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Mechanisms by Which Exercise Training Attenuates Blood Pressure in Animals: Roles of Cytokines, Oxidative Stress, and Glycogen Synthase Kinase-3â

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MECHANISMS BY WHICH EXERCISE TRAINING ATTENUATES BLOOD PRESSURE IN ANIMALS: ROLES OF CYTOKINES, OXIDATIVE STRESS, AND GLYCOGEN SYNTHASE KINASE-3β

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LIST OF ABBREVIATIONS

%FS	% fractional shortening
.ClO^-	hypochlorite
H_2O_2	hydrogen peroxide
.O_2	superoxide
.OH	hydroxyl moiety
.OH^-	hydroxyl radical
$OONO^-$	peroxynitrite
i.m.	intramuscular
i.p.	intraperitoneal
s.c.	subcutaneously
ACE	angiotensin converting enzyme
ACE2	angiotensin converting enzyme 2
aCSF	artificial cerebrospinal fluid
AHA	American Heart Association
Ang(1-7)	angiotensin (1-7)
Ang(1-9)	angiotensin (1-9)
Ang I	angiotensin I
AngII	angiotensin II
ANOVA	analysis of variance
ARB	angiotensin receptor blocker
AT1R	angiotensin II type-1 receptor
AT2R	angiotensin II type-2 receptor

ATP	adenosine triphosphate
BBB	blood brain barrier
BP	blood pressure
BSA	bovine serum albumin
BW	body weight
CDC	Centers for Disease Control
cDNA	complimentary DNA
CMH	1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine
CNS	central nervous system
CPH	1-hydroxy-3-carboxypyrrolidine
Cu/Zn-SOD	copper/zinc SOD
CVDs	cardiovascular diseases
CVO	circumventricular organ
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
eNOS	endothelial nitric oxide synthase
EPR	electron paramagnetic resonance
ExT	exercise training
GABA	*gamma*-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPx	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidized glutathione

HPLC	high performance liquid chromatography
HR	heart rate
HW	heart weight
HW/BW	heart weight/body weight
IACUC	Institutional Animal Care and Use Committee
ICAM1	intercellular adhesion molecule 1
ICV	intracerebroventricular
IL-1β	interleukin-1 *beta*
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IVSTd	interventricular septal thickness in diastole
IVSTs	interventricular septal thickness in systole
IκB	inhibitor of kappa B
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	c-Jun N-terminal kinase
KHB	Krebs-Hepes buffer
LV	left ventricle
LVIDd	left ventricular internal diameter in diastole
LVIDs	left ventricular internal diameter in systole
LVPWTd	left ventricular posterior wall thickness in diastole
LVPWTs	left ventricular posterior wall thickness in systole
LW	lung weight
LW/BW	lung weight/body weight

MAP	mean arterial pressure
MAPK	mitogen activated protein kinase
MasR	M*as* receptor
MCP-1	monocyte chemotactant protein-1
MEF2	monocyte enhancer factor-2
MHC	myosine heavy chain
MHz	megahertz
mmHg	millimeters of mercury
mRNA	messanger RNA
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NE	norepinephrine
NEP	neutral endopeptidase
NF-κB	nuclear factor-kappa B
NIH	National Institute of Health
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
NOX 1	NADPH oxidase with gp91 phox homologue
NOX2	NADPH oxidase with gp91 phox
NOX 4	NADPH oxidase with gp91 phox homologue
NT	nitrotyrosine
NTS	nucleus tractus solitaries
OVLT	organum vasculosum lamina terminalis

OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDTC	pyrrolidine dithiocarbamate
PEG-SOD	polyethylene glycol-conjugated superoxide dismutase
PICs	proinflammatory cytokines
PVN	paraventricular nucleus
RAAS	renin- angiotensin-aldosterone system
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
RVLM	rostral ventrolateral medulla
SBP	systolic blood pressure
SDS-PAGE	sodium dodecyl sulphate-polyacrilamide gel electrophoresis
SEM	standard error of the mean
SFO	subfornical organ
SHR	spontaneously hypertensive rat
SHRSP	SHR stroke-prone
siRNA	small interefering RNA
SNS	sympathetic nervous system
SOD	superoxide dismutase
SON	supraoptic nucleus

Tei	(isovolumic contraction time + isovolumic relaxation time)/ejection time
Temp	Tempol
TNF-α	tumor necrosis factor-alpha
TNFR1	TNF receptor type-1
TNFR2	TNF receptor type-2
VCAM1	vascular cell adhesion molecule 1
VDAC	voltage dependent anion channel
VLM	ventrolateral medulla
WHO	World Health Organization
WKY	Wistar-Kyoto

ABSTRACT

Hypertension is a chronic multifactorial condition with high morbidity and mortality rates, currently affecting about one billion people worldwide. Currently available anti-hypertensive medications are found to be effective in reducing blood pressure (BP), but still more than 50% of those diagnosed with hypertension fail to respond to these anti-hypertensive regimens. Although hypertension has multiple etiologies, physical inactivity has been found to have strong correlation with the disease, so exercise has recently been recommended as a part of lifestyle modifications for all hypertensive patients. Therefore, the present series of *in vivo* and *in vitro* studies were undertaken to gain more insight into the effects of regular long-term exercise training (ExT) within the heart and brain of hypertensive animals with the specific aim of investigating the molecular mechanisms underlying the exercise-induced beneficial effects.

In the first study, we subjected young spontaneously hypertensive rats (SHRs) to moderate-intensity exercise for 16 weeks. Regular exercise delayed progression of hypertension and improved cardiac function in SHRs, and these effects were mediated by reduced myocardial pro-inflammatory cytokines (PICs), NFκB activity, and improved redox homeostasis. In the second study, we found that chronic exercise not only reduces PICs and vasoconstrictor components of the renin-angiotensin system (RAS) but also improved anti-inflammatory cytokines (AIC) and vasodilatory axis of the RAS within the brain of SHRs. In the third study, we explored the effects of cessation of exercise (physical detraining) on these parameters. Next, we examined the role of GSK-3β in dysregulation of PICs and AIC *in vitro* using neuronal cell culture and *in vivo* using angiotensin II-induced hypertensive rat model. Finally, we investigated the effects of ExT on brain GSK-3β in hypertension and whether central GSK-3β mediates exercise-induced beneficial effects in hypertension. Collectively, these studies demonstrate that

unlike pharmacological therapies, chronic regular exercise is a non-pharmacological cost-effective tool that has the capability to positively modulate several components of signaling pathways involved in pathogenesis of hypertension. These findings provide greater insight into the molecular mechanisms underlying the exercise-induced beneficial effects and will ultimately lead us to refine the current guidelines for the treatment of hypertension on the basis of scientific evidence.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

HYPERTENSION

According to the World Health Organization (WHO), hypertension is one of the world's great public health problems and the leading cause of death worldwide (2002). The National Health and Nutrition Examination Survey (NHANES) 2005–2008 indicated that within the United States, 33.5% of adults ages 20 and above have hypertension. This amounts to an estimated 76.4 million US adults with hypertension (Roger, Go et al. 2011). Among hypertensive adults, ≈80% are aware of their condition, 71% are using antihypertensive medication, and only 48% of those aware that they have hypertension have their condition controlled. The estimated direct and indirect cost of high blood pressure (HBP) was $43.5 billion in the year 2007. Hypertension is a substantial risk factor for cardiovascular disease, and efforts to attain a blood pressure (BP) target of 140 mmHg would result in a reduction of 28–44% in stroke and 20–35% in ischaemic heart disease, depending upon the age (He and MacGregor 2003).

Hypertension is defined as a systolic/diastolic blood pressure (SBP/DBP) of 140/90 mmHg or higher. However, data from NHANES 1999 to 2006 estimate 29.7% of adults ≥20 years of age have prehypertension (Ogunniyi, Croft et al. 2010), a condition characterized by SBP of 120 to 139 or DBP of 80-89 mmHg. Hypertension can also be classified as either primary/essential or secondary hypertension. Primary, essential, or idiopathic hypertension refers to an increased BP of an unknown etiology, which constitutes about 95% of all hypertensive cases (Carretero and Oparil 2000). The remaining 5-10% cases are designated as secondary hypertension, which is caused by known conditions that affect the kidney, heart and arteries. Hypertension has multiple etiologies, including high salt intake, increased sympathetic activity, genetic predisposition, and physical inactivity amongst others (Chiong 2008).

Although, various currently available pharmacological therapies such as β-blockers, α-blockers, Ca^{2+} channel blockers, angiotensin converting enzyme (ACE) inhibitors, and angiotensin receptor blockers (ARBs), have been proven to reduce BP; the morbidity and mortality caused by hypertension is still on the rise. According to current "Heart Disease and Stroke Statistics" the death rate from hypertension increased 9.0% from 1997 to 2007, and the actual number of deaths increased 35.6% (Roger, Go et al. 2011). More disturbing is the fact that only one-third of hypertensive patients can be successfully treated with one antihypertensive agent, the other two-thirds require two or more agents for effective BP control (Marc and Llorens-Cortes 2011). These alarming statistics suggest that pharmacological therapies targeting only one component of the signaling pathways involved in hypertension is not sufficient enough to control the disease, rather there is a need for therapeutic approaches that have the capability to target multiple components at the same time.

PATHOGENESIS OF HYPERTENSION

The pathogenesis of hypertension is highly complex and after a century of research, even today, there is no unifying hypothesis for the pathogenesis of hypertension. Alterations in renin-angiotensin system (RAS) (Welch 2008), increases sympathetic activity, inflammatory cytokines, and oxidative stress (Welch 2008) have all been implicated in its development and progression. Moreover, although hypertension has multiple etiologies, physical inactivity has been found to have strong correlation with the disease. Hypertension was initially considered a disease of the circulation, but it has now been established that it is a multifaceted disorder involving both circulating and systemic components.

The RAS in Hypertension. Besides elevated BP and cardiac and arterial hypertrophy and remodeling, hypertension is often characterized by an overactivation of the RAS. The RAS is

critically involved in the physiological regulation of BP and volume homeostasis as well as in the pathogenesis of hypertension (Ferrario 2010; Singh, Mensah et al. 2010). Since its discovery in the 1890s the RAS was considered as an endocrine system whereby circulating kidney-derived renin regulates cardiovascular function through Angiotensin II (Ang II) binding to its receptors on target tissues (Cat and Touyz 2011). AngII is the primary effector molecule of the RAS and is formed from enzymatic cleavage of angiotensinogen to angiotensin I (AngI) by the aspartyl protease renin. AngI is then converted into AngII by angiotensin converting enzyme (ACE). AngII exerts vasoconstrictor, signaling and remodeling effects. Acute stimulation with AngII causes vasoconstriction and regulates salt/water homeostasis leading to increased BP, whereas chronic stimulation promotes hyperplasia and hypertrophy of vascular smooth muscle cells (Geisterfer, Peach et al. 1988; Xi, Graf et al. 1999). Chronic exposure to AngII also plays an important role in cardiac hypertrophy, fibrosis, and remodeling as evident in hypertensive hearts (Mehta and Griendling 2007). Most of the known effects of AngII are mediated by angiotensin type 1 receptor (AT1R), and AngII-ATIR-ACE forms the classical pathway of the RAS. However, a recently discovered carboxypeptidase, ACE2, cleaves one amino acid from either AngI or AngII, producing another metabolite Ang(1-7), which has vasodilator properties. Besides the classical pathway of RAS (ACE, AngII, and AT1R), newly discovered RAS components such as ACE2, Ang1-7, and its receptor Mas have been shown to play an important role in BP regulation, by counteracting the classical pathway. In fact, research over the past decade has suggested that the balance between ACE and ACE2 is an important factor determining the outcome of hypertension (Danilczyk and Penninger 2006).

Once AngII binds to the AT1R, it activates a series of signaling cascades, which in turn regulate various physiological effects of AngII. One well established mechanism by which AngII

signaling occurs involves the classic G-protein mediated pathways. Upon activation, AT1R can activate several tyrosine kinases and serine/threonine kinases (such as mitogen activated protein kinases, MAPKs). However, a growing body of evidence suggests that many of the AngII-induced pathological effects occur via oxidative stress. AngII activates membrane NAD(P)H oxidases, that in turn produces reactive oxygen species (ROS), leading to increased oxidative stress. Although ROS were previously considered to be toxic byproducts of metabolism, they are now known to be potent intercellular and intracellular second messengers that mediate signaling pathways involved in the pathogenesis of inflammation and hypertension (Griendling, Sorescu et al. 2000). Moreover, recent findings from our laboratory (Sriramula, Haque et al. 2008) and others have shown that the RAS can interact with the inflammatory cytokines such as tumor necrosis factor-*alpha* (TNF-α), leading to excessive production of ROS, thereby contributing to hypertension.

Inflammatory Cytokines in Hypertension. One of the hallmarks of hypertension is chronic low-grade inflammation. Pro-inflammatory cytokines (PICs) such as TNF-α (Dorffel, Latsch et al. 1999), interleukin (IL)-1β (Dorffel, Latsch et al. 1999; Peeters, Netea et al. 2001), and IL-6 (Chae, Lee et al. 2001; Peeters, Netea et al. 2001), have been reported to be elevated with the severity of hypertension and are of prognostic significance. Among all PICs, TNF-α is one of the primary cytokines that plays key roles in inflammation and cardiovascular dysfunction associated with hypertension. For instance, TNF-α stimulation evokes cardiomyocyte hypertrophy (Yokoyama, Nakano et al. 1997), progressive myocyte cell death (Krown, Page et al. 1996), myocardial contractile defects (Kubota, McTiernan et al. 1997), and produces vasoconstriction (Nakamura, Yoshida et al. 2000).

TNF-α is a pleiotropic cytokine and it belongs to a family of signaling molecules that exist as type II membrane proteins characterized by the C-terminus being extra-cytoplasmic. TNF-α has 157 amino acids and is produced in response to various inflammatory stimuli. Two distinct surface receptors mediate the effects of TNF-α, TNFR1 and TNFR2; however transduction of signals from TNFR1 and its role in TNF-α signaling is the most studied and well characterized (Sack, Smith et al. 2000). TNF-α receptors signal as homotrimers and can exist either as membrane-bound or as truncated soluble forms (Baker and Reddy 1996). The binding of TNF-α to its homotrimeric TNFR1 activates several divergent downstream signaling pathways, with the activation of nuclear factor kappaB (NFκB)-dependent pathway being one of the most important pathways involved in the pathogenesis of hypertension. TNF-α has also been found to activate ROS (Cai and Harrison 2000; Mariappan, Soorappan et al. 2007; Neri, Cerretani et al. 2007), which in turn can activate various intracellular signaling pathways, including that of nuclear factor-kappa B (NFκB). Activation of NFκB induces gene transcription of PICs, which leads to a further increase in ROS production, fostering a positive feedback mechanism, and eventually leading to the progression of hypertension.

Recent discoveries indicate that besides elevated levels of PICs (Peeters, Netea et al. 2001; Shi, Raizada et al. 2010), anti-inflammatory cytokine, IL-10 has a significant impact on sympathetic outflow, arterial pressure and cardiac remodeling in experimental models of hypertension (Shi, Raizada et al. 2010). In fact, much evidence postulates that the balance between PICs and AICs, such as IL-10, determine the outcome of hypertension. However, the mechanism by which this dysregulation occurs in hypertension is currently unknown.

Oxidative Stress in Hypertension. Besides the RAS and PICs, oxidative stress has been implicated in the pathogenesis of hypertension. Oxidative stress is defined as the imbalanced

redox state where pro-oxidants overwhelm intrinsic anti-oxidant systems, resulting in an increased production of ROS. Increased oxidative stress has been shown to be critically involved in various cardiovascular diseases such as diabetes mellitus, atherosclerosis, heart failure, and hypertension (Griendling, Sorescu et al. 2000; Touyz 2000). During normal physiological conditions, ROS are produced in a controlled manner at low concentrations and function as normal by-products of cellular metabolism. However, under pathological circumstances, increased ROS function as signaling molecules contributing to cell dysfunction, cell apoptosis, lipid peroxidation, inflammation, and increased deposition of extracellular matrix proteins, which all are important factors in vascular, renal, and cardiac damage during the pathogenesis of hypertension (Sawyer, Siwik et al. 2002; Murdoch, Zhang et al. 2006; Papaharalambus and Griendling 2007).

ROS are produced by numerous enzymes in many cell types, including endothelial, vascular smooth muscle, neuronal, microglial, cardiac, and various renal cells. The major ROS produced are the free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl moiety (.OH), alkoxyl (RO-), peroxyl (ROO-), and hydroperoxyl (ROOH-). Other ROS (example, hydrogen peroxide (H2O2) and lipid peroxides) can be converted into free radicals by transition metals, either free in the cell or protein-bound (Cooper, Vollaard et al. 2002). Emerging evidence indicates that AngII-induced membrane-bound NADPH oxidases (NOXs) are the main source of ROS in hypertension. NOX is composed of multiple subunits, including two membrane-bound subunits, p22*phox* and gp91*phox* (also known as NOX2, or the homologues of NOX1 and NOX4), and the cytosolic subunits p47*phox*, p40*phox*, p67*phox* and Rac1 (a small G-protein) (Lassegue and Clempus 2003; Lambeth 2004). Upon stimulation, NOXs produce superoxide anion ($O_2^{\bullet-}$) by the one electron reduction of oxygen using NADPH as the electron donor: $2O_2 + NADPH \rightarrow 2O_2^{-} +$

$NADP^+$ (Rajagopalan, Kurz et al. 1996; Ushio-Fukai, Zafari et al. 1996; Zafari, Ushio-Fukai et al. 1998; Griendling, Sorescu et al. 2000; Lambeth 2004). Other enzymatic sources of ROS are xanthine oxidases, uncoupling of the mitochondrial respiratory chain, cytochrome p450, and uncoupling of endothelial nitric oxide synthase (eNOS).

Although excessive production of ROS is the most common cause of oxidative stress in hypertension, decreased local antioxidative protection is another potential cause of oxidative stress (Kobayashi, Inoue et al. 2002; Wei and Lee 2002; Ahamed and Siddiqui 2007). Under normal physiological conditions, ROS are produced inadvertently in the body by a variety of mechanisms and majority of the free radicals produced *in vivo* are oxidants and are capable of oxidizing a range of biological molecules, including carbohydrate, amino acids, fatty acids, and nucleotides. Therefore, to protect against tissue damage caused by these oxidants, a range of anti-oxidant defenses have evolved in the body that consist of both enzymatic and non-enzymatic antioxidants. Antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. The main non-enzymatic antioxidants include reduced glutathione (GSH), vitamin C and vitamin E. Therefore, not only the increased production of oxidants, but also downregulation of anti-oxidant defense mechanisms in the body, may lead to imbalance in redox homeostasis, ultimately causing oxidative stress.

One of the consequences of increased oxidative stress in hypertension is a functional inactivation of nitric oxide (NO) by high concentrations of superoxide anion, resulting in enhanced formation of the highly-toxic reactive compound peroxynitrite ($OONO^-$). NO is a potent vasodilator and is enzymatically generated from L-arginine by a unique family of calcium/calmodulin-binding NO synthases (NOS) now identified as neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms (Messmer, Lapetina et al. 1995). nNOS and

eNOS are constitutively expressed in tissues whereas iNOS is present in all nucleated mammalian cells and is inducible by endotoxins or cytokines. NO effects its principle biological actions, including that of vascular smooth muscle relaxation and vasodilation, via soluble guanylate cyclase and production of the second messenger c-GMP (Bredt and Snyder 1994). However, NO also mediates tissue injury in pathophysiological states including hypertension (Klinger 2007), mainly by producing $OONO^-$ (Pacher, Beckman et al. 2007). Inhibition of NOS (particularly eNOS) results in vasoconstriction and a rise in systemic BP in animals (Vallance, Collier et al. 1989; Huang, Leblanc et al. 1994) and human beings (Haynes, Noon et al. 1993; Castellano, Rizzoni et al. 1995), increased production of iNOS has been documented to be associated with the development of hypertension (Hong, Loh et al. 2000; Escames, Khaldy et al. 2004).

ROLE OF THE BRAIN IN THE PATHOGENESIS OF HYPERTENSION

Past several decades of research has established that the central nervous system (CNS) plays an important role in regulating cardiovascular function. Several brain regions have been shown to play homeostatic roles and influence cardiovascular function, including the brainstem, pons, hypothalamus, amygdale, and others (Kramer, Plowey et al. 2000). Among all the brain regions, paraventricular nucleus (PVN) of the hypothalamus and rostral ventrolateral medulla (RVLM) of the brain stem are known to be the most important cardiovascular regulatory centers playing role in cardiovascular regulation, fluid homeostasis, and maintenance of tonic sympathetic nerve activity (Gao, Wang et al. 2008).

The RAS was classically considered as a circulating signaling system, however, it has now been shown that in addition to the classic humoral-endocrine system, local RASs are present in various tissues throughout the body, interacting with each other and the endocrine RAS (Xia

and Lazartigues 2010). Studies over the last decades have established that an over-activity of the RAS within the brain plays a vital role in development and maintenance of hypertension (Cuadra, Shan et al. 2010). All known components of the RAS, including the precursor and enzymes required for the production and metabolism of angiotensin peptides, have been identified in the brain nuclei involved in the central regulation of cardiovascular function such as PVN, RVLM, subfornical organ and nucleus of the tractus solitaries (NTS) (Saavedra 1992; Wright and Harding 1994; Lenkei, Corvol et al. 1995; Xia and Lazartigues 2010). In addition to AngII generated in the brain, blood-borne AngII can also enter the CNS via the circumventricular organs (CVO) and contribute to the regulation of BP and volume homeostasis (Davisson 2003).

In the CNS, AngII through AT1R causes increased sympathetic outflow, cardiac baroreflex desensitization, vasopressin release and stimulation of water and salt intake, leading to increased BP (Phillips and Sumners 1998). Increased levels of ACE, AngII, and ATIR in the brain have been found in hypertension (Phillips and de Oliveira 2008). It has been shown that the intracerebroventricular (i.c.v.) injection of AngII causes dose-dependent increase in BP in experimental animals (Phillips 1987; Wright and Harding 1992). Similarly, alterations in expression or activity of vasodilatory components of RAS (ACE2, Ang(1-7), Mas) in the brain has been found in hypertension. For instance, decreased ACE2 activity in the PVN of a chronically hypertensive mouse model has been shown (Xia, Feng et al. 2009). In addition, overexpression of brain ACE2 has recently been shown to prevent the development of AngII-induced hypertension (Sriramula, Cardinale et al. ; Feng, Xia et al. 2010) and reduces BP in SHR rats (Yamazato, Yamazato et al. 2007).

Like RAS, localized production of inflammatory cytokines in almost all the organs of the body, including heart, kidney and brain, suggest their autocrine or paracrine roles. In the last decade, brain inflammatory cytokines, either directly or indirectly through the RAS, have been shown to be involved in the pathogenesis of hypertension. Increased levels of PICs such as TNF-α, IL-1β and IL-6 have been found in the PVN and RVLM of hypertensive rats (Kang, Ma et al. 2009; Agarwal, Welsch et al. 2011). Moreover, infusion of IL-1β intracerebroventricularly (Kimura, Yamamoto et al. 1993; Kannan, Tanaka et al. 1996) or microinjection into the PVN (Lu, Chen et al. 2009) increases sympathetic activity and resting arterial BP in conscious animals. It is apparent from previous studies that within the brain, PICs such as TNF-α and IL-1β act as neuromodulators and play pivotal roles in sympathetic regulation of BP (Shi, Raizada et al. 2010).

Recent discoveries indicate that besides elevated levels of circulating and brain PICs (Peeters, Netea et al. 2001; Shi, Raizada et al. 2010), anti-inflammatory cytokine IL-10 have a significant impact on sympathetic outflow and BP in hypertension (Shi, Raizada et al. 2010). AIC are known to exert inhibitory effects on PICs in the peripheral immune system and have also shown to have a similar role in the CNS (Murray 2005). Overexpression of IL-10 in the brain (particularly within the PVN) ameliorates hypertension and associated organ damage in hypertensive rats (Nonaka-Sarukawa, Okada et al. 2008; Nomoto, Okada et al. 2009).

Like RAS components, TNF-α and other cytokines can be synthesized in the CNS by microglia, astrocytes, and neurons (Lieberman, Pitha et al. 1989). For example, mRNA and protein for TNF-α, IL-1β and IL-6 have been detected in different brain regions including PVN and RVLM. Inflammatory cytokines are polypeptides of molecular weights of greater than 10 kDa and hence peripherally produced cytokines are unable to cross the blood blood brain barrier

(BBB) (Quan and Herkenham 2002). However, these cytokines can penetrate into the brain at the CVO such as subfornical organ, the organum vasculosum of the lamina terminalis and the area postrema, areas that do not have a BBB (Shi, Raizada et al. 2010). Moreover, cytokines can activate afferent nerves that pass information to brain centers such as NTS, which then relays the information to other cardiovascular regulatory centers (PVN and RVLM) (Raison, Capuron et al. 2006). Recently it has been suggested that dysfunction of the BBB in diseased conditions (i.e. hypertension) may allow passage of cytokines into the CNS (Waki, Gouraud et al. 2008).

Within the CNS, cytokines bind with their receptors that are located on multiple cell types (e.g. microglia, astrocytes, and neurons) and initiate a cascade of signaling events that are responsible for development and progression of hypertension. For example, TNF-α and IL-1β increases neuronal activity (Viviani, Bartesaghi et al. 2003; Ishinaga, Jono et al. 2009), possibly by increasing Ca^{2+} influx into the neurons. TNF-α has also been found to activate ROS (Cai and Harrison 2000; Mariappan, Soorappan et al. 2007; Neri, Cerretani et al. 2007), which in turn can activate various intracellular signaling pathways, including that of nuclear factor-kappa B (NFκB), and eventually leading to the progression of hypertension.

NUCLEAR FACTOR *kappa*B (NFκB) IN HYPERTENSION

NFκB is a transcription factor that regulates gene involved in immune and inflammatory responses, cell apoptosis, and cell survival. NFκB is formed by homo- and hetero-dimers of a group of related DNA-binding proteins belonging to the Rel family. The NFκB subunits are RelA (p65), RelB, c-Rel, p50 and p52 and the respective precursors, p105, and p100. The NFκB heterodimer consisting of p50 and p65, is thought to be a key regulator of genes involved in responses to infection, inflammation and stress. In the cytoplasm, NFκB exists in a latent state and is bound to its inhibitory molecule, IκB that prevents its translocation to nucleus. The NFκB

activating signals such as PICs and/or ROS activate the IκB kinase (IKK) complex that induces site-specific phosphorylation of IκB leading to its ubiquitination and proteasomal degradation. NFκB can now translocate to the nucleus to regulate transcription.

Though phosphorylation and subsequent degradation of IκB is the most important step in the activation of NFκB, its activity can also be regulated by IκB-independent mechanisms, for example, post-translational modifications of p65 subunit and stimulus-induced RelB degradation. A number of protein kinases phosphorylate the strongly transactivating p65 subunit at ser276, ser311, ser529, and ser536 leading to increased NFκB activity (Buss, Dorrie et al. 2004). RelB degradation is another way by which NFκB gets activated. RelB degradation is phosphorylation dependent, particularly phosphorylation of Thr84 and Ser552 has been shown to be critical in its degradation (Neumann, Klar et al. 2011).

In addition to above mentioned mechanisms, NFκB activity is regulated by a co-activator, CREB-binding protein (CBP) (Du, Wei et al. 2007). CBP is a highly conserved family of multifunctional proteins, and its binding with p65 increases NFκB activity (Takahashi, Tetsuka et al. 2002). The NFκB activity is also regulated by phosphorylation of precursor molecule (p105) of p50 subunit, and p105 phosphorylation on ser927 and Ser932 is required for its ubiquitination and processing into p50 (Lang, Janzen et al. 2003).

Upon activation, NFκB promotes transcription of several genes involved in the hypertension-associated inflammatory response, such as TNF-α, inducible NOS, and inflammatory adhesion molecules (Sriramula, Haque et al. 2008; Agarwal, Haque et al. 2009). Besides, NFκB activation also leads to increases in ROS production and pro-hypertensive components of RAS, such as AT1R and ACE, thereby contributing to hypertensive response (Cardinale, Sriramula et al. 2011).

CYCLIC AMP RESPONSE ELEMENT BINDING PROTEIN (CREB) IN HYPERTENSION

Another transcription factor, cyclic AMP response element binding protein (CREB) has also been shown to be a key player in regulation of cytokines involved in pathogenesis of hypertension. CREB is a 43 kDa phosphoprotein that plays an important role in transcription of anti-inflammatory cytokines such as IL-10. CREB is a phosphorylation-dependent transcription factor. Several different serine-threonine kinases have been shown to promote phosphorylation of CREB at its transcription activating site, serine 133 (Ser-133), including cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), calmodulin kinases (CaMKs), and ribosomal S6 kinase. CREB phosphorylation on Ser133 causes increased interaction of CREB with its coactivator protein, CBP leading to gene transcription (Gonzalez and Montminy 1989). The CREB activity is also regulated by phosphorylation at other residues that are not completely characterized yet.

PHYSICAL ACTIVITY AND HYPERTENSION

Physical inactivity is the fourth leading risk factor for global death, after high blood pressure, smoking and high blood glucose, accounting for 3.2 million deaths per year (WHO, 2011). The average amount of human daily physical activity has drastically declined over the past century. For example, it is estimated that children today spend 600 kcal/day less on physical activity than their counterparts 50 years ago (Boreham and Riddoch 2001). Physical activity beneficially affects the human body in a multifactorial manner; however, the number of chronic diseases and associated financial costs potentially produced by physical inactivity is still much larger than generally appreciated (Booth, Gordon et al. 2000). It has been found that there is a strong inverse relationship between energy expenditure and various cardiovascular diseases (CVDs) such as coronary heart disease, type 2 diabetes and hypertension (Manson, Nathan et al.

1992; Manson, Hu et al. 1999). Based on a meta-analysis of 44 randomized trials of physical activity, the sedentary population has been shown to have blood pressures that were higher by 2/3 mmHg (SBP/DBP) in normotensive subjects and by 7/6 mmHg in hypertensive patients compared to physically active groups (Fagard 1999). It has been proposed that physical inactivity should be targeted at the primary prevention level (Koplan and Dietz 1999). In contrary, the available data suggest that moderate-intensity (40–70% VO_2 max; VO_2 max also known as maximal oxygen consumption or maximal aerobic capacity is defined as the maximum capacity of an individual's body to transport and use oxygen during incremental exercise) aerobic exercise is associated with a significant reduction of BP in hypertensive and normotensive participants and in overweight, as well as normal-weight participants (Whelton, Chin et al. 2002). Interestingly, increasing exercise intensity to above 70% VO_2 max did not have any additional impact on BP reduction (Lee and Lip 2003; Lee and Lip 2004). According to the Centers for Disease Control (CDC) and the American College of Sports Medicine (ACSM), moderate-intensity exercise is defined as the activity performed at three to six times the basal metabolic rate, which is the equivalent of brisk walking at 3-4 miles/h for most healthy adults or the activity that is enough to expend 200 calories per day (Pate, Pratt et al. 1995).

The beneficial effect of regular exercise in hypertension is not limited to reduction of BP only. It has also been shown to reduce left ventricular hypertrophy (Hinderliter, Sherwood et al. 2002), improve exercise capacity and quality of life (Tsai, Yang et al. 2004). When combined with dietary alterations, regular exercise causes reduction of oxidative stress, increases nitric oxide availability and improves the overall metabolic profile (Roberts, Vaziri et al. 2002).

Recently, physical activity has been recommended as a first line intervention for preventing and treating patients with hypertension (Chobanian, Bakris et al. 2003). However, the

specific quantity and quality of physical activity necessary for the attainment of the health benefits, particularly in diseased condition are less clear. Based on a large prospective cohort studies of diverse populations (Sesso, Paffenbarger et al. 2000; Manson, Greenland et al. 2002), the recent guidelines of the ACSM recommends that healthy adults should engage in moderate-intensity cardiorespiratory exercise training for ≥30 min·d^{-1} on ≥5 d·wk^{-1} for a total of ≥150 min·wk^{-1}, vigorous-intensity cardiorespiratory exercise training for ≥20 min·d^{-1} on ≥3 d·wk^{-1} (≥75 min·wk^{-1}), or a combination of moderate- and vigorous-intensity exercise to achieve a total energy expenditure of ≥500-1000 kcal·wk^{-1} (Garber, Blissmer et al. 2011). However, several studies have shown that significant risk reductions for CVD disease and premature mortality begin to be observed at volumes *below* (about one-half) these recommended targets (Sesso, Paffenbarger et al. 2000; Tanasescu, Leitzmann et al. 2002). Whereas, with the goal of lowering BP to less than 140/90 mmHg, the International Society of Hypertension (2007) has recommended at least 30 minutes of moderate physical activity (e.g. brisk walking) a day, through leisure time, daily tasks and work-related physical activity to all individuals. Though, these guidelines provide a basis for an individual to perform necessary physical activity, the amount/intensity and type of exercise necessary to induce most satisfactory improvement in BP is still not clear. More importantly, the molecular mechanisms underlying the effects of exercise interventions on hypertension are far from understood. Because of the increased risk of exercise for the hypertensive patients, there is a need to refine the current exercise guidelines that can lead to maximum benefit without any adverse outcomes.

Various proposed (mechanisms of the anti-hypertensive effects of exercise training are reduced cardiac output, reduced peripheral vascular resistance (Pescatello, Guidry et al. 2004; Spier, Delp et al. 2004; Sun, Qian et al. 2008), alterations in autonomic nervous system activity

(Sigvardsson, Svanfeldt et al. 1977), reduced circulating norepinephrine (NE) levels (Mitchell, Flynn et al. 1990) and hypothalamic-pituitary-adrenal axis responsiveness (Johnson, Kamilaris et al. 1992), but a complete understanding of the molecular mechanisms underlying the exercise-induced reduction of blood pressure is still lacking.

PHYSICAL DETRAINING AND HYPERTENSION

Although, past several years of research has proven that regular physical activity reduces BP and delays the progression of hypertension in animals and humans, the compliance with the recommended treatment has been found to be very low. For instance, non-compliance with exercise has recently been reported to be closely associated with poor outcomes of the disease (Ahmed, Abdul Khaliq et al. 2008). When compliance to exercise was assessed in patients with controlled and uncontrolled hypertension, the authors found that 43.5% patients with controlled hypertension were compliant with exercise, whereas, only 16.7% of those with uncontrolled hypertension were compliant. Despite these alarming statistics, the effects of cessation of exercise (physical detraining) at the physiological and molecular levels in hypertension are far from understood. Although the reasons for the non-compliance with the recommended exercise regimen are not always within the patient's control (for example the inability to perform physical activity, the onset of chronic illness, etc), it is imperative to understand the effects of cessation of exercise (physical training) on the effects of regular long-term exercise. A few previous studies have examined the effects of detraining on heart and skeletal muscle of hypertensive and normal rats, particularly in relation to insulin sensitivity (Neufer, Shinebarger et al. 1992; Kump and Booth 2005; Lehnen, Leguisamo et al. 2010). The available literature on effects of detraining has been controversial. For instance, 10 weeks of exercise attenuated BP in spontaneously hypertensive rats (SHRs) and 1 or 2 weeks of detraining did not affect attenuated BP in these rats

(Lehnen, Leguisamo et al. 2010). Whereas, 2 weeks of detraining was sufficient to reverse LVPWT in healthy female rats (Bocalini, Carvalho et al. 2010). Similarly, resting cardiac output was found to be reduced in trained SHRs, but it returned to sedentary values only after 5 weeks of detraining (Pavlik 1985). Additionally, 5 weeks of detraining in these SHRs led to reversal of resting HR and peripheral vascular resistance to pre-training levels(Pavlik 1985). Furthermore, Mostarda *et al*. (Mostarda, Rogow et al. 2009) has also demonstrated that 3 weeks of detraining did not cause reversal of hemodynamic benefits in diabetic animals. Most of the previous studies have been performed either on healthy subjects or in diabetic patients and the effects of detraining in hypertension, both at patho-physiological and cellular levels are still lacking.

GLYCOGEN SYNTHASE KINASE (GSK)-3β AND HYPERTENSION

Recently, glycogen synthase kinase (GSK)-3 has gained increasing attention from the scientific community due to its role in many biological processes. GSK-3 was originally identified in 1980 as a serine/threonine kinase involved in insulin-mediated glycogen metabolism (Embi, Rylatt et al. 1980; Ali, Hoeflich et al. 2001; Woodgett 2001; Doble and Woodgett 2003). In mammals, two genes encode for two distinct, but closely related GSK-3 isoforms, GSK-3α (51 kDa) and GSK-3β (47 kDa) (Meijer, Flajolet et al. 2004). GSK-3β is active under resting conditions and its activity is predominantly regulated by post-translational phosphorylation of the serine-9 (inhibitory) and tyrosine-216 (activating) amino acids. GSK-3β is particularly abundant in the CNS and is neuron-specific (Leroy and Brion 1999). Recently, GSK-3β has been reported to modulate the production of inflammatory cytokines, particularly in immune-mediated diseases (Martin, Rehani et al. 2005; Vines, Cahoon et al. 2006; Beurel and Jope 2009). More recently GSK-3β has been found to modulate the activity of CREB (Fiol, Williams et al. 1994). On one hand, GSK-3β is known to regulate downstream transcription factors. On the other hand,

it is perhaps the best known target for various upstream signaling molecules involved in hypertension and activated by the RAS, such as phosphatidylinositol 3-kinase (PI3K) and the kinase Akt (also known as protein kinase B) (Datta, Brunet et al. 1999). The PI3K/Akt signaling pathway regulates GSK-3β activity by its phosphorylation on Ser-9, predicted to reduce its activity (Cross, Alessi et al. 1995).

Role of GSK-3β in Regulating NFκB Activity. Past ten years of research suggest that NFκB activity is subject to regulation by GSK-3β. The first evidence of link between GSK-3β and NFκB was reported in 2000 when Hoeflich *et al* (Hoeflich, Luo et al. 2000) found that phenotype of GSK-3β-deficient mice is strikingly similar to the phenotype displayed by IKKβ (Li, Van Antwerp et al. 1999) or RelA (Beg, Sha et al. 1995) deficient mice and it was characterized by embryonic death due to increased hepatocyte apoptosis. Subsequent studies since then has unraveled that GSK-3β modulates NFκB-mediated transcription at several levels of its activation.

In their study, Hoeflich *et al* (2000) reported that the early steps leading to NFκB activation (IκB degradation and translocation of NFκB to the nucleus) were unaffected by the loss of GSK-3β, and he suggested that NFκB activity is in fact regulated by GSK-3β at the level of the transcriptional complex (Hoeflich, Luo et al. 2000). Later, Steinbrecher et al (2005) provided evidence to this effect by demonstrating that GSK-3β regulates NFκB-mediated transcription in gene-specific manner through a mechanism involving control of promoter-specific recruitment of NFκB (Steinbrecher, Wilson et al. 2005). In contrary, several studies suggested that active form of GSK-3β greatly suppresses NFκB activity by inhibiting IKK and subsequent degradation of IκB in neuronal cells (Bournat, Brown et al. 2000; Sanchez, Sniderhan

et al. 2003). These observations indicate that GSK-3β-mediated NFκB regulation could be tissue-specific.

The past few years of research have provided evidence that is suggestive of a direct enzyme-substrate relationship between GSK-3β and NFκB subunit p65 and p105 (precursor of p50 subunit). The first evidence that GSK-3β targets p65 phosphorylation leading to its enhanced transactivation potential came from the study of Hoeflich *et al* (2000). They reported that fibroblasts lacking GSK-3β have defects in NFκB activation in response to TNF-α despite an unaltered phosphorylation status of IκB. Later, using the GSK-3β inhibitor lithium chloride (LiCl), Schwabe et al (2002) demonstrated that GSK-3β does not alter IKK activity, IκB degradation, or p65 nuclear import or export in TNF-stimulated hepatocytes (Schwabe and Brenner 2002). By incubating recombinant GSK-3β with different p65 substrates in an *in vitro* kinase assay, they further revealed that GSK-3β phosphorylates the COOH terminus of p65. Buss *et al* (2004) using a peptide-array based approach emphasized that GSK-3β is a Ser468 kinase and physiologically phosphorylates serine 468 of p65 and negatively regulates its activity (Buss, Dorrie et al. 2004). Though these studies confirmed that p65 is a physiological substrate for GSK-3β in vitro, further validation of GSK-3β -mediated p65 phosphorylation is warranted by *in vivo* studies.

Not only p65, most recently, GSK-3β has been shown to be a kinase for RelB, degradation of which leads to reduced NFκB-activity. Using IP kinase assays with full-length human RelB (hRelB) as a substrate, Neumann *et al* (2011) demonstrated that hRelB is indeed inducibly phosphorylated at Ser552 by GSK-3β *in vitro* as well as *in vivo* (Neumann, Klar et al. 2011). Furthermore, GSK-3β phosphorylates p105 on Ser903 and Ser907, leading to its

stabilization and subsequent prevention of its conversion to p50 subunit of NFκB (Demarchi, Bertoli et al. 2003).

Role of GSK-3β in Regulating CREB Activity. It is only recently that GSK-3β has been found to have the capability to modulate the activity of CREB. Fiol *et al* (1994) for the first time demonstrated that GSK-3β facilitates activation of CREB. Furthermore, using recombinant CREB proteins in *in vitro* enzymatic reactions, they revealed CREB as a substrate for GSK-3β whereby GSK-3β has ability to phosphorylate CREB at Ser129, but only after it is pre-phosphorylated (primed) at Ser133 by other kinases such as PKA (hierarchical phosphorylation) (Fiol, Williams et al. 1994). Though GSK-3β can phosphorylate both primed and unprimed substrates, its ability to phosphorylate primed substrates is many fold greater (Rayasam, Tulasi et al. 2009). It is noteworthy that GSK-3β is unique in requiring a priming phosphate at *n*-4 (where *n* is the site of phosphorylation by GSK-3β) in order to phosphorylate many of its substrates, with the optimal consensus site for phosphorylation being Ser/Thr-Xaa-Xaa-Xaa-pSer/pThr (where pSer and pThr are phosphoserine and phosphothreonine respectively and Xaa is any amino acid) (Frame and Cohen 2001). This motif is found in several well established substrates of GSK-3β including CREB. Perhaps, CREB phosphorylation at Ser133 creates a consensus site for phosphorylation by GSK-3β at Ser129 leading to increased transactivation (Fiol, Williams et al. 1994). These observations are interesting, but no evidence has yet been presented that Ser129 is phosphorylated *in vivo* or that GSK-3β is a CREB kinase *in vivo*.

GSK-3β and Physical Activity. Since its discovery as a key enzyme involved in insulin-mediated glycogen metabolism, the role of GSK-3β has been studied in relation to diabetes and exercise-induced changes in glycogen synthesis. Few studies have examined the role of GSK-3β in exercise-induced activation of glycogen synthesis in skeletal muscle of healthy animals

(Markuns, Wojtaszewski et al. 1999). Results from the past several years of research have now established GSK-3 as a regulatory switch that can modulate numerous signaling pathways initiated by diverse stimuli (Frame and Cohen 2001; Grimes and Jope 2001; Woodgett 2001). Although the role of GSK-3β in many biological processes including cardiac development, hypertrophy, and function is now becoming evident, its role in pathogenesis of hypertension is not being studied.

STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

Inflammation is a well-known risk factor for various cardiovascular diseases (CVD), such as congestive heart failure, ischemic heart disease, coronary artery disease and hypertension. Increased activation of the RAS along with increased sympathetic activity plays a major role in the development and progression of hypertension. Several drugs targeting various components of the RAS and sympathetic nervous system have been developed and found to be effective in reducing BP; however, the morbidity and mortality caused by hypertension is still on the rise. This has guided the focus of scientific community towards elucidating novel therapeutics and investigating the importance of lifestyle modifications, such as exercise as an adjunct to current therapies, in ameliorating the hypertension. Evidence that exercise can attenuate BP has become increasingly clarified over past few years, providing a foundation for identifying more precisely the underlying mechanisms through which exercise modulates hypertensive response.

Recent research implicated brain in the initiation of all forms of hypertension, including essential hypertension (Reis 1984; Jennings and Zanstra 2009). We and others have shown that the balance between PICs and AIC within cardioregulatory centers of the brain, such as PVN, plays an important role in the pathogenesis of hypertension. At the cellular level, alterations in RAS components induce oxidative stress and may cause an imbalance between PICs and AICs,

however, the exact mechanism by which this dysregulation occurs and whether exercise training can improve this dysregulation and thereby lead to attenuated BP is not yet known.

GSK-3 is a serine/threonine kinase which, in addition to phosphorylating glycogen synthase, has numerous other substrates including several transcription factors, particularly NFκB and CREB, the two most important transcription factors known to play a central role in modulating the gene expression of inflammatory mediators involved in hypertension. On one hand, GSK-3β is known to regulate downstream transcription factors; on the other hand, it is perhaps best known target for various upstream signaling molecules involved in cardiovascular diseases, such as phosphatidylinositol 3-kinase (PI3K) and the kinase Akt (also known as protein kinase B) (Datta, Brunet et al. 1999). It is clear that AngII (or in case of hypertension), possibly by ROS-mediated mechanisms, activates several upstream signaling molecules such as PI3K/Akt, PKC, MAPK, and that exercise has the ability to modulates several of these pathways. Further, exercise has the ability to modulate important downstream transcription factors implicated in the pathogenesis of hypertension. However, the missing link connecting the exercise-mediated alterations in upstream signaling pathways and downstream transcriptional regulation in hypertension is not clear. Therefore, in light of the role of GSK-3β as a target for upstream signaling components and as a regulator of downstream transcription factors, it is plausible to suggest that GSK-3β could be the missing link in the signaling pathway involved in cardiovascular diseases and exercise-mediated reversal of these pathways.

We therefore hypothesized that regular long-term ExT would delay the progression of hypertension, reduce PICs and improve redox status within the heart and PVN of hypertensive rats; that the AngII-induced imbalance between PICs and AICs within the PVN would be modulated by activation of GSK-3β; and that ExT would restore this balance by attenuating

GSK-3β activation and thereby reduce blood pressure and hypertensive-induced changes. We also hypothesized that the exercise-induced beneficial effects in hypertension would be mediated by reduced activation of central GSK-3β.

The main goal of this proposed work is to investigate the effects of regular long-term ExT in hypertension and to understand the basic underlying mechanisms behind these effects. The specific aims are:

Aim 1: To investigate whether regular long-term moderate-intensity exercise training (ExT) delays the progression of hypertension, reduces pro-inflammatory cytokines, and improves redox status in the heart and brain (PVN) of hypertensive rats.

Aim 2: To investigate the effects of physical detraining on pressure-lowering and cardio-protective effects of regular exercise, and on inflammatory cytokines and oxidative stress within the PVN of hypertensive rats.

