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Chronic exercise modulates RAS components and improves balance between pro-and anti-inflammatory cytokines in the brain of SHR

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Abstract

Recently, exercise has been recommended as a part of lifestyle modification for all hypertensive patients; however, the precise mechanisms of its effects on hypertension are largely unknown. Therefore, this study aimed to investigate the mechanisms within the brain that can influence exercise-induced effects in an animal model of human essential hypertension. Young normotensive WKY and SHR rats were given moderate-intensity exercise for 16 weeks. Blood pressure was measured bi-weekly by tail-cuff method. Animals were then euthanized; paraventricular nucleus (PVN) and rostral ventrolateral medulla (RVLM), important cardiovascular regulatory centers in the brain, were collected and analyzed by Real-time RT-PCR, western blot, EIA, and fluorescent microscopy. Exercise of 16 weeks duration attenuated systolic, diastolic, and mean arterial pressure in SHR. Sedentary SHR exhibited increased proinflammatory cytokines (PICs) and decreased anti-inflammatory IL-10 levels in the PVN and RVLM. Furthermore, SHRsed rats exhibited elevated levels of ACE, AT1R, and decreased levels of ACE2 and receptor Mas in the PVN and RVLM. Chronic exercise not only prevented the increase in PICs (TNF- α , IL-1 β), ACE, and AT1R protein expression in the brain of SHR, but also dramatically upregulated IL-10, ACE2, and Mas receptor expression in SHR. In addition, these changes were associated with reduced plasma AngII levels, reduced neuronal activity, reduced NADPH-oxidase subunit gp91^{phox} and inducible NO synthase (iNOS) in trained SHRs indicating reduced oxidative stress. These results suggest 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, which, at least in part, explains the blood pressure-lowering effects of exercise in hypertension.

Keywords

Exercise; cytokines; angiotensin; hypertension; oxidative stress; brain

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2. Introduction

Hypertension is the most common chronic disease in the United States, currently affecting more than 33% of US adults[36]. 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[25]. 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)[53]. In addition, recent discoveries indicate that besides elevated levels of circulating and brain PICs[48, 53], 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[53]. 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 [62]. 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[4, 43, 53, 66].

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[7]. Several experimental, clinical, and epidemiological studies have clearly shown that physical activity reduces BP in animals and humans[3, 58]. 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[13, 38, 65] and diabetic patients[19] 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 vasoconstrictory 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.

3. Materials and Methods

3.1 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- ($25\pm 1^{\circ}\text{C}$) 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.

3.2 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 RVLN 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.

3.3 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° 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 [6, 58]. 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.

3.4 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. Soleus 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 [47]. CS activity was measured from whole muscle homogenate by using commercially 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.

3.5 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[3]. 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.

3.6 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- α (TNF- α), and interleukin (IL)-1 β ; oxidative stress markers viz. gp91^{phox}, and iNOS in the PVN and RVLM by using specific primers. Rat primers used are listed in the Table 2. 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 μ m thickness, and the PVN and RVLM were punched from each brain according to the methods described by Palkovits and Brownstein [15]. Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described [57]. Gene expression was measured by the $\Delta\Delta$ CT 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.

3.7 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-1 β), 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 μ l 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 μ g) were combined with an equal volume of $\times$ 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°C with the primary antibodies. Specific antibodies used included TNF- α , IL-1 β , gp91^{phox}, iNOS, GAPDH, AT1R, ACE, and ACE2, GAD₆₇, 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, GAD₆₇ (Abcam Inc, MA); IL-1 β , 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 ($\times$ 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.

3.8 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* [5]. 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 μ m) 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), at 1:50 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.

3.9 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 [3, 26].

3.10 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).

3.11 Statistical Analysis

All data are presented as means $\pm$ 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 within group changes over time. Results were considered significant when $p < 0.05$.

4. Results

4.1 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 (Fig1). CS activity was higher in WKY rats compared with SHR both in the exercise and the sedentary group.

4.2 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 (Table1).

4.3 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 (Fig 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 (Fig 2).

