. Similar to CHF studies, systemic TNF was able to increase Nox expression concurrently with superoxide and peroxynitrite production in the PVN. Inhibition of these TNF-induced changes by tempol and losartan demonstrate that the actions of TNF are mediated through AngII and ROS.

SIGNIFICANCE OF RESEARCH

Despite so many advances in therapeutic strategies, cardiovascular disease remains the foremost cause of death in the United States. According to the American Heart Association 2009 statistical update, 1 out of 8 deaths reported are due to CHF (Lloyd-Jones *et al.*, 2009). Recent clinical trials reported an annual sudden-death risk of 8-12% in 3 months after MI, even with optimal medical therapy including β-adrenoceptor blockade and angiotensin converting enzyme inhibitor/angiotensin receptor blocker therapy (Pitt *et al.*, 2003). It is well established that sympathetic over activity is the major factor contributing to the progression of CHF. Although all the current therapies modulate sympathetic activity, there still is a need to find a better treatment strategy, yet owing to the continued increasing mortality rate in CHF patients.

There is abundant evidence that pro-inflammatory cytokines are increased after myocardial injury and can eventually lead to the pathology of CHF. Previous reports have demonstrated that brain pro-inflammatory cytokines are increased within minutes after MI (Francis *et al.*, 2004) and contribute to sympathetic hyperactivity (Zhang *et al.*, 2003). Therefore, understanding the mechanism by which TNF contributes to sympathoexcitation is important in the development of new therapies.

We believe that our studies, utilizing transgenic animals or gain-of-function strategies, have identified new targets in the brain for the treatment of CHF and demonstrated the beneficial effects of TNF inhibition on the CHF. Systemic blockade of TNF/superoxide can also lead to neurohormonal feedback decreasing the hyperactivity of the sympathetic nervous system and the HPA axis in CHF. In addition, targeting the NADPH oxidase complex and subsequent superoxide production may be an alternative treatment strategy. From a clinical point of view, however, it is clear that for chronic diseases such as CHF, restraint of uncontrolled sympathoexcitation is needed, and this can be accomplished through targeting the production of TNF in the brain. It is possible that in the past, anti-cytokine agents in the clinical trials might not have crossed the blood-brain-barrier to target the brain cytokines. Our studies suggest that inclusion of an anti-cytokine agent that crosses the blood brain barrier in the treatment regimen of CHF patients may change the clinical outcome. Overall, we believe that our studies yielded an important proof-of-concept work and opened new doors for future studies that will further the understanding of TNF-induced oxidative stress in contributing to sympathoexcitation in the pathogenesis of CHF.

FUTURE DIRECTIONS

Although we believe that our aforementioned studies have made significant contributions in identifying the novel role of elevated TNF in the autonomic regulatory regions of the brain, further studies are required to better understand the underlying mechanisms of TNF-dependent sympathoexcitation in CHF. Our results showed that blocking ROS by tempol resulted in decreased neuronal excitation in the PVN suggesting the role of superoxide in sympathoexcitaion. Future studies are needed to examine the transcription factors involved in superoxide-induced chronic neuronal excitation. Neuronal cultures incubated with AngII exhibited a steady increase in the levels of tyrosine hydroxylase (TH) and dopamine β-

hydroxylase, both of which are involved in the biosynthesis of norepinephrine involved in the regulation of sympathetic activity, mediated via the AT1Rs (Yu *et al.*, 1996). These actions were inhibited by treatment with extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) (Yang *et al.*, 1996). Moreover, in CHF, MAPK plays an important role in the activation of AT1R in the PVN and SFO (Wei *et al.*, 2008). In Alzheimer's disease, iNOS-induction by TNF was shown to involve JNK/c-Jun and NF-κB transcription factors (Medeiros *et al.*, 2007) in the CNS. Furthermore, an oligonucleotide against c-Fos attenuated Ang-II induced increase in TH (Yu *et al.*, 1996). We therefore hypothesize that in CHF, AT1R might be increased by TNF via a MAPK pathway, and both TNF and AT1R might be linked in the dimerization of c-Fos and c-Jun to form the transcription factor AP-1 and further the biosynthesis of norepinephrine. Moreover, recent reports also illustrated that redox mechanisms are involved in the AngII-induced activation of AP-1 (Puri *et al.*, 1995; Viedt *et al.*, 2004), further confirming the critical role of superoxide in the modulation of sympathoexcitation. In addition, understanding the temporal sequence of events following the activation of TNF in these autonomic regulatory neurons that lead to increased sympathetic activity may help us define better interventions aimed at blocking the deleterious effects of TNF.

In summary, we believe that our studies identified potential new targets in the CNS involved in sympathoexcitation in CHF and laid a foundation for a collection of future studies in understanding the role of TNF-induced oxidative stress in autonomic neurons.

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VITA

Anuradha Guggilam was born in Guntur, Andhra Pradesh, India. After testing into the top 1% of all secondary school medical entrance test examinees in India, she chose to receive her Bachelor of Veterinary Science and Animal Husbandry (B.V.Sc. & A.H.) degree in 2001 from Acharya N.G. Ranga Agricultural University, Hyderabad, India. After receiving her veterinary medicine doctoral degree, and knowing that discovery, not diagnostics, truly interested her, she then went to the College of Veterinary Science, Tirupati, India, where she earned a Master of Veterinary Science (M.V.Sc.) in veterinary pharmacology and toxicology in 2003. In August 2004, she was fortunate enough to be accepted into the doctoral program in the Department of Comparative Biomedical Sciences of the School of Veterinary Medicine at Louisiana State University, Baton Rouge, Louisiana, under the mentorship of Associate Professor Joseph Francis (B.V.Sc. & A.H., M.V.Sc. and Ph.D.) to begin her career as a research scientist, specializing in cardiovascular pathophysiology. She is interested in applying her combined knowledge in medicine and cardiovascular pathophysiology towards the development of novel heart failure therapeutics. She will receive her Doctor of Philosophy degree (Ph.D.) in veterinary medical sciences in the Spring commencement, 2009.