Aim 3: To determine the role of GSK-3β in the angiotensinII-induced imbalance between PICs and AICs in hypertension: *in vitro* and *in vivo* studies.

- 3a: To determine the role of GSK-3β in the angiotensin II-induced imbalance between PICs and AICs in neuronal cells
- 3b: To determine the effects of regular exercise on GSK-3β within the PVN of angiotensin II-induced hypertensive rats

Aim 4: To determine whether the exercise-induced beneficial effects in hypertension are mediated by central GSK-3β.

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CHAPTER 2

ROLE OF PROINFLAMMATORY CYTOKINES AND REDOX HOMEOSTASIS IN EXERCISE-INDUCED DELAYED PROGRESSION OF HYPERTENSION IN SPONTANEOUSLY HYPERTENSIVE RATS*

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INTRODUCTION

Hypertension is a well-known risk factor for various cardiovascular diseases and currently, it is estimated that more than 72 million American adults have hypertension (Lloyd-Jones, Adams et al. 2009). One of the hallmarks of hypertension is chronic low-grade inflammation. In several previous studies, pro-inflammatory cytokines (PICs) such as tumor necrosis factor alpha (TNF-α) (Dorffel, Latsch et al. 1999), interleukin (IL)-1β (Dorffel, Latsch et al. 1999; Peeters, Netea et al. 2001), and IL-6 (Chae, Lee et al. 2001; Peeters, Netea et al. 2001) have been reported to be elevated with the severity of hypertension and are of prognostic significance. In addition to PICs, free radicals such as reactive oxygen species (ROS) and superoxide ($O_2^{\bullet -}$), contribute to the pathogenesis of hypertension. More importantly, PICs have been found to activate ROS (Cai and Harrison 2000; Mariappan, Soorappan et al. 2007; Neri, Cerretani et al. 2007), which in turn can activate various intracellular signaling pathways, including that of nuclear factor-kappa B (NF-κB). Activation of NF-κB induces gene transcription of PICs, which leads to further increase in ROS production, fostering a positive feedback mechanism, and eventually leading to the progression of hypertension.

Recently, exercise has been recommended as a part of lifestyle modification for all patients diagnosed with hypertension (Chobanian, Bakris et al. 2003). Although several previous studies investigated the effects of exercise on hypertension, most of the studies are done on severely hypertensive patients or animal models by using short-term exercise protocols or exercise combined with dietary interventions (Graham and Rush 2004; Emter, McCune et al. 2005; Boissiere, Eder et al. 2008; Chicco, McCune et al. 2008; Sun, Qian et al. 2008). However, the effects of long-term exercise training (ExT) in the progression of hypertension are not clearly known. More importantly, the mechanisms by which ExT exerts its effects are largely unknown.

Various proposed mechanisms of the anti-hypertensive effects of exercise training are reduced cardiac output, reduced peripheral vascular resistance (Sun, Qian et al. 2008), alterations in autonomic nervous system activity (Sigvardsson, Svanfeldt et al. 1977), and reduced circulating norepinephrine (NE) levels (Mitchell, Flynn et al. 1990) and hypothalamic-pituitary-adrenal axis responsiveness (Johnson, Kamilaris et al. 1992), but a complete understanding of the molecular mechanisms underlying the exercise-induced reduction of blood pressure is still lacking.

The purpose of the present study was to investigate the effects of regular long-term moderate-intensity exercise training (ExT) in young spontaneously hypertensive rats and to elucidate the mechanisms behind these effects. We hypothesized that: 1) regular chronic moderate-intensity ExT would delay the progression of hypertension in young spontaneously hypertensive rats; 2) the beneficial effects of chronic ExT in young hypertensive rats would be mediated by reduced myocardial PICs and improved myocardial redox homeostasis; and 3) downregulation of NF-κB by decreased ROS generation may be a possible mechanism of exercise-induced effects. These studies will help us to further understand the mechanism by which exercise training ameliorates hypertension.

MATERIALS AND METHODS

All procedures in this study were approved by the Louisiana State University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and Experimental Design. Seven week old male normotensive Wistar-Kyoto (WK) and spontaneously hypertensive (SHR) rats were used in this study. All animals were housed in temperature ($23 \pm 2^{o}C$), and light-controlled (lights on between 7:00 A.M. and 7:00 P.M.) animal quarters and were provided with water and rat chow *ad libitum*. Animals were randomly

assigned either to the sedentary group (SHRsed; n=10 and WKsed; n=10) or to the exercise group (SHRex; n=10 and WKex; n=10). Exercise groups were subjected to moderate-intensity exercise on a motor-driven treadmill continuously for a period of 16 weeks (5 days per week; 60 min per day at 18 m/min, 0^{o} inclination) which includes an acclimation period of 2 weeks. Animals ran an average distance of 1093 m/day. Animals were euthanized twenty-four hours after the last exercise session at the age of 23 weeks. The hearts were excised, weighed, and left ventricle (LV) tissue was separated for later analysis. We performed the following experimental procedures: plethysmography, echocardiographic analysis, real time RT-PCR, western blot analysis, electron paramagnetic resonance (EPR) studies, antioxidant assays, electrophoretic mobility shift assay (EMSA), reverse-phase high-performance liquid chromatography (HPLC), and statistical analysis.

Blood Pressure Measurements. Systolic, diastolic, and mean arterial blood pressure (BP) were measured noninvasively by the tail-cuff plethysmography method using a Coda 6 Blood Pressure System (Kent Scientific, Torrington, CT). Unanesthesized rats from each group were warmed to an ambient temperature of 30^{o}C by placing them in a holding device mounted on a thermostatically controlled warming plate. BP was measured on three consecutive days, and values were averaged from at least six consecutive cycles. BP was measured at baseline (7 weeks of age) and then every two weeks until the end of the study period.

M-mode and Doppler Echocardiography. Left ventricular morphology and function were evaluated noninvasively by transthoracic echocardiography at the end of 16-week study period. Short-axis M-mode echocardiograms on the left ventricle (LV) were performed under inhaled isoflurane anesthesia (5% initial and 2% maintenance) using a Toshiba Aplio SSH770 (Toshiba Medical, Tustin, CA) fitted with a PST 65A sector scanner (8-MHz probe). The following

measurements were obtained: LV internal diameters at diastole and systole (LVIDd and LVIDs, respectively), interventricular septal thickness at diastole and systole (IVSd and IVSs, respectively), and posterior wall thickness at diastole and systole (PWTd and PWTs, respectively). Cardiac hypertrophy was evaluated by left ventricular mass index (LVMI) and relative wall thickness (RWT). The LV mass (LVM) was calculated according to the American Society of Echocardiographic conventions(Wallerson and Devereux 1987) using the following equation: $LVM=0.80[1.04(PWTd+IVSd+LVIDd)^3 – (LVIDd)^3]+0.6$. The LVM index was calculated as the LVM divided by the body weight. Relative wall thickness (RWT) as an indicator of concentric hypertrophy was calculated as 2*PWTd/LVIDd. LV systolic function was estimated by LV fractional shortening (FS%) using equation, FS (%) = [(LVIDd – LVIDs) / LVIDd] X 100. Tei index (an indicator of diastolic dysfunction) was determined from Doppler recordings of LV inflow and outflow as described previously (Dujardin, Tei et al. 1998). In brief, from mitral flow, isovolumetric relaxation time and isovolumetric contraction time were measured. Ejection time was measured from the LV outflow velocity curve recorded from the long-axis view and then the Tei index was calculated using the equation, Tei index = [(isovolumetric relaxation time + isovolumetric contraction time)/ ventricular ejection time].

Analysis of mRNA Expression by Real-Time PCR. Real time RT-PCR was used to determine the expression levels of left ventricular (LV) pro-inflammatory cytokines (PICs); tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-1β, and $gp91^{phox}$, and iNOS genes by using specific primers. Rat primers used are listed in supplementary Table. Total RNA isolation, cDNA synthesis and real-time RT-PCR were performed as previously described (Sriramula, Haque et al. 2008). In brief, total RNA was isolated from LV tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. The RNA concentration was

calculated from the absorbance at 260 nm and RNA quality was assured by 260/280 ratio. The RNA samples were treated with DNase I (Ambion) to remove any genomic DNA. First strand cDNA was synthesized from 2μg RNA with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time RT-PCR was performed in 384-well PCR plates using iTaq SYBR Green Super mix with ROX (Bio-Rad) in triplicate using the ABI Prism 7900 sequence detection system (Applied Biosystems, Fostercity, CA). The PCR cycling conditions were as follows: 50^{o}C for 2 min, 95^{o}C for 3 min, followed for 45 cycles (15 s at 95^{o}C, and 1 min at 60^{o}C). To confirm the specific PCR product, a dissociation step (15 s at 95^{o}C, 15 s at 60^{o}C, and 15 s at 95^{o}C) was added to check the melting temperature. Gene expression was measured by the ΔΔCT method and was normalized to GAPDH mRNA levels. The data are presented as the fold change of the gene of interest relative to that of control animals.

Table 2.1. Sequence of rat primers used for real-time RT-PCR.

Gene	**Sense**	**Antisense**
GAPDH	agacagccgcatcttcttgt	cttgccgtgggtagagtcat
TNF-α	gtcgtagcaaaccaccaagc	tgtgggtgaggagcacatag
IL-1β	gcaatggtcgggacatagtt	agacctgacttggcagaga
gp91^{phox}	cggaatctcctctccttcct	gcattcacacaccactccac
iNOS	ccttgttcagctacgccttc	Ggtatgcccgagttctttca

Western Blot Analysis. Protein expression in LV tissue was analyzed by western blot analysis as described previously (Sriramula, Haque et al. 2008). We used antibodies against TNF-α, IL-1β, gp91^{phox}, and iNOS (Santa Cruz Biotechnology). Protein extracts (25 μg) from the LV of all the experimental rats were combined with an equal volume of 2X Laemmli loading buffer, boiled for 5 minutes and electrophoresed on 10-15% SDS-polyacrylamide gels. The proteins

were then electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore). The membranes were blocked at room temperature for 1 h in 1% casein in phosphate-buffered saline-Tween. Blots were then incubated overnight at 4^{o}C with the primary antibodies TNF-α (1:1,000 dilution), IL-1β (1:1,000 dilution), gp91^{phox} (1:1,000 dilution), iNOS (1:1,000 dilution), and GAPDH (1:1,000 dilution). After washing with wash buffer (1X TBS, 0.1% Tween-20) four times for 10 min each time at room temperature, blots were then incubated for 1 h with secondary antibody (1:20,000 dilution) labeled with horseradish peroxidase. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham), band intensities were quantified using VersaDoc MP 5000 imaging system (Bio-Rad), and were normalized with GAPDH.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed for assessment of NF-kB activity in the LV tissue. Nuclear extracts were prepared using nuclear extract kit (Active Motif, Carlsbad, CA).The single-stranded oligonucleotides (sense, AGT TGA GGG GAC TTT CCC AGG C; antisense, GCC TGG GAA AGT CCC CTC AAC T, Sigma-Aldrich, MO) were end-labeled with biotin using the Biotin 3' End DNA Labeling Kit (Pierce Biotechnology, Rockford, IL). Then, annealing was done by mixing equal amounts of both complementary end-labeled oligos and incubating the mixture for 1 h at room temperature.

The labeled DNA fragment containing the sequence of interest was mixed on ice with LV nuclear extract (20 μg of protein), binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5), and poly (dI.dC), 1 M KCl, 100 mM MgCl2, and 1% NP-40 using LightShift EMSA Optimization and Control Kit (Pierce Biotechnology, Rockford, IL). Poly (dI.dC) was used as a nonspecific competitor of DNA of interest. The reaction mixture was incubated for 30 min at room temperature, loaded onto a pre-electrophoresed native polyacrylamide (6%) gel, and

subjected to electrophoresis. After electrophoresis, the oligonucleotide-protein complexes were transferred onto nylon membrane (Whatman, Inc, Sanford, ME), and crosslinked at 120 mJ/cm^2 using a UV-light cross-linker instrument (CL-1000 Ultraviolet Crosslinker, Entela, Upland, CA). The membrane was then incubated in blocking buffer for 15 min at room temperature, incubated with Streptavidin Peroxidase Conjugate/blocking buffer solution (1:300) for 15 min, washed four times with wash buffer, incubated with substrate equilibration buffer for 5 min, with gentle shaking at every step using chemiluminescent Nucleic Acid Detection Module (Pierce Biotechnology, Rockford, IL). Then, the membrane was incubated with substrate working solution for 5 min and chemiluminescence signals were recorded, and quantified using VersaDoc MP 5000 detection system (BioRad).

Electron Paramagnetic Resonance Studies. Total reactive oxygen species (ROS), superoxide ($O_2^{\bullet -}$), and peroxynitrite ($OONO^-$)were measured in the LV using a BenchTop Electron Paramagnetic Resonance (EPR) spectrophotometer e-scan R (Noxygen Science Transfer and Diagnostics, Elzach, Germany) as described previously (Elks, Mariappan et al. 2009).

Antioxidants Assay. Various enzymatic and nonenzymatic antioxidants were measured in LV tissue. Superoxide dismutase (SOD) activity was measured spectrophotometrically in heart homogenates by rate inhibition of a tetrazolium salt, WST-1 using a SOD assay kit (Dojindo Molecular Technologies) as per the manufacturer's specifications. Enzyme activity was reported as U/mg protein. One unit SOD is defined as a point where a sample gives 50 % inhibition of a colorimetric reaction between reactive dye (WST-1) and superoxide anion. Protein concentration was determined according to the Bradford method by using BSA as the standard. Reduced glutathione (GSH) and oxidized disulfide glutathione (GSSG) concentrations were determined spectrophotometrically in tissue homogenates by using glutathione assay kit (Cayman

Chemical). GSH to GSSG ratio was calculated. All assays were run in triplicate and averaged to obtain a mean value per sample.

Reverse-Phase High-Performance Liquid Chromatography. Plasma norepinephrine (NE) level was measured using reverse-phase high-performance liquid chromatography with electrochemical detection (ECD) using an Eicom HTEC-500 system fitted with HPLC-ECD as described previously (Guggilam, Haque et al. 2007). Isocratic separation was obtained using a CA-5ODS separation column (2.1 x 150 mm; Eicom corp, Japan), and prepackset-CA precolumn (3.0 x 4.0 mm; Eicom, Japan). Elution was done with the following mobile phase: 0.1 M phosphate buffer, pH 6.0, containing 12 % methanol, 600 mg/L sodium 1-octanesulfonate, and 50 mg/L EDTA.2Na. The quantification of NE was done by comparing the peak areas of samples with those of standard and using 3,4-dihydroxybenzylamine as an internal standard. Catecholamine extraction from fresh plasma samples was done using activated alumina as per the Eicom's application manual. 20 μl of extracted sample was injected into the HTEC-500 system. All samples were run in duplicate, averaged, and results were reported as pg/μl.

Myocardial Total Nitrate/Nitrite Concentration. Myocardial total nitrate/nitrite concentration, an index of total nitric oxide (NO) production, was determined spectrophometrically in LV tissue homogenates by using a nitrate/nitrite assay kit (Cayman Chemical).

ELISA. Plasma circulating levels of IL-1β were measured using a commercially available rat IL-1β ELISA kit (Invitrogen).

Statistical Analysis. All data are presented as means ± SE. Statistical analysis was done by either two-way ANOVA, or one-way ANOVA with Bonferroni post hoc test using Graph Pad Prism software (version 5.0). Blood pressure data were analyzed by repeated-measures ANOVA to examine with-in group changes over time. Results were considered significant when $p<0.05$.

Table 2.2. Weights and blood pressure parameters at the end of the training period.				
Parameters	**WKsed**	**WKex**	**SHRsed**	**SHRex**
N	10	10	10	10
BW (g)	365.7±8.9	327.6±9.7‡	387.9±3.4	327.2±9.7#
HW (mg)	1.01±0.04	1.21±0.04‡	1.44±0.02*	1.24±0.05#
HW/BW (mg/kg)	2.88±0.04	3.18±0.1‡	3.45±0.06*	3.68±0.02
SBP (mm Hg)	134±3	140±7	233±2*	202±0.5#
DBP (mm Hg)	89±2	98±6	176±2*	152±5#
MAP (mm Hg)	101±2	112±6	191±0.5*	168±3#
Values are mean±SE. *p<0.05 WKsed vs SHRsed rats. # p<0.05 SHRsed vs SHRex rats. ‡p<0.05 WKsed vs WKex rats.				

RESULTS

Body Weight. Body weight (BW) did not differ among groups at the start of the experiment. At the end of the study period, BW was not significantly different between WKsed and SHRsed. Chronic ExT resulted in reduction in BW both in WK and SHR rats (Table 2.2).

ExT Reduces Blood Pressure in SHRs. Systolic, diastolic, and mean arterial blood pressure (SBP, DBP, and MAP, respectively) were significantly higher in SHRsed than WKsed at the beginning of the experiment (at age 7 weeks) and remained increased for the duration of the study (Figure 2.1). At the end of the study, chronic ExT was found to have significantly reduced SBP, DBP and MAP in trained SHR compared to SHRsed (Table 2.2). Interestingly, BP in SHRex group began to decrease significantly from 8 weeks of ExT; this trend remained until study end. ExT did not affect BP in WK rats (Figure 2.1).

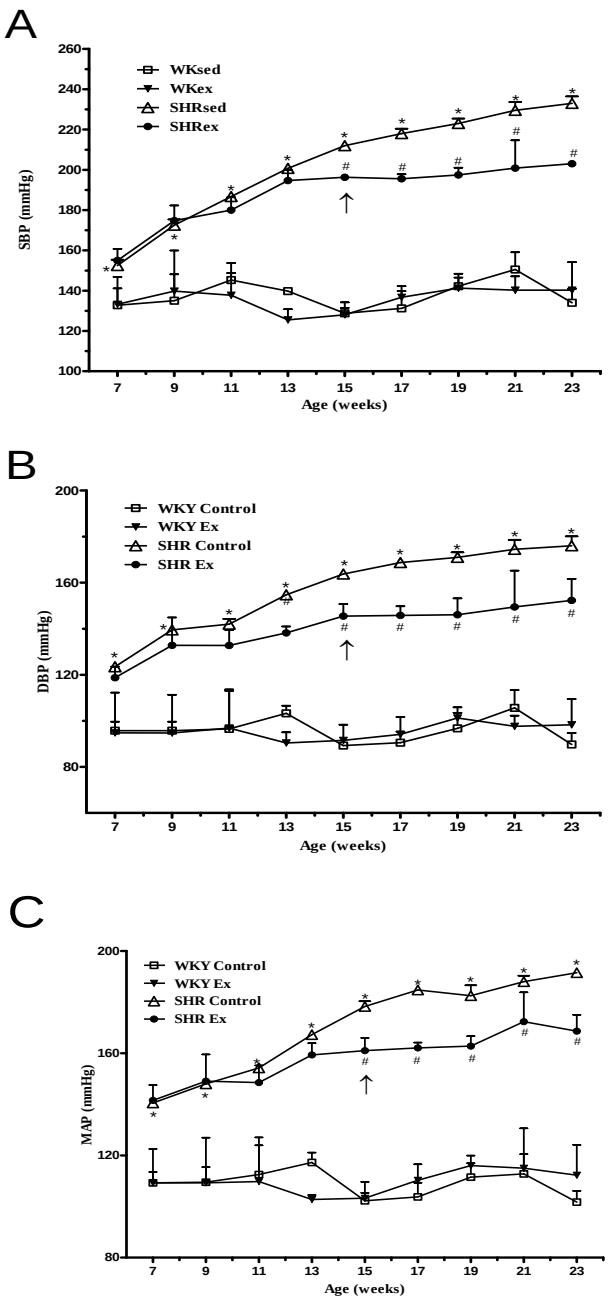

Figure 2.1. Time course of blood pressure (mmHg) in normotensive WKY and SHR rats. A, Systolic blood pressure (SBP). **B**, Diastolic blood pressure (DBP). **C**, Mean arterial pressure (MAP). BP was significantly reduced in SHRex compared to SHRsed from 8 weeks of exercise (arrow). Values are means±SE. n = 10 in each group. * $p<0.05$ WKsed vs SHRsed; # $p<0.05$ SHRsed vs SHRex.

Table 2.3. Echocardiographic analysis of cardiac hypertrophy and function.

Parameters	WKsed	WKex	SHRsed	SHRex
N	10	10	10	10
LVIDd, mm	8.050 ± 0.14	8.320 ± 0.40	7.543 ± 0.20	8.009 ± 0.08
LVIDs, mm	4.933 ± 0.18	5.150 ± 0.38	5.143 ± 0.26	5.809 ± 0.09
IVSd, mm	1.550 ± 0.05	1.7 ± 0.05	2.071 ± 0.08*	1.745 ± 0.06$^{\#}$
IVSs, mm	2.367 ± 0.12	2.540 ± 0.04	2.843 ± 0.13*	2.480 ± 0.05$^{\#}$
PWTd, mm	1.525 ± 0.06	1.7 ± 0.07	1.925 ± 0.07*	1.627 ± 0.04$^{\#}$
PWTs, mm	2.225 ± 0.105	2.3 ± 0.13	2.683 ± 0.11*	2.273 ± 0.05$^{\#}$
FS (%)	36.85 ± 3.0	36 ± 2.8	29.80 ± 1.2	28.34 ± 0.4
EF (%)	73.23 ± 4.9	73.10 ± 4.1	65.12 ± 2.2	61 ± 1.5
Tei index	0.280 ± 0.04	0.345 ± 0.04	0.699 ± 0.04*	0.534 ± 0.04$^{\#}$
LVMI	2.00 ± 0.17	2.31 ± 0.25	2.67 ± 0.08*	2.34 ± 0.16
RWT	0.380 ± 0.02	0.450 ± 0.02	0.530 ± 0.03*	0.407 ± 0.01$^{\#}$
IVRT, ms	18.75 ± 1.4	19.27 ± 1.0	30.55 ± 4.5*	23.96 ± 3.5$^{\#}$

Values are means±SE. LVIDd and LVIDs indicate left ventricular internal diameter at diastole and systole, respectively; IVSd and IVDs, interventricular septal thickness at diastole and systole, respectively; PWTd and PWTd, posterior wall thickness at diastole and systole, respectively; FS, fractional shortening (%); EF (%), ejection fraction; LVMI, left ventricular mass index; RWT, relative wall thickness. *$p<0.05$ WKsed vs SHRsed; $^{\#}p<0.05$ SHRsed vs SHRex.

ExT Reduces Pathological Cardiac Hypertrophy in SHRs. At the end of the study period, SHRsed had higher heart weight (HW) and HW/BW ratio compared to WK rats (Table 2.2). Echocardiographic studies (Table 2.3) revealed that SHRsed rats had significantly higher interventricular septal thickness, posterior wall thickness, relative wall thickness (RWT), and left ventricular mass index (LVMI), without modification of LV chamber size compared to WKsed.

These echocardiographic changes suggest the presence of concentric hypertrophy in SHRsed. Chronic ExT significantly reduced interventricular septal thickness, posterior wall thickness, and RWT in hypertensive rats when compared to their sedentary controls, indicating reduced concentric hypertrophy with ExT. In addition, moderate ExT increased LV chamber size and decreased LVMI in these animals, though values did not reach statistical significance. However,

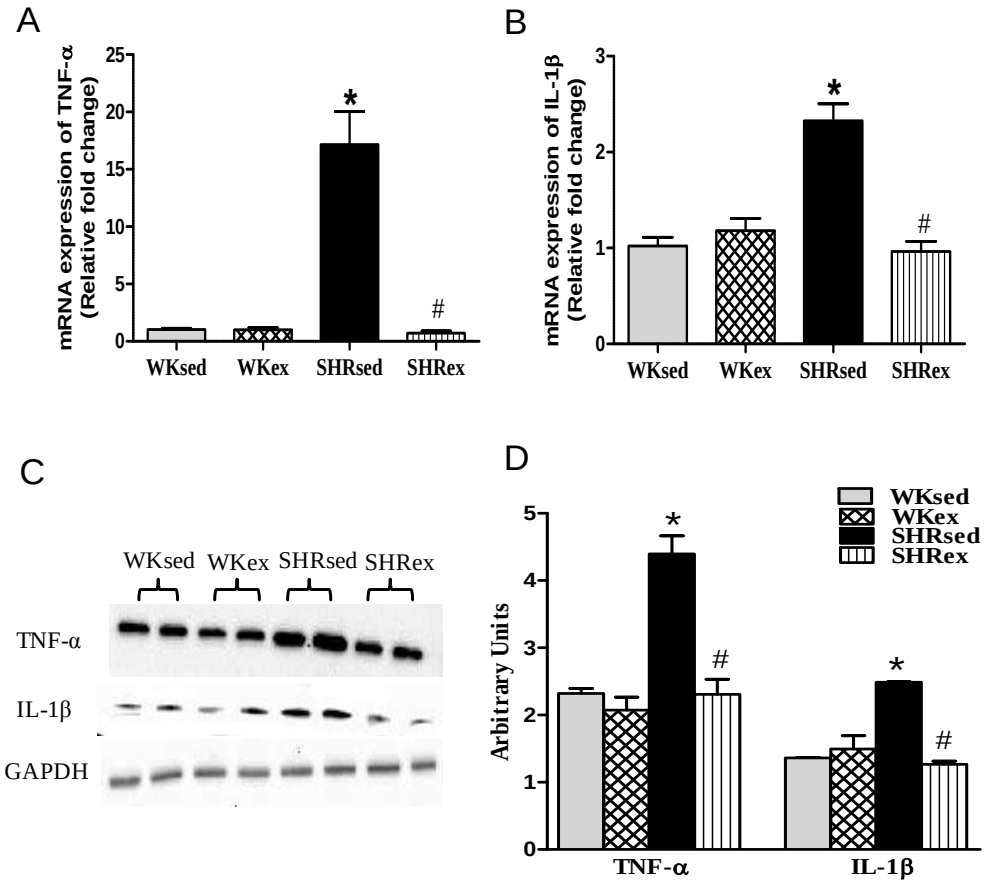

Figure 2.2. Effect of exercise training on myocardial pro-inflammatory cytokines (PICs) in WKY and SHR rats. A, mRNA expression of TNF-α. **B**, mRNA expression of IL-1β. **C**, a representative Western blots for TNF-α and IL-1β. **D**, Quantification of TNF-α and IL-1β protein expression. Values are means±SE. n=10 in each group. * $p<0.05$ WKsed vs SHRsed; # $p<0.05$ SHRsed vs SHRex.

ExT induced eccentric hypertrophy in WK rats as indicated by increased HW and HW/BW ratio without significant changes in septal and posterior wall thickness in WKex compared to WKsed.

ExT Improves LV Diastolic Function in SHRs. We evaluated the cardiac performance of all rats using M-mode and Doppler echocardiography (Table 2.3). LV systolic function was not altered in hypertensive rats, as indicated by the lack of significant changes in LV ejection fraction (EF) and fractional shortening (FS) in SHRsed compared to WKsed. However, diastolic function was severely impaired in SHR as indicated by significantly increased Tei index and increased isovolumic relaxation time (an indicator of impaired LV relaxation) in SHRsed compared to WKsed. Chronic moderate-intensity ExT significantly reduced Tei index in isovolumic relaxation time SHRs, indicative of improved diastolic function. ExT did not alter systolic function in SHRs or in WK rats.

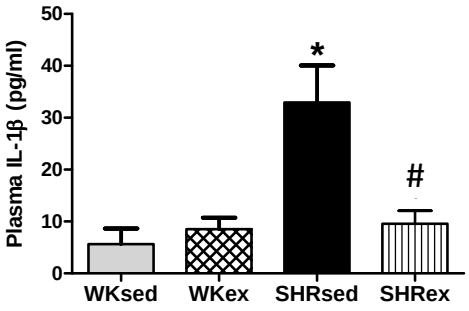

Figure 2.3. Effect of exercise training on plasma circulating levels of IL-1β in WK and SHR rats. Values are means±SE. N=6 in each group. *p<0.05 WKsed vs SHRsed; #p<0.05 SHRsed vs SHRex.

ExT Reduces Myocardial and Circulating PICs in SHRs. To determine whether the effects of chronic ExT were mediated by PICs, we examined TNF-α and IL-1β levels in the LV (Figure 2.2) and plasma (Figure 2.3). SHRsed rats exhibited marked increases in expression of TNF-α and IL-1β in the LV compared to WKsed. This upregulation of TNF-α and IL-1β was significantly attenuated by chronic ExT in SHRs. However, ExT did not change PIC levels in WK rats.

ExT Improves Myocardial Redox Homeostasis in SHRs. To elucidate the potential role of improved redox status in the beneficial effects of chronic ExT, we measured and quantified total ROS and $O_2^{\bullet-}$ production in the LV by EPR. We also examined the mRNA and protein levels of $gp91^{phox}$ by RT-PCR and western blotting. Sedentary SHR had significantly increased levels of total ROS (Figure 2.4A) and $O_2^{\bullet-}$ (Figure 2.4B) production compared to WKsed. Chronic ExT significantly attenuated total ROS and $O_2^{\bullet-}$ production in SHR. ExT did not affect free radical production in WK rats. Furthermore, $gp91^{phox}$ expression was markedly higher in SHRsed when compared to WKsed rats; this expression was significantly reduced by chronic ExT (Figure 2.5A-C).

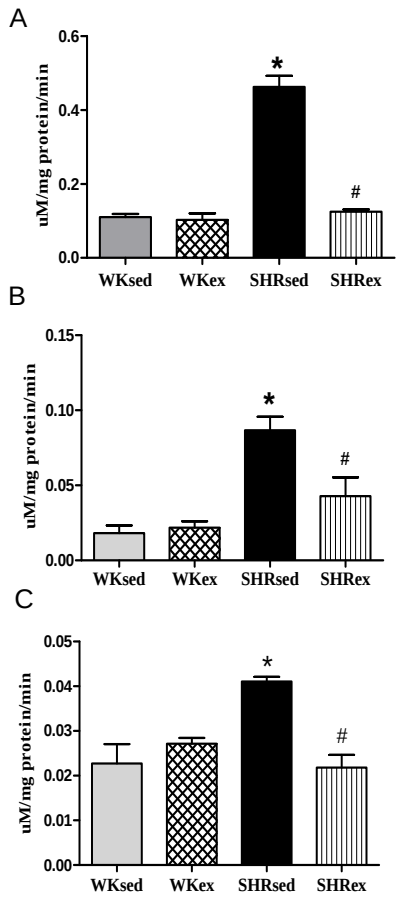

Figure 2.4. Effects of exercise training on myocardial free radical production in WKY and SHR rats. A, Total reactive oxygen species (ROS). **B**, Superoxide. **C**, Peroxynitrite (OONO). Values are means±SE. n=10 in each group. * $p<0.05$ WKsed vs SHRsed; # $p<0.05$ SHRsed vs SHRex.

Because decreased local antioxidative protection is one of the potential sources of ROS formation,(Kobayashi, Inoue et al. 2002) we analyzed various enzymatic and nonenzymatic antioxidant levels in the LV tissue. We observed that SHRsed rats had no changes in myocardial superoxide dismutase (SOD, Figure 2.6A) and reduced glutathione (GSH, Figure 2.6B) concentration when compared to WKsed. In addition, SHRsed exhibited significantly increased GSSG (oxidized disulfide glutathione, Figure 2.6C)

concentration, and reduced GSH/GSSG (an important marker of cellular redox balance (Lew, Pyke et al. 1985; Powers and Jackson 2008)) ratio (Figure 2.6D) in comparison to WKsed rats. Chronic ExT in SHR rats resulted in significantly increased GSH/GSSG ratio, decreased level of GSSG, and increased SOD activity, indicative of improvements in antioxidative defense by ExT. ExT did not affect antioxidant levels in WK rats.

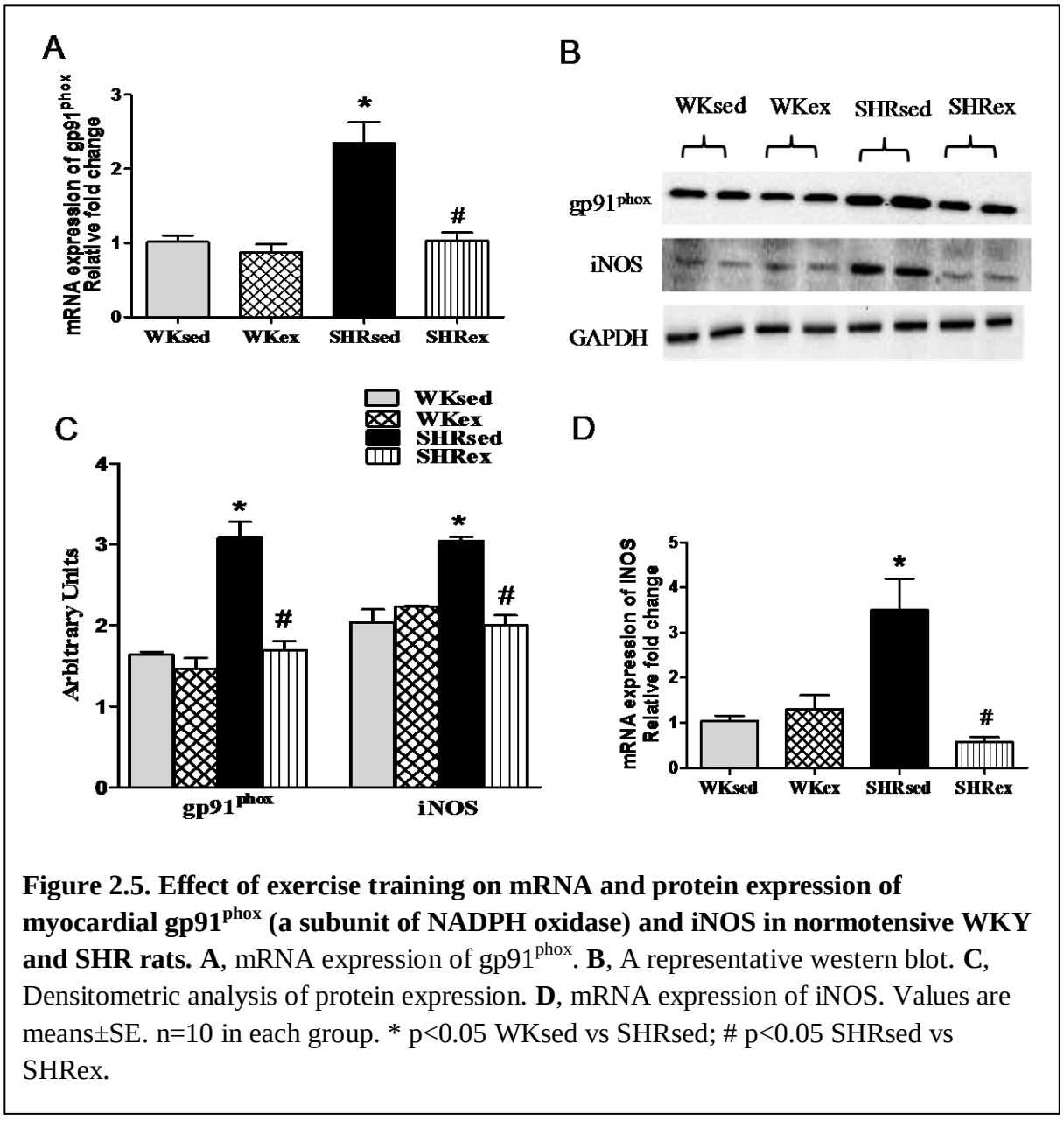

Figure 2.5. Effect of exercise training on mRNA and protein expression of myocardial gp91^{phox} (a subunit of NADPH oxidase) and iNOS in normotensive WKY and SHR rats. A, mRNA expression of gp91^{phox}. **B**, A representative western blot. **C**, Densitometric analysis of protein expression. **D**, mRNA expression of iNOS. Values are means±SE. n=10 in each group. * $p<0.05$ WKsed vs SHRsed; # $p<0.05$ SHRsed vs SHRex.

ExT Reduces Myocardial NO Synthase and Peroxynitrite in SHRs. Hypertensive rats showed significantly higher mRNA and protein expression of myocardial inducible-nitric oxide synthase (iNOS) when compared to WKsed. Chronic ExT resulted in significantly decreased iNOS expression in SHR. ExT did not affect iNOS expression in WK rats (Figure 2.5B-D). Direct measurement and quantification of peroxynitrite ($OONO^-$) by EPR studies revealed that SHRsed rats had significantly increased myocardial production of $OONO^-$ in comparison to WKsed. Interestingly, chronic ExT in SHR resulted in significantly decreased $OONO^-$ production (Figure 2.4C).

ExT Normalizes Myocardial NO Level in SHRs. Myocardial total nitrate/nitrite concentration,

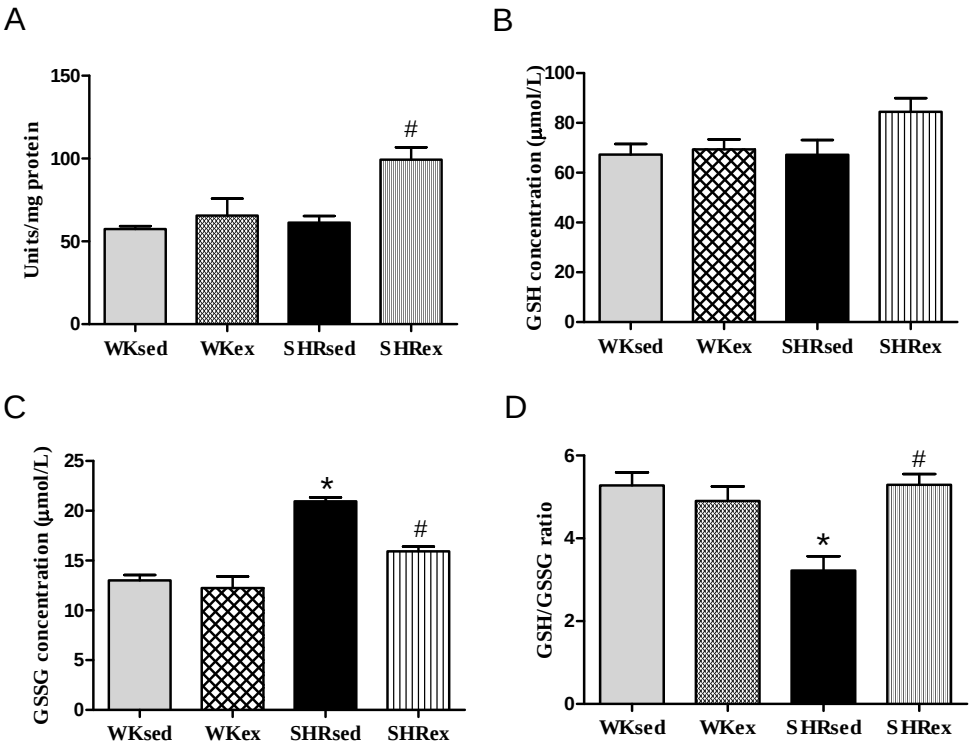

Figure 2.6. Antioxidants level in normotensive WKY and SHR rats. A, Superoxide dismutase (SOD). **B**, Reduced glutathione (GSH). **C**, oxidized glutathione disulphide (GSSG). **D**, GSH to GSSG ratio. Values are means±SE. n=10 in each group. * $p<0.05$ WKsed vs SHRsed; # $p<0.05$ SHRsed vs SHRex.

a marker of NO production, was significantly lower in SHRsed compared with WKsed rats. Chronic ExT normalized myocardial total nitrate/nitrite concentration in SHRs (Figure 2.7).

ExT Attenuates NF-κB Binding Activity in SHRs. SHRsed had significantly higher myocardial NF-κB binding than WKsed. Chronic ExT resulted in a significant decrease in NF-κB binding activity in SHR. NF-κB binding activity was unaltered by ExT in WK rats (Figure 2.8).

ExT Decreases Plasma Norepinephrine Levels in SHRs. At the end of the study, plasma NE levels were found to be significantly higher in SHRsed compared to WKsed (Figure 2.9). Chronic ExT resulted in significantly decreased plasma NE concentrations in SHR, but did not change plasma NE level in WK rats.

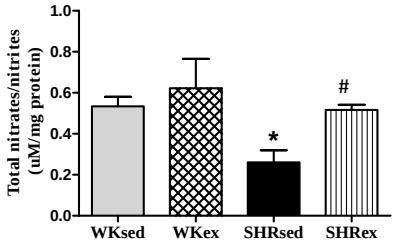

Figure 2.7. Effect of exercise training on myocardial total nitrate/nitrite concentrations in normotensive WK and SHR rats. N=6 in each group. *p<0.05 WKsed vs SHRsed; †p<0.05 SHRsed vs SHRex.

DISCUSSION

In this study, we investigated the effects of chronic moderate-intensity ExT and possible mechanisms of these effects in young spontaneously hypertensive rats (SHR), a congenital model of hypertension which shares many common features of human essential hypertension. The salient findings of this study are: 1) regular long-term moderate-intensity ExT delays the progression of hypertension, reduces cardiac hypertrophy and improves diastolic cardiac function in young SHR; 2) training-induced beneficial effects in SHR rats are mediated by decreased myocardial and circulating TNF-α and IL-1β, and reduced myocardial NF-κB activity; and 3) chronic ExT exerts its effects via improved myocardial redox status and NO production in SHRs. These

findings provide evidence for the involvement of PICs, redox homeostasis, and NF-κB in exercise-induced delayed progression of hypertension and cardiac improvements in SHRs.

At the end of the study, we observed significant reductions in SBP, DBP and MAP in trained SHR compared to SHRsed, and saw no comparable changes in trained WK rats. The pressure-lowering effect of ExT was significant starting from 8 weeks of regular exercise, and continued until the end of the study emphasizing the importance of long-term exercise in patients with hypertension. Previously, it has been reported that exercise training did not reduce BP in severely hypertensive patients and rats (Graham and Rush 2004; Chicco, McCune et al. 2008). The discrepancies in results could be because of low-intensity and/or shorter duration of exercise protocols used in those studies. Also, most of the previous studies were done in severely hypertensive rats (Graham and Rush 2004; Bertagnolli, Schenkel et al. 2008; Chicco, McCune et al. 2008). Nonetheless, results of our study suggest that regular moderate-intensity ExT delays the progression of hypertension.

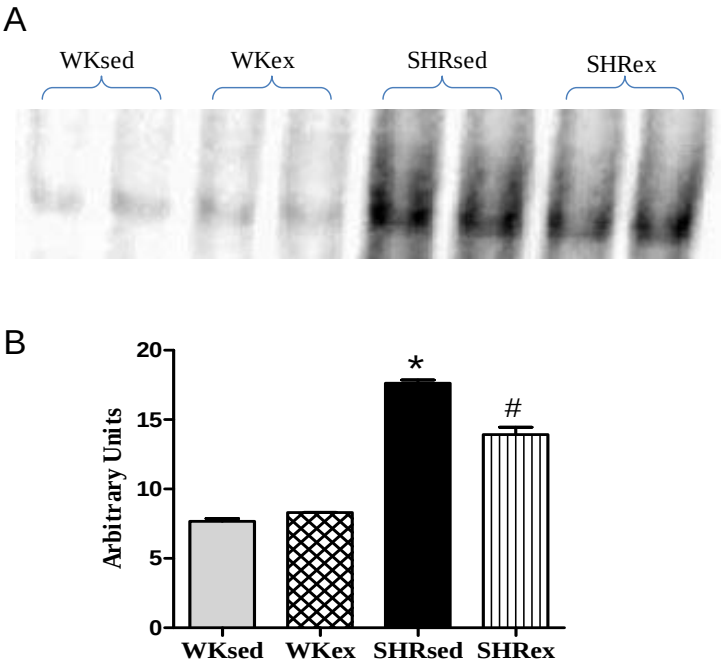

Figure 2.8. Effects of exercise training on LV NF-κB binding activity in WK rats and SHRs. A, A representative electrophoretic mobility shift assay results of NF-κB binding activity; **B**, Densitometric analysis of NF-κB binding intensity. Values are means±SE; n=6 in each group. * $p<0.05$ WKsed vs SHRsed; #$p<0.05$ SHRsed vs SHRex.

Our echocardiographic data showed that chronic ExT resulted in improved cardiac diastolic function in SHRs, as indicated by decreased isovolumic relaxation time. Evidence from previous studies indicates that the beneficial effects of ExT on diastolic function were blunted in rats with severe hypertension (Palmer, Lynch et al. 2001; Boissiere, Eder et al. 2008). To the best of our knowledge, this is the first animal study to report the effects of chronic ExT on diastolic function in young SHRs with early hypertension. Our findings, together with previous reports, suggest that moderate-intensity ExT, when initiated in the early stages of hypertension, can maximize its own cardioprotective effects. Furthermore, ExT did not alter cardiac function in WK rats as assessed by ejection fraction and fractional shortening; however, LV internal dimension was found to be slightly increased, although changes did not reach statistical significance. This observation is in accordance with several previous studies (Pluim, Zwinderman et al. 2000; Boissiere, Eder et al. 2008). Pluim et al (Pluim, Zwinderman et al. 2000), in their meta-analysis encompassing 59 studies and 1451 athletes, have reported normal cardiac function in endurance- and strength-trained athletes and concluded that there is no relationship between cardiac geometry and systolic function.

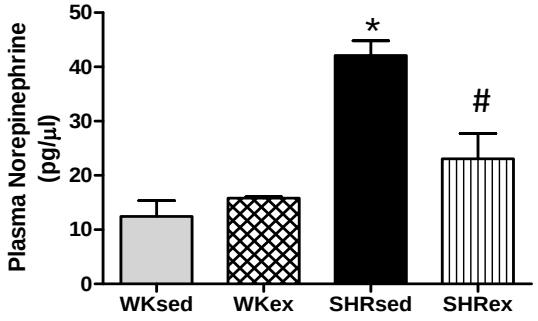

Figure 2.9. Effects of exercise training on plasma norepinephrine levels in normotensive WK and SHRs. Values are means±SE; n=10 in each group. * $p<0.05$ WKsed vs SHRsed; #$p<0.05$ SHRsed vs SHRex.

In the present study, chronic ExT also resulted in reduced cardiac hypertrophy in SHRs. This finding is significant from a clinical perspective, because pathological cardiac hypertrophy

is known to lead to cardiac failure (Rysa, Leskinen et al. 2005). Conversely, ExT in WK rats resulted in eccentric hypertrophy. Eccentric hypertrophy, also known as physiological hypertrophy, is mainly related to training-induced volume-overload (Lorell and Carabello 2000) and is considered as a cardiac adaptation of ExT (Pluim, Zwinderman et al. 2000). These results were in accordance with previous studies (Bertagnolli, Schenkel et al. 2008).