4.4 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-1 β in the PVN and RVLM. We

observed that SHRsed rats exhibited marked increases in TNF- α and IL-1 β expression in the PVN (Fig 3a, 3b, and 6) as well as in the RVLM (Fig 3c, 3d) compared to WKsed. This upregulation of TNF- α and IL-1 β 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-1 β expression in the PVN (Fig 3a), whereas these changes were 6 fold and 4 fold, respectively in the RVLM of SHRex rats (Fig 3c). Exercise did not change PIC levels in WK rats.

4.5 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 (Fig 3a, 3b) as well as RVLM (Fig 3c, 3d, and 7). Moreover, SHRsed rats had higher TNF- α /IL-10 ratio in the PVN (Fig 3b) and RVLM (Fig 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.

4.6 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 (Fig 4 and 6) and RVLM (Fig 5 and 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 RVLM, respectively when compared to SHRsed (Fig 4a and 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 1).

4.7 Chronic exercise reduces 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 control groups. Expression of gp91^{phox} was markedly higher in SHRsed when compared to WKsed rats; this expression was significantly reduced by chronic exercise (Fig 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 (Fig 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 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 (Fig 8). These results further confirm that exercise has ability to reduced oxidative stress in the brain of hypertensive rats.

4.8 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 familygene; 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 (GAD₆₇) 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 (Fig 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 GAD₆₇ in the PVN when compared to WKsed rats (Fig 9b). Interestingly, exercise significantly reduces TH levels, whereas, GAD₆₇ levels were upregulated in SHRex rats compared to SHRsed. However, in the RVLM, there was no significant difference of TH and GAD₆₇ levels among all groups (Fig 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 not change plasma NE level in WK rats (Table 1). Taken together, these results indicate that chronic exercise attenuates sympathoexcitation, possibly by altering neurotransmitter levels in the PVN of hypertensive rats.

4 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-1 β) 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 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 SHR rats compared with SHR sed 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 SHR rats. 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 [48, 53]. 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 [53]. More importantly, several cross-sectional studies demonstrated an association between physical inactivity and low-grade systemic inflammation [1, 18]. 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 [3, 39, 49], 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-1 β) in hypertensive rats. More importantly, brain IL-10 levels were dramatically upregulated and TNF- α /IL-10 ratio was reduced in trained SHR rats. 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) [46]. 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 [55]. Since, the two TNF- α receptors, TNFR1 and TNFR2, have been shown to differentially regulate cardiac and endothelial function *in vitro* and *in vivo* [17, 52], 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 [11]. Therefore, it is possible that exercise induced increase in IL-10 may be responsible for the observed decrease in TNF- α and IL-1 β . 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 cardiomyocyte hypertrophy, left ventricular dilation, and diastolic dysfunction; whereas, anti-TNF- α treatment preserves diastolic dysfunction [52]. Therefore, it can be speculated that exercise-induced reduction in TNF- α may be responsible for improved diastolic dysfunction in trained SHR rats as reported previously [3]. 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 [44, 45]. 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 [9]. We and others have previously shown that PICs, particularly TNF- α , mediates AngII-induced hypertension, cardiac hypertrophy [57], endothelial and cardiac dysfunction [31], and modulates RAS components in the PVN in rats with heart failure (HF) [27]. 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 SHR. 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 [16, 35], and increases Mas receptor expression in the left ventricle of SHR [14]. 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 [29]. 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 SHR (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 SHR, however, the effects were more pronounced in young rats (60% and 39% reduction in plasma AngII in young and adult SHR, respectively) [63]. 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 [8]. 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 [32]. 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 SHR 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 [56]. 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 [12, 22]. Hypertension is often found to be associated with increased levels of excitatory neurotransmitter, norepinephrine (NE) [3] and deficit in inhibitory GABAergic system in the cardiovascular regulatory regions of the brain [24]. In this study, we demonstrated that SHR had significantly reduced levels of GAD₆₇, a 67-kDa isoform of GAD, and increased tyrosine hydroxylase (TH) when compared to WK. Concomitantly, when compared to WK, SHR 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 GAD₆₇ in the PVN of SHR, 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 [27, 37, 50, 60, 64]. In addition, it has been suggested that TNF-induced imbalance in neurotransmitters in the PVN and RVLM, possibly via oxidative stress, contributes to sympathoexcitation [21, 28]. 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 [40, 66]. 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 [41, 43, 54]. Of various isoforms of NOX, role of NOX2 (also known as gp91^{phox}) in AngII-induced hypertension and endothelial dysfunction is well established [42]. 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 SHR. 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 [51], thoracic aorta of SHR [20], aging arteries of rat [34], and human mammary arteries [2]. 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 [41], 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 [23]; 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 [59]. 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 [10]. 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 [61].