Recent evidence suggests that PICs play important roles in hypertension-induced cardiac hypertrophy. Various PICs such as TNF-α, IL-6, and IL-1β, have reported to increase with the severity of hypertension (Edwards, Ziegler et al. 2007). Few studies have documented the reduction in inflammatory markers by exercise training on obese individuals (Esposito, Pontillo et al. 2003; Marfella, Esposito et al. 2004) and diabetic patients (Giannopoulou, Fernhall et al. 2005). Several randomized clinical trials have shown reduced plasma TNF-α and/or IL-6 levels by physical training with or without dietary interventions in patients with chronic heart failure (Adamopoulos, Parissis et al. 2002) and coronary heart disease (Goldhammer, Tanchilevitch et al. 2005), accompanied by various degrees of treated hypertension. However, until now, no studies have examined the effect of chronic ExT on left ventricular PICs in hypertension. In our study, we found that chronic ExT resulted in decreased myocardial TNF-α and IL-1β in SHRs, suggesting that the beneficial effects of chronic ExT in hypertensive rats are mediated by reduced myocardial and circulating PICs.

A growing body of evidence indicates that hypertension is also characterized by increased sympathetic activity (Donohue, Stitzel et al. 1988; Guyenet 2006).The success of beta-blockers in reducing hypertension-induced cardiac hypertrophy suggests that sympathetic hyperactivity plays an important role in cardiac hypertrophy and cardiac damage in hypertension. In this study, the increases in circulating plasma NE (an indirect marker of sympathetic activity)

seen in SHRs compared with WK rats were found to be significantly decreased by chronic ExT. the finding was in agreement with recently published data showing significantly reduced cardiac NE concentrations in trained SHRs (Bertagnolli, Schenkel et al. 2008). These findings, together with previous findings from our laboratory that TNF-α blockade attenuates sympathoexcitation in heart failure (Guggilam, Patel et al. 2008), provide strong evidence of an association between PICs and sympathetic hyperactivity and reinforce the idea that ExT causes a reduction in PICs by attenuating sympathetic activity in SHRs. Therefore, it can be concluded that reduced sympathetic activity may contribute, at least in part, to exercise-induced reduced PICs in young SHRs.

Previous studies have shown that oxidative stress plays a key role in the development of hypertension and cardiac hypertrophy (Bertagnolli, Schenkel et al. 2008).We reported previously that cytokines and their transcription factor, NF-κB, contribute to the induction of oxidative stress in heart failure (Guggilam, Haque et al. 2007) and hypertension (Elks, Mariappan et al. 2009). Given the current finding that ExT reduces PICs in SHRs, we further examined the effect of chronic ExT on redox balance in hypertensive animals. Our electron paramagnetic resonance studies revealed that myocardial total ROS and $O_2^{\bullet-}$ production was significantly higher in SHRsed as compared to WKsed; however, the antioxidant defense system was unaltered. These data suggest that an imbalance in redox homeostasis plays an important role in the progression of hypertension. More importantly, chronic ExT not only reduced myocardial total ROS and $O_2^{\bullet-}$ production rates but also increased antioxidants, leading to restoration of cellular redox homeostasis. Previous evidence that TNF-α is an important contributor to oxidative stress (Guggilam, Haque et al. 2007), and our finding that decreased oxidative stress is associated with decreased PICs in SHRex rats, raises the possibility that decreased PICs might be responsible for

the exercise-induced decrease in oxidative stress in SHRs. In addition, we observed that ExT resulted in attenuation of increased expression of $gp91^{phox}$ (a subunit of NAD(P)H oxidase, a major source of ROS) in SHRs. Angiotensin II is a major regulator of NAD(P)H oxidase activity, therefore, the possible contribution of the renin-angiotensin system to exercise-mediated effects cannot be overlooked. Nonetheless, our data lead us to conclude that pressure-lowering and anti-hypertrophic effects of regular long-term moderate-intensity ExT in unestablished hypertension are mediated by improved redox status in the body rather than the attenuation of oxidant production alone.

In last few years, iNOS has been documented to be associated with the development of hypertension (Hong, Loh et al. 2000; Escames, Khaldy et al. 2004). The evidence that iNOS is predominantly induced by cytokines (Cotton, Kearney et al. 2002), and our finding that ExT reduces PICs in SHR, led us to explore whether ExT results in decreased myocardial iNOS expression. We observed that mRNA and protein expression of myocardial iNOS was markedly higher in SHRsed compared to WKsed; these levels were significantly decreased by chronic ExT in SHRs. Furthermore, reduced myocardial total nitrate/nitrite levels in SHRsed rats were normalized in SHRex rats, which is indicative of increased NO bioavailability by chronic ExT in SHRs. The decrease in iNOS level by ExT suggests lowered NO production; however, the concomitant decrease in $O_2^{\bullet-}$ in SHRex rats seems responsible for attenuated $O_2^{\bullet-}$ mediated degradation of NO, leading to increased NO bioavailability. This was further supported by our finding that ExT significantly attenuated increased $OONO^-$ production in SHRs. Therefore, the results of this study suggest that chronic moderate-intensity ExT not only decreases iNOS expression but also decreases $OONO^-$-induced tissue damage and increases NO bioavailability in SHRs. Also, in support of our results, recent studies have demonstrated that iNOS gene deletion

reduces oxidative stress and preserves cardiac function in mice with hypertension (Sun, Carretero et al. 2009).

Recent work from our laboratory suggests that NF-κB blockade reduces cytosolic and mitochondrial oxidative stress and attenuates hypertension in SHRs (Elks, Mariappan et al. 2009). PICs have also been shown to act via NF-κB-mediated signaling pathways. Therefore, one possible mechanism by which exercise exerts its beneficial effects could be via down regulation of NF-κB activity. Our present observation that NF-κB activity was increased in SHRsed compared with WKsed rats further support this hypothesis. More importantly, chronic ExT resulted in downregulation of NF-κB activity in SHRs. In our study, reduced NF-κB activity was also associated with reduced PICs and oxidative stress, suggesting that attenuation of NF-κB activity by ExT might be attributable to exercise-mediated reduced PICs and oxidative stress, which in turn leads to disruption of detrimental positive feed-back cycle involved in the progression of hypertension.

PERSPECTIVES

The findings of this study indicate that regular moderate-intensity ExT delays the progression of hypertension, reduces cardiac hypertrophy, and improves diastolic function in rats with developing hypertension. More importantly, this study provides mechanistic evidence that the pressure-lowering and cardioprotective effects of chronic exercise are mediated by reduced myocardial PICs, improved cellular redox homeostasis, increased NO production, and downregulation of NF-κB activity. We also observed that decreases in PICs by ExT were associated with reduced plasma NE levels. Although a direct cause/effect relationship could not be established in this study, we can attribute the beneficial effects of ExT on hypertension to an altered interplay between sympathetic activity, PICs, and oxidative stress via NF-κB mediated

signaling pathways. Further studies could be directed toward providing more direct evidence to support the cause/effect relationship between various parameters. Furthermore, here, we chose a moderate-intensity exercise protocol to elucidate the mechanisms of the beneficial effects of ExT in SHRs. However, the comparison of different intensities of ExT with regard to parameter studied could certainly be an important perspective of this study.

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CHAPTER 3

CHRONIC EXERCISE MODULATES RAS COMPONENTS AND IMPROVES BALANCE BETWEEN PRO-AND ANTI-INFLAMMATORY CYTOKINES IN THE BRAIN OF SHR*

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INTRODUCTION

Hypertension is the most common chronic disease in the United States, currently affecting more than 33% of US adults (Lloyd-Jones, Adams et al. 2010). Uncontrolled hypertension may lead to coronary heart disease, heart failure, chronic renal failure, and stroke. Though, the brain has typically been considered as a target for late stage hypertensive disease, a growing body of evidence has implicated brain in the initiation of all forms of hypertension including essential hypertension (Jennings and Zanstra 2009). One of the important hallmarks of hypertension is chronic low-grade inflammation. Past few years of research has implicated brain cytokines in the pathogenesis of hypertension. It is apparent from these studies that pro-inflammatory cytokines (PICs) such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 act as neuromodulators and play a pivotal role in sympathetic regulation of blood pressure (BP) (Shi, Raizada et al. 2010). In addition, recent discoveries indicate that besides elevated levels of circulating and brain PICs (Peeters, Netea et al. 2001; Shi, Raizada et al. 2010), anti-inflammatory cytokines (AICs) such as IL-10 has a significant impact on sympathetic outflow, arterial pressure and cardiac remodeling in experimental models of hypertension (Shi, Raizada et al. 2010). In the brain, paraventricular nucleus (PVN) and rostral ventrolateral medulla (RVLM) are known to be the most important cardiovascular regulatory centers playing role in sympathetic regulation of BP. Studies over the last several decades have also established that an over-activity of the brain/central renin-angiotensin system (RAS) plays a vital role in development and maintenance of genetic hypertension (Xia and Lazartigues 2010). Interestingly, it is becoming clear from previous studies that cytokines and RAS interacts with each other, possibly via production of reactive oxygen species (ROS), and thereby regulate BP (Zimmerman, Lazartigues et al. 2004; Bai, Jabbari et al. 2009; Nagae, Fujita et al. 2009; Shi, Raizada et al. 2010).

Although, several currently available anti-hypertensive medications targeting RAS and sympathetic nervous system have been found to be effective in reducing BP, still more than 50% of those diagnosed with hypertension fail to respond to these anti-hypertensive regimens. Moreover, recent epidemiological studies suggest a strong relationship between sedentary behavior and hypertension and therefore, physical activity has been recommended as a first line intervention for preventing and treating patients with hypertension (Chobanian, Bakris et al. 2003). Several experimental, clinical, and epidemiological studies have clearly shown that physical activity reduces BP in animals and humans (Sun, Qian et al. 2008; Agarwal, Haque et al. 2009). Recent studies have explored the possible mechanisms underlying the exercise-induced attenuation of BP; however, the exact mechanisms of exercise-induced effects in hypertension are still poorly understood. Few studies on obese individuals (Ziccardi, Nappo et al. 2002; Esposito, Pontillo et al. 2003; Marfella, Esposito et al. 2004) and diabetic patients (Giannopoulou, Fernhall et al. 2005) have documented the reduction in inflammatory markers by exercise. However, until now, no studies have examined the effect of chronic exercise on brain pro- and anti-inflammatory cytokines in hypertension. In addition, effects of physical activity on sympathetic activity and vasodilatory or vascoconstrictory components of central RAS in setting of hypertension has never been investigated yet.

Therefore, this study was undertaken to gain more insight into the effects of regular long-term exercise within the brain (PVN and RVLM) of hypertensive animals. We hypothesize that 1) chronic, regular moderate intensity exercise training would attenuate blood pressure in spontaneously hypertensive rats (SHRs) 2) regular exercise would improve the balance between central anti- and pro-inflammatory cytokines in SHRs; 3) chronic exercise would modulate components of RAS and reduce oxidative stress in the brain of SHRs; and 4) exercise would

attenuate neuronal excitation in the brain of SHRs. Understanding of mechanisms of exercise-induced benefits in hypertension may lead us to develop most efficient exercise regimen for hypertensive patients.

MATERIALS AND METHODS

Animals. Seven-week–old male normotensive Wistar-Kyoto (WK) and spontaneously hypertensive (SHR) rats were used in this study. Animals were housed in a temperature-($25\pm1^{o}C$) and light-controlled (12:12 hour light:dark cycle) room with free access to food and water. All of the procedures in this study were approved by the Louisiana State University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Protocol. WK and SHR rats were randomly assigned either to the sedentary group (SHRsed, n=20; WKsed, n=20) or to the exercise group (SHRex, n=20; WKex, n=20). Exercise groups were subjected to moderate-intensity exercise for 16 weeks. Body weight was recorded 24 hours after the last exercise session, animals were then euthanized, and brain tissue was harvested. PVN and RVLM tissues were punched from the excised brain for later analyses. We performed the following experimental procedures: real time RT-PCR, Western blot analysis, immunofluorescence, EIA, and statistical analysis.

Exercise Protocol. Exercise groups were subjected to moderate-intensity exercise on a motor-driven treadmill continuously for a period of 16 weeks (5 days per week; 60 min per day at 18 m/min, 0^{o} inclination) which includes an acclimation period of 2 weeks. After acclimation, training intensity was set at approximately 60% of maximal aerobic velocity (MAV), which corresponds to moderate intensity exercise (18-20m/min). This training intensity was maintained throughout the study period. The MAV was evaluated from an incremental exercise test as

reported previously (Boissiere, Eder et al. 2008; Sun, Qian et al. 2008). The rats in sedentary groups were kept in the treadmill for the same duration as exercising rats except that the treadmill was not turned on.

Assessment of Efficacy of the Exercise Protocol. Citrate synthase (CS), a respiratory enzyme which has been shown to undergo adaptive increases due to exercise in skeletal muscle fibers, was used as a marker of training efficacy. *S*oleus muscles from both legs of each animal were collected and stored at −80 °C for determination of CS activity (n=8 per group), a measure of muscle oxidative capacity, to determine the efficacy of the training protocol (Ogihara, Schoorlemmer et al. 2010). CS activity was measured from whole muscle homogenate by using commerically available citrate synthase activity assay kit (Sigma Aldrich). Briefly, muscle tissue from each animal were homogenized in an extraction buffer (50 mM Tris·HCl and 1 mM EDTA, pH 7.4). After centrifugation at 13,000 rpm, for 1 min, at 4° C, aliquots of supernatants were used for the measurement of the enzyme activity. The activity of CS was expressed as nanomoles per minute per milligram of protein. Protein content of muscle homogenate was determined as described by Bradford using bovine serum albumin as a standard.

Blood Pressure Measurements. Systolic, diastolic, and mean arterial blood pressure (BP) were measured noninvasively using a Coda 6 Blood Pressure System (Kent Scientific, Torrington, CT), as described previously (Agarwal, Haque et al. 2009). BP was measured at baseline (7 weeks of age) and then every two weeks until the end of the study period. BP was measured on three consecutive days, and values were averaged from at least six consecutive cycles.

Real-time RT-PCR Analysis. Semi-quantitative real-time RT-PCR (n=9 per group) was used to determine the mRNA levels of RAS components viz. angiotensin converting enzyme (ACE), ACE2 AT1R, and receptor Mas; PICs *viz.* tumor necrosis factor-alpha (TNF-α), and interleukin

(IL)-1β; oxidative stress markers *viz.* $gp91^{phox}$, and iNOS in the PVN and RVLM by using specific primers. Rat primers used are listed in the Table2. Briefly, the rats were euthanized, the brains were quickly removed, immediately frozen on dry ice, and blocked in the coronal plane. Brains were then sectioned at 100 um thickness, and the PVN and RVLM were punched from each brain according to the methods described by Palkovits and Brownstein (Gao, Wang et al. 2005). Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (Sriramula, Haque et al. 2008). Gene expression was measured by the ΔΔCT method and was normalized to GAPDH mRNA levels. The data are presented as the fold change of the gene of interest relative to that of control animals.

Western Blot Analysis. The tissue homogenate from the PVN and RVLM were subjected to western blot analysis (n=6 per group) for determination of protein levels of tyrosine hydroxylase (TH), 67-kDa isoform of glutamate decarboxylase (GAD67), RAS components (ACE, ACE2 AT1R, and Mas), PICs (TNF-α, IL-1β), oxidative stress markers ($gp91^{phox}$, iNOS), and GAPDH. The PVN and RVLM tissues were collected as described under the section 'real-time RT-PCR analysis'. The tissues were then homogenized in 100 µl of RIPA lysis buffer (Cell Signaling Technology, Inc., MA) containing protease inhibitor cocktail. The protein was extracted from the homogenates, and the protein concentration in the lysate was measured using a Bradford assay using BSA standards. Protein extracts (30 µg) were combined with an equal volume of 2X Laemmli loading buffer, boiled for 5 minutes and electrophoresed on 10-15% SDS-polyacrylamide gels. The proteins were then electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore). Non-specific binding was blocked by incubating the membranes in 1% casein in phosphate-buffered saline-Tween for 1 h at room temperature (RT). Blots were then incubated overnight at $4^{o}C$ with the primary antibodies. Specific antibodies used

included TNF-α, IL-1β, $gp91^{phox}$, iNOS, GAPDH, AT1R, ACE, and ACE2, GAD_{67}, at 1:1000 dilution; TH and receptor Mas, at 1:200 dilution; and IL-10, at 1;500 dilutions. Antibodies were commercially obtained: TNF-α, AT1R, TH, GAD_{67} (Abcam Inc, MA); IL-1β, iNOS, GAPDH, ACE, and ACE2 (Santa Cruz Biotechnology, Santa Cruz, CA); IL-10 (Abbiotec, CA); receptor Mas (Alomone Labs Ltd., Jerusalem, Israel), and $gp91^{phox}$ (BD biosciences, USA). After washing with wash buffer (1X TBS, 0.1% Tween-20) four times for 10 min each time at RT, blots were then incubated for 1 h with secondary antibody (1:10,000 dilution, Santa Cruz Biotechnology) labeled with horseradish peroxidase. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham), band intensities were quantified using VersaDoc MP 5000 imaging system (Bio-Rad), and were normalized with GAPDH.

Immunofluorescence Staining. Immunofluorescence technique was used to determine the protein expression of PICs, RAS components and oxidative stress markers. The immunostaining protocol used was modified from Block *et al* (Block, Santos et al. 1988). Briefly, the rats (n=5 per group) were deeply anesthetized with carbon dioxide and perfused transcardially with PBS (pH 7.4), followed by 4% paraformaldehyde in PBS. The brain was then removed, postfixed for 2 h in 10% paraformaldehyde in PBS, and coronal sections (20 μm) were made in a cryostat. The sections were incubated in xylene solution for 15 minutes at RT, two times followed by dehydration in ethanol. The sections were then washed in PBS, three times, 5 minutes each. Antigen retrieval was then performed using citrate target retrieval solution (Biocare Medical, CA). Slides were then washed with PBS and the nonspecific staining was blocked with 2% normal donkey serum containing 1% bovine serum albumin (BSA) for 1 h at RT. Sequentially, the tissues were incubated with the primary antibodies TNF-α (Abcam Inc, MA), IL-10 (Abbiotec, CA), ACE (Santa Cruz, CA), ACE2 (Santa Cruz, CA), and AT1R (Abcam Inc, MA),

at1:50 dilution for each, overnight at 4°C. The sections were then incubated either with Alexa 594-labeled anti-mouse secondary antibody (red fluorescence), Alexa 488-labeled anti-rabbit secondary antibody (green fluorescence), or Alexa 594-labeled anti-rabbit secondary antibody (red fluorescence) (Invitrogen, CA), at 1:500 dilution for 2 h at RT. The sections were rinsed 3 times in PBS and mounted in ProLong® Gold antifade reagent (Invitrogen). The stained sections were photographed with a confocal laser-scanning microscope.

Reverse-Phase High-Performance Liquid Chromatography. Plasma norepinephrine (NE) levels were measured using reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) using an Eicom HTEC-500 system fitted with HPLC-ECD as described previously (Agarwal, Haque et al. 2009; Kang, He et al. 2009).

Measurement of Plasma Angiotensin II Levels. Plasma angiotensin II (AngII) levels were determined by using commercially available enzyme immunoassay (EIA) kit (Phoenix pharmaceuticals, Inc, CA).

Statistical Analysis. All data are presented as means±SE. Statistical analysis was done by either two-way ANOVA or one-way ANOVA with a Bonferroni post hoc test using Graph Pad Prism software (version 5.0). Blood pressure data were analyzed by repeated-measures ANOVA to examine with-in group changes over time. Results were considered significant when $p<0.05$.

RESULTS

Assessment of Training Efficacy. Citrate synthase (CS) activity in soleus muscle was used as a marker of training efficacy. After the period of 16 weeks of exercise, the CS activity was significantly higher in SHR and WKY rats compared with their sedentary control groups indicating the efficacy of the exercise protocol (Figure 3.1). CS activity was higher in WKY rats compared with SHR both in the exercise and the sedentary group.

Body Weight. Body weight (BW) did not differ among groups at the start of the experiment. At the end of the study period, BW was not significantly different between the WKsed and SHRsed groups. Chronic exercise resulted in reduction in BW in both WK and SHR rats (Table 3.1)

Chronic Exercise Reduces Blood Pressure in SHRs. Systolic, diastolic, and mean arterial blood pressure (SBP, DBP, and MAP, respectively) were significantly higher in SHRsed than in. WKsed rats at the beginning of the experiment (at age 7 weeks) and remained increased for the duration of the study (Figure 3.2). Chronic exercise resulted in significantly reduced SBP, DBP, and MAP in SHRex rats when compared with SHRsed rats; the significant difference in BP was observed beginning from 8 weeks of exercise. Exercise did not affect BP in WK rats (Figure 3.2).

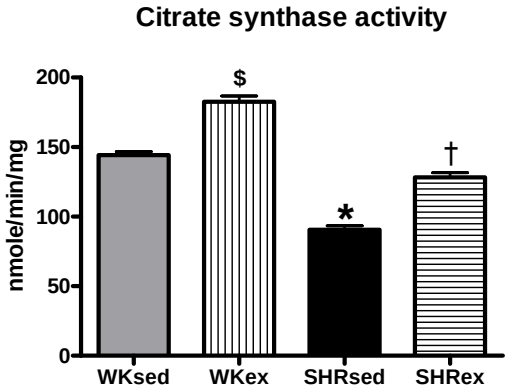

Figure 3.1.Citrate synthase activity (mol/min1/mg of protein) in soleus muscle of sedentary or exercised SHR and WKY as measured by citrate synthase activity assay kit. After the period of 16 weeks of exercise, the activity of citrate synthase in the soleus muscle was significantly higher in SHR as well as in WKY rats compared with their sedentary control groups indicating the efficacy of the exercise protocol. $p<0.05 WKsed vs WKex; *p<0.05 WKsed vs SHRsed; †p<0.05 SHRsed vs SHRex.

Table 3.1. Effect of exercise training on body weight and plasma norepinephrine and plasma AngII levels in normotensive WK and SHR rats.

Parameters	WKsed	WKex	SHRsed	SHRex
Number of animals (n)	10	10	10	10
Body weight (grams)	377.3 ± 7.3	333.3 ± 8.4$	380.5 ± 3.5	327.6 ± 6.2†
Plasma Norepinephrine (pg/µl)	16.50 ± 1.2	13.50 ± 1.1	69.56 ± 3.9*	18.59 ± 0.5†
Plasma AngII (ng/ml)	88.26 ± 3.6	90.83 ± 3.2	127.6 ± 5.4*	94.30 ± 3.5†

Data are mean±SE. *p<0.05 WKsed vs SHRsed; †p<0.05 SHRsed vs SHRex; $ p<0.05 WKsed vs WKex.

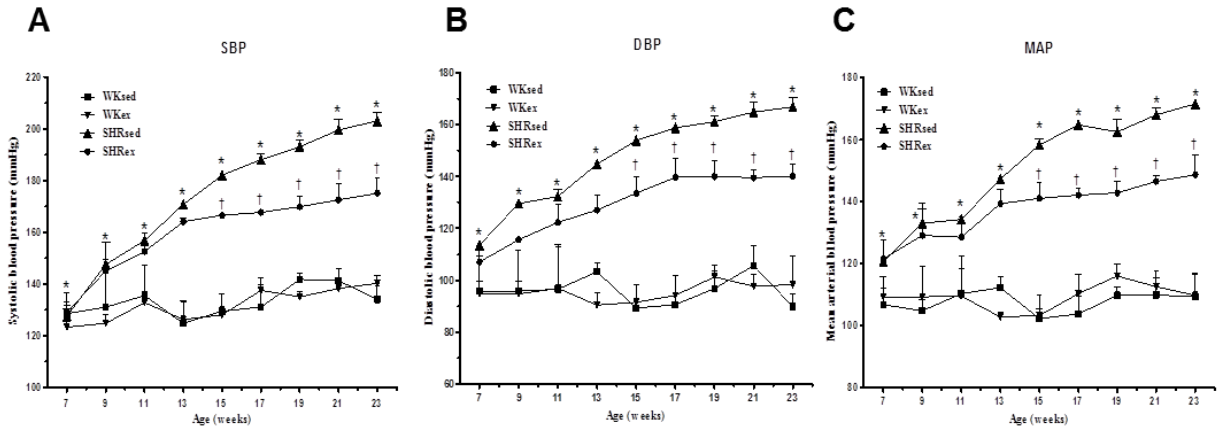

Figure 3.2. Effect of chronic exercise on time course of blood pressure (in millimeters of mercury) in WK rats and SHRs. A, systolic blood pressure (SBP). **B**, diastolic blood pressure (DBP). **C**, mean arterial pressure (MAP). Exercise significantly reduced SBP, DBP, and MAP in SHRex compared with SHRsed rats from 8 weeks of exercise (at 15 weeks of age). Values are mean±SE; n=10 in each group. *p<0.05 WKsed vs SHRsed rats; †p<0.05 SHRsed vs SHRex rats.

Chronic Exercise Attenuates Pro-inflammatory Cytokines in the PVN and RVLM of SHRs. To investigate the influence of exercise on PICs within the brain of hypertensive rats, we examined the mRNA and protein levels of TNF-α, and IL-1β in the PVN and RVLM. We observed that SHRsed rats exhibited marked increases in TNF-α and IL-1β expression in the PVN (Figure 3.3A, 3.3B, and 3.6) as well as in the RVLM (Figure 3.3C, 3.3D) compared to WKsed. This upregulation of TNF-α and IL-1β was significantly attenuated by chronic exercise in SHRs. At the mRNA level, chronic exercise in SHR resulted in 7 fold decrease in TNF-α and 5 fold decrease in IL-1β expression in the PVN (Figure 3.3A), whereas these changes were 6 fold and 4 fold, respectively in the RVLM of SHRex rats (Figure 3.3C). Exercise did not change PIC levels in WK rats.

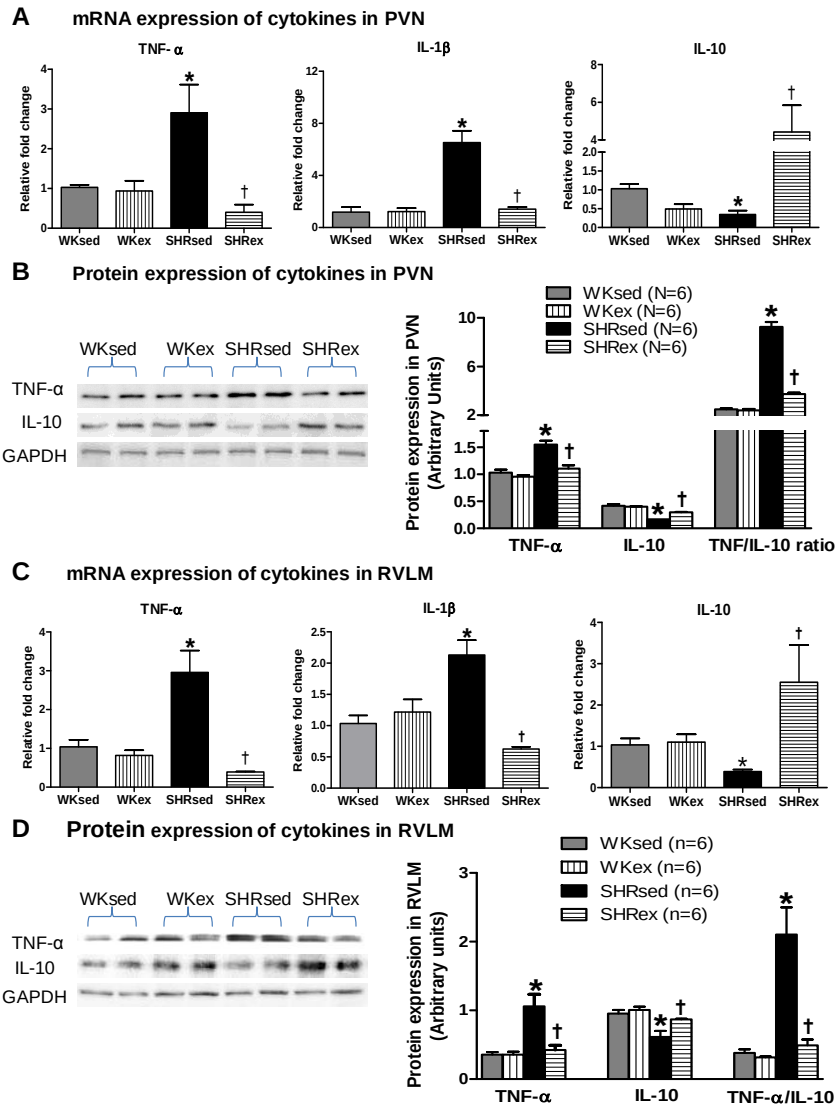

Figure 3.3. Effects of ExT on tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-10 expression in the PVN and RVLM of WK rats and SHRs. A, mRNA expression of TNF-α, IL-1β, and IL-10 in the PVN. **B**, A representative Western blot (left panel) and densitometric analysis (right pane) of TNF-α and IL-10 protein expression in the PVN. **C**, mRNA expression of TNF-α, IL-1β, and IL-10 in the RVLM. **D**, densitometric analysis of TNF-α, IL-1β, and IL-10 in the RVLM. Values are mean±SE. *p<0.05 vs. WKsed; †p<0.05 vs SHRsed. n=9 per group for mRNA analysis and n=6 per group for protein analysis.

Exercise Improves Balance Between Pro- and Anti-inflammatory Cytokines in the PVN and RVLM of SHRs. To investigate the influence of exercise on anti-inflammatory status within the PVN and RVLM, we determined the mRNA and protein levels of IL-10, a potent AIC. To further investigate whether chronic exercise has ability to improve the balance between PICs and AICs in the brain of hypertensive rats, we determined the alterations in ratio of TNF-α to IL-10 protein levels in these rats. A significant attenuation in the levels of IL-10 in the hypertensive sedentary rats compared with the WKsed rats was evident within the PVN (Figure 3.3A, 3.3B) as well as RVLM (Figure 3.3C, 3.3D, and 3.7). Moreover, SHRsed rats had higher TNF-α/IL-10 ratio in the PVN (Figure 3.3B) and RVLM (Figure 3.3D) compared to WKsed. These results provide further evidence that an imbalance between PICs and AICs plays role in pathogenesis of hypertension. Interestingly, exercise resulted in significantly increased levels of IL-10 in the brain of SHRs. We observed 93% and 85% increase in IL-10 mRNA levels in the PVN and RVLM of trained SHRs, respectively. These results were also accompanied by dramatic decrease in TNF-α/IL-10 ratio in SHRs indicating an improvement in balance between PICs and AIC by chronic exercise.

Chronic Exercise Modulates RAS Components in the PVN and RVLM of SHRs. To determine whether chronic exercise modulates vasoconstrictory and vasodilatory components of RAS, we examined the mRNA and protein levels of ACE, AT1R, ACE2, and Mas (receptor of Ang(1-7), an AngII metabolite with vasodilator properties) in the PVN and RVLM. At basal conditions, sedentary SHRs exhibited marked increase in ACE and AT1R in the PVN (Figure 3.4 and 3.6) and RVLM (Figure 3.5 and 3.7) compared to WKsed. In addition, ACE2 and Mas levels were significantly reduced in SHRsed compared to WKsed. These results indicate the existence of an imbalance between vasoconstrictor and vasodilatory axis of RAS in hypertensive rats.

Interestingly, chronic exercise prevented the increase in ACE and AT1R expression in SHR. At the mRNA level, ACE expression in SHRex was lowered by 85% and 77% in the PVN and

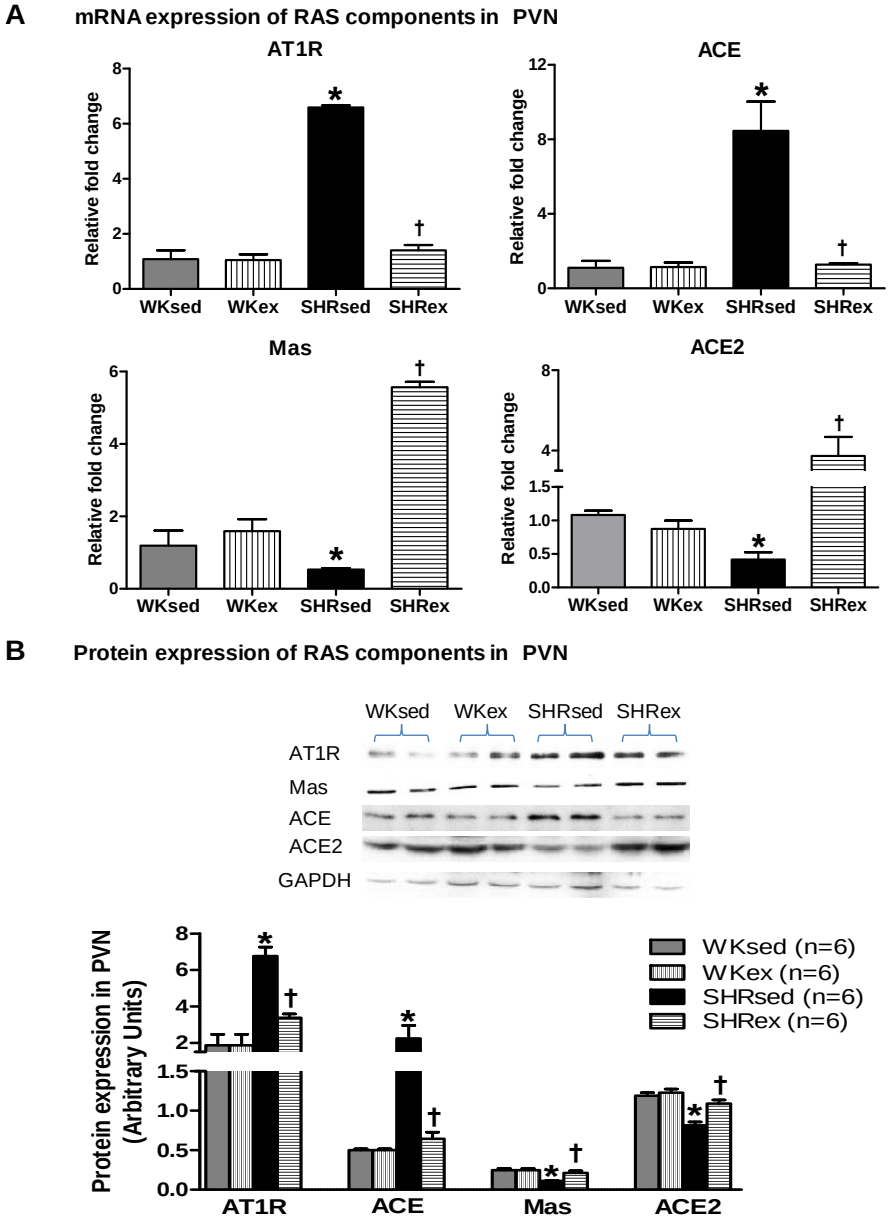

Figure 3.4. Effects of exercise on RAS components (AT1R, ACE, Mas, and ACE2) in the PVN of WK rats and SHRs. **A**, mRNA expression. **B**, densitometric analysis of protein expression and a representative Western blot. Values are mean±SE. *p<0.05 WKsed vs. SHRsed; †p<0.05 SHRsed vs SHRex. n=9/group for mRNA analysis and n=6/group for protein analysis.

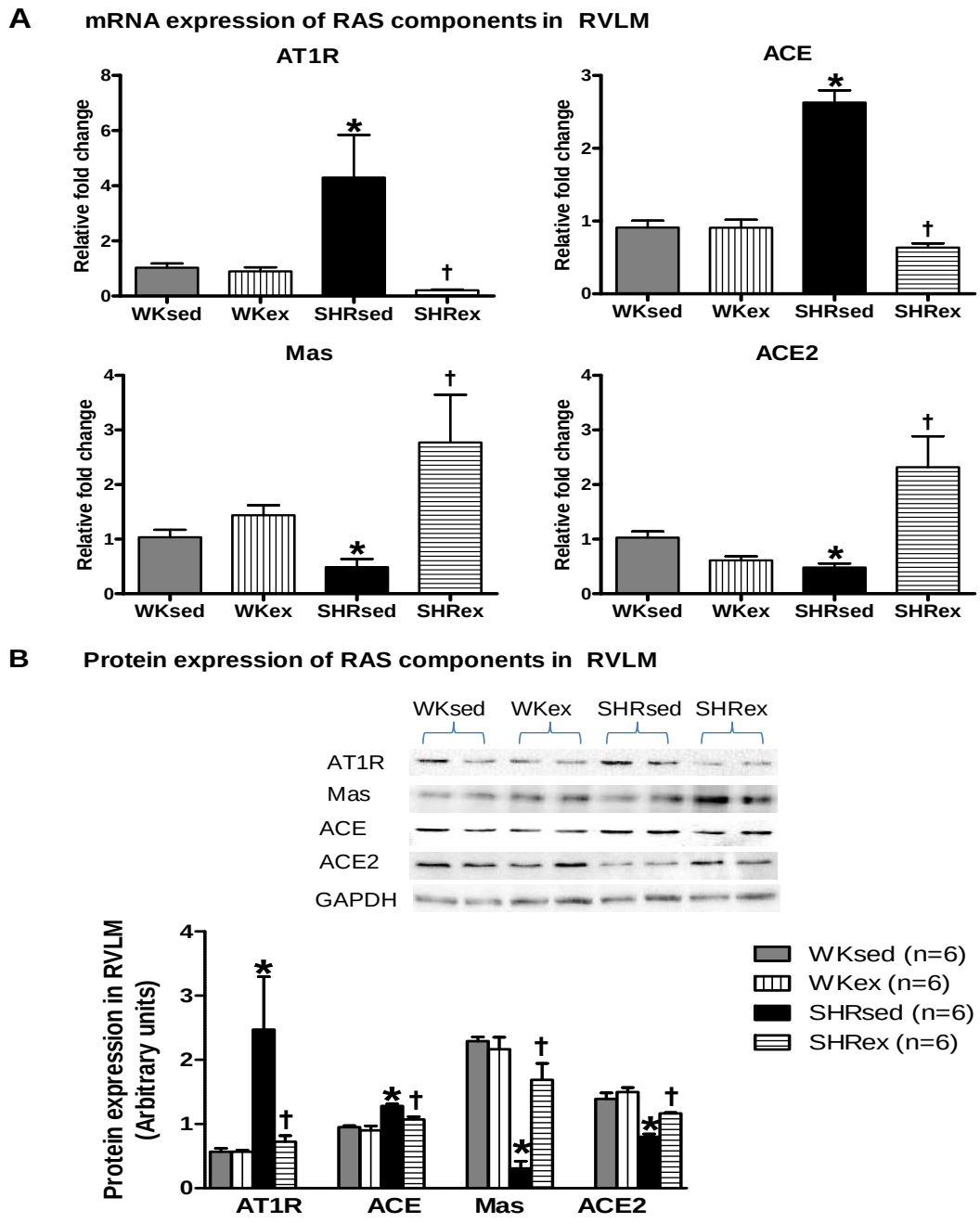

Figure 3.5. Effects of exercise on RAS components (AT1R, ACE, Mas, and ACE2) in the RVLM of WK rats and SHRs. A, mRNA expression. **B**, densitometric analysis of protein expression and a representative Western blot. Values are mean±SE. *p<0.05 WKsed vs. SHRsed; †p<0.05 SHRsed vs SHRex. n=9/group for mRNA analysis and n=6/group for protein analysis.

RVLM, respectively when compared to SHRsed (Figure 3.4A and 3.5A). Furthermore,

expression of ACE2 and Mas, were dramatically upregulated in trained SHR. ACE2 expression was elevated by 9 fold in the PVN and 5 fold in the RVLM of SHRex compared to SHRsed. Similarly, Mas levels were increased by about 82% in the SHRex group. However, exercise did not change levels of these RAS components in WK rats. Additionally, plasma angiotensin II (AngII) levels were found to be significantly higher in SHRsed compared with WKsed rats, whereas, chronic exercise resulted in significantly decreased plasma AngII concentrations in SHRs but did not change plasma AngII level in WK rats (Table 3.1).

Chronic Exercise R**educes Oxidative Stress in the PVN and RVLM of SHR.** Because Ang II, through activation of AT1R, regulates NAD(P)H oxidase and contributes to oxidative stress, the expression of $gp91^{phox}$, a subunit of NAD(P)H oxidase, was analyzed in the brain of training and

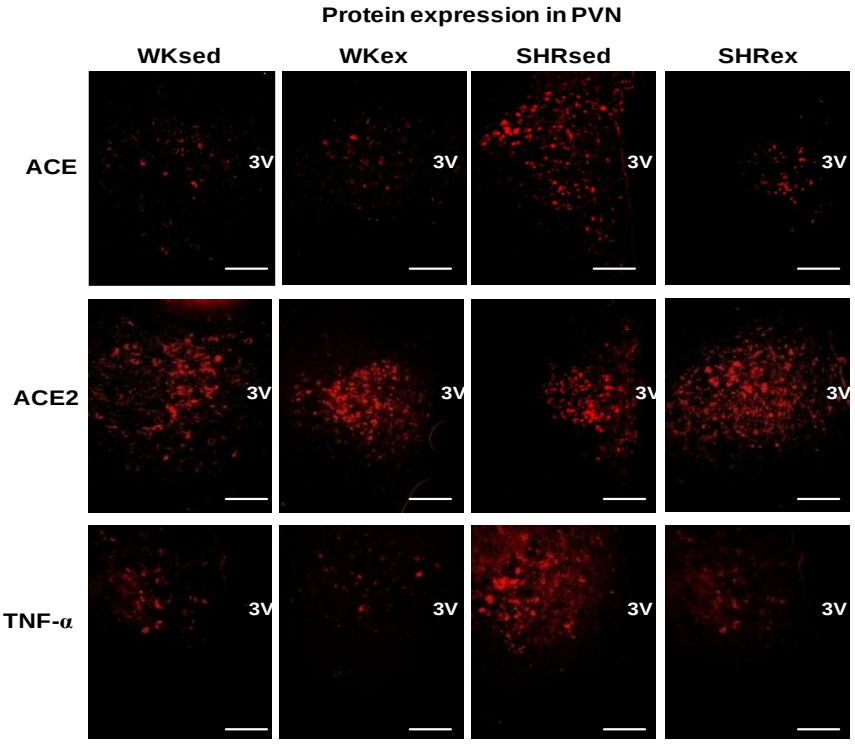

Figure 3.6. A representative confocal photomicrographs (X20) showing the effects of exercise on protein expression of ACE, ACE2, and TNF-α in the PVN of WK rats and SHRs (n=5/group). Scale bar=100 μm. 3V,third ventricle.

control groups. Expression of gp91^{phox} was markedly higher in SHRsed when compared to WKsed rats; this expression was significantly reduced by chronic exercise (Figure 3.8). In trained SHRs, a diminished mRNA expression of gp91^{phox} by 59% in the PVN and 77% in the RVLM was observed compared with the SHRsed group. The training-associated lower mRNA expression was linked to a significant reduction in protein expression as well (Figure 3.8).

Inducible nitric oxide synthase (iNOS) has been considered another marker of oxidative stress because of its ability to sequester excess superoxide leading to formation of more toxic reactive oxygen species, peroxynitrite. Therefore, we investigated whether exercise has any influence on iNOS levels within the brain. Our mRNA analysis demonstrated that SHRsed rats

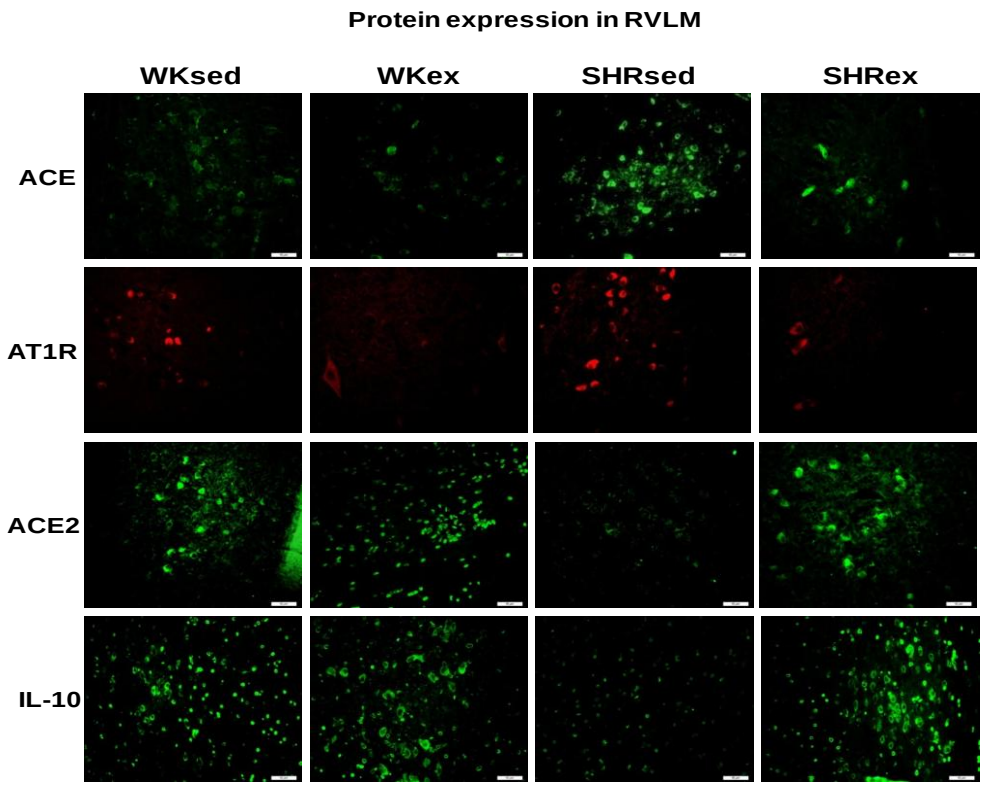

Figure 3.7. A representative confocal photomicrographs (X20) showing the effects of ExT on protein expression of ACE, AT1R, ACE2, and IL-10 in the RVLM of WK rats and SHRs. n=5 per group; Scale bar=100 μm.

had marked increase in iNOS within the PVN and RVLM. Surprisingly, exercise in SHR caused an 8 and 5 fold decrease in iNOS expression in the PVN and RVLM, respectively (Figure 3.8). These results further confirm that exercise has ability to reduce oxidative stress in the brain of hypertensive rats.

Chronic Exercise Attenuates Neuronal Excitation in the Brain of SHRs. To determine whether chronic exercise influences sympathoexcitation in the brain, we examined the protein expression of Fra-like (Fra-LI, fos family gene; indicating chronic neuronal excitation) by immunofluorescence staining. To further determine whether exercise-induced effects are mediated by alterations in neurotransmitter in the brain, we determined the levels of tyrosine hydroxylase (TH) and 67-kDa isoform of glutamate decarboxylase (GAD_{67}) in the brain. TH is a rate-limiting enzyme in the synthesis of the catecholamines, Norepinephrine (NE) and Epinephrine. GAD is the rate-limiting enzyme in the synthesis of inhibitory neurotransmitter GABA. We observed that SHRsed rats exhibited increased Fra-LI activity in the PVN neurons compared to WKsed (Figure 3.9A). Notably, this upregulation of Fra-LI activity was significantly attenuated by chronic exercise in SHR. However, Exercise did not change Fra-LI activity in WK rats.

Furthermore, we found that sedentary SHRs exhibited higher levels of TH and significantly lower levels of GAD_{67} in the PVN when compared to WKsed rats (Figure 3.9B). Interestingly, exercise significantly reduces TH levels, whereas, GAD_{67} levels were upregulated in SHRex rats compared to SHRsed. However, in the RVLM, there was no significant difference of TH and GAD_{67} levels among all groups (Figure 3.10). Additionally, plasma NE levels were found to be significantly higher in SHRsed compared with WKsed rats, whereas, chronic exercise resulted in significantly decreased plasma NE concentrations in SHRs but did

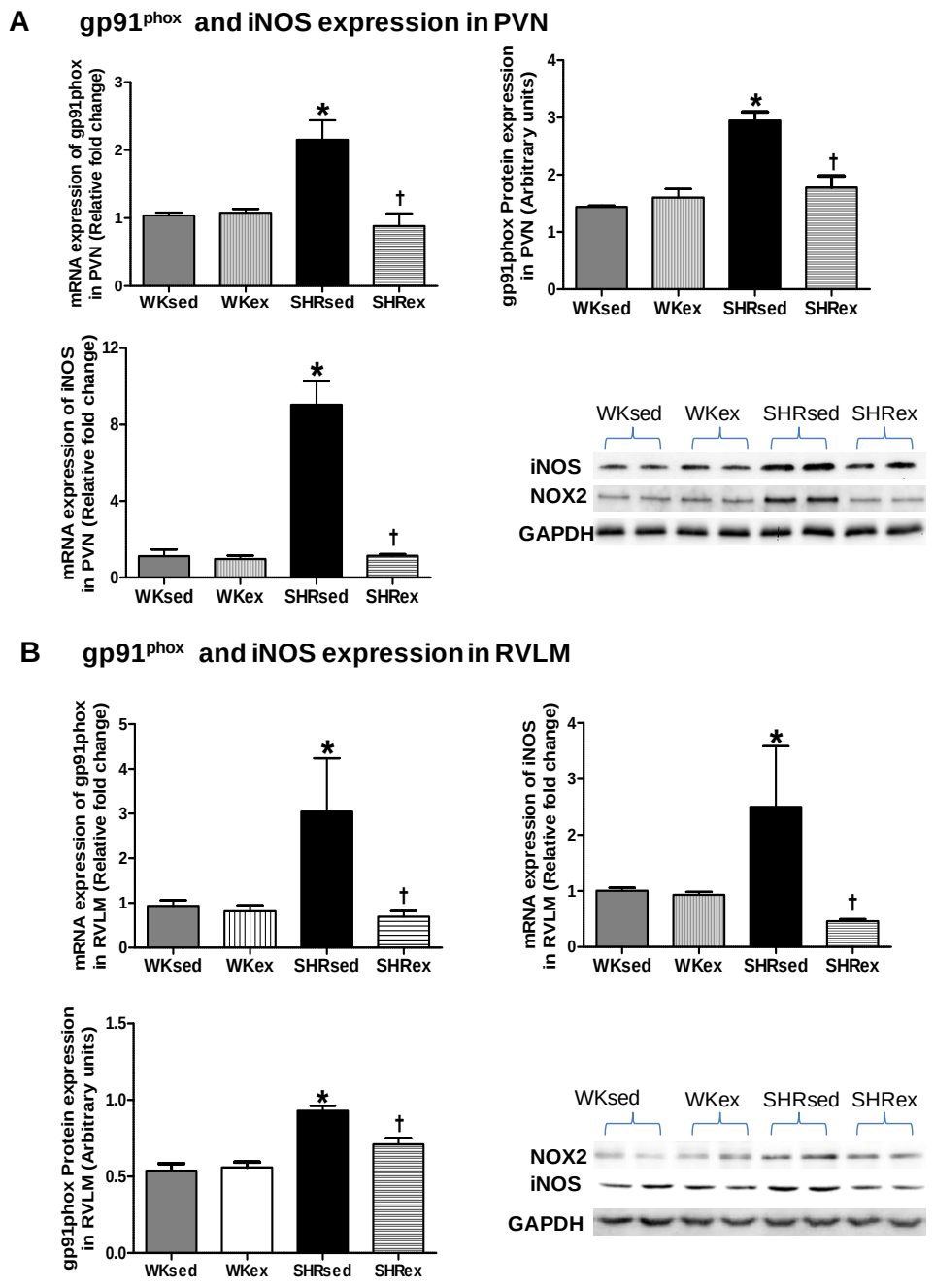

Figure 3.8. Effects of ExT on gp91^{phox} and iNOS expression in the PVN and RVLM of WK rats and SHRs. A, mRNA and protein expression of gp91^{phox} and iNOS in the PVN. **B**, mRNA and protein expression of gp91^{phox} and iNOS in the RVLM. Values are mean±SE. *p<0.05 vs. WKsed; †p<0.05 vs SHRsed. N=9 per group for mRNA analysis and n=6 per group for protein analysis.

not change plasma NE level in WK rats (Table 3.1). Taken together, these results indicate that chronic exercise attenuates sympathoexcitation, possibly by altering neurotransmitter levels in the PVN of hypertensive rats.

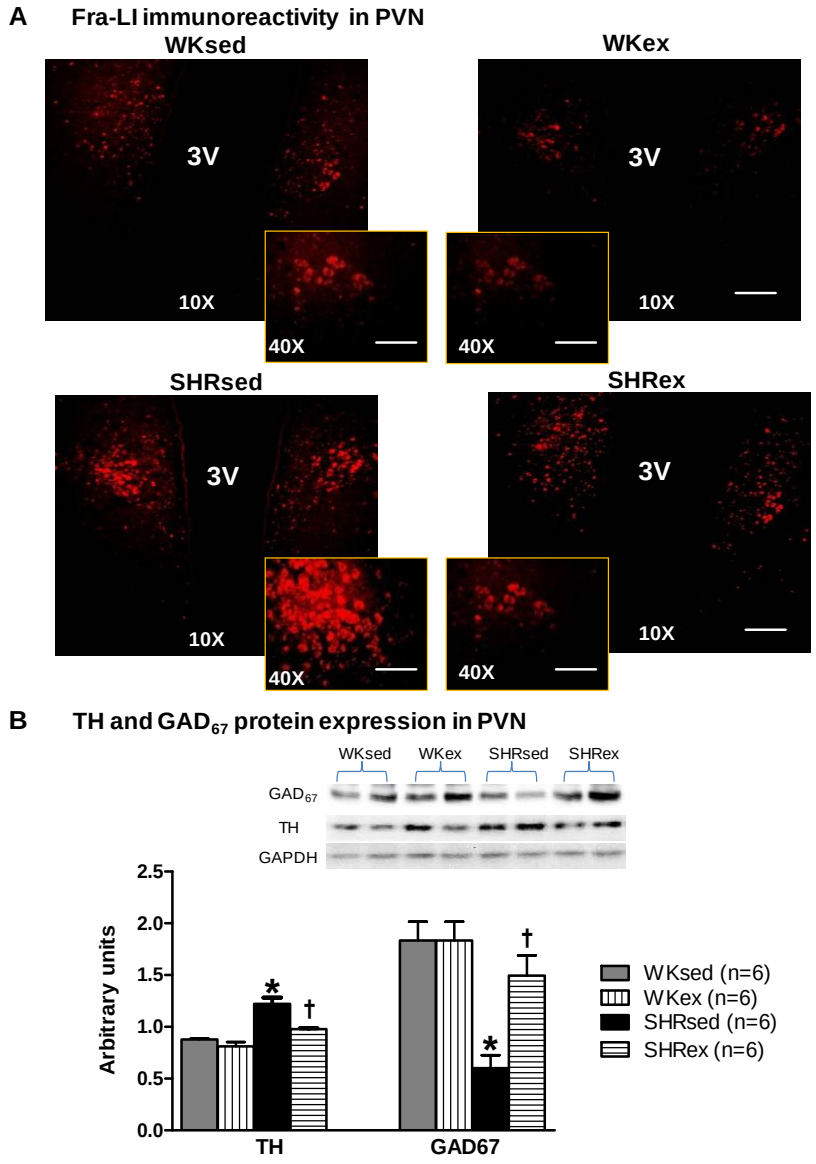

Figure 3.9. Effects of ExT on sympathoexcitation in the PVN of WK rats and SHRs. A, A representative confocal photomicrographs (X10, scale bar = 200 μm; and X40, scale bar=50 μm) from each group showing Fra-LI immunoreactivity in PVN neurons (n=5/group). **B**, Densitometric analysis (n=6/group) of protein expression of TH and GAD_{67} in the PVN accompanied with a representative western blot. Values are mean±SE; *p<0.05 vs. WKsed; †p<0.05 vs SHRsed. 3V, third ventricle.

DISCUSSION

The present study sought to evaluate the impact of chronic moderate intensity exercise of 16 weeks duration on blood pressure, pro- and anti-inflammatory cytokines, RAS components, neuronal activity, and oxidative stress, within the brain of spontaneously hypertensive rats (SHRs), a genetic model of hypertension. Three major novel findings emerge from this study. First, chronic exercise improves balance between pro- and anti-inflammatory cytokines by attenuating PICs (TNF-α, IL-1β) and upregulating anti-inflammatory IL-10 expression in the PVN and RVLM of SHR. Second, effects of chronic exercise in hypertensive rats were modulated by both vasoconstrictor as well as the vasoprotective components of RAS in the PVN and RVLM. Finally, exercise attenuated oxidative stress in the PVN and RVLM of SHRs, possibly by reducing sympathoexcitation. These results suggest that chronic exercise not only attenuates PICs and the vasoconstrictor axis of the RAS but also

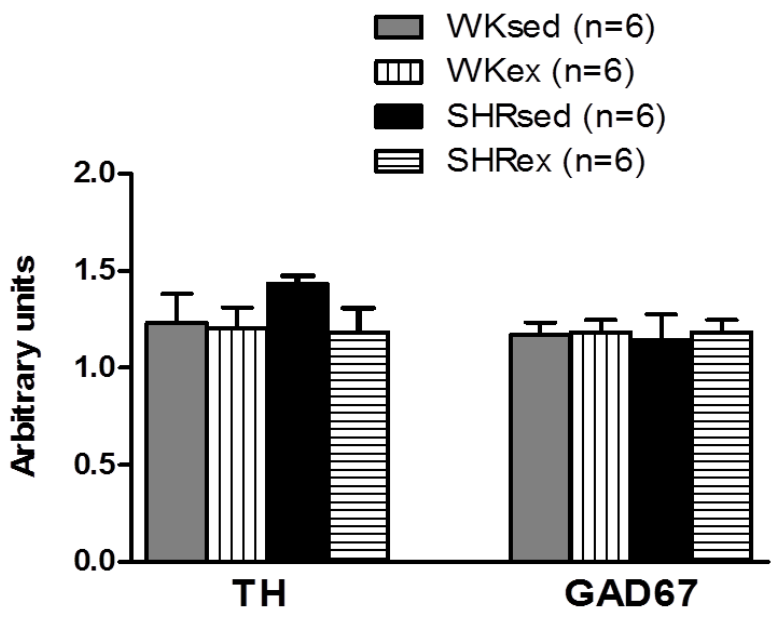

Figure 3.10. Densitometric analysis (n=6/group) of protein expression of TH and GAD_{67} in the RVLM of WK rats and SHRs. In the RVLM, there was no significant difference of TH and GAD_{67} levels among all groups. Values are mean ± SE.

attenuates sympathoexcitation, improves anti-inflammatory defense mechanisms and vasoprotective axis of the RAS in the brain, which, at least in part, explains the blood pressure-lowering effects of exercise in hypertension.

At the end of the study, we observed significant reductions in SBP, DBP, and MAP in trained SHRs compared with SHRsed rats and saw no comparable changes in trained WK rats. The pressure-lowering effect of ExT was significant starting from 8 weeks of regular exercise and continued until the end of the study, emphasizing the importance of long-term exercise in patients with hypertension. Additionally, we observed that chronic exercise caused significant reduction in body weight (BW) both in WK as well as in SHR rats, whereas, reduction in BP was observed only in SHRs. This excludes the possibility that exercise-induced reduction in BP observed in this study was due to reduction in BW. Hypertension is characterized by chronic low-grade inflammation which is reflected by a two- to threefold increase in circulating levels of several PICs (Peeters, Netea et al. 2001; Shi, Raizada et al. 2010). Interestingly, recent discoveries indicate that anti-inflammatory cytokines (AICs) such as IL-10, exerts inhibitory effects on PICs and therefore, has a significant impact on sympathetic outflow, arterial pressure, and cardiac remodeling in experimental models of hypertension (Shi, Raizada et al. 2010). More importantly, several cross-sectional studies demonstrated an association between physical inactivity and low-grade systemic inflammation (Geffken, Cushman et al. 2001; Abramson and Vaccarino 2002). Our current findings together with previous other studies clearly suggest that physical activity reduces BP in hypertensive humans and animals. These findings led us to explore the role of brain pro- and anti-inflammatory cytokines in pressure-lowering effects of exercise. Although, very few studies have shown that exercise reduces circulating levels of PICs (Mattusch, Dufaux et al. 2000; Petersen and Pedersen 2005; Agarwal, Haque et al. 2009),

influence of exercise on brain inflammatory status has never been investigated. In this study, we found that regular exercise resulted in robust decrease in brain PICs (TNF-α and IL-1β) in hypertensive rats. More importantly, brain IL-10 levels were dramatically upregulated and TNF-α/IL-10 ratio was reduced in trained SHRs. Though not in the brain, a recent study reported similar improvement in plasma IL-10 levels by physical exercise of 8-weeks duration in rats with chronic heart failure (CHF) (Nunes, Tonetto et al. 2008). Similarly, Smith *et al*. (1999) showed that the ability of blood mononuclear cells to produce IL-10 increased by 36% in trained individuals at risk of ischemic heart disease (Smith, Dykes et al. 1999). Since, the two TNF-α receptors , TNFR1 and TNFR2, have been shown to differentially regulate cardiac and endothelial function *in vitro* and *in vivo* (Schulz and Heusch 2009; Garlie, Hamid et al. 2011), it may be interesting to investigate the role played by these receptors in exercise-induced effects on hypertension as well. Nevertheless, taken together, the results of this study provide evidence of a shift in the balance between PIC and AIC by physical training, favoring anti-inflammatory response.

IL-10 has been shown to inhibit the production of various PICs as well as chemokines from LPS-activated human monocytes (Das 1994). Therefore, it is possible that exercise induced increase in IL-10 may be responsible for the observed decrease in TNF-α and IL-1β. However, the possibility of direct effects of exercise on production of PIC cannot be ignored. Schulz and Heusch (2009) have summarized in their recent article that TNF-α overexpression in mice leads to progressive cardiomycoyte hypertrophy, left ventricular dilation, and diastolic dysfunction; whereas, anti-TNF-α treatment preserves diastolic dysfunction (Schulz and Heusch 2009). Therefore, it can be speculated that exercise-induced reduction in TNF-α may be responsible for improved diastolic dysfunction in trained SHRs as reported previously (Agarwal, Haque et al.

2009). Nonetheless, the ability of exercise to improve IL-10 levels in the PVN and RVLM is noteworthy, because overexpression of brain IL-10 has been shown to preserve cardiac function and prevent cardiac damage and hypertension (Nonaka-Sarukawa, Okada et al. 2008; Nomoto, Okada et al. 2009). Therefore, based on the results of this study, exercise-induced improvement in overall immune condition of the brain in hypertensive rats, explains, at least in part, the underlying mechanisms of exercise-mediated reduction in BP.

Hypertension is also characterized by an overactivation of central/brain RAS. Besides, classical pathway of RAS (ACE, AngII, and AT1R), newly discovered RAS components such as ACE2, Ang1-7, and receptor Mas have been shown to play an important role in BP regulation, by counteracting the classical pathway. Research over the past decade has suggested that the balance between ACE and ACE2, particularly within the brain, is an important factor determining the outcome of hypertension (Danilczyk and Penninger 2006). We and others have previously shown that PICs, particularly TNF-α, mediates AngII-induced hypertension, cardiac hypertrophy (Sriramula, Haque et al. 2008), endothelial and cardiac dysfunction (Kleinbongard, Heusch et al. 2010), and modulates RAS components in the PVN in rats with heart failure (HF) (Kang, Wang et al. 2010). Interestingly, findings of this study revealed that chronic exercise not only reduced ACE and AT1R levels, but also dramatically upregulated expression levels of ACE2 and Mas receptor within the PVN and RVLM of SHRs. These findings provide evidence that effects of chronic exercise in hypertension are modulated by both vasodilatory and vasoconstrictor arms of central RAS. These results extended the observations of previous studies showing that physical activity reduces plasma levels of AngII and AT1R in rabbits with CHF (Liu, Kulakofsky et al. 2002; Gao, Wang et al. 2007), and increases Mas receptor expression in the left ventricle of SHR (Filho, Ferreira et al. 2008). Our results were also in agreement with a

recent report that demonstrated normalization of ACE and ACE2 levels by exercise in the RVLM of rabbits with CHF (Kar, Gao et al. 2010). Besides RVLM, they have demonstrated similar changes in hypothalamus, cerebellum, and NTS suggesting the involvement of brain regions other than RVLM and PVN in exercise-induced effects, at least, in animals with HF. In addition to central RAS, we observed almost complete normalization of plasma AngII levels in trained SHRs (Table 1). In a recent publication Zamo *et al* (2011) reported that low-intensity swimming exercise of 8 weeks duration caused marked differences in systemic and cardiac RAS in young as well as adult SHRs, however, the effects were more pronounced in young rats (60% and 39% reduction in plasma AngII in young and adult SHRs, respectively) (Zamo, Barauna et al. 2011). The difference in degree of improvement in plasma AngII levels could be attributed to the longer duration of exercise protocol used in the present study. Because role of AngII is well established in regulation of renal excretion of water and electrolyte, exercise-induced increase in urinary sodium excretion could also attribute to pressure-lowering effects of exercise. For instance, Ciampone *et al* (2011) have recently reported an association between reduced BP, increased natriuresis, and improvement in renal RAS components (Ciampone, Borges et al. 2011). It is also important to discuss that adipocytes are known to play an important role in cytokine production and a recent study reported increased ACE expression by adipocyte-derived lipid mediators in macrophages (Kohlstedt, Trouvain et al. 2011). Although, we observed that exercise significantly reduced body weight in SHR as well as in WK rats, the role of adipose tissue in exercise-induced reduction in ACE in SHRs is not clear.

A recently published report from our lab demonstrated that overexpression of ACE2 within the PVN by bilateral microinjection of an adenovirus encoding human ACE2 reduces BP in AngII- induced hypertensive rats (Sriramula, Cardinale et al. 2011). The results of

this study also revealed that attenuation of PICs in the PVN in combination with the shift of the RAS towards the anti-hypertensive axis (ACE2/Ang-(1-7)/Mas) may be responsible for the overall beneficial effects of ACE2 overexpression. Our current findings together with the previous reports from our lab clearly suggest that exercise has capability to not only improve the systemic RAS but also central RAS, which, at least in part, explains the pressure-lowering effects of chronic exercise in hypertension.

Besides PICs and RAS, sympathetic nervous system plays an important role in cardiovascular regulation of BP (Esler, Straznicky et al. 2006; Guyenet 2006). Hypertension is often found to be associated with increased levels of excitatory neurotransmitter, norepinephrine (NE) (Agarwal, Haque et al. 2009) and deficit in inhibitory GABAergic system in the cardiovascular regulatory regions of the brain(Horn, Shonis et al. 1998).

In this study, we demonstrated that SHRsed had significantly reduced levels of GAD_{67}, a 67-kDa isoform of GAD, and increased tyrosine hydroxylase (TH) when compared to WKsed. Concomitantly, when compared to WKsed, SHRsed rats exhibited increased circulating plasma levels of NE (an indirect marker of sympathetic activity) as well as increased expression of Fra-LI in the PVN (indicative of increased neuronal activity). These results provide further evidence that neurotransmitters mechanisms within the cardiovascular regulatory centers in the brain contribute to sympathoexcitation and plays an important role in the pathogenesis of essential hypertension.

More importantly, exercise caused reduction in Fra-LI staining and prevented the increase in TH and decrease in GAD_{67} in the PVN of SHRs, suggesting exercise-induced reduction in sympathoexcitation in hypertensive rats. Taken together, this study provide sufficient evidence that chronic exercise may cause alteration in excitatory and inhibitory

neurotransmitter in the brain leading to reduced sympathoexcitation in hypertensive rats. It is now well established that PICs and RAS modulate sympathetic neuronal outflow in the CNS leading to elevated resting BP in conscious animal (Kang, Wang et al. 2010; Zhang, Wei et al. 2003; Ufnal, Zera et al. 2005; Phillips and de Oliveira 2008; Lu, Chen et al. 2009). In addition, it has been suggested that TNF-induced imbalance in neurotransmitters in the PVN and RVLM, possibly via oxidative stress, contributes to sympathoexcitation (Guggilam, Cardinale et al. 2011; Kang, Zhang et al. 2011). Therefore, current findings taken together with previous studies raise the possibility that improved balance between PIC and AIC in trained hypertensive rats either alone or in combination with improved RAS components may have contributed to exercise-induced attenuation in sympathoexcitation observed in this study.

Research over past several decades has established that cytokines and RAS alter neuronal activity via induction of oxidative stress (Mayorov, Head et al. 2004; Zimmerman, Lazartigues et al. 2004). Of particular importance, NADPH oxidase (NOX)derived reactive oxygen species (ROS) act as potent intra- and inter-cellular second messengers in signaling pathways causing hypertension (Mehta and Griendling 2007; Nagae, Fujita et al. 2009; Sirker, Zhang et al. 2011). Of various isoforms of NOX, role of NOX2 (also known as $gp91^{phox}$) in AngII-induced hypertension and endothelial dysfunction is well established (Murdoch, Alom-Ruiz et al. 2011). Given the role of AngII-induced ROS generation in the brain in hypertension, it is interesting to investigate whether exercise has ability to attenuate ROS generation within the brain of hypertensive rats. Our data illustrated that moderate-intensity exercise reduces brain oxidative stress in hypertensive rats as indicated by reduced levels of $gp91^{phox}$ and iNOS within the brain of SHRs. In accordance with these findings, previous studies have shown that exercise causes reductions in various subunits of NADPH oxidase in isolated porcine aortic endothelial cells

(Rush, Turk et al. 2003), thoracic aorta of SHR (Graham and Rush 2004), aging arteries of rat (Li, Xiong et al. 2009), and human mammary arteries (Adams, Linke et al. 2005). However, our results are first to provide evidence that exercise can attenuate oxidative stress in the PVN and RVLM neurons of hypertensive rats. In light of our findings that exercise causes reduction in ACE and AT1R levels and because RAS is a potent mediator of activation of NADPH oxidase (Mehta and Griendling 2007), it is plausible to suggest that exercise-induced reduction in ACE and AT1R might be responsible for attenuation of $gp91^{phox}$. In addition, catecholamines and cytokines have also known to promote ROS formation from NADPH oxidases (Heusch and Schulz 2011); therefore, role of exercise-induced improvement in neurotransmitters and cytokine levels in attenuation of oxidative stress cannot be ignored. Recently, it has been reported that inhibition of the cannabinoid receptor CB1 (CB1-R), which is mainly localized in the central nervous system, positively affects BP, endothelial function, and reduces aortic ROS production and NADPH oxidase activity (Tiyerili, Zimmer et al. 2010). These findings together with our current findings indicate that there exists a cross-talk between these various pathways within the brain that can influence exercise-induced attenuation of oxidative stress and BP. Paradoxically, however, exercise has been shown to induce oxidative stress in some cases. However, exercise-induced oxidative stress has been seen mainly after vigorous exercise and is more frequent in long-distance runners and/or long bursts of severe and unaccustomed exercise (Das 2004).On the other hand, regular and moderate intensity exercise seems more effective in reducing oxidative stress in hypertensive rats as evident from our findings. This can be further explained by a recent report of Craenenbroeck *et al* (2010) where they have demonstrated that acute exercise-induced functional changes in circulating angiogenic cells (CAC, known to contribute to endothelial

repair) declined with exercise training in CHF patients, suggesting that repetitive exercise bouts progressively lead to functional endothelial repair (Van Craenenbroeck, Hoymans et al. 2010).

In summary, the present study shows that chronic exercise not only attenuates PICs and the vasoconstrictor axis of the RAS but also improves the anti-inflammatory defense mechanisms and vasoprotective axis of the RAS in the brain. Also, exercise alters the adrenergic and GABAergic system and reduces oxidative stress in the brain of hypertensive rats. These results provide mechanistic evidence that unlike currently available pharmacological anti-hypertensive therapies, regular moderate intensity exercise has ability to favorably affect multiple pathways involved in pathogenesis of hypertension. The results of this study provide greater insight into the mechanisms by which exercise exerts beneficial effects in hypertensions and therefore, may lead us to design an exercise regimen resulting in maximum cardio-protective benefits in hypertensive patients.

PERSPECTIVES

The present study provides insights into the mechanisms within the brain that can influence exercise-mediated effects in SHR. Our data demonstrated that chronic moderate-intensity exercise attenuates sympathoexcitation, modulates RAS components, improves the balance between PIC and AIC, and reduces oxidative stress in the PVN as well as RVLM of hypertensive rats. Since, RAS is thought to be a driving force in increased sympathetic activation and reduced oxidative stress; our findings suggest that exercise-induced reduction in BP could be mediated, at least in part by, improvement in central vasodilatory RAS components. In addition, increased IL-10 levels could be responsible for additional benefits. This is the first evidence to our knowledge showing the effectiveness of exercise in ameliorating the hypertensive components within the brain of SHR. These results further support the hypothesis that exercise

can affect cardiovascular regulation by specifically impacting regions in the central nervous system (Kramer, Plowey et al. 2000).

Since, brain RAS has been implicated in the initiation of various forms of hypertension, therapeutically targeting the brain RAS could be one of the strategies to treat hypertension. However, systemically administered pharmacological therapies such as ACE inhibitors have very less access to central ACE compared with circulating ACE due to the presence of blood brain barrier. Therefore, the results of this study are important from clinical perspective, because it suggests that regular long-term exercise could be one of the non-pharmacological yet cost effective tools in shifting the balance between vasoconstrictor RAS components to vasodilator components towards the vasodilatory and hence protective effects in hypertensive rats.

Although much progress has been made in animal studies, there is a need for rigorous clinical intervention trials on exercise that are guided by this knowledge from animal studies. The extent and frequency of exercise that result in maximum functional benefits in hypertensive patients must be determined. In this regard, it is worth mentioning that Kemi *et al*., have made an attempt to address this question (Kemi, Haram et al. 2005). They demonstrated that cardiovascular adaptations to training are intensity-dependent. However, further studies in relation to the parameters studied in the present study are still warranted. Furthermore, here, we chose SHR rat model of hypertension to elucidate the mechanisms of the beneficial effects of exercise in hypertension. However, the validation of results of this study in other animal models of hypertension could certainly be an important perspective.

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CHAPTER 4

DETRAINING DIFFERENTIALLY PRESERVED BENEFICIAL EFFECTS OF EXERCISE ON HYPERTENSION: EFFECTS ON BLOOD PRESSURE, CARDIAC FUNCTION, BRAIN INFLAMMATORY CYTOKINES AND OXIDATIVE STRESS

INTRODUCTION

Systemic arterial hypertension is a clinical condition associated with high morbidity and mortality (Lehnen, Leguisamo et al. 2010). Hypertension is characterized by cardiac hypertrophy and dysfunction, and overactivation of the renin-angiotensin-aldosterone system (RAAS) (Agarwal, Welsch et al. 2011). Chronic low-grade inflammation is another hallmark of hypertension (Agarwal, Welsch et al. 2011). Pro-inflammatory cytokines (PICs), such as tumor necrosis factor-alpha (TNF-α) (Dorffel, Latsch et al. 1999) and interleukin-1β (IL-1β) (Dorffel, Latsch et al. 1999; Peeters, Netea et al. 2001), have also been shown to correlate with the severity of hypertension and are of prognostic significance. In addition to PICs, several markers of oxidative stress such as NADPH oxidase (NOX) and inducible nitric oxide synthase (iNOS) have been found to be dramatically upregulated in various tissues such as heart (Agarwal, Haque et al. 2009), kidney (Vaziri, Lin et al. 2003; Agarwal, Elks et al. 2012), and brain (Fujita, Ando et al. 2007; Agarwal, Welsch et al. 2011) of hypertensive animals. A growing body of evidence suggest that the brain not only plays role in normal regulation of blood pressure (BP) but it can also initiate all forms of hypertension, including essential hypertension (Jennings and Zanstra 2009). Previous reports from our laboratory and others have demonstrated that increased levels of PICs (Shi, Raizada et al. 2010) and oxidative stress (Kang, Ma et al. 2009; Xia, Suda et al. 2011) within the paraventricular nucleus (PVN), most important cardiovascular regulatory centers of the brain, contribute to the development of hypertension.

Although, various currently available pharmacological therapies targeting the components of the RAAS have been proven to reduce BP; the morbidity and mortality caused by hypertension is still on the rise. According to current “Heart Disease and Stroke Statistics” the death rate caused by hypertension increased 9.0% from 1997 to 2007, and the actual number of

deaths increased 35.6% (Roger, Go et al. 2011). Therefore, physical activity has recently been recommended as a non-pharmacological approach for the treatment and control of hypertension. Although, past several years of research has proven that regular physical activity reduces BP and delays the progression of hypertension in animals and humans, the compliance with the recommended treatment has been found to be very low. For instance, non-compliance with exercise has recently been reported to be closely associated with poor outcomes of the disease (Ahmed, Abdul Khaliq et al. 2008). When compliance to exercise was assessed in patients with controlled and uncontrolled hypertension, the authors found that 43.5% patients with controlled hypertension were compliant with exercise, whereas, only 16.7% of those with uncontrolled hypertension were compliant. Despite these alarming statistics, the effects of cessation of exercise (physical detraining) at the physiological and molecular levels in hypertension are far from understood. A few previous studies have examined the effects of detraining on heart and skeletal muscle of hypertensive and normal rats, particularly in relation to insulin sensitivity (Neufer, Shinebarger et al. 1992; Kump and Booth 2005; Lehnen, Leguisamo et al. 2010). However, no studies, to date, have examined the effects of detraining on inflammatory cytokines and oxidative stress, particularly, within the cardiovascular regulatory centers of the brain in hypertension. Also, the effects of detraining on cardiac morphology and function in hypertension are poorly understood.

Therefore, this study was designed to investigate the effects of detraining on mean arterial blood pressure (MAP) using radiotelemetry, and cardiac morphology and function in hypertension. We also aimed to investigate the effects of detraining on pro- and anti-inflammatory cytokines (PICs and AIC) and oxidative stress within the PVN of hypertensive rats.

MATERIALS AND METHODS

Animals. In this study, we used a Angiotensin II (AngII)-induced hypertensive rat model, a well-established model of neurogenic hypertension. A total of 90 adult male Sprague-Dawley rats (250-350 grams) were studied, of which 45 rats were infused with AngII dissolved in 0.9% saline, at a subpressor concentration of 200ng/kg/min via osmotic minipumps (Alzet, model 2006; 0.15ul/hr). This AngII dose was based on previous publications from our laboratory and others (Cardinale, Sriramula et al. 2012). The other 45 rats were infused with saline (Sal) in place of AngII and were used as normotensive controls. The pumps were implanted subcutaneously for 42 days (6 weeks). Animals were randomized into six groups (n = 15 per group): saline+sedentary (Sal+Sed), saline+exercise (Sal+Ex), saline+detraining (Sal+Det), angiotensin II+sedentary (AngII+Sed), angiotensin II+exercise (AngII+Ex), and angiotensin II+detraining (AngII+Det) (Figure 4.1). The animals in exercise groups were subjected to moderate intensity exercise for 42 days. Animals in detraining groups were given exercise for a period of 28 days (4 weeks) followed by 14 days (2 weeks) of detraining. Echocardiographic assessment was carried out at baseline and at the conclusion of the study. After 42 days, the rats were euthanized, the brains were collected, and immediately frozen on dry ice. The paraventricular nucleus (PVN) tissues were punched out from the brain for further analysis.

Animals were housed in a temperature-controlled room (25 ± 1^{o}C) and maintained on a 12:12 hour light:dark cycle with free access to water and food. All animal and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University in compliance with NIH guidelines.

Exercise and Detraining Protocol. Rats in exercise groups (Sal+Ex and AngII+Ex) underwent moderate-intensity exercise (5 days per week; 60 min per day at 18 m/min, 0^{o} inclination) on a

motor-driven treadmill continuously for a period of 42 days. Animals in detraining groups (Sal+Det and AngII+Det) were given moderate-intensity exercise of a period of 28 days and remained sedentary for next 14 days (i.e. detraining). All the animals were acclimatized to treadmill for 2 weeks prior to osmotic mini-pump implantation. After acclimation, training intensity was set at approximately 60% of maximal aerobic velocity (MAV), which corresponds to moderate intensity exercise (18-20m/min). This training intensity was maintained throughout the study period. The MAV was evaluated from an incremental exercise test as reported previously (Boissiere, Eder et al. 2008; Sun, Qian et al. 2008). The rats in sedentary groups (Sal+Sed and AngII+Sed) were placed on a nonmoving treadmill during the training sessions.

Blood Pressure Measurement. MAP was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN) 7 days prior to implantation of the osmotic minipumps (Figure 4.1). Rats (n = 6 per group) were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and placed dorsally on a heated surgical table. An incision was made on the medial surface of the left leg, the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and another suture was placed proximally to temporarily interrupt the blood flow. The catheter tip of the radio-telemetry transmitter was introduced through a small hole in the femoral artery, advanced ~6 cm into the abdominal aorta such that the tip was distal to the origin of the renal arteries, and sutured into place. The probe body was placed into the abdominal cavity and sutured to the abdominal wall. The abdominal musculature was sutured and the skin layer closed following implantation. Rats received enrofloxacin (approximately 10 mg/kg) and buprenorphine (0.1 mg/kg, s.c.) immediately following surgery and 12 hours postoperatively and allowed to recover for seven days.

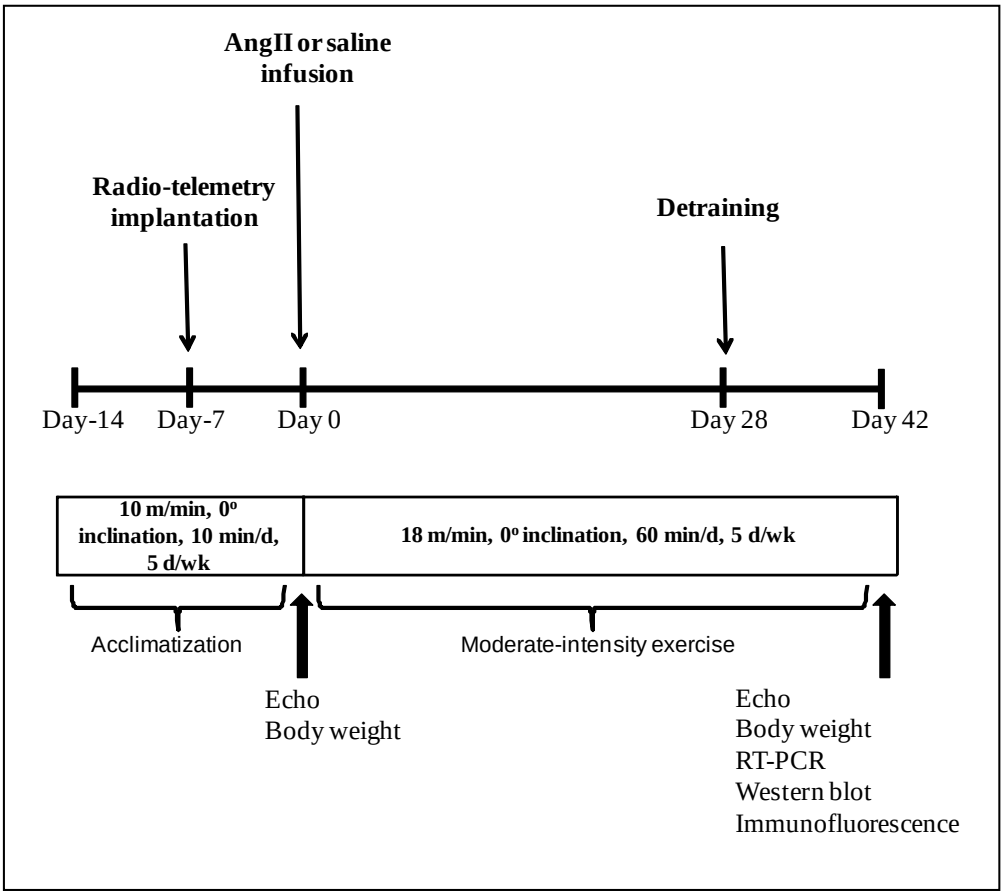

Figure 4.1. **Experimental protocol:** Rats were first acclimatized to the treadmill for 14 days before the start of the experiment. After 7 days of acclimation, rats were implanted with radio-telemetry probes for continuous recording of MAP and then were allowed to recover for next 7 days. Then miniosmotic pumps (42 days) filled with AngII or saline were subcutaneously implanted. Before AngII pump implantation, animals were weighed and a baseline echo was performed. Animals in exercise groups were allowed to run for 42 days, whereas, animals in sedentary groups were placed on non-running treadmill for the exercise sessions. Animals in detraining groups underwent exercise for 28 days and were kept sedentary for the rest of the14 days. 24 hours after the last exercise session, animals were weighed and an echo was done. Animals were then euthanized and the brains were collected for real-time RT-PCR and Western blot analysis.

Echocardiographic Assessment of Cardiac Function and Hypertrophy. Echocardiography (n = 8 per group) was performed at baseline and at the end of the 42-day study period, as described previously (Agarwal, Haque et al. 2009). Briefly, transthoracic echocardiography was performed

under isoflurane 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. Short-axis M-mode echocardiography was performed and the following measurements were obtained as an average of at least three cardiac cycles: Left ventricular internal diameter at diastole and systole (LVIDd and LVIDs, respectively), left ventricular posterior wall thickness at diastole and systole (LVPWTd and LVPWTs, respectively), interventricular septal thickness at diastole and systole (IVSTd and IVSTs, respectively), and fractional shortening (%FS) was calculated using the equation, FS= [(LVIDd- LVIDs)/ LVIDd] X 100. Tei index was determined from left ventricular inflow and outflow Doppler recordings as previously described (Pellett, Tolar et al. 2004).

Real-time RT-PCR Analysis. Semi-quantitative real-time RT-PCR (n=6 per group) was used to determine the mRNA levels of PICs *viz.* TNF-α and IL-1β, AIC (IL-10), and oxidative stress markers *viz.* $gp91^{phox}$ (also known as NOX2), and iNOS in the PVN by using specific primers. Rat primers used are listed in Table 4.1. In Brief, the rats were euthanized, the brains were quickly removed and immediately frozen on dry ice. The brains were blocked in the coronal plane, sectioned at 100 μm thickness, and the PVN were punched from each brain according to the methods described by Palkovits and Brownstein (Gao, Wang et al. 2005). Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (Agarwal, Welsch et al. 2011). Gene expression was measured by the ΔΔCT method and was normalized to GAPDH mRNA levels. The data is presented as the fold change of the gene of interest relative to that of control animals.

Table 4.1. Rat primers used for real-time RT-PCR

Gene	Sense	Antisense
GAPDH	agacagccgcatcttcttgt	cttgccgtgggtagagtcat
TNF-α	gtcgtagcaaaccaccaagc	tgtgggtgaggagcacatag
IL-1β	gcaatggtcgggacatagtt	agacctgacttggcagaga
IL-10	gggaagcaactgaaacttcg	atcatggaaggagcaacctg
$gp91^{phox}$	cggaatctcctctccttcct	gcattcacacaccactccac
Inos	ccttgttcagctacgccttc	ggtatgcccgagttctttca

Western Blot Analysis. The tissue homogenates from the PVN were subjected to Western blot analysis (n = 5 per group) for the determination of protein levels of PICs (TNF-α, IL-1β), IL-10, $gp91^{phox}$, iNOS, and GAPDH. The extraction of protein and Western blot was performed as described before (Agarwal, Welsch et al. 2011). Specific antibodies used included: TNF-α, IL-1β, $gp91^{phox}$, iNOS, and GAPDH, at 1:1,000 dilution; and IL-10, at 1:500 dilution. Antibodies were commercially obtained: TNF-α (Abcam Inc, MA, USA); IL-1β, iNOS, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA); IL-10 (Abbiotec, CA,USA); and $gp91^{phox}$ (BD biosciences, USA). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham), band intensities were quantified using Versa Doc MP 5000 imaging system (Bio-Rad), and were normalized with GAPDH.

Statistical Analysis. All data are presented as means±SE. Statistical analysis was done by either two-way ANOVA or one-way ANOVA with a Tukey's post hoc test using Graph Pad Prism software (version 5.0). Blood pressure data were analyzed by repeated-measures ANOVA to examine with-in group changes over time. Results were considered significant when $p<0.05$.

Table 4.2. Baseline characteristic of studied rats: BW, MAP, and echocardiographic analysis of cardiac hypertrophy and function.

Parameters	Sal+Sed	Sal+Ex	Sal+Det	AngII+Sed	AngII+Ex	AngII+Det
BW (g)	270.7±7.5	269.8±4.8	270.0±5	272.9±1.7	271.0±6.4	273.0±6.2
MAP (mmHg)	109.0±1.9	105.6±2.2	106.5±2	103.6±5.4	102.3±2.8	105.3±2.8
IVSTd, mm	1.7±0.04	1.6±0.03	1.6±0.02	1.7±0.03	1.6±0.04	1.6±0.05
IVSTs, mm	2.9±.12	2.6±0.06	2.6±0.02	2.9±0.05	2.7±0.07	2.9±0.06
LVIDd, mm	7.4±0.13	7.5±0.17	7.7±0.15	7.5±0.16	7.5±0.07	7.3±0.18
LVIDs, mm	4.2±0.14	4.2±0.10	4.3±0.09	4.4±0.15	4.3±0.11	4.0±0.09
LVPWTd, mm	1.6±0.06	1.6±0.05	1.6±0.04	1.7±0.06	1.5±0.06	1.6±0.11
LVPWTs, mm	2.6±0.06	2.8±0.13	2.9±0.16	2.8±0.05	2.7±0.15	2.7±0.14
FS, %	43.4±2.3	44.1±0.5	44.0±0.7	42.7±1.0	42.8±1.7	44.8±0.8
EF, %	77.0±1.2	80.2±1.8	80.4±1.0	77.5±2.6	80.0±1.8	82.8±2.2
HR	356±3	358±5	354±5	344±7	361±6	365±6
Tei index	0.516±0.06	0.494±0.04	0.486±0.01	0.564±0.03	0.514±0.04	0.414±0.02

Values are mean ±SE. Sal+Sed , saline+sedentary; Sal+Ex, saline+exercise; Sal+Det, saline+detraining; AngII+Sed, angiotensionII+sedentary; AngII+Ex, angiotensinII+exercise; AngII+Det, angiotensinII+detraining. BW(g), body weight (grams); MAP, mean arterial pressure (mmHg). LVIDd and LVIDs indicate left ventricular internal diameter at diastole and systole, respectively; IVSTd and IVSTs, interventricular septal thickness at diastole and systole, respectively; LVPWTd and LVPWTd, left ventricle posterior wall thickness at diastole and systole, respectively; FS, fractional shortening (%); EF (%), ejection fraction; HR, heart rate.

RESULTS

Baseline Characteristics. Table 4.2 shows the baseline characteristics of the studied animals. At the beginning of the study, the body weight and echocardiographic parameters were similar between groups and all rats had normal MAP.

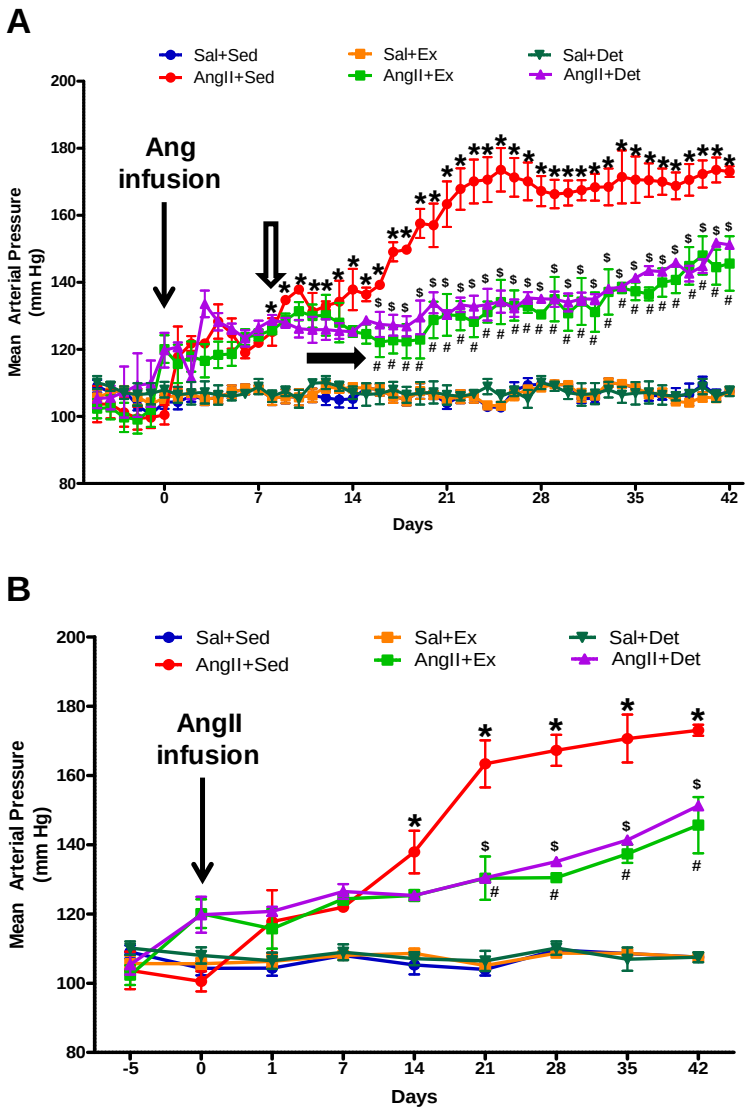

Figure 4.2. Time course of mean arterial pressure (MAP, in millimeters of mercury) in normotensive and hypertensive rats. A, average daily pressure. **B,** average weekly pressure. MAP was significantly increased in AngII+Sed compared with Sal+Sed rats from day 8 of Ang infusion (empty arrow). MAP was significantly reduced in AngII+Ex compared with AngII+Sed rats from day 16 of exercise (filled arrow). 2 weeks of detraining did not abolish the exercise-induced reduction in MAP in AngII-infused rats. Values are mean±SE; n=6 per group. *p<0.05 Sal+Sed versus AngII+Sed; #p<0.05 AngII+Sed versus AngII+Ex; $p<0.05 AngII+Sed versus AngII+Det.