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 hypertension and therefore, may lead us to design an exercise regimen resulting in maximum cardio-protective benefits in hypertensive patients.

5 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 [33].

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 [30]. 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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Fig 1.

Citrate synthase activity (nmol/min/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

Fig 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 SHRsed rats from 8 weeks of exercise (at 15 weeks of age). Values are mean $\pm$ SE; n=10 in each group. *p0.05 WKsed vs SHRsed; †p0.05 SHRsed vs SHRex

Fig 3.

Effects of exercise on TNF- α , IL-1 β , and IL-10 expression in the PVN and RVLM of WK rats and SHRs. mRNA expression in the PVN (a); A representative Western blot (left panel, b) and densitometric analysis (right panel, b) of TNF- α and IL-10 protein expression in the PVN; mRNA expression (c) and densitometric analysis (d) of these cytokines in the RVLM. Values are mean $\pm$ 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

Fig 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

Fig 6.

A representative confocal photomicrographs ($\times 20$) 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 μ m. 3V, third ventricle

Fig 7.
A representative confocal photomicrographs ($\times 20$) showing the effects of exercise on protein expression of ACE, AT1R, ACE2, and IL-10 in the RVLM of WK rats and SHRs (n=5/group). Scale bar=100 μ m

Fig 8.

Effects of exercise on gp91^{phox} and iNOS expression in the PVN and RVLM of WK rats and SHRs. mRNA and protein expression of gp91^{phox} and iNOS in the PVN (a) and RVLM (b). Values are mean±SE. *p<0.05 WKsed vs. SHRsed; †p<0.05 SHRsed vs SHRex. N=9 per group for mRNA analysis and n=6 per group for protein analysis

Fig 9.

Effects of exercise on sympathoexcitation in the PVN of WK rats and SHRs. a, A representative confocal photomicrographs ($\times 10$, scale bar=200 μ m; and $\times 40$, scale bar=50 μ m) 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 GAD₆₇ in the PVN accompanied with a representative Western blot. Values are mean $\pm$ SE; *p<0.05 WKsed vs. SHRsed; †p<0.05 SHRsed vs SHRex. 3V, third ventricle

TH and GAD₆₇ protein expression in RVLM **Fig 10.**

Densitometric analysis (n=6/group) of protein expression of TH and GAD₆₇ in the RVLM of WK rats and SHRs. In the RVLM, there was no significant difference of TH and GAD₆₇ levels among all groups. Values are mean±SE

Table 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.

Table 2
Rat primers used for real-time RT-PCR

Gene	Sense	Antisense
GAPDH	agacagccgcatcttctgt	cttgccgtgggtagagtcac
TNF- α	gtcgtagcaaccaccaagc	tgtgggtgaggagcacatag
IL-1 β	gcaatggtcgggacatagtt	agacctgacttggcagaga
IL-10	gggaagcaactgaaacttcg	atcatggaaggcaacctg
ACE	ttgacgtgagcaactccag	cagatcaggctccagtaca
ACE2	acccttctacatcagccctactg	tgtccaaaacctacccacatat
AT1R	cacgagacacagccttc	ttaaaatcgaccgtgtgcag
Mas	tccaccaagacctgctagga	tcttgtctggaccattca
gp91 ^{phox}	cggaatctctctctctctct	gcattcacaccactccac
iNOS	ccttggtcagctacgccttc	ggatgcccggagtctttca