Effects of Exercise and Detraining on MAP. As shown in Figure 4.2, AngII infusion in sedentary rats caused increase in MAP starting at day 5 of AngII infusion, the increase being significant starting at day 8 of infusion when compared to Sal+Sed and remained for the duration of the study. The maximum increase in MAP in AngII+Sed rats was observed at day 23 of infusion after which it reached to plateau. Regular exercise resulted in significant reduction in MAP in AngII+Ex when compared with AngII+Sed rats; the values were found significant beginning from day 16 of exercise. Similarly, in AngII+Det group, exercise caused significant reduction in MAP beginning from day 16 when compared with AngII+Sed. There was no difference in MAP between AngII+Ex and AngII+Det rats. However, in AngII+Det group, MAP appears to slightly increase towards the end of study, although the difference was insignificant in comparison with AngII+Ex. Exercise did not affect MAP in normotensive rats.

Effects of Exercise and Detraining on Cardiac Hypertrophy and Cardiac Function. At the end of the study period, AngII+Sed had higher heart weight (HW) and HW:BW ratio compared with Sal+Sed rats (Table 4.3). Echocardiographic studies (Figure 4.3A-C) revealed that when compared with Sal+Sed, AngII+Sed rats had significantly higher interventricular septal thickness (IVSTd) and left ventricular posterior wall thickness at diastole (LVPWTd), without modification of LV chamber size. These echocardiographic changes indicate the presence of concentric cardiac hypertrophy and suggest diastolic dysfunction in AngII-induced hypertensive rats. Furthermore, the increased Tei index (Figure 4.3D) in AngII+Sed when compared with Sal+Sed rats confirms the presence of diastolic dysfunction in hypertensive rats. Regular exercise significantly reduced IVSTd, LVPWTd, and Tei index in AngII infused rats, indicating attenuated cardiac hypertrophy and improved diastolic function.

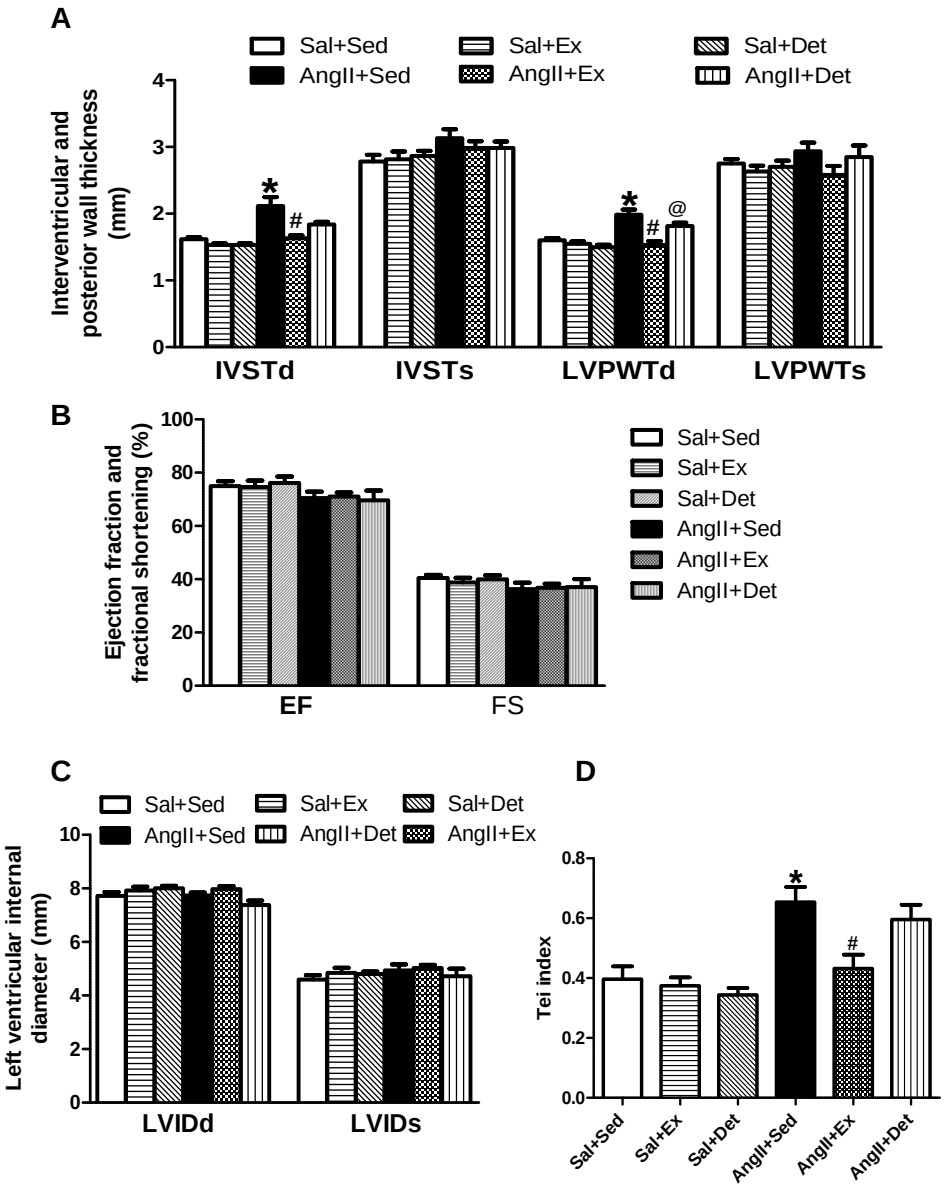

Figure 4.3. Effect of exercise and detraining on cardiac hypertrophy and cardiac function in normotensive and hypertensive rats as measured by M-mode and Doppler echocardiography. AngII+Sed rats had significantly higher levels of IVSTd, LVPWTd, and Tei index when compared to Sal+Sed. Exercise caused significant reduction in these variables in AngII+Sed rats. 2 weeks of detraining resulted in significantly increased LVPWTd in comparison with AngII+Ex; whereas, IVSTd and Tei index values were considerably but insignificantly increased in AngII+Det versus AngII+Ex. These data suggest that detraining caused partial reversal of exercise-induced changes in hypertensive rats. Values are means±SE. n=8 per group. *p<0.05 Sal+Sed versus AngII+Sed; #p<0.05 AngII+Sed versus AngII+Ex; @p<0.05 AngII+Ex versus AngII+Det.

Exercise caused reduction in HW:BW ratio as well, although, the values did not reach significance. Interestingly, AngII+Det rats exhibited higher IVSTd and Tei index in comparison with AngII+Ex, although, values were statistically not significant between AngII+Det and AngII+Ex and between AngII+Det and AngII+Sed. However, The LVPWTd was significantly increased in the AngII+Det rats when compared to AngII+Ex, and there was no difference when compared to the AngII+Sed rats. AngII+Det rats had significant increase in LVPWTd and a slight but insignificant increase in IVSTd and Tei index when compared to AngII+Ex, suggesting that 2 weeks of detraining may not be sufficient to completely reverse the exercise-induced changes in cardiac hypertrophy and function but it may lead to complete reversal if continued for longer than 2 weeks.

Effects of Exercise and Detraining on Pro-inflammatory Cytokines in the PVN of Hypertensive Rats. To investigate the influence of exercise and detraining on PICs within the PVN of hypertensive rats, we examined the mRNA (Figure 4.4A-B) and protein (Figure 4.4D-E) levels of TNF-α and IL-1β. AngII+Sed rats exhibited marked increases in TNF-α and IL-1β expression in the PVN compared to Sal+Sed. This upregulation was significantly attenuated by regular exercise in AngII-induced hypertensive rats. Interestingly, two weeks of detraining did not reverse the effects of exercise on PICs. There was significant difference in TNF-α and IL-1β levels between AngII+Sed and AngII+Det rats, while, there was no difference in AngII+Ex and AngII+Det groups.

Effects of Exercise and Detraining on Anti-inflammatory Cytokines in the PVN of Hypertensive Rats. To investigate the influence of exercise and detraining on anti-inflammatory status within the PVN, we determined the mRNA (Figure 4.4C) and protein (Figure 4.4D-E) levels of IL-10, a potent AIC. A significant decrease in IL-10 levels in the PVN was observed in

Table 4. 3. Effect of exercise and detraining on weights, MAP, and HR of rats.

Parameter	Sal+Sed	Sal+Ex	Sal+Det	AngII+Sed	AngII+Ex	AngII+Det
BW (g)	368.9±9.1	386.6±5.9	380.8±6.0	383.8±6.4	373.8±8.9	373.7±10.7
HW (g)	1.124±0.04	1.219±0.03	1.180±0.04	1.473±0.07*	1.354±0.10	1.214±0.04
HW/BW (mg/g)	3.09±0.04	3.02±0.08	3.10±0.02	3.70±0.12*	3.17±0.11	3.30±0.20
MAP (mmHg)	107.5±0.80	108.0±0.6	108.5±0.7	173.0±1.6*	145.6±8.1$^{\#}$	151.2±0.61
HR	357±10	331±4	351±4	355±10	330±5	347±9

Values are mean ±SE. Sal+Sed , saline+sedentary; Sal+Ex, saline+exercise; Sal+Det, saline+detraining; AngII+Sed, angiotensionII+sedentary; AngII+Ex, angiotensinII+exercise; AngII+Det, angiotensinII+detraining. BW(g), body weight (grams); HW (g), heart weight (grams); HW/BW (mg/g), heart weight to body weight ratio; MAP, mean arterial pressure (mmHg); HR, heart rate. *$p<0.05$ Sal+Sed vs AngII+Sed; $^{\#}p<0.05$ AngII+Sed vs AngII+Ex.

AngII+Sed when compared with Sal+Sed rats. Regular exercise resulted in significant upregulation of IL-10 levels in AngII-induced hypertensive rats. Interestingly, IL-10 levels in AngII+Det group were significantly lower than the AngII+Ex and they were not significantly different from the AngII+Sed group.

Effects of Exercise and Detraining on Oxidative Stress Markers in the PVN of Hypertensive Rats. To assess whether training and detraining can modulate oxidative stress within the PVN, we examined the expression levels of $gp91^{phox}$, (a subunit of NADPH Oxidase, major source of AngII-induced ROS production) and inducible NOS (iNOS). Both protein and gene expression levels of iNOS (Figure 4.5A, C-D) were significantly elevated in AngII+Sed when compared to Sal+Sed rats. Exercise caused significant reduction in iNOS expression in the PVN of hypertensive rats. Importantly, iNOS levels in AngII+Det group were significantly higher than the AngII+Ex rats.

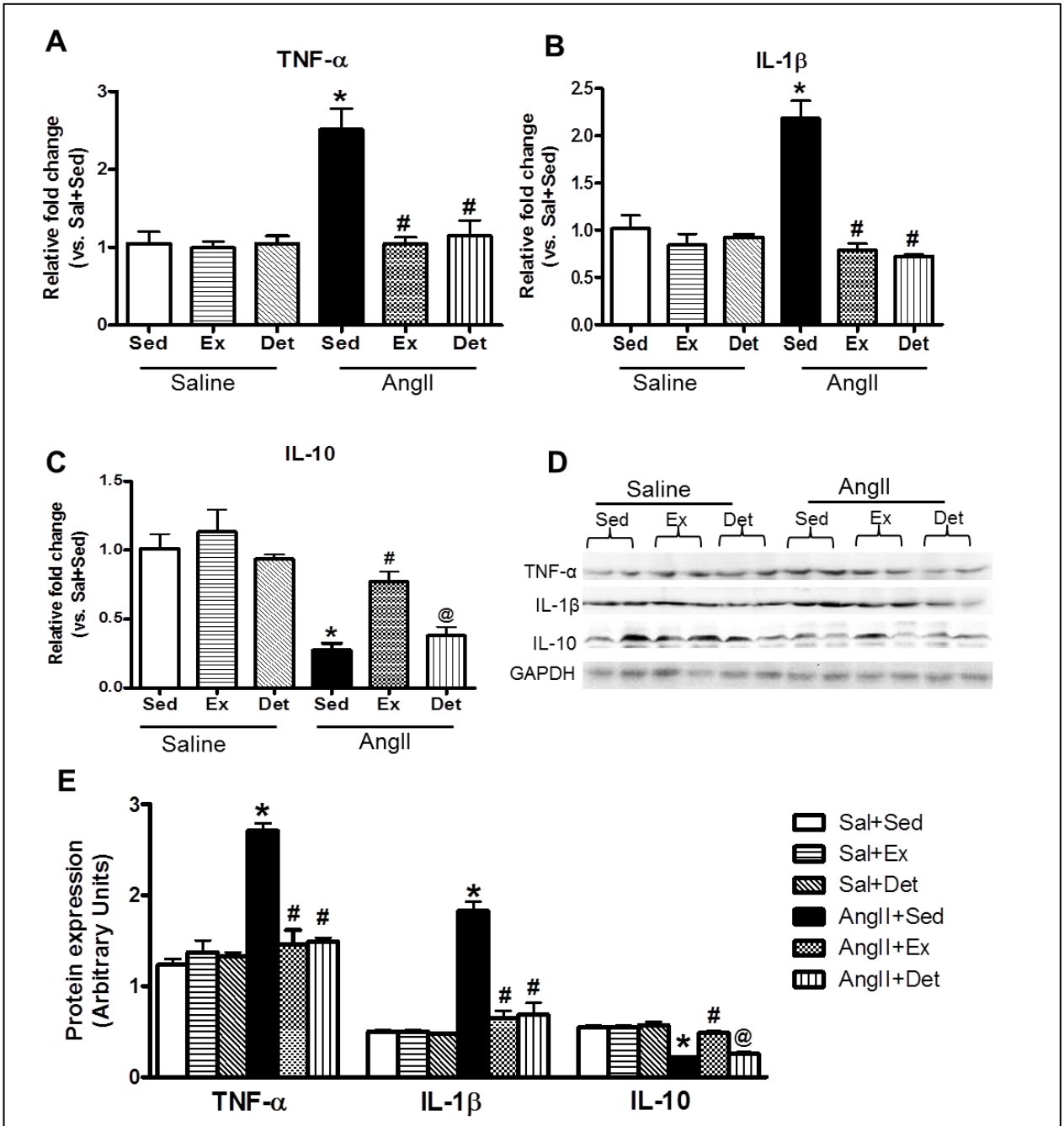

Figure 4.4. Effects of exercise on TNF-α, IL-1β, and IL-10 in the PVN of normotensive and hypertensive rats. A, mRNA expression of TNF-α. **B,** mRNA expression of IL-1β. **C,** mRNA expression of IL-10. **D**, a representative Western blot. **E,** densitometric analysis of protein expression. Detraining did not alter exercise-induced reduction in TNF-α and IL-1β levels in the PVN of Ang-infused animals; whereas, it did abolish exercise-mediated increase in IL-10 levels. Values are means±SE. n=6 per group for mRNA and n=5 per group for protein analysis. *p<0.05 Sal+Sed versus AngII+Sed; #p<0.05 AngII+Sed versus AngII+Ex and AngII+Sed versus AngII+Det; @p<0.05 AngII+Ex versus AngII+Det.

Similarly, as shown in Figure 4.5B-D, gp91^{phox} expression was much higher in AngII+Sed than Sal+Sed rats within the PVN. Exercise caused significant reduction in gp91^{phox} expression in the PVN of hypertensive rats. Similar reduction was observed in AngII+Det

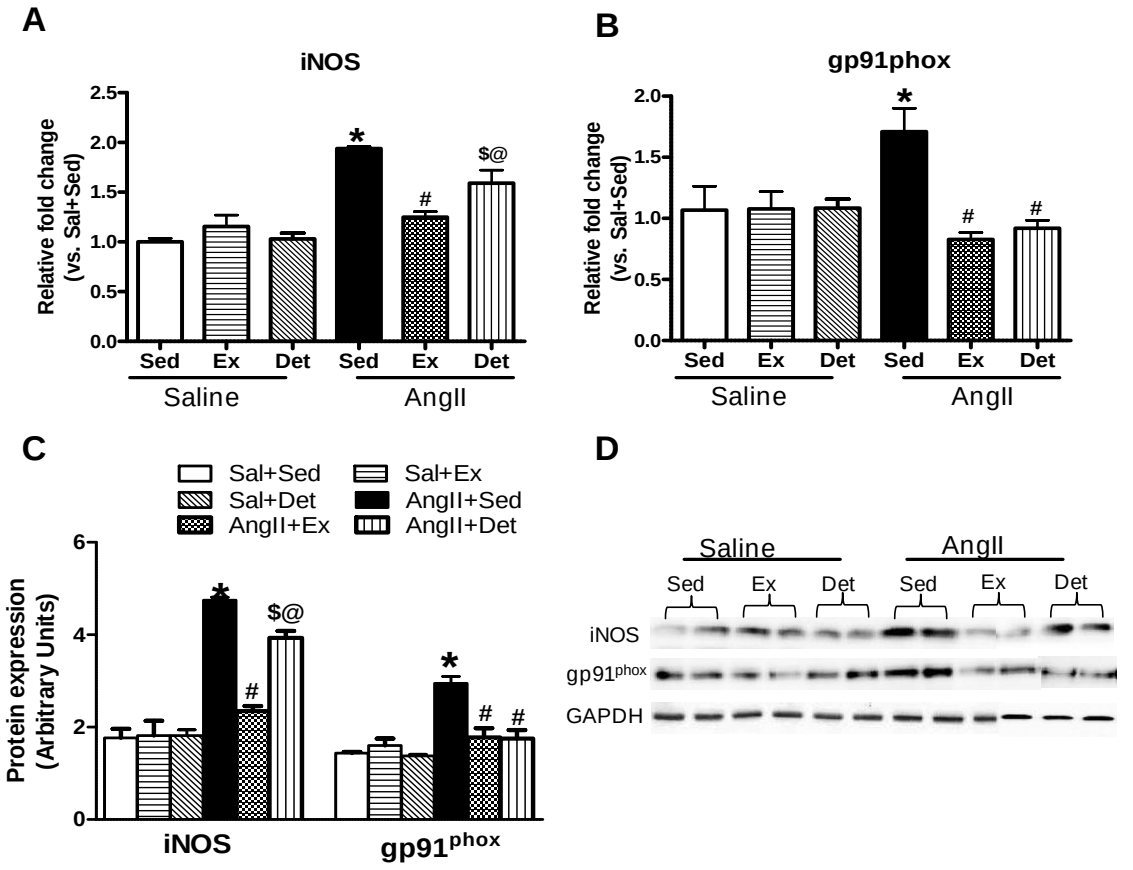

Figure 4.5. Effects of exercise on iNOS and gp91^{phox} in the PVN of normotensive and hypertensive rats. A, mRNA expression of iNOS. **B,** mRNA expression of gp91^{phox}. **C**, densitometric analysis of protein expression. D**,** a representative Western blot. Detraining did not alter exercise-induced reduction in gp91^{phox} levels in the PVN of Ang-infused animals; whereas, it partially abolished exercise-mediated reduction in iNOS levels. Values are means±SE. n=6 per group for mRNA and n=5 per group for protein analysis. *p<0.05 Sal+Sed versus AngII+Sed; #p<0.05 AngII+Sed versus AngII+Ex and AngII+Sed versus AngII+Det; @p<0.05 AngII+Ex versus AngII+Det; $AngII+Sed versus AngII+Det.

compared to AngII+Sed group. Among hypertensive rats, there were no significant differences in $gp91^{phox}$ expression between detraining and exercise group.

DISCUSSION

The present study sought to evaluate the impact of regular exercise and 2 weeks of detraining on blood pressure, cardiac hypertrophy and cardiac function in an AngII-induced hypertensive rat model. Also, we investigated the impact of exercise and detraining on pro- and anti-inflammatory cytokines and oxidative stress within the brain of these hypertensive rats. Three novel and important findings emerge from this study. First, two weeks of detraining did not abolish the exercise-induced attenuation in MAP in hypertensive rats, whereas, detraining failed to completely preserve the exercise-mediated improvement in cardiac hypertrophy and diastolic function in these rats. Second, two weeks of detraining does not have any detrimental effects on exercise-induced improvement in PICs and $gp91^{phox}$ levels in the PVN of hypertensive rats. Third, 2 weeks of detraining in exercising hypertensive rats abolished the exercise-induced improvement in IL-10 and iNOS levels in the brain, the two important molecules contributing to the pathogenesis of hypertension. Collectively, these results led us to conclude that 2 weeks of detraining is not long enough to completely abolish the exercise-induced beneficial effects; however, further cessation of exercise may lead to complete reversal of the beneficial effects. These results suggest that the exercise-induced improvement in MAP in hypertensive rats is indeed preceded by changes in cardiac morphology and function, IL-10 and iNOS levels in the brain. In addition, these results indicate IL-10 and iNOS are two of the most sensitive parameters to assess the effects of exercise or detraining.

At the end of the study, we observed significant reduction in MAP in trained hypertensive rats compared with their sedentary counterparts and saw no comparable changes in

trained normotensive controls. As depicted in Figure 4.2, the continuous recording of MAP in conscious rats by implanted telemetry device showed that AngII infusion resulted in significant increase in MAP in sedentary rats beginning from day 8 of infusion and this increase in MAP reached to plateau at day 23 of infusion. Regular exercise resulted in significant reduction in MAP beginning from day 16 of training and remained significant until the end of the study. Interestingly, 2 weeks of detraining preceded by 4 weeks of exercise in AngII-induced hypertensive rats was found to be insufficient to abolish exercise-induced attenuation in MAP as indicated by no significant difference in MAP between AngII+Ex and AngII+Det rats. In accordance with these findings, previous reports have demonstrated that 10 weeks of exercise attenuated BP in spontaneously hypertensive rats (SHRs) and 1 or 2 weeks of detraining did not affect attenuated BP in these rats (Lehnen, Leguisamo et al. 2010). However, in the present study, towards the end of two weeks of detraining, MAP appears slightly higher in AngII+Det when compared with AngII+Ex rats (mean±SE, 144+5 vs. 151+1 on day 41 and 145+8 vs. 151+0.6 on day 42, Figure 4.2A). The discrepancies in results could be because of the disease model, exercise protocol, and/or methodological differences among various studies. It is noteworthy that previous studies from our lab and others have used tail-cuff method for BP measurements and most of those studies reported BP as measured only before and/or after the study. Whereas, to best of our knowledge, this is the first study that has employed telemetry recording of MAP in conscious sedentary and exercising animals without causing any undue stress on animals. This methodological improvement in the present study not only allowed us to obtain the most accurate measurements but also allowed us to monitor day-to-day changes in BP in relation to exercise and detraining. Nonetheless, the data suggests that although two weeks of

detraining may not be long enough to revert MAP back to sedentary values, continuing detraining may lead to complete reversal.

Our echocardiographic data showed that regular moderate-intensity exercise resulted in reduced cardiac hypertrophy and improved diastolic function in hypertensive rats Interestingly, 2 weeks of detraining failed to completely preserve this exercise-induced improvements in cardiac hypertrophy and function as suggested by significant increase in LVPWTd and a not significant but considerable increase in IVSTd and Tei index in AngII+Det when compared to AngII+Ex rats. These results extended the observations of Bocalini *et al*, who demonstrated that 2 weeks of detraining was sufficient to reverse LVPWT in healthy female rats (Bocalini, Carvalho et al. 2010). However, our study examined in detail cardiac function using M-mode and Doppler echocardiography performed in the same animal at baseline and at the end of the study, thus providing greater insight into the effects of detraining on cardiac function and morphology.

In the present study, the detraining could not fully preserve the cardioprotective effects of exercise; however, it is noteworthy that the 2 weeks of detraining were not sufficient to completely reverse the benefits either. Therefore, it is plausible to suggest that cessation of exercise for more than 2 weeks may lead to complete reversal of the cardioprotection offered by regular exercise. In support of this, it has previously been reported that resting cardiac output is reduced in trained SHRs, and that it returns to sedentary values only after 5 weeks of detraining (Pavlik 1985). Additionally, 5 weeks of detraining in these SHRs led to reversal of resting HR and peripheral vascular resistance to pre-training levels (Pavlik 1985). Furthermore, Mostarda *et al*. (Mostarda, Rogow et al. 2009) has also demonstrated that 3 weeks of detraining did not cause reversal of hemodynamic benefits in diabetic animals. Taken together, the current findings along with previous studies clearly suggest that shorter periods of detraining may prove to be

insufficient in abolishing the beneficial effects of exercise in hypertension. Continued absence of exercise can certainly have detrimental effects and hence emphasis should be given to regular active life-style to maintain the benefits.

Besides cardiac hypertrophy and diastolic dysfunction, hypertension is characterized by chronic inflammation which is reflected by a two- to threefold increase in circulating levels of several PICs (Peeters, Netea et al. 2001). In addition, the past few years of research have implicated brain cytokines, particularly in the PVN of the brain, in the pathogenesis of hypertension as well. It is apparent from these studies that PICs such as TNF-α and IL-1β act as neuromodulators and play a pivotal role in sympathetic regulation of BP (Shi, Raizada et al. 2010). Additionally, anti-inflammatory cytokines (AIC) such as IL-10 have a significant impact on arterial pressure (Shi, Raizada et al. 2010). We have recently reported that chronic regular exercise of 16 weeks duration decreases PICs and upregulates IL-10 levels in the brain of SHRs (Agarwal, Welsch et al. 2011). In the present study, we found that regular exercise induces similar improvements in PIC and AIC in the PVN of AngII-induced hypertensive rats. Interestingly, 2 weeks of detraining did not abolish the exercise-mediated improvement in TNF-α and IL-1β levels in the PVN. In contrary, detraining reversed the IL-10 levels back to near sedentary values in hypertensive rats. Given that it is not only the PICs but the balance between PIC and AIC that determines the outcome of the disease, there is a possibility that the reduction of IL-10 levels by detraining may ultimately lead to upregulation of PICs, if continued longer than 2 weeks. Nevertheless, our data suggest that the anti-inflammatory defense system of the body is vulnerable and sensitive to detraining. These data also emphasize the importance of regular physical activity in improving the anti-inflammatory status in hypertension.

Research over past several decades has established that PICs contribute to the increase in BP via induction of oxidative stress (Mayorov, Head et al. 2004; Zimmerman, Lazartigues et al. 2004). Of particular importance, NADPH oxidase (NOX)-derived reactive oxygen species (ROS) act as potent intra- and intercellular second messengers in signaling pathways causing hypertension (Sirker, Zhang et al. 2011). Of the various isoforms of NOX, the role of NOX2 ($gp91^{phox}$) in AngII-induced hypertension is well established (Murdoch, Alom-Ruiz et al. 2011). Given the role of AngII-induced oxidative stress in the brain in hypertension, it is interesting to investigate whether training and detraining has the ability to influence ROS generation within the brain of hypertensive rats. Our data illustrates that regular exercise dramatically downregulated increased levels of $gp91^{phox}$ and iNOS in hypertensive rats. Interestingly, 2 weeks of detraining abolished the effects of exercise on iNOS; whereas, $gp91^{phox}$ levels remained unchanged in detrained animals when compared with trained hypertensive rats. These data suggest that like IL-10, iNOS could be another sensitive parameter to evaluate the effects of training or detraining in hypertension.

Previous studies have investigated the effects of detraining on heart and skeletal muscle of hypertensive and normal rats in relation to insulin sensitivity (Neufer, Shinebarger et al. 1992; Kump and Booth 2005; Lehnen, Leguisamo et al. 2010). For instance, 48 hours (Kump and Booth 2005) to 1 week (Neufer, Shinebarger et al. 1992) of detraining was found to reduce GLUT4 gene expression in the skeletal muscle of normotensive rats. In another study, cessation of training for 1 week resulted in reduced levels of GLUT4 in the heart and white fat tissue in both normotensive and hypertensive rats (Lehnen, Leguisamo et al. 2010). However, to the best of our knowledge, the present study is the first to demonstrate the effects of detraining on inflammatory cytokines and oxidative stress, in particular within the brain of AngII-induced

hypertensive animals. Also, the effects of detraining on cardiac morphology and function in hypertension have rarely been studied before.

In summary, this study demonstrated that 2 weeks of detraining could partially revert the exercise-induced improvements in cardiac hypertrophy, cardiac function, and IL-10 and iNOS levels in the brain of hypertensive rats, although, positive effects in MAP, PICs, and $gp91^{phox}$ remained unchanged. In addition, among hypertensive rats a slight increase in MAP was observed in detrained when compared to trained group during the last two days of the study period. This observed phenomenon led us to suggest that the alterations in IL-10 and iNOS probably preceded the changes in MAP induced by exercise or detraining. In other words, anti-inflammatory effects of exercise and attenuated iNOS seem to be one of the most important factors contributing to reduction in MAP.

PERSPECTIVES

Given that exercise is recommended as a current guideline for the treatment of hypertension and non-compliance with the recommended treatment is a universal phenomenon, it is imperative to understand the cardiac and molecular changes associated with detraining. A few previous studies have examined the effects of detraining on heart and skeletal muscle of hypertensive and normal rats in relation to insulin sensitivity (Neufer, Shinebarger et al. 1992; Kump and Booth 2005; Lehnen, Leguisamo et al. 2010).The results of the current study provides a greater insight in to how detraining can influence the mean arterial blood pressure, cardiac function, inflammatory cytokines, and redox status within the brain of hypertensive rats. The understanding underlying molecular mechanisms and the time taken for each signaling pathway to lose adaptation induced by regular exercise will lead us to improve the current guidelines for the treatment of hypertension on the basis of scientific evidence.

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CHAPTER 5

ANGIOTENSIN II CAUSES IMBALANCE BETWEEN PRO- AND ANTI-INFLAMMATORY CYTOKINES BY MODULATING GSK-3B IN RAT NEURONAL CELLS

INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of death in the United States and of all the CVD conditions, hypertension has the highest prevalence. According to most recent report from the American Heart Association, an estimated 76.4 million adults ≥ 20 years of age have high blood pressure (Roger, Go et al.). Despite of success of several anti-hypertensive medications such as angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs) and β-adrenergic receptor blockers in reducing blood pressure (BP), the incidence and prevalence of hypertension is on the rise. These statistics clearly suggest the need of novel therapeutic strategies for the treatment of hypertension.

Inflammation is a well-known risk factor for various CVDs including hypertension (Agarwal, Welsch et al. ; Guggilam, Cardinale et al.). Pro-inflammatory cytokines (PICs), such as tumor necrosis factor-α (TNF-α) (Dorffel, Latsch et al. 1999), interleukin (IL)-1β (Dorffel, Latsch et al. 1999; Peeters, Netea et al. 2001), and IL-6 (Chae, Lee et al. 2001; Peeters, Netea et al. 2001), have been reported to increase with the severity of hypertension and are of prognostic significance. Besides circulating cytokines, recently brain cytokines have also been implicated in the pathogenesis of the disease (Guggilam, Cardinale et al. ; Kang, Zhang et al.). Recent discoveries indicate that besides elevated levels of circulating and brain PICs (Peeters, Netea et al. 2001; Shi, Raizada et al. 2010), anti-inflammatory cytokines (AICs) such as IL-10 has a significant impact on arterial pressure and cardiac remodeling in experimental models of hypertension (Shi, Raizada et al. 2010). Additionally, an overactivation of the renin-angiotensin system (RAS) directly or indirectly through PIC plays a vital role in the pathogenesis of hypertension.

The most important transcription factors, nuclear factor *kappa*B (NFκB) and cyclic AMP response element binding protein (CREB) are known to play a central role in modulating the gene expression of inflammatory mediators involved in hypertension. However, unlike NFκB, which positively regulates gene expression of PICs (Kang, Gao et al.), activation of CREB positively regulates expression of anti-inflammatory cytokines such as IL-10 (Avni, Ernst et al.). Competition between NFκB and CREB for binding to the co-activator CREB-binding protein (CBP), is important in regulating their transcriptional activity (Grimes and Jope 2001; Shenkar, Yum et al. 2001). Although, Angiotensin II (AngII), a major effector molecule of RAS, has been shown to elevate PIC levels in the brain, the effects of overactivation of RAS on anti-inflammatory cytokines are not very well understood. Also, the exact mechanisms underlying AngII-induced effects on inflammatory cytokines are still poorly understood.

Recently, glycogen synthase kinase (GSK)-3 has gained increasing attention from the scientific community due to its role in many biological processes. Past several years of research has now established that GSK-3 acts as a regulatory switch that determines the output of numerous signaling pathways initiated by diverse stimuli (Frame and Cohen 2001; Grimes and Jope 2001; Woodgett 2001). Of the two isoforms-*alpha* and -*beta*, GSK-3β is particularly abundant in the central nervous system (CNS) and is neuron-specific (Leroy and Brion 1999). Recently, GSK-3β has been reported to modulate the production of inflammatory cytokines in an NFκB-dependent manner (Martin, Rehani et al. 2005; Steinbrecher, Wilson et al. 2005; Vines, Cahoon et al. 2006; Beurel and Jope 2009). However, the role of GSK-3β in AngII-induced dysregulation of inflammatory molecules within the brain has not been explored yet.

Therefore, the present series of studies were undertaken to investigate the novel role of GSK-3β in AngII-induced dysregulation of PICs and AICs in rat neuronal cells. We

hypothesized that 1) AngII causes an imbalance between PIC and AIC in rat neuronal cells; 2) AngII-induced imbalance in PIC and AIC is modulated by downstream transcription factors, NFκB and CREB; and that 3) dysregulation in PICs and AICs in neuronal cells are mediated by GSK-3β. In this study, we constructed highly efficient lentiviral short interfering RNA (siRNA) targeting GSK-3β to examine the role of GSK-3β in AII-mediated effects in rat neuronal cells. The results of this study will help us to develop newer therapeutics targets for the treatment of hypertension.

MATERIALS AND METHODS

Neuronal Cell Culture. The rat neuronal PC12 cells were obtained from American Type Culture Collection. Unless otherwise stated, cells were plated at a density of $4x10^6$ cells/60mm dish for 18 h in 5% (v/v) fetal bovine serum and 15% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37^oC in a humidified atmosphere of 95% air and 5% CO_2.The serum was withdrawn for the 24 hours (h) before experimentation. To investigate the effects of AngII on inflammatory cytokines, PC12 cells were exposed to 10 μM Ang II or vehicle for 1h, 6h, 12h, and 24h. In subsequent experiments, cells were exposed to AngII for 6h. In another set of experiments, PC12 cells were transduced with lentiviral short interfering RNA targeting GSK-3β (L-si-GSK3β) for 48h before AngII exposure for 6h. Following exposure to agonists, cells were harvested for real-time RT-PCR, western blot, immunoprecipitation, and immunoflurorescence analysis. Results are presented as the mean ± SD and represent set of three different experiments in PC12 cells. In each experiment, n=6 per treatment groups were used.

Lentiviral Construction and Transduction. We explored the effects of inhibition of GSK-3β by using gene knock-down approach: RNA interference (RNAi) through delivery of a small interfering RNA (siRNA) against GSK-3β using a lentiviral vector (L-si-GSK3β). The target

sequences for the rat-specific GSK-3β siRNA (L-si-GSK3β) is as follows: TGGTAGCATGAAAGTTAGC. L-si-GSK3β was commercially obtained (NitAn Biotech LLC, Columbus, OH) and these vectors were tagged with EGFP. A scrambled sequence of the same length was used as a control (mentioned as L-control in text). 24 h after plating, PC12 cells were transduced (in triplicates) separately in six-well laminin coated plates with 30 MOI (multiplicity of infection, which is equal to ratio of infectious viral particles to cell) of L-si-GSK3β and scrambled sequence (L-control) viral particles in presence of 8 μg/ml of polybrene. We use 2 ml of viral supernatant, which contain 2×10^7-10^8 viral particles for each transduction experiment. After 48 h, the efficiency of the transduction was measured by monitoring EGFP expression under fluorescence microscope. Additionally, we performed western blotting to further assess the silencing effects of L-si-GSK3β. The transduction efficiency was found to be 80-90% after two successive infections (Figure 5.4). Cells were stimulated with AngII 48 h after transduction. Cells were also transduced with L-control separately in presence of AngII.

RNA Extraction and Real-time RT-PCR. Semi-quantitative real-time RT-PCR was used to determine the mRNA levels of TNF-α, IL-1β, and IL-10 in rat neuronal cells by using specific primers. Rat primers used were: GAPDH, Forward: 5'agacagccgcatcttcttgt-3', Reverse: 5'cttgccgtgggtagagtcat-3'; TNF-α, Forward: 5'gtcgtagcaaaccaccaagc-3', Reverse: 5'tgtgggtgaggagcacatag-3'; IL-1β, Forward: 5'gcaatggtcgggacatagtt-3', Reverse: 5'agacctgacttggcagaga-3'; and IL-10, Forward: 5'gggaagcaactgaaacttcg-3', Reverse: 5'atcatggaaggagcaacctg-3'. Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (Sriramula, Haque et al. 2008). Semilog amplification curves were evaluated by the comparative quantification method ($2^{-\Delta\Delta Ct}$), and GAPDH was used for

normalization of all reported gene expression levels. The data are presented as the fold change of the gene of interest relative to that of control group.

Immunoblot Analysis. For whole cell extracts, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were scraped into in100 µl/dish of cell lysis buffer (Cell Signaling Technology, Inc, MA, USA) containing protease and phosphatase inhibitors. Samples were incubated on ice for 10 min and then centrifuged (10,000 x g, 5 min, 4^{o}C). The supernatants were retained. Protein concentrations were determined by the Bradford method (Bradford 1976). The lysates were stored at -80^{o}C until used for immunoblotting.

Cell lysates were mixed with Laemmli sample buffer (Bio-rad Laboratories, CA, USA) and placed in a boiling water bath for 5 min. Proteins (30 µg) were separated by SDS-polyacrylamide gel electrophoresis using 10-15% (w/v) resolving gels and 6% (w/v) stacking gels, and then transferred to nitrocellulose membrane. Non-specific binding sites were blocked with 1% (w/v) casein (for non-phosphorylated antibodies) in PBS or 1% (w/v) bovine serum albumin (for phosphorylated antibodies) in TBST [20mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20]. Blots were probed (overnight, 4^{o}C) with the primary antibodies. Specific antibodies used included: TNF-α, IL-10, GSK-3β, p-GSK3β(Ser-9), p-GSK3β(Tyr-216), p-CREB(Ser-133), and CBP at 1:1000 dilution. Antibodies were commercially obtained: TNF-α (Abcam Inc, MA, USA); IL-10 (Abbiotec, CA, USA); GSK-3β (BD Transduction laboratories, USA), p-GSK3β(Ser-9), p-GSK3β(Tyr-216), p-CREB(Ser-133), and p-p65(Ser-276) (Cell Signaling Technology, Inc, MA, USA); and CBP (Santa Cruz Biotechnology, CA, USA). Blots were washed in TBST, incubated (60 min, room temperature) with horseradish peroxidase-(HRP-) conjugated secondary antibodies (1:10,000) in blocking solution. Immunoreactive bands were visualized using enhanced chemiluminescence (ELC Plus, Amersham), band intensities

were quantified using VersaDoc MP 5000 imaging system (Bio-rad, CA, USA), and were normalized with GAPDH.

Immunoprecipitation. To determine the role of GSK-3β in regulating downstream transcription factors, we used the catch and release immunoprecipitation system (Upstate Biotechnology, MA, USA) as described previously (Martin, Rehani et al. 2005). For these experiments, protein CBP was immunoprecipitated by incubating cell lysates with 2 μg of CBP monoclonal antibody (Pharmingen, USA) overnight at 4^{o}C. Samples were incubated with 60 μL of protein G sepharose beads (Amersham, NJ, USA) for 1h at 4^{o}C with gentle agitation. The immune complexes were washed three times with lysis buffer. Samples in Laemmli buffer were placed in a boiling water bath, proteins were separated by SDS-PAGE, and samples were immunoblotted with anti-p-CREB(Ser-133) or anti- p-p65(Ser-276). The membranes were reprobed with an anti-CBP antibody to confirm the efficiency and specificity of immunoprecipitation (IP).

Statistical Analysis. Statistical analysis was completed by either unpaired t-test or one-way ANOVA with Bonferroni post hoc test using Graph Pad Prism software (version 5.0). Data are presented as the fold change of each gene of interest relative to controls. Results were considered significant when $p<0.05$.

RESULTS

AngII Causes an Imbalance Between Pro- and Anti-inflammatory Cytokines in Rat Neuronal Cells. To investigate the influence of AngII on PICs and AIC in the neuronal cells, rat PC12 cells were exposed to AngII (10 μM) for indicated time and then we examined the mRNA (Figure 5.1A) and protein (Figure 5.1B) levels of TNF-α and IL-10 in whole cell extracts. We observed that AngII treated cells exhibited time-dependent increase in TNF-α level with maximal effects at 6h of exposure. At mRNA level, AngII exposure (6h) resulted in fivefold

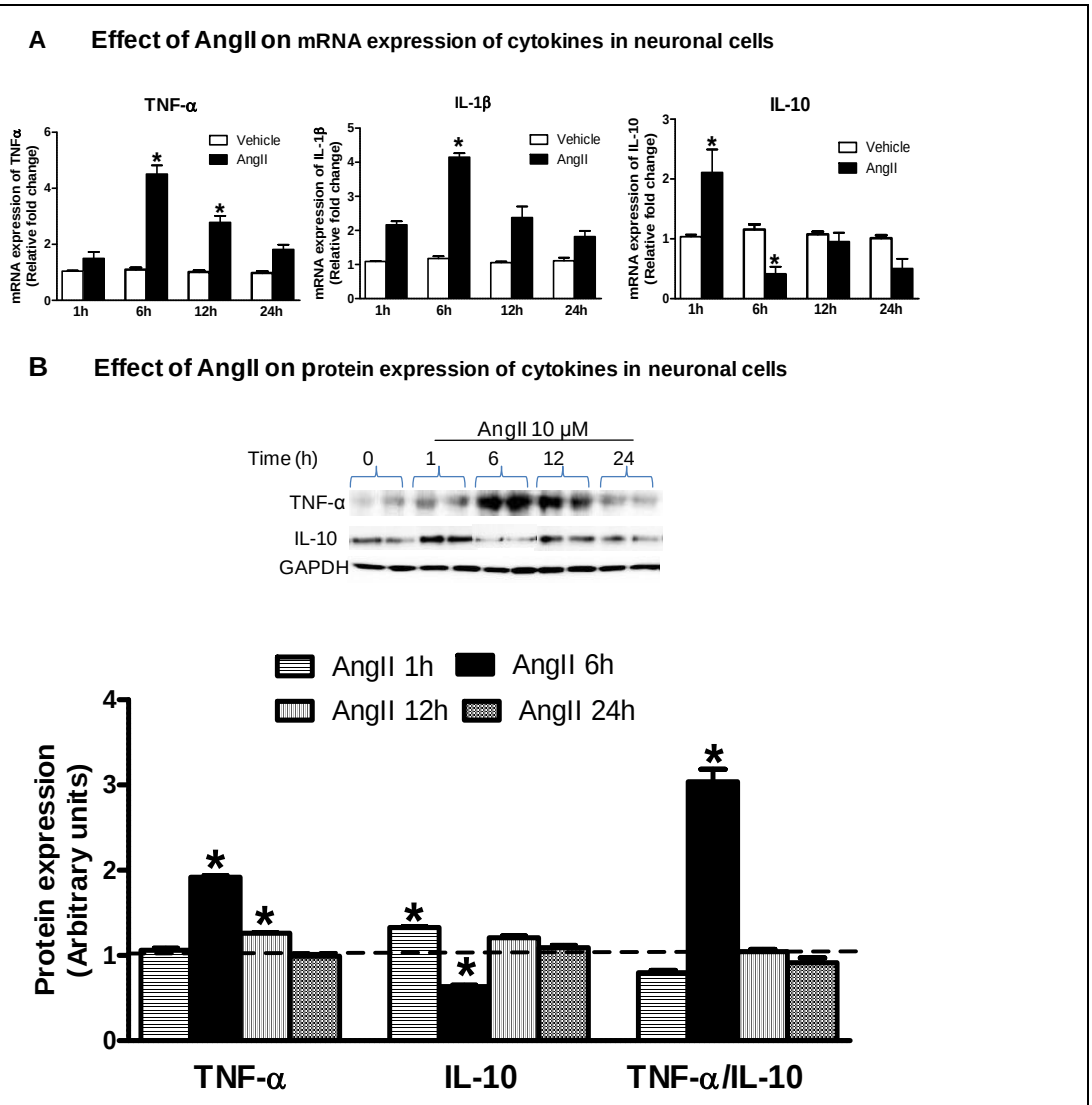

Figure 5.1. Effects of AngII treatment on TNF-α, IL-1β, and IL-10 expression levels in neuronal cells. Serum starved PC12 cells were stimulated with 10 μM AngII for the indicated time. Unstimulated control cells were treated with vehicle (saline) in place of AngII. **A,** mRNA expression of TNF-α, IL-1β, and IL-10. **B,** A representative western blot and densitometric analysis of protein expression of TNF-α and IL-10. Quantitative western blot analysis is shown as the ratio of intensities of the protein of interest and GAPDH, relative to unstimulated control cells (represented as one as shown by the dashed line). AngII resulted in increased TNF-α and IL-1β and reduced IL-10 levels indicating an imbalance between PICs and AIC in the PC12 cells in time-dependent manner with maximum alterations at 6h of AngII treatment. The results are means±SD of three independent experiments. * $p < 0.05$ compared to their respective vehicle-treated group.

increase in TNF-α and more than twofold decrease in IL-10 expression in PC12 cells (Figure

5.1A). TNF-α levels in AngII exposed cells were reduced after 12 and 24h of exposure when compared with 6h, however, it remains elevated in comparison with vehicle treated cells. In contrary, IL-10 levels in cells treated with AngII for 6h were significantly lower when compared to vehicle treated cells. At 12h and 24h, IL-10 levels remained lower in comparison with vehicle groups, although the differences were not significant. Noteworthy, AngII exposure for 1h significantly upregulated IL-10 levels, whereas, TNF-α level was slightly higher at this time point.

To further confirm that AngII causes an imbalance between PIC and AIC in neuronal cells, we determined the alterations in ratio of TNF-α to IL-10 protein levels in cells treated with AngII or vehicle. A significant increase of threefold in TNF-α /IL-10 protein ratio was observed upon 6h of AngII exposure when compared to all other groups (Figure 5.1B). These data provide evidence that AngII exposure results in an imbalance between PIC and AIC in favor of PIC in rat neuronal cells.

AngII Induces Activation of GSK-3β in Neuronal Cells. To investigate whether AngII exposure for 6h (maximal effective exposure time) activates GSK-3β, we determined the protein expression levels of p-GSK3β(Ser-9) and p-GSK3β(Tyr-216) by immunoblot analysis in neuronal cells exposed with AngII or vehicle. Immunoblot analysis demonstrated that GSK-3β is expressed in rat neuronal (PC12) cells and there was a slight but significant increase in the phosphorylation of GSK3β(Ser-9) (Figure 5.2B; quantitation in Figure 5.2E). Simultaneously, however, AngII dramatically upregulated phosphorylation of GSK3β(Tyr-216), in PC12 cells (Figure 5.2C; quantitation in Figure 5.2E). Densitometric analysis further revealed that the ratio of protein expression of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) is significantly upregulated on AngII exposure (Figure 5.2D). Since, phosphorylation of GSK3β(Tyr-216) is essential for their

catalytic activity, these results indicate overall activation of GSK-3β upon AngII (10μM) exposure for 6h. The same blots were stripped and reprobed for native GSK-3β showing no significant difference on native GSK-3β expression between the vehicle and AngII treated

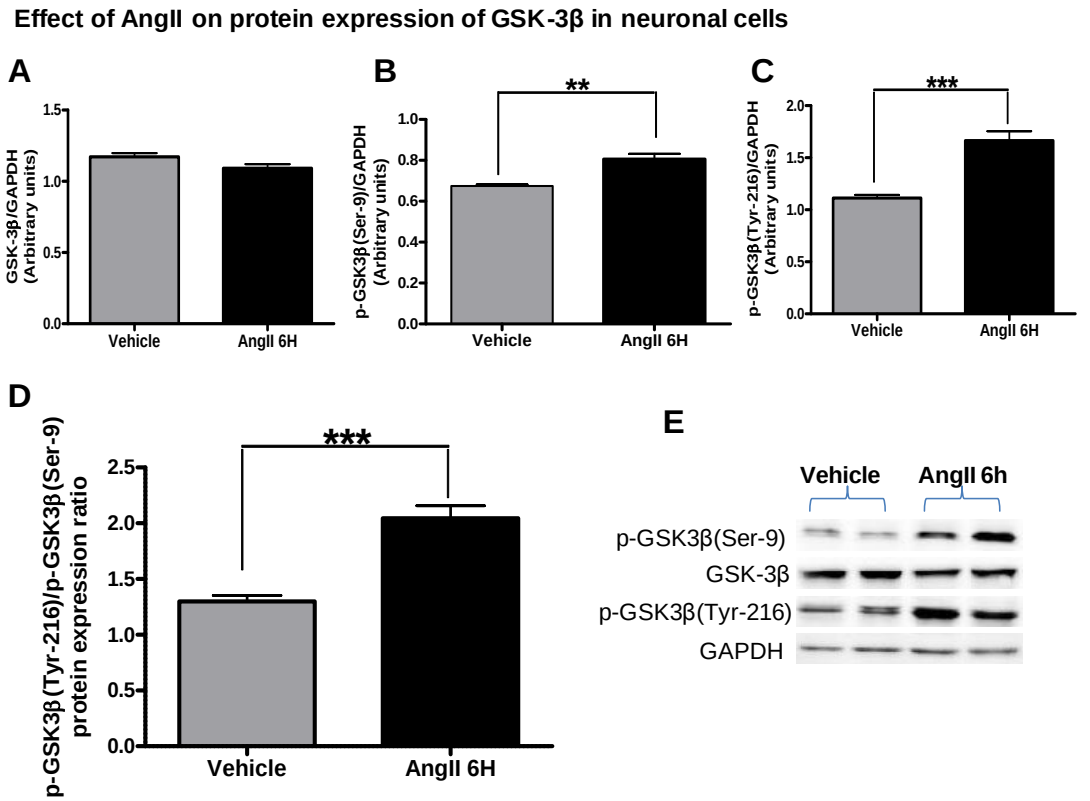

Figure 5.2. Effects of AngII treatment on total and phosphorylated GSK-3β expression in neuronal cells. Serum starved PC12 cells were stimulated with 10μM AngII for 6 hours and cell extracts were then subjected to protein analysis by Western blot. Densitometric analysis of western blot results showing protein expression of **A** total GSK-3β, **B** p-GSK3β(Ser-9), **C** p-GSK3β(Tyr-216), **D** p-GSK3β(Tyr-216)/p-GSK3β(Ser-9) ratio, and **E** A representative western blot. AngII caused significant activation of GSK-3β as indicated by reduced p-GSK3β(Ser-9), increased p-GSK3β(Tyr-216), and increased ratio of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) protein expression in PC12 cells. AngII exposure did not alter total GSK-3β protein levels. The results are means±SD of three independent experiments. ** $p < 0.01$; *** $p < 0.001$ compared with cells treated with vehicle.

groups (Figure 5.2A). These findings suggest that AngII-induced effects in neuronal cells could be mediated by activation of GSK-3β.

AngII Exposure Resulted in Altered Binding of CBP With CREB and NFκB in Rat Neuronal Cells. To investigate whether AngII induced imbalance in PIC and AIC is mediated by alterations in downstream transcription factors NFκB subunit p65 and CREB, we assessed the binding of CBP (co-activator protein) with p65 and CREB by immunoprecipitation analysis of vehicle and AngII treated groups. Since, phosphorylation of CREB at Ser-133 and p65 at Ser-276 have been shown to be essential for their binding with CBP, we also determined the protein levels of p-CREB(Ser-133) and p-p65(Ser-276). AngII exposure resulted in significant reduction in p-CREB(Ser-133) expression and increased p-p65(Ser-276) (Figure 5.3A), leading to

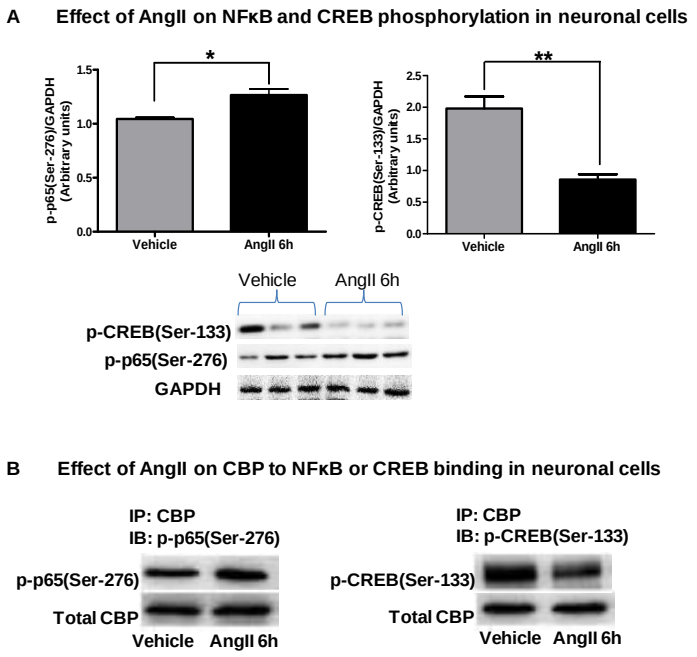

Figure 5.3. Effects of AngII treatment on CBP to NFκB and CREB binding in neuronal cells. The serum-starved (24h) PC12 cells were treated without or with AngII (10μM; 6h). **A,** Densitometric analysis and a representative immunoblot showing increased expression levels of p-p65(Ser-276) and decreased p-CREB(Ser-133) in AngII treated cells when compared to vehicle. **B,** Immunoblot showing increased CBP to p-p65(Ser-276) binding and decreased CBP to p-CREB(Ser-133) binding in AngII stimulated cells as measured by immunoprecipitation analysis. The results are means±SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$, compared with cells treated with vehicle.

decreased CREB-CBP binding and increased NFκB-CBP binding as confirmed by immunoprecipitation analysis. As demonstrated in Figure 5.3B, in rat neuronal cells, binding between CBP and p65 has been increased and binding between CBP and CREB has been decreased, as reflected by increased presence of p-p65(Ser-276) and decreased presence of p-CREB(Ser-133) in CBP immunoprecipitates of AngII exposed cells when compared to cells

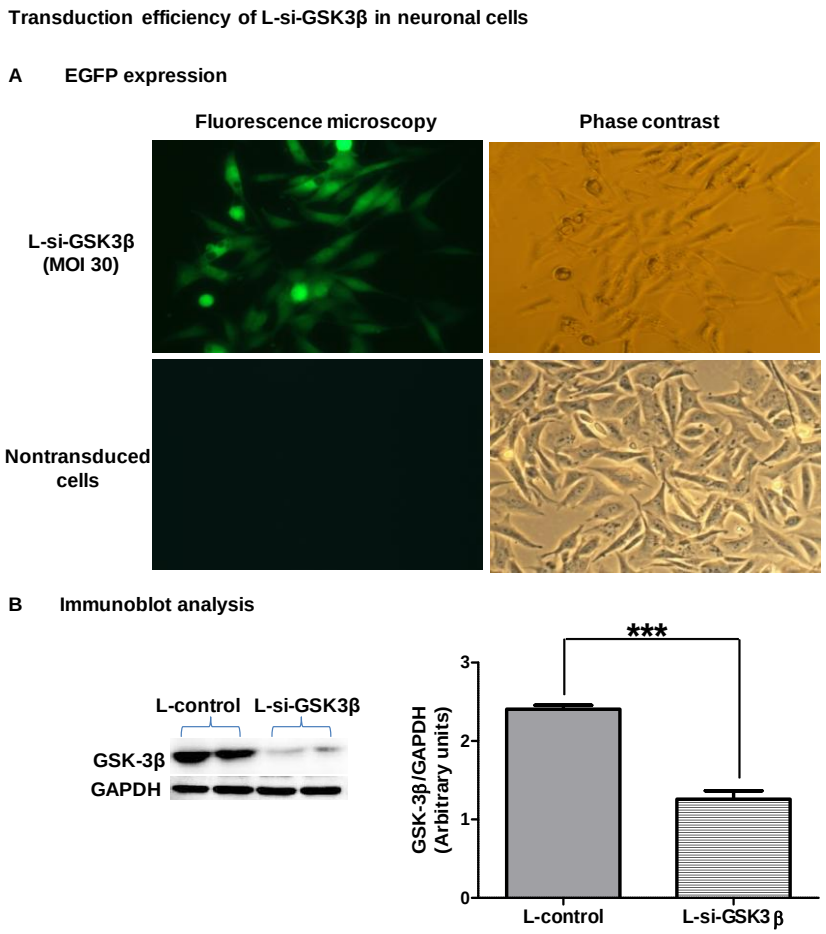

Figure 5.4. Transduction efficiency of lentiviral siRNA targeting GSK3β (L-si-GSK3β) in PC12 cells. Serum starved PC12 cells were transduced with L-si-GSK3β at a multiplicity of infection (MOI) of 30 for 48 hours. **A,** An immunofluorescence and phase contrast microscopy showing EGFP expression. **B,** an immunoblot analysis and a representative blot showing efficient suppression of GSK-3β protein expression by L-si-GSK3β. The results are means±SD of three independent experiments. *** $p < 0.001$, compared with cells tranduced with the scrambled sequence (L-control).

treated with vehicle. These data suggest that AngII causes an imbalance in cytokine levels by modulating the downstream transcription factors.

Lentiviral-mediated RNAi Suppressed GSK-3β Expression. To elucidate whether effects of AngII on rat neuronal cells are mediated by GSK-3β, we employed highly efficient lentiviral short interfering RNA (siRNA) targeting GSK-3β (L-si-GSK3β). Efficiency of L-si-GSK3β was assessed by monitoring EGFP expression under fluorescence microscope and western blot analysis. As depicted in Figure 5.4, L-si-GSK3β caused efficient suppression of GSK-3β. Fluorescence and phase-contrast microscopy results demonstrated that more than 90% cells were transduced with L-si-GSK3β at MOI 30 (Figure 5.4A). Furthermore, densitometric analysis of immunoblot showed that cells transduced with L-si-GSK3β (MOI 30) had significantly lower protein expression of GSK-3β when compared to cells transduced with scrambled sequence (Figure 5.4B). These results confirmed efficient suppression of GSK-3β by L-si-GSK3β in neuronal cells.

Inhibition of GSK-3β by Lentivirus Reversed AngII-mediated Imbalance in PIC and AIC in Rat Neuronal Cells. As shown in Figure 5.5, AngII exposed cells had significantly increased levels of TNF-α and decreased levels of IL-10 when compared to vehicle treated cells. Interestingly, pretreatment of cells with L-si-GSK3β resulted in significant reduction in mRNA and protein levels of TNF-α when compared to AngII treated cells. In addition, IL-10 mRNA and protein levels were significantly higher in AngII+L-si-GSK3β compared with AngII groups. There were no significant differences between vehicle treated and L-si-GSK3β+AngII treated groups. Furthermore, densitometric analysis showed that TNF-α /IL-10 protein ratio was significantly higher in AngII groups in comparison with vehicle, whereas, significant reduction in TNF-α /IL-10 ratio was observed in L-si-GSK3β+AngII when compared to AngII treated.

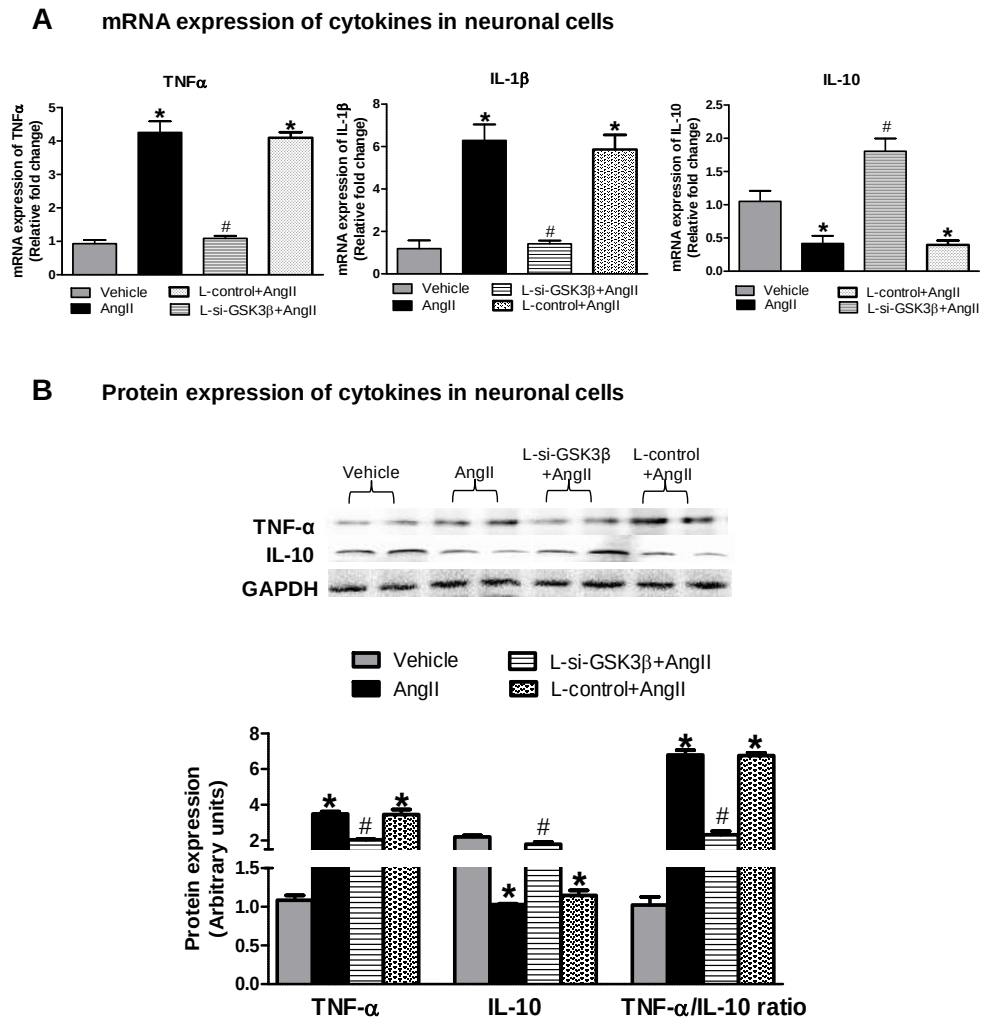

Figure 5.5. Inhibitory effects of L-si-GSK3β on AngII-induced imbalance between PIC and AIC in neuronal cells. Serum starved PC12 cells were transduced with L-si-GSK3β at a multiplicity of infection (MOI) of 30 for 48 hours and were stimulated with 10μM AngII for 6 h. **A**, mRNA expression of TNF-α, IL-1β, and IL-10. **B,** Densitometric analysis and a representative immunoblot showing protein expression of TNF-α and IL-10. Pre-treatment with L-si-GSK3β resulted in reversal of AngII-induced increase in TNF-α and IL-1β and attenuation in IL-10 levels indicating improved balance between PIC and AIC by GSK-3β inhibition. Cells transduced with scrambled sequences (L-control) in presence of AngII did not show any effect. The results are means±SD of three independent experiments. *$p < 0.05$ versus vehicle-treated cells; # $p < 0.05$ versus L-si-GSK3β+AngII treated cells.

cells. These results demonstrate that pretreatment of cells of lentiviral silencing GSK-3β

causes reversal of AngII-induced imbalance between PIC and AIC in neuronal cells

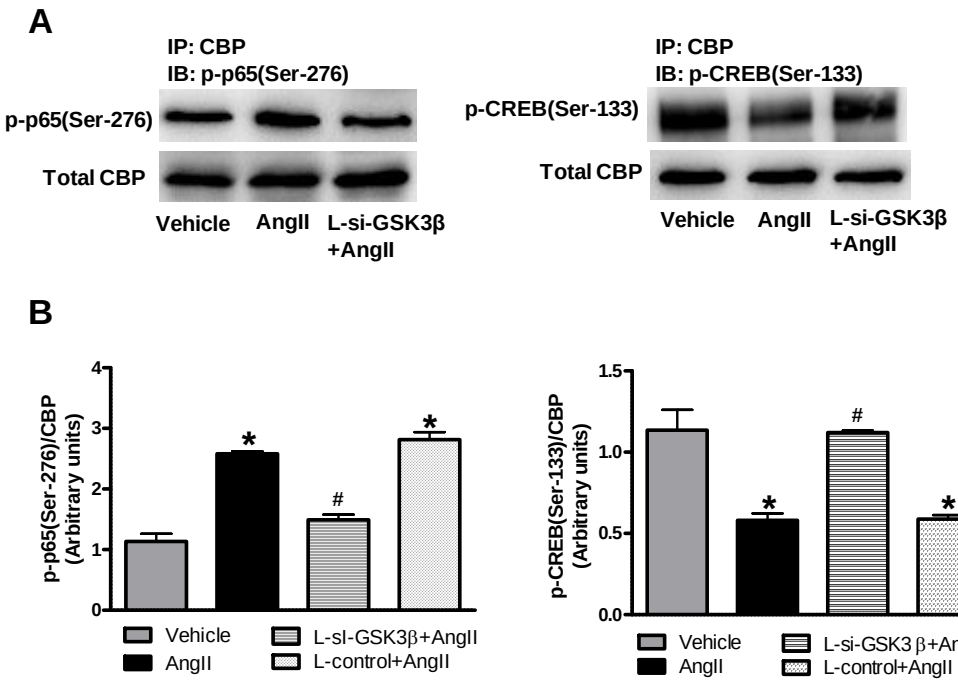

Figure 5.6. Effect of GSK-3β knockdown by lentiviral siRNA (L-si-GSK3β) on AngII induced alterations in transcription factors. Serum starved PC12 cells were transduced with L-si-GSK3β at a multiplicity of infection (MOI) of 30 for 48 hours and were stimulated with 10μM AngII for 6 h. Cell extracts were then immunoprecipitated with CBP and immunoblotted either with p-p65(Ser-276) or p-CREB(Ser-133). **A,** An immunoblot and **B,** densitometric analysis showing reversal of AngII-induced increased CBP to p-p65(Ser-276) and reduced CBP to p-CREB(Ser-133) binding in neuronal cells. Cells transduced with scrambled sequences (L-control) in presence of AngII did not show any effect. The results are means±SD of three independent experiments. . *$p < 0.05$ versus vehicle-treated cells; # $p < 0.05$ versus AngII treated cells.

Inhibition of GSK-3β by Lentivirus Reversed AngII-mediated Altered Binding of CBP to NFκB or CREB in Rat Neuronal Cells. As shown in Figure 5.6, AngII exposure resulted in increased CBP to p65 binding and decreased CBP to CREB binding in PC12 cells. Interestingly, pretreatment of cells with L-si-GSK3β caused significant reduction in AngII-induced elevation in CBP to p65 binding. In addition, CBP-CREB binding was found to be significantly higher in L-si-GSK3β+AngII group when compared to AngII exposed cells. Furthermore, L-si-

GSK3β+AngII treated cells exhibited significantly elevated levels of p-CREB(Ser-133) and reduced levels of p-p65(Ser-276), in comparison with AngII group. There were no significant differences between vehicle treated and L-si-GSK3β+AngII treated groups. These results indicate that AngII-induced imbalance in cytokine levels and transcription factors are mediated by GSK-3β in neuronal cells.

DISCUSSION

The main aim of the present study was to investigate the underlying molecular mechanisms by which AngII causes an imbalance between PIC and AIC, and to elucidate the role of GSK-3β in mediating this dysregulation. Three novel findings emerge from this study. First, AngII causes an imbalance between PIC and AIC in rat neuronal cells by upregulating binding of CBP to NFκB and downregulating binding of CBP to CREB. These data explains the increased NFκB-mediated transcription of PIC and decreased CREB-mediated transcription of IL-10 on AngII stimulation. Second, AngII causes significantly increased phosphorylation of GSK-3β at Tyr-216 and increased p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio indicating increased activation of GSK-3β in neuronal cells. Final, AngII-induced effects in neuronal cells were reversed by lentiviral-mediated silencing of GSK-3β, suggesting that AngII-induced effect are indeed mediated by GSK-3β in rat neuronal cells. The results of this study reveal a novel molecular mechanism that AngII-induced increased activation of GSK-3β leads to altered activity of downstream transcription factors, NFκB and CREB, in favor of NFκB -mediated gene transcription, thereby, causing an imbalance between PIC and AIC in rat neuronal cells (Figure. 5.7). Our data also showed that AngII-induced effects in neuronal cells could be alleviated by GSK-3β inhibition suggesting GSK-3β as an important therapeutic target in various CVDs, particularly hypertension which is characterized by increased PICs and NFκB activation.

Chronic low-grade inflammation is one of the hallmarks of hypertension. PICs, such as TNF-α (Dorffel, Latsch et al. 1999), IL-1β (Dorffel, Latsch et al. 1999; Peeters, Netea et al. 2001), and IL-6 (Chae, Lee et al. 2001; Peeters, Netea et al. 2001), have been reported to increase with the severity of hypertension and are of prognostic significance. Besides circulating cytokines, recently brain cytokines have also been implicated in the pathogenesis of hypertension. However, emerging evidence indicates that it is not only the PIC (Peeters, Netea et al. 2001; Shi, Raizada et al. 2010) but the balance between pro- and anti-inflammatory cytokines that determines the outcome of the disease, and that these PICs can cross-talk with components of the RAS during hypertensive response. In the present study, we observed that AngII exposure resulted in upregulation of TNF-α expression in a time-dependent manner in rat neuronal cells with maximal effects at 6h after AngII exposure. In line with our results, previous studies from our lab have shown that infusion of AngII in the

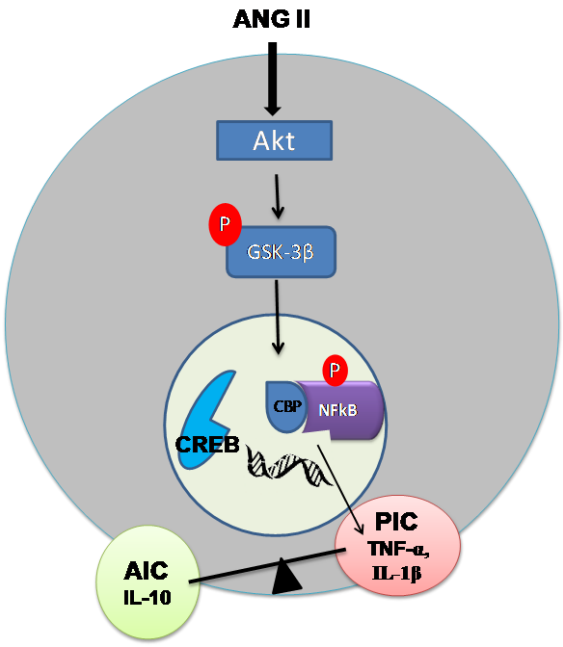

Figure 5.7. A schematic representing the proposed mechanisms of AngII-induced dysregulation in inflammatory cytokines in neuronal cells. The results of this study reveal a novel molecular mechanism that AngII-induced increased phosphorylation of GSK-3β(Tyr-216) and increased p-GSK3β(Tyr-216) to p-GSK-3β(Ser-9) ratio leads to altered activity of downstream transcription factors, NFκB and CREB, in favor of NFκB -mediated gene transcription, thereby, causing an imbalance between PIC and AIC in rat neuronal cells.

paraventricular nucleus (PVN), an important cardiovascular regulatory center in the brain, increases production of TNF-α and IL-1β in rats (Cardinale, Sriramula et al. 2011). Although most of these previous studies have examined the effects of AngII on brain PICs, the effects of overactivation of RAS on anti-inflammatory cytokines are not very well understood. In the present study, we observed a significant reduction in IL-10 levels by AngII exposure (6h). Furthermore, ratio of TNF-α/IL-10 protein expression was found to be dramatically upregulated in AngII treated cells. Also, we observed an initial increase in IL-10 mRNA level at 1h of AngII exposure which could be due to compensatory and protective response to initial increase in TNF-α. Taken together, these results suggest that, at the cellular level, alterations in RAS components not only increases PIC but also causes an imbalance between PIC and AIC in favor of PIC. However, these results raise another question, what are the exact mechanisms by which alterations in RAS components cause this dysregulation?

Accumulating evidence has suggested that NFκB -signaling pathway is activated by AngII via the G-protein coupled angiotensin type I receptor (Wolf and Wenzel 2004). In the unstimulated cells, NFκB is sequestered in the cytoplasm as inactive complex with inhibitors of NFκB (IκB) (Ghosh and Karin 2002). Upon stimulation by some inducers such as AngII, IκB is phosphorylated and degraded, leading to translocation of p65 subunit of NFκB into the nucleus where it activates gene transcription of TNF-α and IL-1β. In addition to the nuclear translocation of NFκB, its transcriptional activity is regulated by a co-activator CREB-binding protein (CBP), that associates with the *C*-terminal transactivation domain of p65 (Takahashi, Tetsuka et al. 2002). Phosphorylation of p65 at Ser-276 has been shown to be required for recruitment of the CBP and transcriptional activity. Besides NFκB, another transcription factor, CREB has been shown to be involved in the pathogenesis of hypertension. CREB is a 43kDa phosphoprotein that

positively regulates expression of anti-inflammatory cytokines such as IL-10 (Avni, Ernst et al. 2010). Although, activity of CREB is regulated by complex phosphorylation mechanisms that are not yet completely understood, phosphorylation of CREB at Ser-133 has been shown to be required for recruitment of the CBP and transcriptional activity (Chrivia, Kwok et al. 1993). Due to limited availability of CBP in the nucleus, competition for CBP by diverse transcription factors is inevitable (Yang, Jiang et al. 2010). Since, NFκB and CREB are the key transcription factors in the production of cytokines, it is plausible to investigate whether AngII-induced dysregulation in PIC and AIC is mediated by them. In the present study, we observed that AngII exposure resulted in increased phosphorylation of p65 at Ser-276 and reduced phosphorylation of CREB at Ser-133. Moreover, our immunoprecipitation analysis showed that CBP to NFκB binding was increased in AngII (6h) exposed neuronal cells, whereas, CBP to CREB binding was reduced. These results were also associated with elevated levels of TNF-α and reduced IL-10 levels in AngII (6h) exposed cells. Collectively, these results suggest that alterations in phosphorylation status of NFκB and CREB by AngII lead to their altered binding with co-activator CBP, leading to an imbalance between PIC and AIC production.

Recently, glycogen synthase kinase-3 (GSK-3), an enzyme which was originally discovered for its role in insulin-mediated glycogen metabolism (Embi, Rylatt et al. 1980; Rylatt, Aitken et al. 1980; Woodgett and Cohen 1984; Hughes, Nikolakaki et al. 1993; Ali, Hoeflich et al. 2001; Woodgett 2001; Doble and Woodgett 2003), has now been shown to regulate activity of several metabolic, signaling, and structural proteins (Frame and Cohen 2001; Woodgett 2001; MacAulay and Woodgett 2008). Not only the activity of GSK3 is regulated by its post-translational phosphorylation, it itself phosphorylates broad range of substrates and thereby regulates their function (Frame and Cohen 2001; Woodgett 2001). Among the signaling proteins

regulated by GSK-3β are many transcription factors including CREB and NFκB (Plyte, Hughes et al. 1992; Grimes and Jope 2001). Therefore, we postulated that AngII-induced alterations in phosphorylation status of NFκB and CREB as observed in the present study could be mediated by GSK-3β (Figure 5.7). To investigate this hypothesis, we first examined whether AngII perhaps has any effect on GSK-3β expression. Interestingly, our immunoblot analysis showed that AngII exposure resulted in a slight increase in p-GSK3β(Ser-9) levels in neuronal cells suggesting inactivation of GSK-3β at first sight. Surprisingly, however, p-GSK3β(Tyr-216) levels were found to be significantly elevated in AngII treated cells. Moreover, ratio of p-GSK3β(Tyr-216) to p-GSK3β (Ser-9) was higher in AngII exposed cells when compared to vehicle treated cells. Since N-terminal phosphorylation of GSK-3β at Ser-9 has an inhibitory effect, whereas, phosphorylation of Tyr-216 activates it (Forde and Dale 2007), these results clearly suggested activation of GSK-3β upon AngII exposure. Although, role of GSK-3β in CVDs is recently becoming a focus of scientific community, to best of our knowledge none of the previous studies have explored effects of AngII, a key mediator of most of the CVDs, on neuronal GSK-3β. Additionally, most of these previous studies have reported the phosphorylation status of GSK-3β at Ser-9 suggesting inhibition of its activity (Javadov, Rajapurohitam et al. 2009; Tateishi, Matsushita et al. 2010). However, these studies have not investigated the phosphorylation level of GSK-3β(Tyr-216) leaving us with insufficient data to conclude whether those stimuli cause inhibition or activation of GSK-3β. In the present study, we observed that AngII exposure caused upregulation of p-GSK3β(Ser-9) with concomitant and much higher increase in p-GSK3β(Tyr-216), indicating activation of GSK-3β.

Various upstream kinases such as phosphatidyl-inositol 3-kinase (PI3K), protein kinase B (PKB), MAP kinases, p70 ribosomal S6 kinase, protein kinase A (PKA), and protein kinase C

(PKC), have been reported to be responsible for phosphorylation of GSK-3β at Ser-9 upon stimulation with insulin and other growth factors (Doble and Woodgett 2003). PKB (also termed Akt), a serine/threonine kinase located downstream of PI3K, has been shown to phosphorylate GSK-3β at Ser-9 in vitro and in vivo (Cross, Alessi et al. 1994; Cross, Alessi et al. 1995). AngII is known to exert its cellular effects via activation of several downstream kinases such as PI3K, Akt, and MAPK (Zhang, Yu et al. 2012; Wei, Yu et al. 2009). Therefore, the observed increase in p-GSK3β(Ser-9) levels in AngII exposed cells in this study could be due to activation of one or more of these kinases. Although, which of these kinases is primarily responsible for AngII-induced phosphorylation of GSK-3β(Ser-9) is not clear at this time. However, we found that AngII-exposed neuronal cells had significantly higher levels of p-Akt(Ser-473) (data not shown), indicating its activation as phosphorylation of Akt at Ser-473 is known to be crucial for its activation (Alessi, Andjelkovic et al. 1996). However, the upstream kinase or kinases responsible for AngII-induced increased phosphorylation of Tyr-216 is not known at this time and could be a focus of future studies.

Since, GSK-3β acts as a key regulator of transcription factors, NFκB and CREB, it is plausible to speculate that GSK-3β could be the missing link in AngII-induced alterations in inflammatory cytokines. In this study, we observed that suppression of GSK-3β by highly efficient lentiviral siRNA prevented AngII-induced increase in TNF-α and decrease in IL-10 levels in neuronal cells. Furthermore, GSK-3β suppression in AngII exposed cells led to increased CBP to CREB binding and attenuated CBP to NFκB binding. The altered binding capability of NFκB and CREB to CBP was observed to be due to altered phosphorylation status of both of these transcription factors. Our results showed that GSK-3β silencing caused reduced phosphorylation of NFκB at Ser-276, whereas, it increased phosphorylation of CREB at Ser-133.

It has been shown previously that phosphorylation of NFκB and CREB at Ser-276 (Reber, Vermeulen et al. 2009) and Ser-133 (Chrivia, Kwok et al. 1993), respectively, is essential for their binding with the CBP and subsequent transactivation. These results suggest that AngII-induced alterations in NFκB and CREB activity are mediated by GSK-3β in neuronal cells. In line with our results, Grimes and Jope (Grimes and Jope 2001) have shown that inhibition of GSK-3β by lithium facilitates CREB activity in human neuroblastoma SH-SY5Y cells. However, activity of NFκB is known to be regulated by phosphorylation of IκB and its subsequent nuclear transport. Therefore, the possibility that suppression of GSK-3β affects NFκB regulation at levels other than CBP binding cannot be ignored. Although, we have not studied the effects of GSK-3β suppression on phosphorylation of IκB, it has been suggested that GSK-3β does not disrupt NFκB nuclear import in embryonic fibroblasts isolated from GSK3β-null mice (Doble and Woodgett 2003). Nonetheless, our current results showed that GSK-3β inhibition in AII-stimulated neuronal cells regulates activity of NFκB and CREB by altering their ability to recruit the co-activator CBP, which explains the AngII-induced dysregulation in PIC and AIC.

In summary, the present study shows that AngII exposure causes upregulation of PIC and downregulation of AIC in rat neuronal cells by increasing CBP to NFκB binding and attenuating CBP to CREB binding, and AngII-induced this dysregulation in inflammatory cytokines is indeed mediated by GSK-3β. The results of this study explain a novel molecular mechanism by which an overactivation of the RAS in the neuronal cells modulates activity of the transcription factors leading to inflammatory alterations. The identification of GSK-3β as a downstream target of AngII in mammalian cells suggests an effector role for GSK-3β in cellular responses to AngII. The results of this study suggest the therapeutic potential of inhibiting GSK-3β in the treatment

of CVDs characterized by chronic inflammation. However, in vivo validation of the data presented here could certainly be an important perspective of this study.

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CHAPTER 6

EXERCISE TRAINING INHIBITS GSK-3β AND INCREASES CREB-MEDIATED GENE EXPRESSION IN THE PARAVENTRICULAR NUCLEUS OF ANGIOTENSINII-INDUCED HYPERTENSIVE RATS

INTRODUCTION

A growing body of evidence indicates that exercise training (ExT) improves the quality of life, decreases cardiovascular events, and increases survival of patients with various cardiovascular diseases (CVDs) including hypertension. It has been known for decades that regular physical activity reduces blood pressure (BP) and hence, ExT has recently been recommended as a part of lifestyle modifications to all the patients diagnosed with hypertension. However, the precise mechanisms of exercise-mediated beneficial effects are largely unknown. Recent findings from our lab and others have demonstrated that chronic ExT delays the progression of hypertension and improves cardiac function in spontaneously hypertensive rats (Agarwal, Haque et al. 2009) and these effects were mediated by attenuated pro-inflammatory cytokines (PICs) and improved renin-angiotensin system (RAS) within the cardiovascular regulatory centers of the brain (Agarwal, Welsch et al. 2011). Furthermore, the study showed that chronic exercise not only decreases PICs but also upregulates anti-inflammatory cytokines in the brain of hypertensive rats. These studies provide evidence that improvements in inflammatory cytokines and the RAS within the brain mediate pressure-lowering and cardio-protective effects of exercise in hypertension. However, further research is still warranted to have a greater understanding of how exactly ExT influences these fundamental processes in hypertension.

Recently, glycogen synthase kinase (GSK)-3 has gained increasing attention from the scientific community due to its role in many biological processes including cardiac development, hypertrophy, and function. Past several years of research has now established that GSK-3 acts as a regulatory switch that determines the output of numerous signaling pathways initiated by diverse stimuli (Frame and Cohen 2001; Grimes and Jope 2001; Woodgett 2001). Of the two

isoforms-*alpha* and -*beta*, GSK-3β is particularly abundant in the central nervous system (CNS) and is neuron-specific (Leroy and Brion 1999). GSK-3 is a serine/threonine kinase which, in addition to phosphorylating glycogen synthase, has numerous other substrates including several transcription factors, particularly nuclear factor *kappa*B (NFκB) and cyclic AMP response element binding protein (CREB), the two most important transcription factors known to play a central role in modulating the gene expression of inflammatory mediators involved in hypertension. It has recently been reported that GSK-3β modulates the production of inflammatory cytokines in an NFκB-dependent manner (Martin, Rehani et al. 2005; Steinbrecher, Wilson et al. 2005; Vines, Cahoon et al. 2006; Beurel and Jope 2009). Consistently, we have recently reported that in cultured neuronal cells, angiotensin II (AngII)-induced dysregulation of inflammatory cytokines is mediated by GSK-3β. These findings raise the possibility that exercise-mediated improvement in PIC and AIC in hypertension could be mediated by GSK-3β. Few studies have examined the role of GSK-3β in exercise-induced activation of glycogen synthesis in skeletal muscle of healthy animals (Markuns, Wojtaszewski et al. 1999). However, to best of our knowledge, role of GSK-3β, as a signaling molecule, in exercise-induced beneficial effects in a disease condition has never been investigated yet.

Therefore, this study was designed to investigate the hypotheses that 1) regular moderate intensity ExT would reduce BP and improve balance between PIC and AIC within the PVN of Angiotensin II (AngII)-induced hypertensive rats and 2) AngII would increase activation of GSK-3β in the PVN of hypertensive rats, leading to alterations in NFκB- and CREB-mediated gene transcription, and 3) ExT would inhibit GSK-3β and augment CREB-mediated gene expression, thereby contributing to improvement in inflammatory cytokines in the paraventricular nucleus (PVN) of AngII-induced hypertensive rats.

MATERIALS AND METHODS

Animals. AngII-induced hypertensive rat model was used in this study. A total of 60 adult male Sprague-Dawley rats (250-350 grams) were studied, of which 30 rats were infused with AngII dissolved in 0.9% saline, at a subpressor concentration of 200ng/kg/min via osmotic minipumps (Alzet, model 2004; 0.25ul/hr). This AngII dose was based on previous publications from our laboratory and others (Cardinale, Sriramula et al. 2012). The other 30 rats were infused with saline (Sal) in place of AngII and were used as normotensive controls. The pumps were implanted subcutaneously for 28 days (4 weeks). Animals were randomized into four groups (n = 15 per group): saline+sedentary (Sal+Sed), saline+exercise (Sal+ExT), angiotensin II+sedentary (AngII+Sed), and angiotensin II+exercise (AngII+ExT). The animals in exercise groups were subjected to moderate intensity exercise for 28 days. 24 hours after the last exercise session, the rats were euthanized; the brains were collected, and immediately frozen on dry ice. The paraventricular nucleus (PVN) tissues were punched out from the brain for further analysis.

Animals were housed in a temperature-controlled room (25 ± 1^{o}C) and maintained on a 12:12 hour light:dark cycle with free access to water and food. All animal and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University in compliance with NIH guidelines.

Exercise and Detraining protocol. Rats in exercise groups (Sal+ExT and AngII+ExT) underwent moderate-intensity exercise (5 days per week; 60 min per day at 18 m/min, 0^{o} inclination) on a motor-driven treadmill continuously for a period of 28 days. All the animals were acclimatized to treadmill for 2 weeks prior to osmotic mini-pump implantation. After acclimation, training intensity was set at approximately 60% of maximal aerobic velocity (MAV), which corresponds to moderate intensity exercise (18-20m/min). This training intensity

was maintained throughout the study period. The MAV was evaluated from an incremental exercise test as reported previously (Boissiere, Eder et al. 2008; Sun, Qian et al. 2008). The rats in sedentary groups (Sal+Sed and AngII+Sed) were placed on a nonmoving treadmill during the training sessions.

Blood Pressure Measurement. Mean arterial blood pressure (MAP) was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN) 7 days prior to implantation of the osmotic minipumps. Rats (n = 6 per group) were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and placed dorsally on a heated surgical table. An incision was made on the medial surface of the left leg, the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and another suture was placed proximally to temporarily interrupt the blood flow. The catheter tip of the radio-telemetry transmitter was introduced through a small hole in the femoral artery, advanced ~6 cm into the abdominal aorta such that the tip was distal to the origin of the renal arteries, and sutured into place. The probe body was placed into the abdominal cavity and sutured to the abdominal wall. The abdominal musculature was sutured and the skin layer closed following implantation. Rats received enrofloxacin (approximately 10mg/kg) and buprenorphine (0.01 mg/kg, s.c.) immediately following surgery and 12 hours postoperatively and allowed to recover for seven days.

Real-time RT-PCR Analysis. Semi-quantitative real-time RT-PCR (n=6 per group) was used to determine the mRNA levels of TNF-α and IL-10 in the PVN by using specific primers. In Brief, the rats were euthanized; the brains and LV were quickly removed, immediately frozen on dry ice. The brains were blocked in the coronal plane, sectioned at 100 μm thickness, and the PVN were punched from each brain according to the methods described by Palkovits and Brownstein

(Gao, Wang et al. 2005). Rat primers used were: GAPDH, Forward: 5'agacagccgcatcttcttgt-3', Reverse: 5'cttgccgtgggtagagtcat-3'; TNF-α, Forward: 5'gtcgtagcaaaccaccaagc-3', Reverse: 5'tgtgggtgaggagcacatag-3'; and IL-10, Forward: 5'gggaagcaactgaaacttcg-3', Reverse: 5'atcatggaaggagcaacctg-3'. Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (Agarwal, Welsch et al. 2011). Gene expression was measured by the ΔΔCT method and was normalized to GAPDH mRNA levels. The data is presented as the fold change of the gene of interest relative to that of control animals.

Western blot analysis. The whole cell extracts obtained from the PVN were subjected to Western blot analysis (n = 5 per group) for the determination of protein levels of TNF-α, IL-10, GSK-3β, p-GSK3β(Ser-9), p-GSK3β(Tyr-216), and GAPDH. For p-p65(Ser-276) and p-CREB(Ser-133) immunoblot, nuclear extracts were obtained using an extraction kit from Active Motif (Carlsband, CA), as described before. The protein concentration in the lysate was measured using a Bradford assay using BSA standards. Protein extracts (30 μg) were combined with an equal volume of 2X Laemmli loading buffer, boiled for 5 min and electrophoresed on 10–15% SDS-polyacrylamide gels. The proteins were then electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore). Non-specific binding sites were blocked with 1% (w/v) casein (for non-phosphorylated antibodies) in PBS or 1% (w/v) bovine serum albumin (for phosphorylated antibodies) in TBST [20mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20]. Blots were probed (overnight, 4^{o}C) with the primary antibodies. Specific antibodies used included: TNF-α, IL-10, GSK-3β, p-GSK3β(Ser-9), p-GSK3β(Tyr-216) at 1:1000 dilution, and p-p65(Ser-276) and p-CREB(Ser-133) at 1:500 dilution. Antibodies were commercially obtained: TNF-α (Abcam Inc, MA, USA); IL-10 (Abbiotec, CA, USA); GSK-3β (BD Transduction laboratories, USA), and p-GSK3β(Ser-9), p-GSK3β(Tyr-216), p-p65(Ser-276), and

p-CREB(Ser-133) (Cell Signaling Technology, Inc, MA, USA). After washing with wash buffer (1X TBS, 0.1% Tween-20) four times for 10 min each time at RT, blots were then incubated for 1 hour with secondary antibody (1:10,000 dilution, Santa Cruz Biotechnology) labeled with horseradish peroxidase. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham), band intensities were quantified using Versa Doc MP 5000 imaging system (Bio-Rad), and were normalized with GAPDH.

Statistical Analysis. All data are presented as means±SE. Statistical analysis was done by either two-way ANOVA or one-way ANOVA with a Tukey's post hoc test using Graph Pad Prism software (version 5.0). Blood pressure data were analyzed by repeated-measures ANOVA to examine with-in group changes over time. Results were considered significant when $p<0.05$.

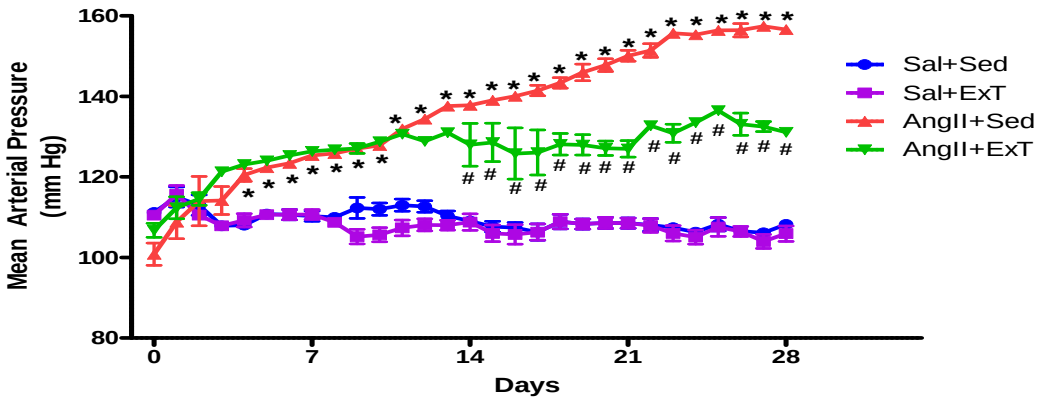

Figure 6.1. Time course of mean arterial pressure (MAP, in millimeters of mercury) in sedentary and exercised normotensive and hypertensive rats. Rats were acclimatized to treadmill 2 weeks before the start of AngII infusion. 28 day osmotic minipump was then implanted subcutaneously on day 0 for chronic infusion. Simultaneously, animals were subjected to moderate-intensity exercise for 28 days. MAP was significantly reduced in AngII+ExT compared with AngII+Sed rats from day 14 of exercise (arrow). Values are mean±SE; n=6 per group. *p<0.05 Sal+Sed versus AngII+Sed; #p<0.05 AngII+Sed versus AngII+ExT.

RESULTS

Exercise Training Reduces Blood Pressure in AngII-induced Hypertensive Rats.

As shown in Figure 6.1, AngII infusion in sedentary rats caused significant increase in MAP starting at day 5 of AngII infusion and remained for the duration of the study. The maximum increase in MAP in AngII+Sed rats was observed at day 23 of infusion after which it reached to plateau. Regular exercise resulted in significant reduction in MAP in AngII+ExT when compared with AngII+Sed rats; the values were found significant beginning from day 14 of exercise.

Exercise Training Prevented AngII-induced Cytokine Imbalance in the PVN of Hypertensive Rats. To investigate whether ExT has ability to improve the balance between PIC and AIC within the PVN of AngII-induced hypertensive rats, we examined the mRNA and protein levels of TNF-α, IL-1β, and IL-10 (Figure 6.2). We observed that AngII+Sed rats exhibited marked increases in TNF-α and IL-1β expression in the PVN compared to Sal+Sed rats. This upregulation of TNF-α and IL-1β was significantly attenuated by regular exercise in AngII-infused rats. On the other hand, the IL-10 levels were significantly lower in AngII+Sed in comparison with Sal+Sed. ExT in AngII-infused rats resulted in dramatic increase in IL-10 levels within the PVN. These results suggest that ExT not only reduces PICs but also improves anti-inflammatory defense within the PVN of AngII-induced hypertensive rats.

Exercise Training Prevented AngII-induced Activation of GSK-3β in the PVN of Hypertensive Rats. To investigate whether AngII infusion causes activation of GSK-3β within the PVN and whether ExT has any effects on AngII-induced GSK-3β activation, we determined the protein expression levels of p-GSK3β(Ser-9) and p-GSK3β(Tyr-216) by immunoblot

analysis. Immunoblot analysis demonstrated that there was a slight but significant increase in the phosphorylation of GSK3β(Ser-9) in AngII+Sed rats when compared to Sal+Sed (Figure 6.3A;

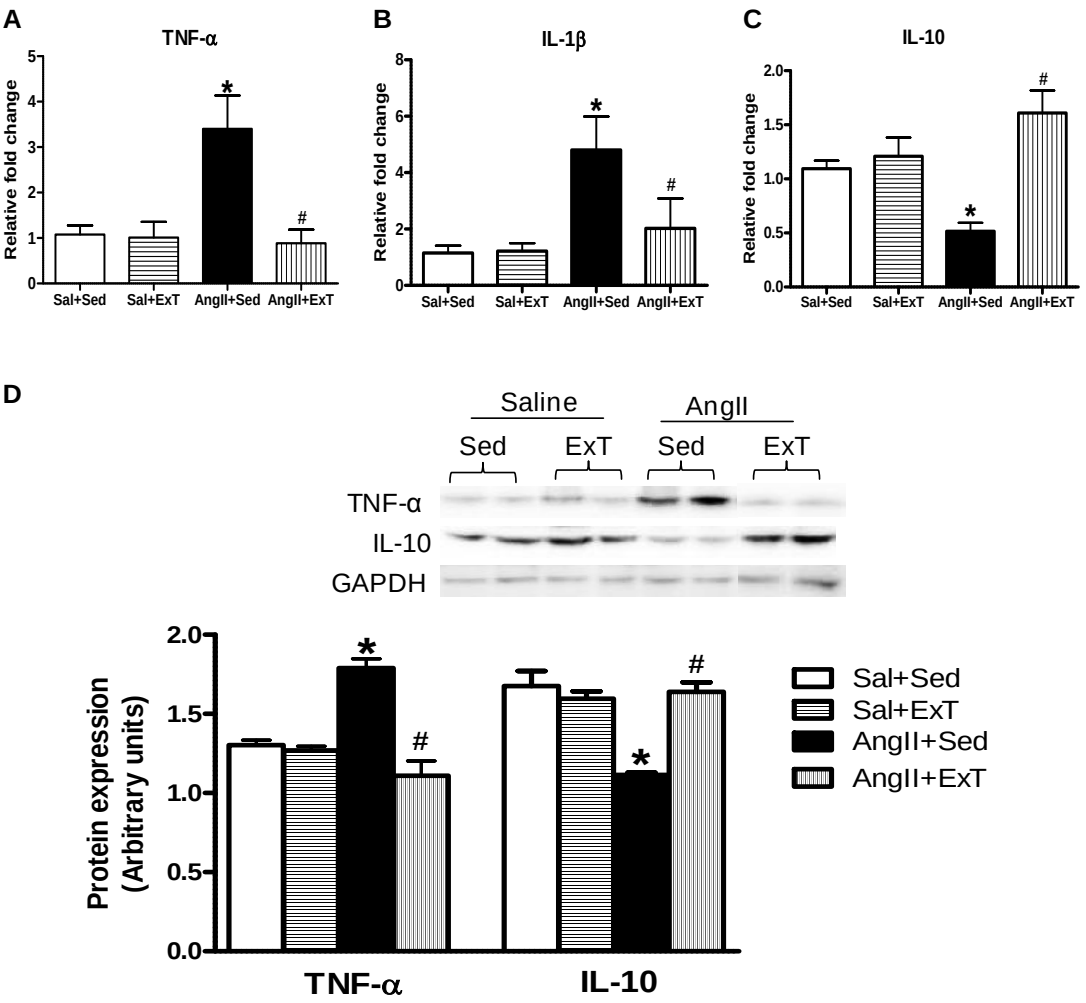

Figure 6.2. Effects of chronic exercise on pro- and anti-inflammatory cytokines within the PVN of normotensive and hypertensive rats. A, mRNA expression of TNF-α. **B**, mRNA expression of IL-1β. **C**, mRNA expression of IL-10. **D** Densitometric analysis and a representative immunoblot showing protein expression of TNF-α and IL-10. Exercise significantly attenuated PICs and upregulated IL-10 levels within the PVN of hypertensive rats. The results are means±SE. n=6 per group for mRNA and n=5 per group for protein analysis. *p < 0.05 Sal+Sed versus AngII+Sed; # p < 0.05 AngII+Sed versus AngII+ExT.

quantitation in Figure 6.3E). Simultaneously, however, AngII+Sed had robustly increased levels of p-GSK3β(Tyr-216) (Figure 6.3B; quantitation in Figure 6.3E). Densitometric analysis further revealed that the ratio of protein expression of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) is significantly upregulated within the PVN of AngII+Sed rats compared to Sal+Sed (Figure 6.3C). Since, phosphorylation of GSK3β(Tyr-216) is essential for their catalytic activity, these results indicate overall activation of GSK-3β in the PVN of AngII-induced hypertensive rats. The same blots were stripped and reprobed for native GSK-3β showing no significant difference on native GSK-3β expression between the Sal+Sed and AngII+Sed groups (Figure 6.3D). Interestingly, ExT resulted in reduced expression of p-GSK3β(S9) as well as p-GSK3β(Tyr-216); however, the ratio of protein expression of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) was found to be significantly attenuated within the PVN of AngII+ExT rats compared to AngII+Sed, indicating decreased activity of GSK-3β in exercising hypertensive rats.

Exercise Training Caused Reversal of AngII-induced Alterations in NFκB and CREB Phosphorylation in the PVN of Hypertensive Rats. To investigate whether ExT affects AngII-induced alterations in phosphorylation of downstream transcription factors NFκB subunit p65 (also known as RelA) and CREB, we determined the protein levels of p-CREB(Ser-133) and p-p65(Ser-276) by immunoblot analysis of PVN tissues of all four groups. As demonstrated in Figure 6.4, we observed increased expression of p-p65(Ser-276) and decreased p-CREB(Ser-133) in the PVN of AngII+Sed when compared to Sal+Sed rats. In contrary, ExT in hypertensive rats increased p-CREB(Ser-133) and decreased p-p65(Ser-276) expression within the PVN. Since, phosphorylation of CREB at Ser-133 and p65 at Ser-276 has been known to be associated with respective increase in their activity; therefore these results suggest that ExT prevents AngII-induced activation of NFκB and deactivation of CREB within the PVN.

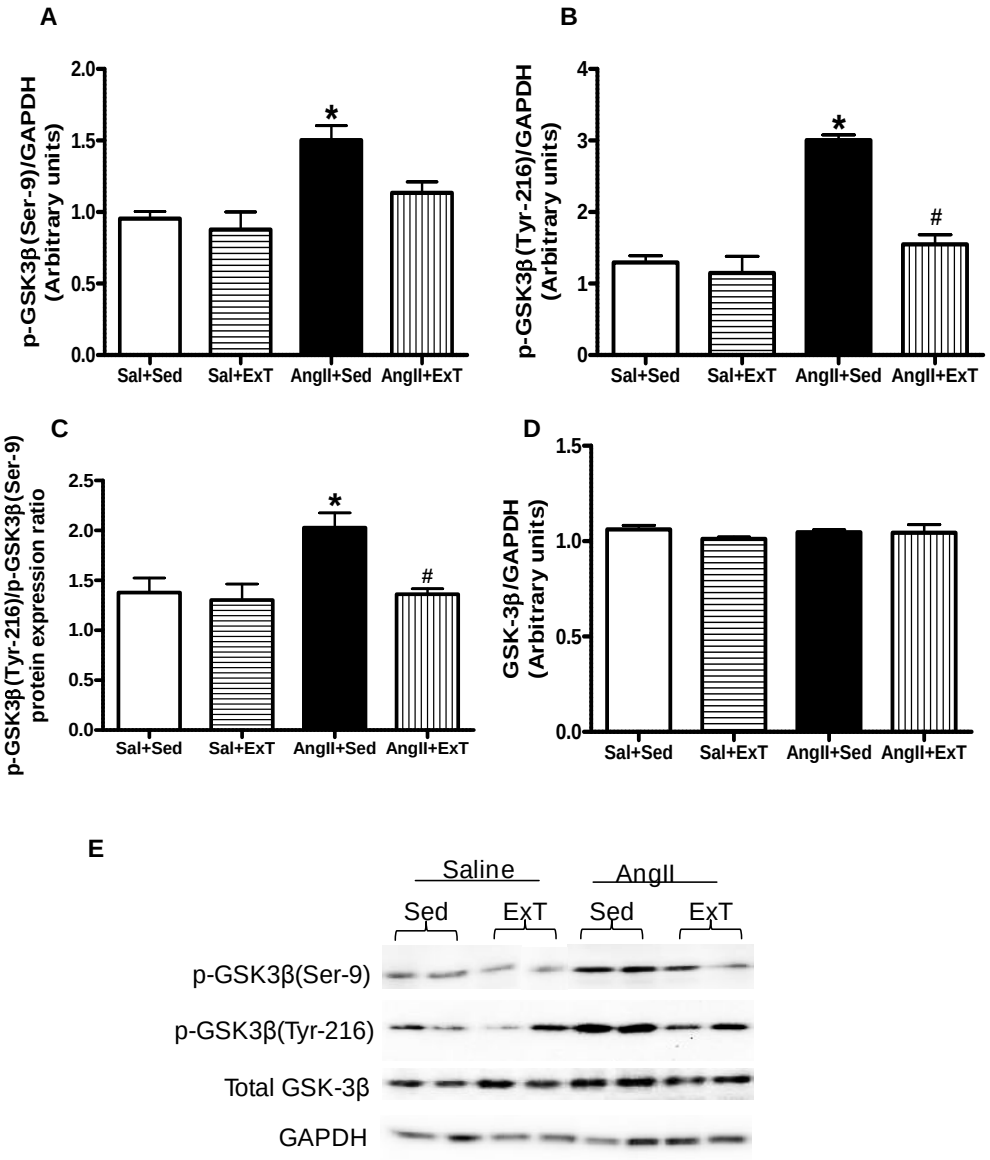

Figure 6.3. Effects of chronic exercise on total and phosphorylated GSK-3β expression within the PVN of normotensive and hypertensive rats.
Densitometric analysis of western blot results showing protein expression of **A** p-GSK3β(Ser-9), **B** p-GSK3β(Tyr-216), **C** p-GSK3β(Tyr-216)/p-GSK3β(Ser-9) ratio, and D total GSK-3β. AngII-induced hypertensive rats exhibited significantly increased levels of p-GSK3β(Ser-9), p-GSK3β(Tyr-216), and p-GSK3β(Tyr-216)/p-GSK3β(Ser-9) ratio, suggesting activation of GSK-3β. Exercise caused reversal of these changes in hypertensive rats, indicating exercise-induced inactivation of GSK-3β within the PVN. The results are means±SE. n=6 per group for mRNA and n=5 per group for protein analysis. *$p < 0.05$ Sal+Sed versus AngII+Sed; # $p < 0.05$ AngII+Sed versus AngII+ExT.

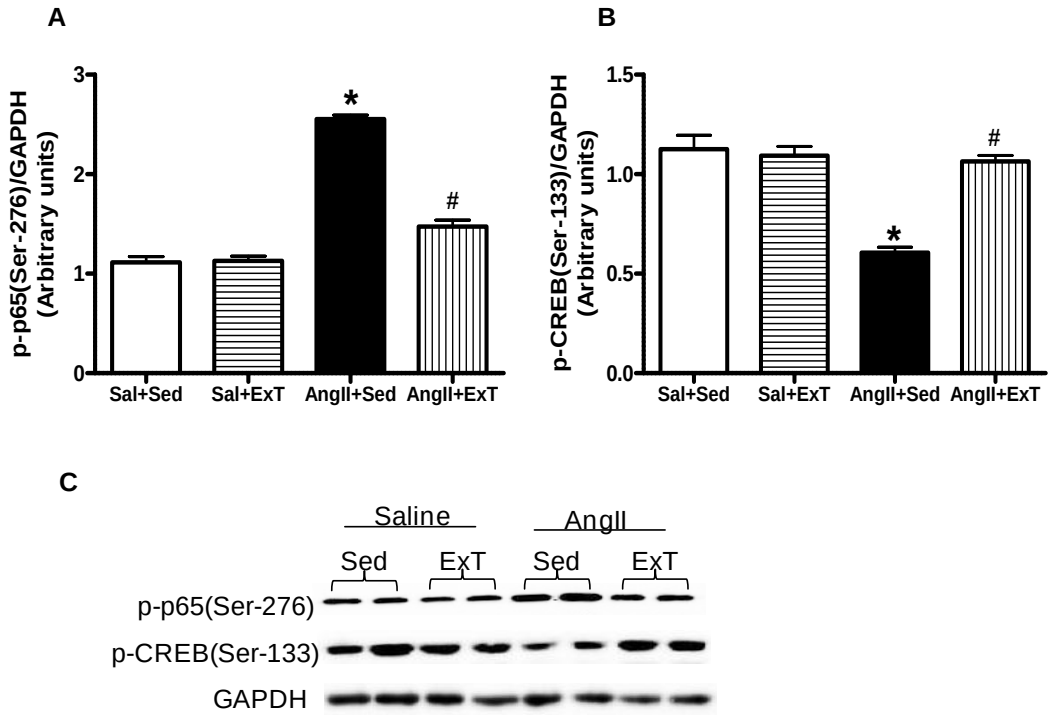

Figure 6.4. Effects of chronic exercise on phosphorylated NFκB(subunit p65) and CREB expression within the PVN of normotensive and hypertensive rats. Nuclear extracts were isolated from the PVN tissues and subjected to western blot using site-specific phospho-antibodies. Densitometric analysis of western blot results showing protein expression of **(A)** p-p65(Ser-276) and **(B)** p-CREB(Ser-133). AngII-induced hypertensive rats exhibited significantly increased levels of p-p65(Ser-276) and decreased p-CREB(Ser-133) levels within the PVN; whereas, chronic exercise reversed these changes. The results are means±SE. n=6 per group for mRNA and n=5 per group for protein analysis. *$p < 0.05$ Sal+Sed versus AngII+Sed; # $p < 0.05$ AngII+Sed versus AngII+ExT.

DISCUSSION

The present study sought to evaluate the possible intracellular mechanisms by which regular moderate-intensity exercise attenuates blood pressure and improves pro- and anti-inflammatory cytokines within the PVN of AngII-induced hypertensive rats. Three major findings emerge from this study. First, regular moderate-intensity exercise attenuates mean

arterial pressure (MAP) and improves the balance between pro- and anti-inflammatory cytokines within the PVN of AngII-induced hypertensive rats. Second, chronic subcutaneous infusion of AngII resulted in increased activation of GSK-3β within the PVN, as reflected by increased ratio of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) protein expression. These results were also associated with increased p-p65(Ser-276) and reduced p-CREB(Ser-133) levels within the PVN, suggesting downregulation of CREB and upregulation of NFκB. Final, regular ExT prevented AngII-induced increase in p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio within the PVN. Concomitantly, trained hypertensive rats exhibited increased phosphorylation of CREB at Ser-133 and reduced p-p65(Ser-276) levels when compared to their sedentary counterparts. These results suggest that regular exercise attenuates AngII-induced GSK-3β activation leading to alterations in NFκB- and CREB-mediated gene transcription in favor of anti-inflammatory cytokines, which explains the underlying mechanisms by which exercise improves balance between PIC and AIC and thereby contributing to attenuated MAP in hypertensive rats. In conclusion, the effects of exercise to inactivate GSK-3β may function as an initial signaling event that results in increased CREB-mediated gene transcription and reduced NFκB -mediated gene transcription within the brain of hypertensive rats. These findings are the first to provide greater insights into the mechanisms by which regular moderate-intensity exercise exerts beneficial effects in hypertension.

At the end of the study, we observed significant reduction in MAP in trained AngII-induced hypertensive rats compared with AngII+Sed rats and saw no comparable changes in trained normotensive rats. The continuous recording of MAP by implanted telemetry probe showed that the reduction in MAP was significant beginning from day 14 of regular exercise and continued until the end of the study, suggesting delayed progression of hypertension by regular

exercise. We have previously reported that chronic exercise causes significant reductions in systolic, diastolic, and mean arterial BP in spontaneously hypertensive rats (SHRs) (Agarwal, Haque et al. 2009; Agarwal, Welsch et al. 2011). The results of the present study extends the observations of those previous studies and suggest that the pressure-lowering effects of regular moderate-intensity exercise is not limited to a genetic model of hypertension.

Recent studies have implicated PICs within the PVN, an important cardiovascular regulatory center of the brain, in the pathogenesis of hypertension. Various PICs such as TNF-α and IL-1β, have reported to increase within the PVN of hypertensive experimental animals (Agarwal, Welsch et al. 2011). In addition to PICs, reduced levels of anti-inflammatory cytokines within the PVN have been shown to be associated with hypertension (Shi, Raizada et al. 2010; Agarwal, Welsch et al. 2011). It has recently been proposed that it is not only the PIC but the balance between PIC and AIC that determines the outcome of the disease (Shi, Diez-Freire et al. 2010; Shi, Raizada et al. 2010). Few studies have documented the reduction in inflammatory markers by ExT in obese individuals (Esposito, Pontillo et al. 2003; Marfella, Esposito et al. 2004) or diabetic patients (Giannopoulou, Fernhall et al. 2005). However, the impact of regular exercise on PIC as well as AIC within the PVN in hypertension is relatively unexplored. In the present study, we found that regular ExT resulted in significant reduction in TNF and IL-1, and dramatic increase in IL-10 levels within the brain of AngII-induced hypertensive rats, suggesting that the exercise not only reduces PICs but also improves AIC and thereby contributing to delayed progression of hypertension.

The two critical transcription factors that govern inflammatory responses in hypertension are Nuclear Factor-*kappa*B (NFκB) and CREB. A recent report from our lab demonstrated that bilateral microinjection of NFκB blocker in the PVN attenuates AngII-induced hypertension and

reduces PICs within the PVN (Cardinale, Sriramula et al. 2012), suggesting that within the PVN, NFκB acts as a potential perpetuator of systemic hypertensive response. However, unlike NFκB, role of CREB in hypertension has recently been examined, and downregulation of CREB has been reported to be associated with various vascular diseases, including hypertension (Schauer, Knaub et al. 2010). It is noteworthy that unlike NFκB, which positively regulates gene expression of PICs (Kang, Gao et al. 2011), activation of CREB positively regulates expression of anti-inflammatory cytokines such as IL-10 (Avni, Ernst et al. 2010). Given the current finding that regular exercise not only reduces PICs but also improves AIC, we explored the possibility that the exercise-induced alterations in inflammatory cytokines could be mediated by alterations in NFκB and CREB. It has been shown that the competition between NFκB and CREB for binding to the co-activator CREB-binding protein (CBP), is important in regulating their transcriptional activity (Grimes and Jope 2001; Shenkar, Yum et al. 2001). In addition, phosphorylation of CREB at Ser-133 and NFκB subunit p65 at Ser-276 (Zhong, SuYang et al. 1997) has been shown to be associated with their respective binding with CBP and transactivating potential. Therefore, we examined the effects of exercise on p-CREB(Ser-133) and p-p65(Ser-276) levels. Our results showed that regular exercise caused reversal of AngII-induced increase in p-p65(Ser-276). Furthermore, trained hypertensive rats had significantly increased levels of p-CREB(Ser-133) within the PVN when compared with sedentary hypertensive rats. These findings clearly suggest that exercise modulates the activity of NFκB and CREB leading to increased CREB-mediated gene transcription of IL-10 and reduced NFκB-mediated gene transcription of PICs. This was further supported by our previous finding that chronic exercise reduced myocardial NFκB activity in SHRs (Agarwal, Haque et al. 2009). Several previous studies have demonstrated anti-inflammatory effect of exercise. However, until

now, no studies have examined the effect of exercise on phosphorylated (i.e. active) CREB and NFκB, particularly within the PVN of hypertensive rats. Nonetheless, our current findings raise another question that how exactly ExT modulates the activity of these transcription factors ultimately leading to altered transcription of inflammatory cytokines.

Recently, glycogen synthase kinase (GSK)-3 has been shown to be a critical player in regulation of many biological processes including cardiac development, hypertrophy, and function. GSK-3 was originally discovered as a serine/threonine kinase which phosphorylates and inhibits glycogen synthase (GS), a key enzyme involved in glycogen metabolism. However, past several years of research has now established GSK-3β as a key regulator of a broad array of metabolic and structural proteins, inflammatory cytokines, and transcription factors including NFκB and CREB (Grimes and Jope 2001). Recently, GSK-3β has been reported to modulate the production of inflammatory cytokines in an NFκB-dependent manner (Martin, Rehani et al. 2005; Steinbrecher, Wilson et al. 2005; Vines, Cahoon et al. 2006; Beurel and Jope 2009). This evidence and our current finding that AngII causes alterations in inflammatory cytokines and transcription factor led us to explore whether AngII has any effects on GSK-3β activity in vivo within the PVN of hypertensive rats. Since, activity of GSK-3β is predominantly regulated by post-translational phosphorylation of the Serine-9 (inhibitory) and Tyrosine-216 (activating) amino acids, we examined the p-GSK3β(Tyr-216) and p-GSK3β(Ser-9) expression by immunoblot analysis. Our results showed that AngII caused significant increase in both p-GSK3β(Tyr-216) as well as p-GSK3β(Ser-9), however, the increase in tyrosine phosphorylation was found to be robust and much more than the serine phosphorylation. We observed significantly elevated levels of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio within the PVN of AngII-induced hypertensive rats, suggesting activation of GSK-3β in hypertension. These results

are in accordance with our recent in vitro study (unpublished) showing increased levels of active GSK-3β with concomitant dysregulation in PIC and AIC in AngII-exposed neuronal cells. Also, the results showed that inhibition of GSK-3β by lentiviral siRNA prevents AngII-induced imbalance in PIC and AIC, suggesting that effects of AngII in neuronal cells are mediated by GSK-3β. It is important to mention here that although few previous studies have reported increased p-GSK3β(Ser-9) in hypertrophic and failing human hearts (Haq, Choukroun et al. 2001), the others did not see any changes in phosphorylation status of GSK-3β. The discrepancies in results could be because of stage of the disease investigated or the methodological differences. Also, the tissue-specific role of GSK-3β cannot be ignored. Nevertheless, our current finding that AngII causes activation of GSK-3β raise the possibility that exercise-mediated improvement in PIC and AIC in hypertension could be mediated by inactivation of GSK-3β.

Interestingly, our results demonstrated that regular moderate-intensity exercise resulted in dramatic decrease in p-GSK3β(Tyr-216) levels within the PVN of hypertensive rats, suggesting inactivation of GSK-3β by chronic ExT in hypertension. Although, exercise also resulted in reduced p-GSK3β(Ser-9) levels, the decrease in tyrosine phosphorylation was much more than decreased in serine phosphorylation, leading to significantly reduced p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio, indicating overall inactivation of GSK-3β. These results are in accordance with a previous study that demonstrated time-dependent deactivation of GSK-3β in the skeletal muscle of healthy exercising rats (10 min to 60 min of high-intensity exercise) (Markuns, Wojtaszewski et al. 1999). However, the previous study did not report any changes in phosphorylation status of GSK3β(Tyr-216). The discrepancies in results could be because of acute, high-intensity exercise protocol used in the previous study. Also, the previous study was

done in skeletal muscle of healthy rats. To best of our knowledge, this is the first animal study to report role of GSK-3β in modulating the beneficial effects of exercise in hypertensive brain. Our findings that exercise downregulates GSK-3β in hypertensive animals is significant from a clinical perspective, because GSK-3β inhibition has recently been proposed for the treatment of various cardiovascular diseases (Hirotani, Zhai et al. 2007; Zhai, Gao et al. 2007). Given the current findings that exercise downregulated GSK-3β within the PVN and that GSK-3β has capability to phosphorylate CREB (Clerk, Cullingford et al. 2007) and NFκB, it is plausible to suggest that exercise-induced improvement in PIC and AIC could be mediated by alterations in NFκB and CREB. In summary, these findings, together with previous reports led us to conclude that regular moderate-intensity exercise improves the balance between PIC and AIC in a manner dependent on inactivation of GSK-3β, and the subsequent alterations in CREB/ NFκB transactivating potential, thereby contributing to improvement in inflammatory cytokines and attenuated blood pressure.

PERSPECTIVES

The findings of this study indicate that AngII-induced dysregulation in inflammatory cytokines within the PVN of hypertensive rats are mediated by activation of GSK-3β/NFκB/CREB signaling pathway. Our recent in vitro studies demonstrating reversal of AngII-induced dysregulation in PIC and AIC by GSK-3β inhibition in rat neuronal cell further support this hypothesis. More importantly, this study provide mechanistic evidence that chronic regular moderate-intensity exercise-induced causes inactivation of GSK-3β leading to improved CREB-mediated and reduced NFκB-mediated gene transcription within the PVN of hypertensive rats, thereby contributing to improvement in balance between PIC and AIC and attenuated MAP. Although, our in vitro studies provide evidence of a direct cause-effect relationship between

activation of GSK-3β and NFκB/CREB, whether inactivation of GSK-3β by ExT is associated in causal way with these transcription factors, remains to be resolved. In addition, future studies could be directed to prove that GSK-3β is the main player in the exercise mediated effects, possibly by GSK-3β overexpression or by using transgenic animals.

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CHAPTER 7

ROLE OF CENTRAL GSK-3β IN MEDIATING EXERCISE-INDUCED IMPROVEMENT IN INFLAMMATORY CYTOKINES AND REDUCTION IN BLOOD PRESSURE IN HYPERTENSIVE RATS

INTRODUCTION

Hypertension is a well-known risk factor for various cardiovascular diseases (CVDs) and is a leading cause of the death worldwide (World Health Organization, 2011). It is well known that the renin-angiotensin system (RAS) plays a major role in central regulation of blood pressure (BP). A growing body of evidence indicates that the RAS can interact with the pro-inflammatory cytokines (PICs) during hypertensive response (Sriramula, Haque et al. 2008; Cardinale, Sriramula et al. 2012). In the last decade, increased levels of PICs such as TNF-α, IL-1β and IL-6 have been found in the paraventricular nucleus (PVN) and other cardiovascular regulatory centers in the brain of hypertensive rats (Kang, Ma et al. 2009; Agarwal, Welsch et al. 2011). It is apparent from previous studies that within the brain, PICs such as TNF-α and IL-1β act as neuromodulators and play pivotal roles in sympathetic regulation of BP (Kimura, Yamamoto et al. 1993; Lu, Chen et al. 2009; Shi, Raizada et al. 2010). Besides PICs, anti-inflammatory cytokines such as IL-10 has recently been shown to have a significant impact on sympathetic outflow, arterial pressure and cardiac remodeling in experimental models of hypertension (Shi, Raizada et al. 2010). At the cellular levels, the RAS directly or indirectly through PICs causes increased oxidative stress leading to activation of several intracellular signaling pathways including that of nuclear factor kappaB, thereby contributing to increased sympathetic activity and BP.

Besides these molecular events, sedentary lifestyle has been found to have a strong association with the hypertension (Booth, Gordon et al. 2000). It has been known for decades that regular physical activity reduces BP and improves cardiac function in hypertensive animals and humans. At the molecular and cellular levels, exercise has been shown to reduce circulating levels of inflammatory molecules in hypertensive patients (Agarwal, Haque et al. 2009). More

recent studies in experimental animal models of hypertension have demonstrated that regular exercise improves inflammatory cytokines within the brain (Agarwal, Welsch et al. 2011). However, the exact mechanisms underlying the exercise-induced improvement in cytokines in the brain, particularly within the PVN, have not been investigated yet.

Accumulating evidence suggest that glycogen synthase kinase (GSK)-3β, a recently discovered serine/threonine kinase, modulates inflammatory processes in various disease conditions, particularly in NFκB-dependent manner (Vines, Cahoon et al. 2006). Moreover, altered activity of GSK-3β has been found to be associated with several CVDs, cardiac hypertrophy and dysfunction (Hardt and Sadoshima 2002; Tong, Imahashi et al. 2002; Kerkela, Woulfe et al. 2007). The activity of GSK-3β is mainly regulated by post-translational phosphorylation with N-terminal phosphorylation of GSK-3β at Ser-9 has an inhibitory effect, whereas, phosphorylation of Tyr-216 activates it (Forde and Dale 2007). Recent findings from our laboratory also suggest that angiotensin II (AngII)-induced dysregulation in inflammatory cytokines in neuronal cells are mediated by increased activation of GSK-3β, suggesting that GSK-3β plays critical role in hypertension. These observations led us to hypothesize that the exercise-induced improvement in inflammatory cytokines in hypertension are mediated by GSK-3β. To investigate this hypothesis, we examined whether increased expression of active form of GSK-3β (p-GSK3β(Tyr-216)) into the brain would prevent exercise-induced reduction in BP and alterations in cytokines within the PVN of AngII-induced hypertensive rats. In order to increase expression of active GSK-3β into the brain, we injected triciribine (TCN), chronically into the brain by intracerebroventicular (ICV) route. Triciribine (TCN) is a cell-permeable and reversible tricyclic nucleoside that selectively inhibits the cellular phosphorylation/activation of Akt kinase

(Yang, Dan et al. 2004), which is a well known kinase responsible for inactivation of GSK-3β, ultimately leading to increased activation of GSK-3β.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (250-350 grams) were used in this study. Animals were housed in a temperature-controlled room (25 ± 1^{o}C) and maintained on a 12:12 hour light:dark cycle with free access to water and food. All animal and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University in compliance with NIH guidelines.

Experimental Protocol. An angiontensin II (AngII)-induced hypertensive rat model was used in this study. A total of 90 rats were randomized into six experimental groups (n = 15 per group): 1) saline+exercise+vehicle (Sal+Ex+Veh); 2) saline+exercise+TCN (Sal+Ex+TCN); 3) angiontensin II+sedentary+vehicle (AngII+Sed+Veh); 4) angiontensin II+sedentary+TCN (AngII+Ex+TCN); 5) angiontensin II+exercise+vehicle (AngII+Ex+Veh); and 6) angiontensin II+exercise+TCN (AngII+Ex+TCN). Animals in group 3 to 6 were infused with AngII dissolved in 0.9% saline, at a subpressor concentration of 200ng/kg/min via osmotic minipumps (Alzet, model 2004; 0.15ul/hr); whereas, animals in group 1 and 2 were infused with saline in place of AngII and were used as normotensive sedentary controls. The pumps were implanted subcutaneously and drugs were infused for 42 days (6 weeks). Rats in group 1, 2, 5, and 6 were subjected to moderate-intensity exercise for a period of 42 days and ICV infusion of triciribine (TCN) (Tocris Bioscience, Bristol, UK) or vehicle for the last 14 days (Figure 7.1). Rats in group 3 and 4 were kept sedentary and subjected to ICV infusion of TCN or vehicle for the last 14 days. TCN was dissolved in 20% DMSO and infused at dose rate of 1 mg/kg/day; whereas, control rats received 20% DMSO (vehicle). Triciribine (TCN) is a cell-permeable and reversible

tricyclic nucleoside that selectively inhibits the cellular phosphorylation/activation of Akt kinase (Yang, Dan et al. 2004), ultimately leading to increased activation of GSK-3β. TCN has also been shown to inhibit serine-9 (inhibitory) phosphorylation of GSK-3β (Yang, Dan et al. 2004). The TCN concentration was determined from a previous pilot study in rats using three different doses, 100μg/kg/day, 1mg/kg/day, and 10mg/kg/day. The 1mg/kg/day dose was found to be optimal, while the highest dose did not cause any additional increase in p-GSK-3β(Tyr-216) expression and the lowest dose did not produce sufficient increase in p-GSK-3β(Tyr-216) expression in the PVN. 24 hours after the last exercise session, echocardiography was performed to evaluate cardiac morphology and function, the rats were then euthanized; the brains were collected, and immediately frozen on dry ice. The paraventricular nucleus (PVN) tissues were punched out from the brain for further analysis.

Blood Pressure Measurement. Mean arterial blood pressure (MAP) was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN) 7 days prior to implantation of the osmotic minipumps. Rats (n = 6 per group) were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and placed dorsally on a heated surgical table. An incision was made on the medial surface of the left leg and the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and another suture was placed proximally to temporarily interrupt the blood flow. The catheter tip of the radio-telemetry transmitter was introduced through a small hole in the femoral artery, advanced ~6 cm into the abdominal aorta such that the tip was distal to the origin of the renal arteries, and sutured into place. The probe body was placed into the abdominal cavity and sutured to the abdominal wall. The abdominal musculature was sutured and the skin layer closed following implantation. Rats received enrofloxacin

(10mg/kg, s.c.) and buprenorphine (0.1 mg/kg, s.c.) immediately following surgery and 12 hours postoperatively and allowed to recover for seven days.

ICV Cannula Implantation. Following the transmitter recovery period, the rats were implanted with ICV cannulae for infusion of TCN or vehicle (Francis, Weiss et al. 2003). The rats were anesthetized and the head was positioned in a Kopf stereotaxic apparatus. An ICV cannula was

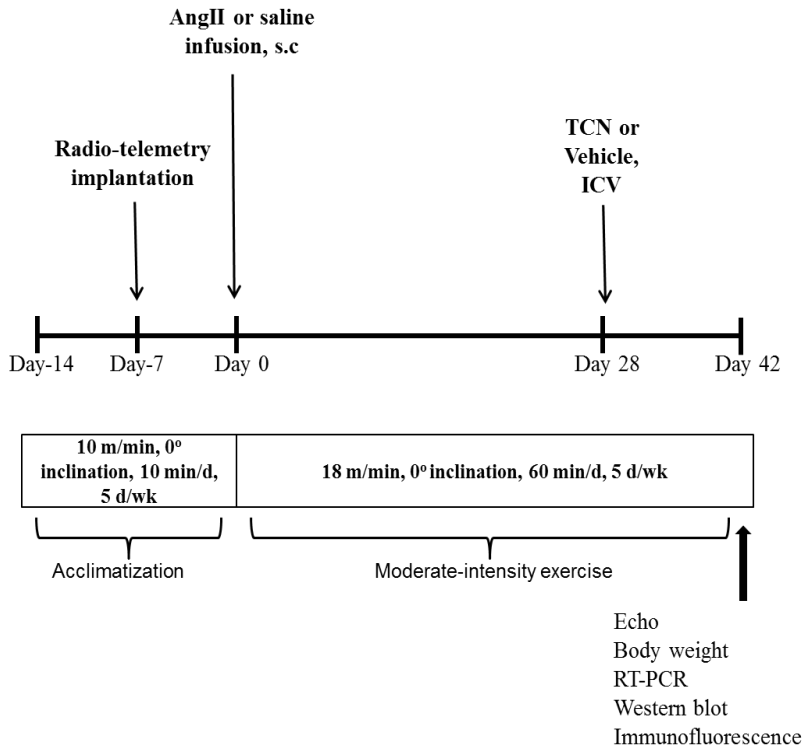

Figure 7.1. **Experimental protocol:** Rats were first acclimatized to the treadmill for 14 days before the start of the experiment. After 7 days of acclimation, rats were implanted with radio-telemetry probes for continuous recording of MAP and then were allowed to recover for next 7 days. Then miniosmotic pumps (42 days) filled with AngII or saline were subcutaneously (s.c.) implanted. Animals in exercise groups were allowed to run for 42 days, whereas, animals in sedentary groups were placed on non-running treadmill for the exercise sessions. Animals in triciribine (TCN) or vehicle groups were subjected to ICV (intracerebroventricular) infusion of either TCN or vehicle after 28 days. 24 hours after the last exercise session, animals were weighed and an echo was done. Animals were then euthanized and the brains were collected for real-time RT-PCR, Western blot analysis and immunofluorescence staining.

implanted into the right lateral cerebroventricle (1.3 mm caudal to bregma, 1.5 mm lateral to the midline, and 3.5 mm ventral to the dura) according to Paxinos and Watson, and fixed to the cranium using small screws and dental cement, a 14-day osmotic minipump was implanted subcutaneously and connected to the infusion cannula via the catheter tube to deliver TCN or vehicle into the brain.

Exercise Protocol. Rats in exercise groups (Sal+Ex+Veh, Sal+Ex+TCN, AngII+Ex+Veh and AngII+Ex+TCN) underwent moderate-intensity exercise (5 days per week; 60 min per day at 18 m/min, 0^{o} inclination) on a motor-driven treadmill continuously for a period of 42 days. All the animals were acclimatized to treadmill for 2 weeks prior to osmotic mini-pump implantation. After acclimation, training intensity was set at approximately 60% of maximal aerobic velocity (MAV), which corresponds to moderate intensity exercise (18-20m/min). This training intensity was maintained throughout the study period. The MAV was evaluated from an incremental exercise test as reported previously (Boissiere, Eder et al. 2008; Sun, Qian et al. 2008). The rats in sedentary groups (AngII+Sed+Veh and AngII+Sed+TCN) were placed on a nonmoving treadmill during the training sessions.

Echocardiographic Assessment of Cardiac Function and Hypertrophy. Echocardiography (n = 8 per group) was performed at baseline and at the end of the 42-day study period, as described previously (Agarwal, Haque et al. 2009). Briefly, transthoracic echocardiography was performed under isoflurane 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. Short-axis M-mode echocardiography was performed and the following measurements were obtained as an average of at least three cardiac cycles: Left ventricular internal diameter at diastole and systole (LVIDd

and LVIDs, respectively), left ventricular posterior wall thickness at diastole and systole (LVPWTd and LVPWTs, respectively), interventricular septal thickness at diastole and systole (IVSTd and IVSTs, respectively), and fractional shortening (%FS) was calculated using the equation, FS= [(LVIDd- LVIDs)/ LVIDd] X 100. Tei index was determined from left ventricular inflow and outflow Doppler recordings as previously described (Pellett, Tolar et al. 2004).

Real-time RT-PCR Analysis. Semi-quantitative real-time RT-PCR (n=6 per group) was used to determine the mRNA levels of PICs *viz.* TNF-α and IL-1β, AIC (IL-10), and oxidative stress markers *viz.* $gp91^{phox}$ (also known as NOX2), and iNOS in the PVN by using specific primers. Rat primers used are listed in Table 1. In Brief, the rats were euthanized, the brains were quickly removed and immediately frozen on dry ice. The brains were blocked in the coronal plane, sectioned at 100 μm thickness, and the PVN were punched from each brain according to the methods described by Palkovits and Brownstein (Gao, Wang et al. 2005). Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (Agarwal, Welsch et al. 2011). Gene expression was measured by the ΔΔCT method and was normalized to GAPDH mRNA levels. The data is presented as the fold change of the gene of interest relative to that of control animals.

Determination of efficacy of TCN infusion (ICV) in causing increased p-GSK3β(Tyr-216) expression in the PVN. To determine the efficacy of TCN infusion (ICV) in causing increased expression of **p-**GSK3β(Tyr-216) within the PVN, we examined the expression of phosphorylated GSK3β(Tyr-216) (activating) and phosphorylated GSK3β (Ser-9) (inhibitory) by immunofluorescence staining and Western blot analysis.

Immunofluorescence Staining. The immunostaining protocol used was modified from Block *et al* (Block, Santos et al. 1988). Briefly, the rats (n=5 per group) were deeply anesthetized with

carbon dioxide and perfused transcardially with PBS (pH 7.4), followed by 4% paraformaldehyde in PBS. The brain was then removed, postfixed for 2 hours in 4% paraformaldehyde in PBS, and coronal sections (10 μm) were made in a cryostat. The sections were incubated in xylene solution for 15 minutes at room temperature (RT) two times followed by dehydration in ethanol. The sections were then washed in PBS three times, 5 minutes each. Antigen retrieval was then performed using citrate target retrieval solution (Biocare Medical, CA). Slides were then washed with PBS and the nonspecific staining was blocked with 2% normal donkey serum containing 1% bovine serum albumin (BSA) for 1 hour at RT. Sequentially, the tissues were incubated with the primary antibody specific to p-GSK3β(Tyr-216) and p-GSK3β (Ser-9) (Cell Signaling Technology, Inc, MA, USA) at 1:50 dilution overnight at 4°C. The sections were then incubated with Alexa 594-labeled anti-rabbit secondary antibody (red fluorescence) (Invitrogen, CA), at 1:500 dilution for 2 hours at RT. The sections were rinsed 3 times in PBS and mounted in ProLong® Gold antifade reagent (Invitrogen). The stained sections were photographed with a confocal laser-scanning microscope.

Western Blot Analysis. The tissue homogenates from the PVN were subjected to Western blot analysis (n = 5 per group) for the determination of protein levels of PICs (TNF-α, IL-1β), IL-10, $gp91^{phox}$, iNOS, GSK-3β, p-GSK3β(Ser-9), p-GSK3β(Tyr-216), and GAPDH. The extraction of protein and Western blot was performed as described before (Agarwal, Welsch et al. 2011). Specific antibodies used included: TNF-α, IL-1β, $gp91^{phox}$, iNOS, GSK-3β, p-GSK3β(Ser-9), p-GSK3β(Tyr-216), and GAPDH, at 1:1,000 dilution; and IL-10, at 1:500 dilution. Antibodies were commercially obtained: TNF-α (Abcam Inc, MA, USA); IL-1β, iNOS, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA); IL-10 (Abbiotec, CA,USA); $gp91^{phox}$ (BD biosciences, USA); GSK-3β (BD Transduction laboratories, USA); and p-GSK3β(Ser-9), p-

GSK3β(Tyr-216) (Cell Signaling Technology, Inc, MA, USA). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham), band intensities were quantified using Versa Doc MP 5000 imaging system (Bio-Rad), and were normalized with GAPDH.

Statistical Analysis. All data are presented as means±SE. Statistical analysis was done by either two-way ANOVA or one-way ANOVA with a Tukey's post hoc test using Graph Pad Prism software (version 5.0). Blood pressure data were analyzed by repeated-measures ANOVA to examine with-in group changes over time. Results were considered significant when $p<0.05$.

RESULTS

Chronic ICV Infusion of TCN Increases p-GSK-3β(Tyr-216) Expression in the PVN. To determine the role of brain GSK-3β in exercise-induced beneficial effects in hypertensive rats, we performed chronic ICV infusion of TCN. To determine the efficacy of TCN in causing increased p-GSK-3β(Tyr-216) expression specifically within the PVN, we measured the protein expression of p-GSK3β(Ser-9), an inactive form, and p-GSK3β(Tyr-216), an active form of GSK-3β, by immunofluorescent staining (Figure 7.2A) and western blot (Figure 7.2B) of the PVN. The results demonstrated that sedentary hypertensive rats (AngII+Sed+Veh and AngII+Sed+TCN) had robustly increased levels of p-GSK3β(Tyr-216) (Figure 7.2A) and p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio (Figure 7.2B) when compared to normotensive rats, suggesting increased activation of GSK-3β in the PVN of sedentary hypertensive rats. There was no difference in p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio between AngII+Sed+Veh and AngII+Sed+TCN rats. Furthermore, trained hypertensive rats that received ICV vehicle (AngII+Ex+Veh) had significantly reduced levels of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio within the PVN of compared to sedentary hypertensive rats, indicating decreased activity of

GSK-3β in exercising hypertensive rats. TCN infusion in trained hypertensive rats was found to be dramatically increase the levels of p-GSK3β(Tyr-216) (Figure 7.2A) and p-GSK3β(Tyr-216)

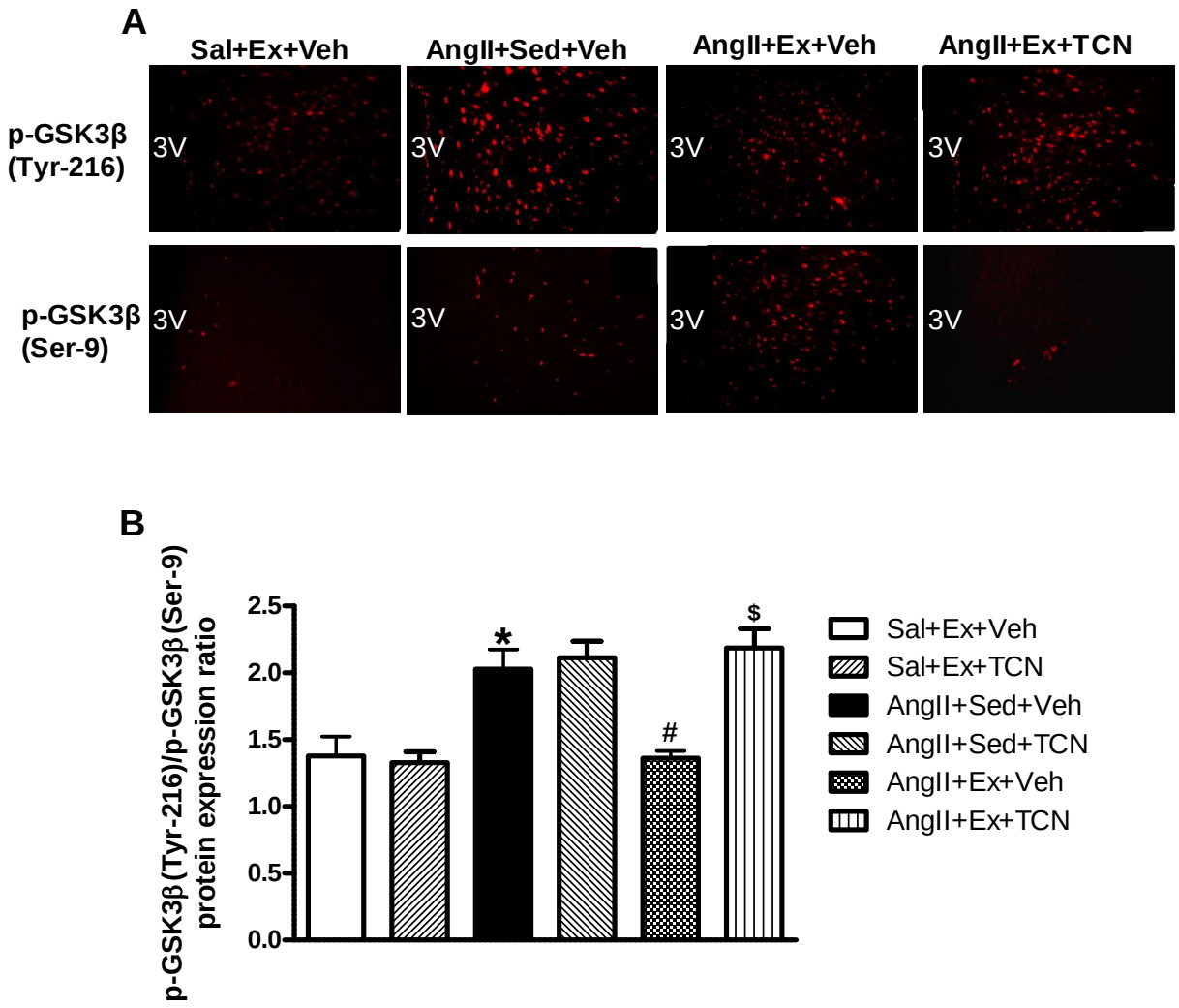

Figure 7.2. Efficacy of TCN infusion (ICV) in causing increased p-GSK3β(Tyr-216) expression in the PVN. A, A Representative immunofluorescence image showing expression of p-GSK-3β(Tyr-216) and p-GSK-3β(Ser-9) in the PVN. There was an increased expression of p-GSK-3β(Tyr-216) and reduced p-GSK-3β(Ser-9) expression in the PVN of TCN treated trained hypertensive rats in comparison with their vehicle treated counterparts. Also, sedentary hypertensive rats had dramatically increased levels of p-GSK-3β(Tyr-216) compared to normotensive rats. **B**, Densitometric analysis of western blot results showing protein expression ratio of p-GSK-3β(Tyr-216) to p-GSK-3β(Ser-9). Values are mean±SE; n=5 per group. *p<0.05 vs Sal+Ex+Veh; #p<0.05 vs AngII+Sed+Veh; $p<0.05 vs AngII+Ex+Veh.

to p-GSK3β(Ser-9) ratio (Figure 7.2B) in the PVN when compared to the AngII+Ex+Veh group. In contrary, expression levels of p-GSK3β(Ser-9) were found to be much lower in AngII+Ex+TCN rats than AngII+Ex+Veh. This data indicate that the ICV infusion of TCN causes marked increase in activated GSK-3β in the PVN of trained hypertensive rats. However, TCN treatment did not affect p-GSK3β(Tyr-216) and p-GSK3β(Ser-9) levels in sedentary hypertensive and trained normotensive rats.

Effects of ICV Infusion of TCN on Exercise-induced Reduction in MAP in Hypertensive Rats. Exercise training in AngII-induced hypertensive rats (AngII+Ex+Veh group) caused significant reduction in MAP starting from day 16 of exercise and remained until study (Figure 7.3). Chronic ICV infusion of TCN in trained hypertensive rats tended to reverse exercise-induced decrease in MAP. This trend was observed beginning from day 34 (or day 6 of TCN infusion) and at 41 and 42 day, there was significant increase in MAP in AngII+Ex+TCN when compared to AngII+Ex+Veh group. Among sedentary hypertensive and normotensive rats, there was no difference between vehicle and TCN treated groups.

Effects of ICV Infusion of TCN on Exercise-induced Improvement in Cardiac Hypertrophy and Cardiac Function in Hypertensive Rats. At the end of the study period, AngII+Ex+Veh had lower heart weight to body weight (HW:BW) ratio compared with AngII+Sed+Veh rats (Figure 7.4).

Echocardiographic studies (Figure 7.5A-C) revealed that when compared with AngII+Sed+Veh, AngII+Ex+Veh rats had significantly lower interventricular septal thickness (IVSTd) and left ventricular posterior wall thickness at diastole (LVPWTd), without modification of LV chamber size. These echocardiographic changes indicate that regular exercise in hypertensive rats attenuates concentric cardiac hypertrophy. Furthermore, the

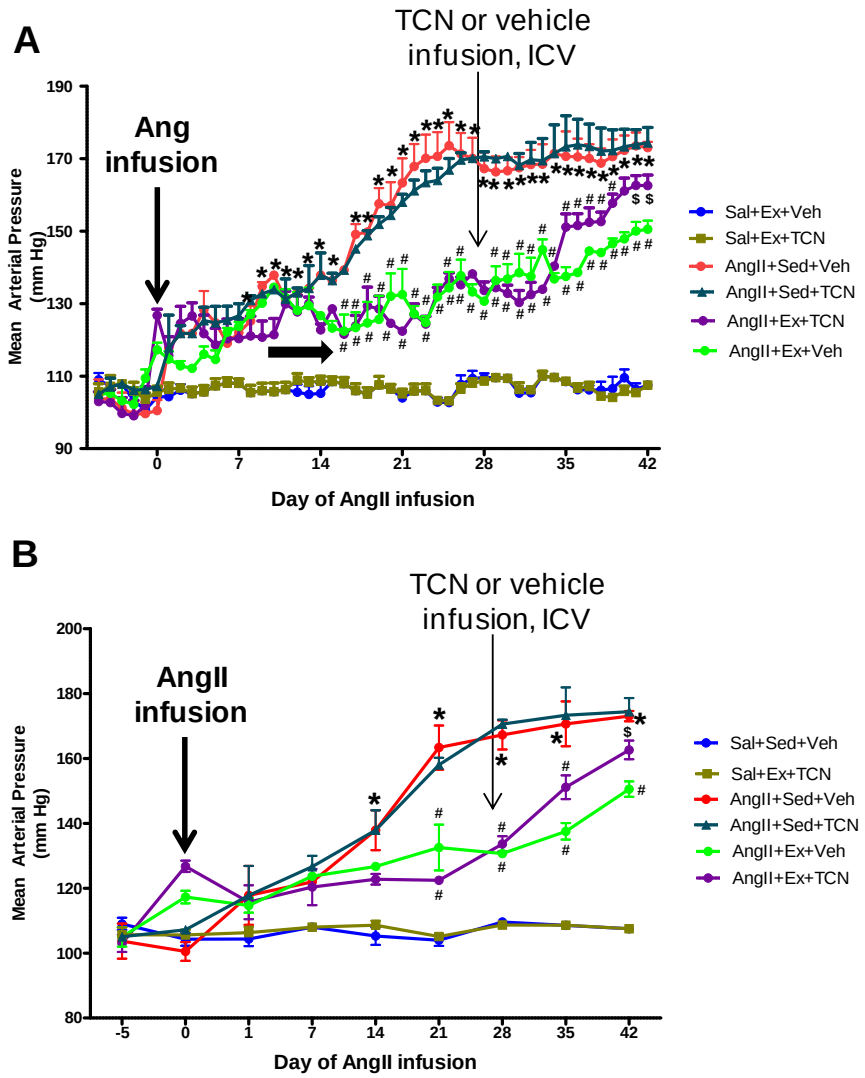

Figure 7.3. Effects of ICV infusion of TCN on the time course of mean arterial pressure (MAP, in millimeters of mercury) in hypertensive rats. A, average daily pressure. **B,** average weekly pressure. MAP was significantly reduced in AngII+Ex+Veh and AngII+Ex+TCN rats beginning from day 16 of exercise; however, TCN treated rats exhibited slow increase in MAP beginning day 36 and the increase was significant at day 41 and 42 when compared to their vehicle treated counterparts. TCN infusion in normotensive rats and sedentary hypertensive rats did not affect MAP when compared to their respective vehicle controls. Values are mean±SE; n=6 per group. *p<0.05 vs Sal+Ex+Veh; #p<0.05 vs AngII+Sed+Veh; $p<0.05 vs AngII+Ex+Veh.

increased Tei index (Figure 7.5C, right panel) in AngII+Sed+Veh were significantly reduced in

AngII+Ex+Veh, indicating improvement in diastolic function in trained hypertensive rats.

Interestingly, AngII+Ex+TCN rats exhibited significantly higher HW:BW ratio, IVSTd, and LVPWTd, in comparison with AngII+Ex+Veh rats; whereas, there was no significant differences in these parameters between AngII+Ex+TCN and sedentary hypertensive rats. These data suggest that ICV infusion of TCN resulted in reversal of exercise-induced reduction in cardiac hypertrophy in hypertensive rats. Moreover, AngII+Ex+TCN rats exhibited significantly higher Tei index, in comparison with AngII+Ex+Veh rats and there was no significant difference between AngII+Ex+TCN and sedentary hypertensive rats, suggesting reversal of exercise-induced improvement in diastolic function by increased p-GSK3β(Tyr-216) levels in the brain. There were no significant differences in these parameters between AngII+Sed+Veh and AngII+Sed+TCN and between Sal+Ex+Veh and Sal+Ex+TCN.

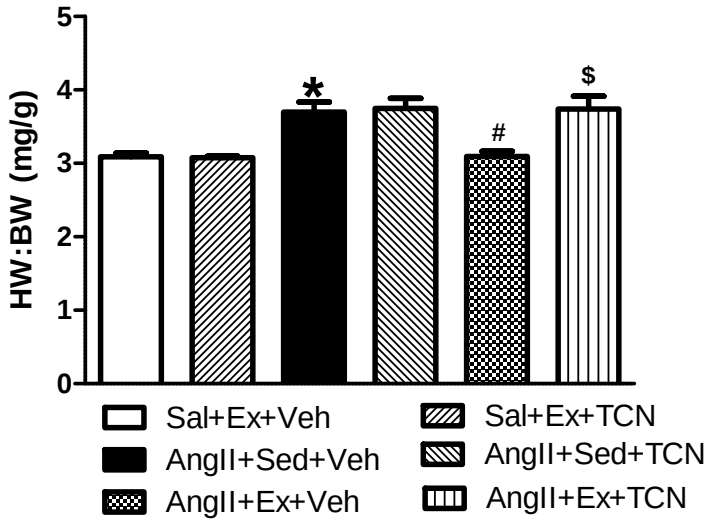

Figure 7.4. Effects of ICV infusion of TCN on HW:BW ratio in hypertensive rats. TCN treatment prevented exercise-induced reduction in HW:BW ratio in hypertensive rats, suggesting reversal of cardiac hypertrophy. Values are means±SE. n=8 per group. *p<0.05 vs Sal+Ex+Veh; #p<0.05 vs AngII+Sed+Veh; $p<0.05 vs AngII+Ex+Veh.

Effects of ICV Infusion of TCN on Exercise-induced Decreases in Pro-inflammatory Cytokines in Hypertensive Rats. As expected, exercise training (AngII+Ex+Veh) resulted in

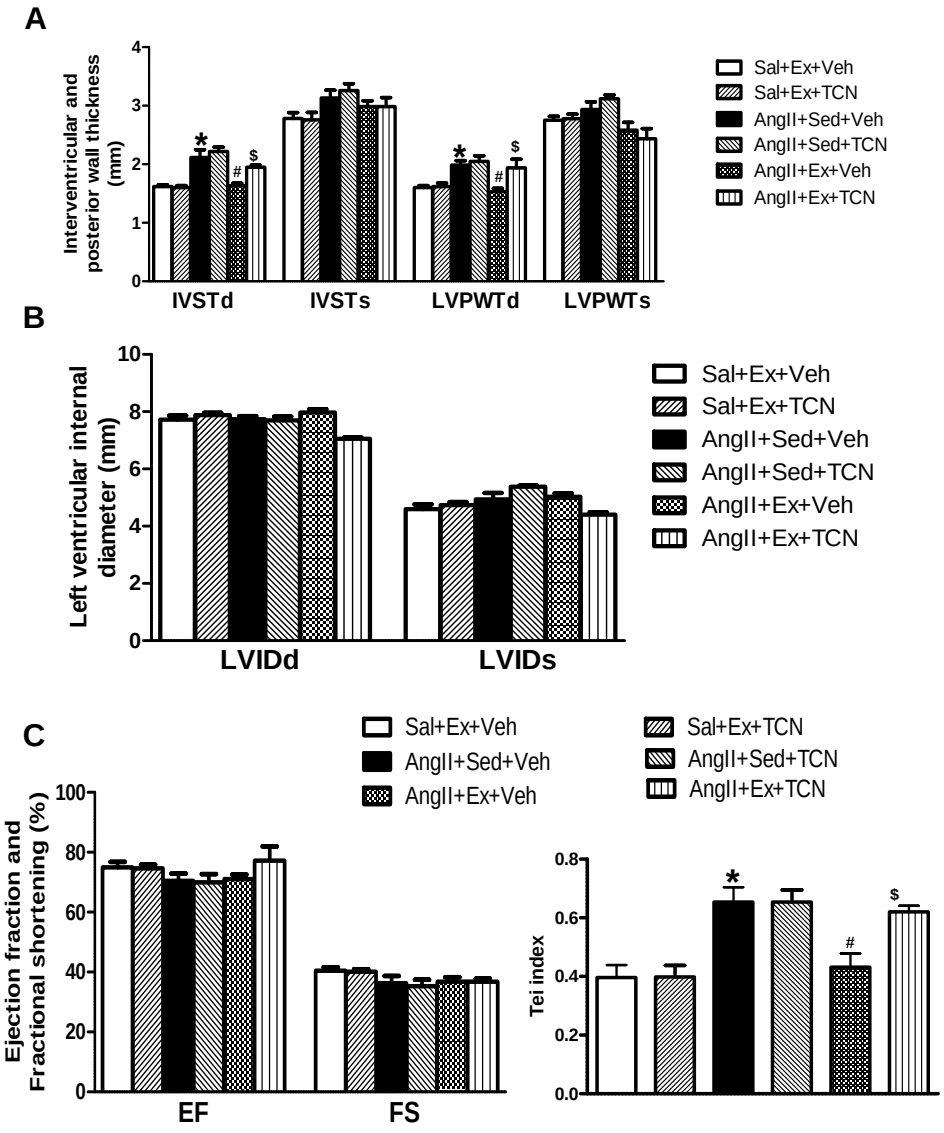

Figure 7.5. Effects of ICV infusion of TCN on Cardiac Hypertrophy and Cardiac Function in hypertensive rats as measured by M-mode and Doppler echocardiography. TCN treatment prevented exercise-induced reduction in IVSTd, LVPWTd, and Tei index in trained hypertensive rats; whereas, TCN treatment did not affects these parameters in sedentary hypertensive and normotensive rats. Values are means±SE. n=8 per group. *p<0.05 vs Sal+Ex+Veh; #p<0.05 vs AngII+Sed+Veh; $p<0.05 vs AngII+Ex+Veh.

significant reduction in TNF-α and IL-1β in the PVN of hypertensive rats (Figure 7.6 A-B).

Interestingly, TCN infusion in the brain of exercising hypertensive rats (AngII+Ex+TCN) caused

significant increase in TNF-α and IL-1β levels within the PVN when compared with AngII+Ex+Veh. At mRNA level, there was about two-fold increase in TNF-α and IL-1β levels in TCN treated trained hypertensive rats when compared with their vehicle treated counterparts. There was no significant difference between AngII+Ex+TCN and sedentary hypertensive rats. TCN infusion did not alter levels of these cytokines in sedentary hypertensive as well as in normotensive rats.

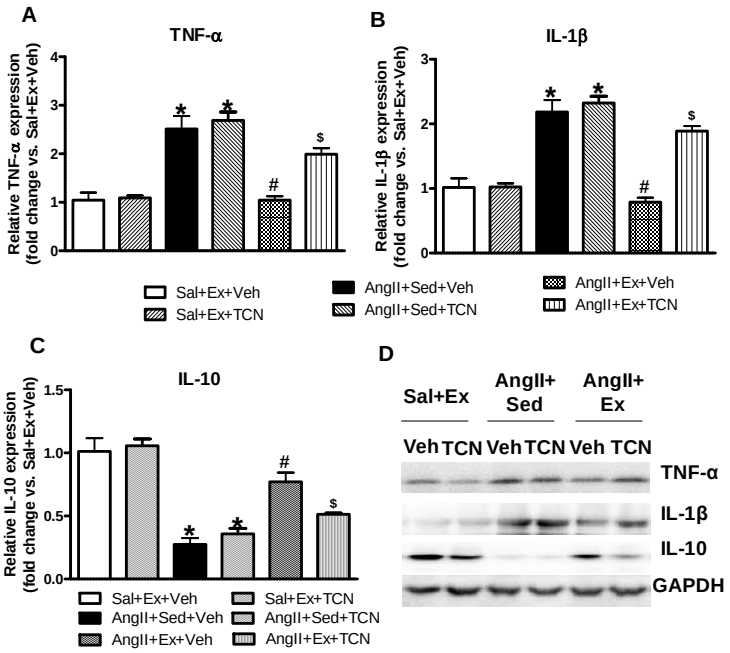

Figure 7.6. Effects of ICV infusion of TCN on TNF-α, IL-1β, and IL-10 in the PVN of hypertensive rats. A, mRNA expression of TNF-α. **B,** mRNA expression of IL-1β. **C,** mRNA expression of IL-10. **D**, A representative Western blot of protein expression. TCN treated trained hypertensive rats had significantly increased levels of TNF-α and IL-1β and decreased IL-10 levels when compared to vehicle treated trained hypertensive rats, indicating reversal of exercise-induced improvements in inflammatory cytokines by TCN infusion. TCN treatment did not have any effects on normotensive and sedentary hypertensive rats. Values are mean±SE; n=6 per group. *p<0.05 vs Sal+Ex+Veh; #p<0.05 vs AngII+Sed+Veh; $p<0.05 vs AngII+Ex+Veh.

Effects of ICV Infusion of TCN on Exercise-induced Upregulation in Anti-inflammatory Cytokine in Hypertensive Rats. Exercise in AngII-infused rats dramatically upregulated IL-10 levels in the PVN (Figure 7.6 C). Interestingly, chronic infusion of TCN by ICV route reversed exercise-induced upregulation of IL-10 in hypertensive rats. As depicted in Figure 7.6C, AngII+Ex+TCN rats had significantly higher IL-10 levels when compared to AngII+Ex+Veh, whereas, there was no significant difference between AngII+Ex+TCN and sedentary hypertensive rats. Among sedentary hypertensive animals, there was no difference in IL-10 levels between vehicle and TCN treated animals.

Effects of ICV Infusion of TCN on Exercise-induced Reduction in Oxidative Stress in Hypertensive Rats. To assess whether effects of exercise on oxidative stress are mediated by brain GSK-3β, we examined the expression levels of $gp91^{phox}$, (a subunit of NADPH oxidase, a major source of AngII-induced ROS production) and inducible NOS (iNOS). Both protein and gene expression levels of iNOS (Figure 7.7A) were significantly reduced in the PVN of AngII+Ex+Veh when compared to their sedentary counterparts. Importantly, ICV infusion of TCN in exercising hypertensive rats resulted in significant increase in iNOS levels when compared with AngII+Ex+Veh rats. There was no significant difference between AngII+Ex+TCN and sedentary hypertensive rats. Among sedentary hypertensive animals, we did not observe any difference in iNOS levels between vehicle and TCN treated animals. Similarly, as shown in Figure 7.7 B, $gp91^{phox}$ expression was much higher in AngII+Sed+Veh than AngII+Ex+Veh rats within the PVN. Whereas, in AngII+Ex+TCN group, $gp91^{phox}$ expression was significantly higher compared to AngII+Ex+Veh, suggesting complete reversal of exercise-induced reduction in $gp91^{phox}$ expression within the PVN by chronic ICV infusion of TCN.

Among sedentary hypertensive animals, we did not observe any difference in gp91^{phox} levels between vehicle and TCN treated animals.

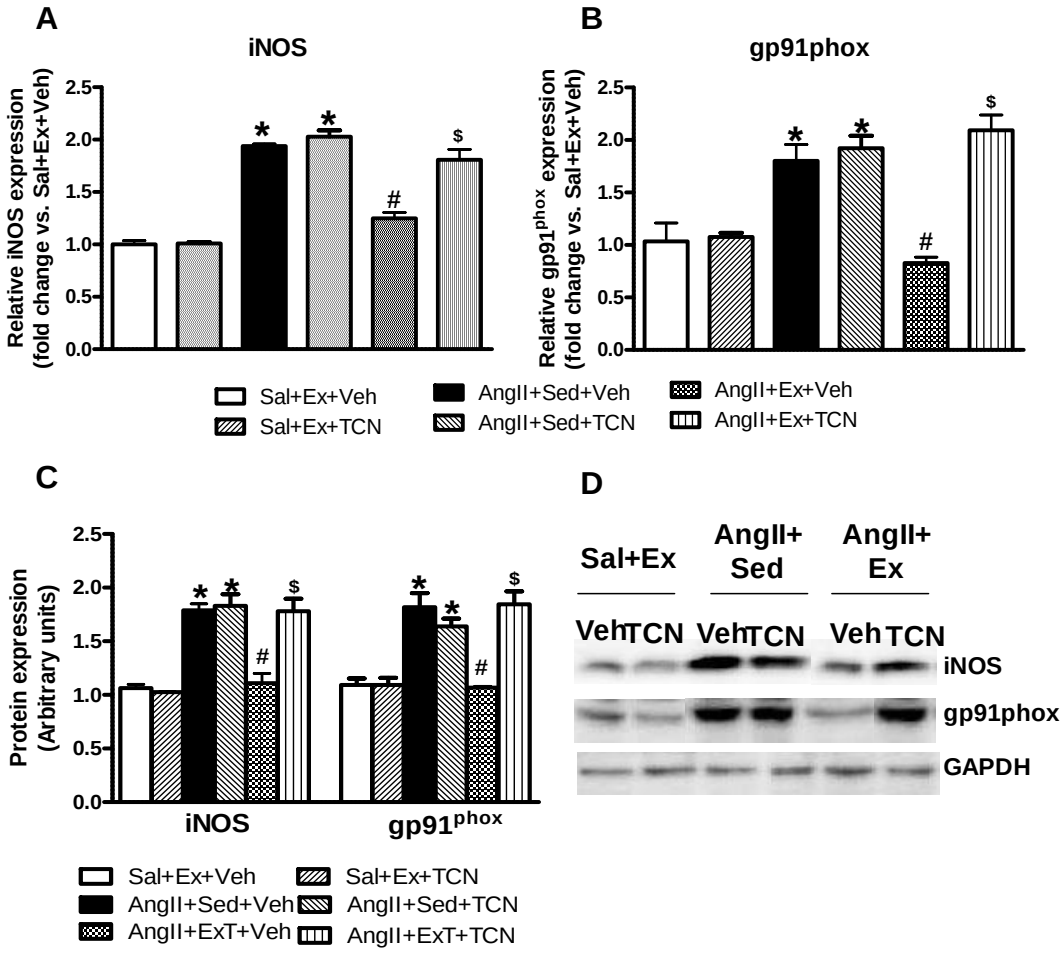

Figure 7.7. Effects of ICV infusion of TCN on oxidative stress markers in the PVN of hypertensive rats. A, mRNA expression of iNOS. **B,** mRNA expression of gp91^{phox}. **C**, Densitometric analysis of protein expression. **D**, A representative western blot. Expression levels of iNOS and gp91^{phox} in the PVN of TCN treated trained hypertensive rats were similar to sedentary hypertensive rats; whereas, these levels were significantly lower in AngII+Ex+Veh in comparison with sedentary hypertensive rats. This data suggest that chronic TCN infusion in the brain causes complete reversal of exercise-induced reduction in oxidative stress in hypertension. TCN treatment did not have any effects on normotensive and sedentary hypertensive rats. Values are mean±SE; n=6 per group. *p<0.05 vs Sal+Ex+Veh; #p<0.05 vs AngII+Sed+Veh; $p<0.05 vs AngII+Ex+Veh.

DISCUSSION

The major findings in this study are as follows: 1) regular moderate-intensity exercise delayed the progression of hypertension, reduced cardiac hypertrophy and improved diastolic function in an AngII-induced hypertensive rat model of hypertension and chronic infusion of TCN in the brain prevented these beneficial changes; 2) TCN treatment prevented exercise-induced improvement in balance between PIC and AIC within the PVN as indicated by significantly increased levels of TNF-α and IL-1β and reduced levels of anti-inflammatory IL-10 in TCN-treated trained hypertensive rats compared to their vehicle-treated controls; 3) TCN infusion in the brain completely reversed the exercise-induced reduction in oxidative stress within the PVN of hypertensive rats, as suggested by the data that iNOS and $gp91^{phox}$ expression in TCN-treated trained hypertensive rats were similar to sedentary hypertensive rats. These findings demonstrate that the beneficial effects of regular moderate-intensity exercise in hypertension are mediated, at least in part, by reduced activation of central GSK-3β and potentially via improvement in inflammatory cytokines and oxidative stress within the PVN.

Our immunofluorescence and western blot results demonstrated that subcutaneous infusion of AngII in sedentary rats resulted in robustly increased levels of p-GSK3β(Tyr-216) and p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio in AngII+Sed+Veh and AngII+Sed+TCN rats when compared to normotensive rats. Furthermore, exercise training in AngII-infused rats (AngII+Ex+Veh) caused significant reduction in p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio within the PVN. Since, N-terminal phosphorylation of GSK-3β at Ser-9 has an inhibitory effect, whereas, phosphorylation of Tyr-216 activates it (Forde and Dale 2007), these results clearly suggest that regular exercise reduces AngII-induced activation of GSK-3β within the PVN. Given the current results and our previous findings that AngII-induced dysregulation in

inflammatory cytokines are mediated by GSK-3β, it was plausible to speculate that the exercise-induced beneficial effects could be mediated by reduced activation of central GSK-3β. Therefore, to investigate this hypothesis, we examined whether increased expression of active form of GSK-3β (p-GSK3β(Tyr-216)) into the brain would prevent exercise-induced beneficial effects in AngII-induced hypertensive rats. In order to increase expression of active GSK-3β into the brain, we chronically infused TCN by ICV route for the last 14 days (2 weeks) of study. TCN selectively inhibits the cellular activation of Akt kinase (Yang, Dan et al. 2004), a well known kinase responsible for inactivation of GSK-3β. Our results demonstrated that chronic ICV infusion of TCN causes marked increase in activated GSK-3β in the PVN of trained hypertensive rats as showed by dramatic increase in the levels of p-GSK3β(Tyr-216) and p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio (Figure 7.2) in the PVN of AngII+Ex+TCN when compared to the AngII+Ex+Veh group. However, TCN treatment did not affect p-GSK3β(Tyr-216) and p-GSK3β(Ser-9) levels in sedentary hypertensive and trained normotensive rats, that could be because TCN does not act on Akt kinase when Akt kinase is either not activated (as in normotensive rats) or when fully active (as in hypertensive rats). In support of this, our results showed that AngII-exposed neuronal cells had significantly higher levels of p-Akt(Ser-473) (activating) in comparison with controls (data not shown). Moreover, TCN infusion in sedentary hypertensive rats did not cause additive effects on any of the parameters studied when compared to their vehicle controls, providing further evidence that AngII exerts its effects via GSK-3β activation.

In this study, we observed that when compared with sedentary hypertensive rats, exercising rats (AngII+Ex+Veh) had significantly reduced MAP beginning from day 16 of exercise and continued until the end of the study, suggesting delayed progression of hypertension

by regular exercise. Interestingly, exercising hypertensive rats that were receiving TCN in the brain (AngII+Ex+TCN) exhibited similar reduction in MAP beginning from day 16 of exercise; however, MAP started to slowly increase from day 34 (or day 6 of TCN infusion) and became significantly higher at the end of the study in comparison with their vehicle-infused counterparts. As evidence, a previous study (unpublished) from our laboratory demonstrated that regular exercise prevented AngII-induced activation of GSK-3β within the PVN of hypertensive rats. Similarly, although not in the brain, a previous study from other laboratory demonstrated time-dependent deactivation of GSK-3β in the skeletal muscle of healthy exercising rats (Markuns, Wojtaszewski et al. 1999). Taken together, the findings of current study provide strong evidence of role of central GSK-3β in mediating exercise-induced delayed progression of hypertension.

Our echocardiographic data showed that exercise training resulted in reduced cardiac hypertrophy and improved cardiac diastolic function in hypertensive rats, as indicated by decreased IVSTd, LVPWTd, HW:BW ratio, and Tei index. More importantly, at the end of the study, trained hypertensive rats infused with TCN exhibited significant increase in all these parameters when compared with their vehicle infused counterparts. These data showed that centrally activated GSK-3β causes complete reversal of exercise-induced improvement in cardiac hypertrophy and function, suggesting inactivation of GSK-3β as an underlying mechanism by which exercise exerts cardio-protective effects in hypertension. A number of previous studies suggest that GSK-3β plays a pivotal role in cardiac hypertrophy and function. Although GSK-3β has been known to be a negative regulator of cardiac hypertrophy (Sugden, Fuller et al. 2008) and inhibition of GSK-3β has been observed in end-stage heart failure (Haq, Choukroun et al. 2001), several studies did not observe such inhibition in human hearts with hypertrophy (Haq, Choukroun et al. 2001). More recent work has shown increased fibrosis, apoptosis, and

decreased contractility in mice in which GSK-3 is activated (Michael, Haq et al. 2004; Hirotani, Zhai et al. 2007; Zhai, Gao et al. 2007). Similarly, in the present study we found increased levels of active form of GSK-3β in the PVN of AngII-induced hypertensive rats. It is noteworthy that the AngII dose used in the present study is a subpressor dose from which end-stage heart failure does not result. Therefore, the discrepancies in results could be attributed to the stage of cardiac dysfunction as well as tissues studied. It is also noteworthy that although GSK-3β is highly expressed in the central nervous system and is neuron-specific (Leroy and Brion 1999), role of brain GSK-3β in pathogenesis of CVDs including hypertension has not been investigated yet. To the best of our knowledge, this is the first study that provides evidence of role of brain GSK-3β in hypertension and in exercise-induced beneficial effects.

Recent studies have implicated PICs within the PVN in the pathogenesis of hypertension. Various PICs, such as TNF-α and IL-1β, have reported to increase within the PVN of hypertensive experimental animals (Agarwal, Welsch et al. 2011). In addition to PICs, reduced levels of anti-inflammatory cytokines within the PVN have been shown to be associated with hypertension (Shi, Raizada et al. 2010; Agarwal, Welsch et al. 2011). It has recently been proposed that it is not only the PIC, but the balance between PIC and AIC that determines the outcome of the disease (Shi, Diez-Freire et al. 2010; Shi, Raizada et al. 2010). Few studies have documented the reduction in inflammatory markers by exercise in obese individuals (Esposito, Pontillo et al. 2003; Marfella, Esposito et al. 2004), diabetic patients (Giannopoulou, Fernhall et al. 2005), and hypertension (Agarwal, Haque et al. 2009). Moreover, recent evidence suggests that regular exercise has capability to improve the balance between PIC and AIC within the heart as well as brain (Agarwal, Haque et al. 2009; Agarwal, Welsch et al. 2011). However, the underlying molecular mechanisms by which exercise improves this balance are still not clear. In

the present study, we observed that exercise training in hypertensive rats resulted in significant reduction in TNF-α and IL-1β; whereas, IL-10 were dramatically upreglated. More importantly, chronic infusion of TCN in the brain prevented these changes in hypertensive rats, suggesting that exercise-induced improvements in inflammatory cytokines are mediated by central GSK-3β.

The two critical transcription factors that regulate gene transcription of TNF-α and IL-10 are nuclear factor-*kappa*B (NFκB) and cyclic AMP response element binding protein (CREB), respectively. Previous studies demonstrated that GSK-3β plays important roles in regulating activity of NFκB and CREB (Clerk, Cullingford et al. 2007). Therefore, there is a possibility that increased activity of GSK-3β in the brain may have caused alterations in NFκB and CREB activity in favor of NFκB, thereby preventing the exercise-induced improvement in PICs and AIC. Although in this study we did not examine the activity of these transcription factors, previous studies (data unpublished) from our laboratory demonstrated that inhibition of GSK-3β prevents Ang-induced activation of NFκB and reduced activity of CREB in rat neuronal cells.

A growing body of evidence suggest role of oxidative stress in the development of hypertension and cardiac hypertrophy (Bertagnolli, Schenkel et al. 2008).We reported previously that cytokines and their transcription factor, NFκB, contribute to the induction of oxidative stress in heart failure (Guggilam, Haque et al. 2007) and hypertension (Elks, Mariappan et al. 2009). Given the current finding that increased levels of activated GSK-3β in the brain prevented effects of exercise on cytokines, we further examined the effect of TCN infusion on oxidative stress makers in exercised hypertensive animals. Our mRNA and protein analysis revealed that regular chronic exercise in hypertensive rats resulted in significant reduction in $gp91^{phox}$ and iNOS, and TCN infusion in the brain completely reversed these changes in hypertensive rats, further

suggesting that GSK-3β is the main player in exercise-induced reduction in oxidative stress in hypertension.

In summary, the present study shows that chronic infusion of TCN (or increased levels of activated GSK-3β) in the brain prevent exercise-induced delayed progression of hypertension and cardioprotection in an AngII-induced hypertensive rat model of hypertension. Also, ICV infusion of TCN prevents exercise-induced improvement in balance between PIC and AIC as well as reduction in oxidative stress within the PVN of hypertensive rats. These data provide direct evidence that reduced activation of central GSK-3β mediates, at least in part, the beneficial effects of regular moderate-intensity exercise on PIC and AIC, possibly via attenuated oxidative stress, thereby contributing to delayed progression of hypertension and improved cardiac function.

PERSPECTIVES

The findings of this study, for the first time, suggest a cause-effect relationship between inactivation of brain GSK-3β and exercise-induced reduction in BP and improvement in cardiac hypertrophy and function in hypertensive animals. Also, this study provide strong evidence that effects of regular moderate-intensity exercise on inflammatory cytokines and oxidative stress in the PVN of hypertensive animals are, at least in part, mediated by brain GSK-3β. Although our *in vitro* studies provide evidence of a direct cause-effect relationship between GSK-3β activation and NFκB/CREB, whether inactivation of GSK-3β by exercise is associated in causal way with these transcription factors remains to be resolved. Additionally, here, we used an AKT inhibitor to increase activated GSK-3β levels. However, use of plasmid vectors to increase cellular levels of GSK-3β or the use of transgenic animals in order to confirm these results could certainly be an important perspective of this study.

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CHAPTER 8

CONCLUDING REMARKS

OVERALL SUMMARY OF FINDINGS

Hypertension is a chronic condition and is an important public-health challenge worldwide. In the year 2000, 26.4% (approximately 972 million) of the adult population had hypertension and this number is projected to increase by about 60% to a total of 1.56 billion by 2025 (Kearney, Whelton et al. 2005). Not only that, hypertension is a major risk factor for various other cardiovascular and renal diseases. Although hypertension is a complex multifactorial disease that results from a complex interaction of genes and environmental factors, physical inactivity has been shown to have strong association with the disease. Therefore, current guidelines for the treatment and prevention of hypertension recommend regular exercise as a part of lifestyle modification for all patients diagnosed with hypertension (Chobanian, Bakris et al. 2003). However, the amount and/or intensity and type of exercise necessary to induce satisfactory improvement in BP, particularly in hypertensive patients, is still not clear. Therefore, the aim of this dissertation was to understand the exact underlying mechanisms by which exercise attenuates BP and improves cardiac function in hypertension.

Hypertension is characterized by chronic low-grade inflammation as reflected by two- to three-fold increase in circulating levels of various pro-inflammatory cytokines (PICs), such as tumor necrosis factor-*alpha* (TNF-α), interleukin (IL)-1β and IL-6 (Dorffel, Latsch et al. 1999; Chae, Lee et al. 2001; Peeters, Netea et al. 2001). Besides increased PICs, downregulation of anti-inflammatory defense mechanisms in the body have recently been shown to contribute to the pathogenesis of hypertension. The renin-angiotensin system (RAS), directly or indirectly through PICs, increases sympathetic activity and modulates the hypertensive response. PICs have been found to activate reactive oxygen species (ROS) (Cai and Harrison 2000; Mariappan, Soorappan et al. 2007; Neri, Cerretani et al. 2007), which in turn can activate various intracellular signaling

pathways, including that of nuclear factor-kappa B (NFκB), and eventually leading to the progression of hypertension. It has been known for decades that regular exercise reduces BP in hypertensive patients; however, the effects of exercise on progression of hypertension have not been investigated before. Importantly, whether exercise modulates the above mentioned signaling pathways is not clearly understood.

In Chapter 2, we aimed to investigate whether chronic moderate-intensity exercise training (ExT) would delay the progression of hypertension in young spontaneously hypertensive rats (SHR), and if so, what are the mechanisms of exercise-induced effects within the heart. Regular moderate-intensity ExT of 16 weeks duration was found to delay the progression of hypertension, reduce cardiac hypertrophy, and improve diastolic cardiac function in young SHR. Our results also showed that training-induced beneficial effects in SHR rats are mediated by decreased myocardial and circulating TNF-α and IL-1β and improved redox homeostasis, possibly via reduced myocardial NFκB activity. These findings provide mechanistic evidence for the involvement of PICs, redox homeostasis, and NFκB in exercise-induced delayed progression of hypertension and cardiac improvements in SHRs.

Chapter 3 acknowledges that the existing local RAS within the brain plays a critical role in the pathogenesis of hypertension and that inflammatory cytokines act as neuromodulators and play a pivotal role in BP regulation (Shi, Raizada et al. 2010). Here, we showed that regular moderate-intensity exercise of 16 weeks duration improves balance between pro- and anti-inflammatory cytokines by attenuating PICs (TNF-α, IL-1β) and upregulating anti-inflammatory IL-10 expression in the cardiovascular regulatory centers of the brain (PVN and RVLM) of SHR. Our results also showed that chronic exercise in hypertensive rats downregulates vasoconstrictor components of RAS and upregulates the vasoprotective components in the brain and that

exercise attenuates oxidative stress in the PVN and RVLM of SHRs, possibly by reducing sympathoexcitation. These results suggest that chronic exercise not only attenuates PICs and the vasoconstrictor axis of the RAS but also attenuates sympathoexcitation, improves anti-inflammatory defense mechanisms and vasoprotective axis of the RAS in the brain, which, at least in part, explains the blood pressure-lowering effects of exercise in hypertension.

In Chapter 4, we acknowledge that despite the known benefits of exercise, non-compliance with exercise has recently been reported to be closely associated with poor outcomes of the disease and is becoming a universal problem (Ahmed, Abdul Khaliq et al. 2008). However, the effects of cessation of exercise (physical detraining) at the physiological and molecular levels in hypertension are far from understood. We demonstrated that two weeks of detraining did not abolish the exercise-induced attenuation in MAP in hypertensive rats, although, it failed to completely preserve the exercise-mediated improvement in cardiac hypertrophy and diastolic function. We also observed that two weeks of detraining does not have any detrimental effects on exercise-induced improvement in PICs and $gp91^{phox}$ levels in the PVN of hypertensive rats, but, improvements in IL-10 and iNOS levels were completely abolished. The findings led us to conclude that 2 weeks of detraining is not long enough to completely abolish the exercise-induced beneficial effects; however, further cessation of exercise may lead to complete reversal of the beneficial effects.

In Chapter 5, using an *in vitro* approach, we demonstrated that AngII causes an imbalance between PIC and AIC in rat neuronal cells by upregulating binding of CBP to NFκB and downregulating binding of CBP to CREB. AngII causes increased phosphorylation of GSK-3β at Tyr-216 and increased p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio, indicating increased activation of GSK-3β. We also demonstrated that AngII-induced effects in neuronal cells were

reversed by lentiviral-mediated silencing of GSK-3β. This data provides evidence that AngII-induced imbalance between PICs and AIC are mediated by GSK-3β, potentially via alterations in activity of downstream transcription factors in rat neuronal cells.

As discussed in Chapter 5 AngII causes an increased activation of GSK-3β in neuronal cells. In Chapter 6 using an *in vivo* approach, we examined the effects of AngII on GSK-3β and transcription factors within the PVN of hypertensive rats. We also examined whether regular exercise reverses AngII-induced aforementioned changes in the PVN. Chronic subcutaneous infusion of AngII resulted in dysregulation between PIC and AIC and an increased activation of GSK-3β within the PVN. These results were also associated with increased p-p65(Ser-276) and reduced p-CREB(Ser-133) levels, suggesting downregulation of CREB and upregulation of NFκB. Importantly, our results demonstrated that regular ExT prevented AngII-induced activation of GSK-3β and altered activity of NFκB and CREB. These results suggest that regular exercise attenuates AngII-induced GSK-3β activation leading to alterations in NFκB- and CREB-mediated gene transcription in favor of anti-inflammatory cytokines, which explains the underlying mechanisms by which exercise improves balance between PIC and AIC and thereby contributes to attenuated MAP in hypertensive rats.

Finally, in Chapter 7, we investigated the effects of increased expression of active GSK-3β (p-GSK3β(Tyr-216)) in the brain on exercise-induced alterations in cytokines in the PVN in AngII-induced hypertension. Our results showed that regular moderate-intensity exercise delayed the progression of hypertension, reduced cardiac hypertrophy, and improved diastolic function in AngII-induced hypertensive rats, and increased levels of active GSK-3β in the brain prevented these beneficial changes. We also observed that increased expression of p-GSK3β(Tyr-216) prevented exercise-induced reduction in TNF-α and IL-1β and increase in anti-inflammatory IL-

10. Furthermore, central increase of active GSK-3β completely reversed the exercise-induced reduction in oxidative stress within the PVN of hypertensive rats. These findings demonstrate that the beneficial effects of regular moderate-intensity exercise in hypertension are mediated, at least in part, by reduced activation of central GSK-3β and potentially via improvement in inflammatory cytokines and oxidative stress within the PVN.

SIGNIFICANCE OF RESEARCH

Various currently available pharmacological therapies targeting the components of the RAS have been proven to reduce BP; however, the morbidity and mortality caused by hypertension is still on the rise. Moreover, only one-third of hypertensive patients can be successfully treated with one antihypertensive agent, the other two-thirds require two or more agents for effective BP control (Marc and Llorens-Cortes 2011), diverting the attention of the scientific community toward other therapeutic approaches that have the capability to target multiple components of the pathogenic signaling pathways at the same time.

Recent guidelines for the treatment and prevention of hypertension recommend regular physical activity as a life-style modification for all patients diagnosed with hypertension. However, the specific quantity and quality of physical activity necessary for the attainment of the health benefits, particularly in the diseased condition, are less clear. Moreover, effects of regular exercise on the progression of hypertension and the exact mechanisms underlying the beneficial effects of exercise are far from completely understood. Thus, understanding cellular and molecular mechanisms of effects of regular exercise would help us to refine the current guidelines that can lead to maximum benefit without any adverse outcomes.

We believe that our studies have provided greater insight into the effects of regular exercise on the progression of hypertension, cardiac function, and elucidated the role of

inflammatory cytokines, oxidative stress, and glycogen synthase kinase (GSK)-3β in exercise-mediated effects in hypertension. It is clear from these studies that exercise-mediated delayed progression of hypertension and improved cardiac function is not only limited to genetic hypertension. We have shown that unlike available pharmacological therapies, exercise has the capability to modulate several components of the signaling pathways, such as inflammatory cytokines, oxidative stress, and downstream transcription factors within the heart and brain, potentially via central GSK-3β, eventually leading to dramatic improvements in BP and cardiac function. It is also clear from these studies that non-compliance with the exercise regimen can lead to slow but complete reversal of beneficial effects both at physiological and molecular levels.

FUTURE DIRECTIONS

The aforementioned studies have made significant contributions to understanding the beneficial effects of regular exercise in hypertension and the basic mechanisms by which exercise exerts those beneficial effects. Although the studies provide strong evidence of role of central GSK-3β in mediating exercise-induced improvements in inflammatory cytokines and oxidative stress, these results should be confirmed by using transgenic animals and by a direct measurement of GSK-3β activity. Also, the *in vitro* studies provide evidence of a direct cause-effect relationship between GSK-3β activation and NFκB and CREB; however, whether inactivation of GSK-3β by exercise is associated in a causal way with these transcription factors remains to be resolved.

Finally, an understanding of the upstream signaling pathways contributing to increased GSK-3β activation in hypertension and exercise-mediated inactivation of GSK-3β will help us to gain more in-depth insight into the underlying mechanisms. In summary, our studies have laid a

foundation for a collection of future studies aimed at better understanding the signal transduction pathways involved in exercise-induced pressure-lowering and cardio-protective effects.

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APPENDIX

LETTER OF PERMISSION

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VITA

Deepmala Agarwal was born in Satna, Madhya Pradesh, India, to Kirtan Prasad Agrawal and Asha Agrawal. Following high school, she chose to pursue Bachelor of Veterinary Science and Animal Husbandry (BVSc&AH) program at Jawaharlal Nehru Krishi Vishwa Vidyalaya, Mhow, India, in 1999. The BVSc&AH program provided her an opportunity to obtain comprehensive knowledge of subjects related to both husbandry and clinical aspects of animal health and disease. She received her Bachelor's degree in 2004 and stood in the top 3% of student at the university. Then, through a highly competitive national level entrance examination, she secured a seat for master study (MVSc) in Veterinary Clinical Medicine at Tamilnadu Veterinary and Animal Sciences University (TANUVAS), Chennai, India. Her clinical and research experience during MVSc program led her to specialize in the field of canine cardiovascular disorders. She earned her Master's in 2006. Thereafter, she worked as a senior research fellow in the Department of Veterinary Pharmacology at TANUVAS, India, until December 2007. In January 2008, 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. She studied under the mentorship of Associate Professor Joseph Francis (BVSc&AH, MVSc, PhD) and began her career as a research scientist, specializing in cardiovascular pathophysiology. During these studies, she married Rahul Dange (BVSc&AH, MVSc) and was blessed with a little angel. Following her graduation with her doctoral degree in May, 2012, she will continue to expand her knowledge in the field of exercise physiology, particularly in relation to cardiovascular diseases, as a postdoctoral fellow. Her long term goal is to blossom as a full fledge researcher in the field of cardiovascular physiology and eventually contribute to improve the quality of life of suffering animals and human beings.