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Original Paper

Exercise Training Attenuates Proinflammatory Cytokines, Oxidative Stress and Modulates Neurotransmitters in the Rostral Ventrolateral Medulla of Salt-Induced Hypertensive Rats

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Key Words
Exercise training • Rostral ventrolateral medulla • Neurotransmitters • Nuclear factor-kappa B • Oxidative stress • Hypertension

Abstract
Background/Aims: Exercise training (ExT) was associated with cardiovascular diseases including hypertension. The rostral ventrolateral medulla (RVLM) is a key region for central control of blood pressure and sympathetic nerve activity. Therefore, this study aimed to investigate the mechanisms within RVLM that can influence exercise training induced effects in salt-induced hypertension. ***Methods:*** Male Wistar rats were fed with a normal salt (0.3%) (NS) or a high salt (8%) (HS) diet for 12 weeks to induce hypertension. Then these rats were given moderate-intensity ExT for a period of 12 weeks. RVLM was used to determine glutamate and gamma-aminobutyric acid (HPLC), phosphorylated IKKβ, Fra-LI, 67-kDa isoform of glutamate decarboxylase (GAD67), proinflammatory cytokines (PIC) and NADPH-oxidase (NOX) subunits expression (Immunohistochemistry and Immunofluorescence, Western blotting). PIC and NF-κB p65 activity in the plasma were evaluated by ELISA studies. Renal sympathetic nerve activity (RSNA) was recorded and analyzed using the PowerLab system. ***Results:*** High salt diet resulted in increased mean arterial pressure and cardiac hypertrophy. These high salt diet rats had higher RVLM levels of glutamate, PIC, phosphorylated IKKβ, NF-κB p65 activity, Fra-LI, superoxide, NOX- 2 ($gp91^{phox}$) and 4, and lower RVLM levels of gamma-aminobutyric acid and GAD67, and higher plasma levels of PIC, norepinephrine, and higher RSNA. ExT attenuated

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these changes in salt-induced hypertensive rats. ***Conclusions:*** These findings suggest that high salt diet increases the activity of NF-κB and the levels of PIC and oxidative stress, and induces an imbalance between excitatory and inhibitory neurotransmitters in the RVLM. ExT attenuates hypertension and cardiac hypertrophy partially mediated by attenuating oxidative stress and modulating neurotransmitters in the RVLM.

Introduction

Hypertension is characterized by cardiac hypertrophy and dysfunction, and elevated levels of blood pressure (BP) and sympathetic tone [1]. It is well known that the rostral ventrolateral medulla (RVLM) is a main active region for central regulation of the cardiovascular function and plays a key role in maintaining resting BP and sympathetic tone [2, 3]. A growing body of evidence indicates that abnormalities in structure and function of the RVLM contribute to the pathogenesis of hypertension [3, 4].

We and others have indicated that increased pro-inflammatory cytokines (PIC) such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6 in the rostral ventrolateral medulla (RVLM) have a significant impact on sympathetic outflow, arterial pressure and cardiac remodeling in experimental models of hypertension [5]. High level of BP and sympathetic overactivity are also closely related to the enhancement of reactive oxygen species (ROS) in the RVLM under the hypertensive state [5, 6]. Over- production of ROS can activate several redox-sensitive signal transduction pathways such as nuclear factor-kappaB (NF-κB). Activation of NF-κB induces transcription of PIC genes, leading to further increases in ROS production and fostering a cyclic positive feedback mechanism, thereby accelerating the progression of hypertension and its associated heart changes [7].

Exercise training (ExT) is an efficient strategy for the prevention and treatment of hypertension, and is acknowledged by all major institutes. It is reported that ExT effectively lowers BP, decreases cardiac output, and enhances the baroreflex sensitivity in patients [8] and animals [6, 9, 10] with hypertension. However, the exact mechanisms of exercise-induced effects in hypertension are still poorly understood. Previous studies often focused on the peripheral mechanisms which ExT may act on, such as reduced peripheral vascular resistance [11], reductions in oxidative stress [12] and predominance of endothelium relaxing over contractile factors [13].

Recent evidence suggested that neural plasticity in the central cardiovascular networks can be more important for the beneficial effects of ExT on cardiovascular dysfunctions [14-17]. Growing evidence has been demonstrated that ExT significantly attenuates increases in BP and sympathetic activity induced by stimulation of the RVLM [18], suggesting the importance of the RVLM in mediating the effects of ExT on cardiovascular regulation. Therefore, this study was designed to investigate whether exercise training attenuates blood pressure, sympathetic nerve activity and cardiac hypertrophy by modulating pro-inflammatory cytokines, oxidative stress and neurotransmitters within RVLM in salt induced hypertension.

Materials and Methods

Animals and Experimental Design

Male Wistar rats were received at 6 weeks of age and were allowed to acclimate for 1 week with standard rat diet (Beijing Vital River Laboratory Animal Technology Co., Ltd, China) and tap water before beginning the experiment. Rats were housed at a constant room temperature, humidity and light cycle (12:12 h light–dark) with free access to tap water and fed with standard rat chow *ad libitum*. During the 24-week experimental period, the NS group received a normal salt (0.3%) diet (Beijing Vital River Laboratory Animal Technology Co., Ltd, China) and the HS group received a high salt (8%) diet (Beijing Vital River Laboratory Animal Technology Co., Ltd, China). At the 12th week, these rats were randomly selected and

received moderate-intensity exercise trained or sedentary for a period of 12 weeks. Therefore, 4 groups (n=30/group) were included in this study: (1) Wistar normal salt diet (0.3% NaCl) + sedentary (NS + Sed) (2) Wistar normal salt diet (0.3% NaCl) + exercise trained (NS + ExT) (3) Wistar high salt diet (8% NaCl) + sedentary (HS + Sed) (4) Wistar high salt diet (8% NaCl) + exercise trained (HS + ExT) (Fig. 1). All animal and experimental procedures in this study were reviewed and approved by the Animal Care and Use Committees of Xi'an Jiaotong University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1996). At the end of the study, rats were euthanized; the blood, brains and heart tissues were collected, and immediately frozen on dry ice. Some rats (n=21 for each group) were used for hemodynamic and anatomical studies. The rostral ventrolateral medulla (RVLM) tissues were punched out from the brain for western blot analysis or for immunohistochemistry assay (n=7 for each group).

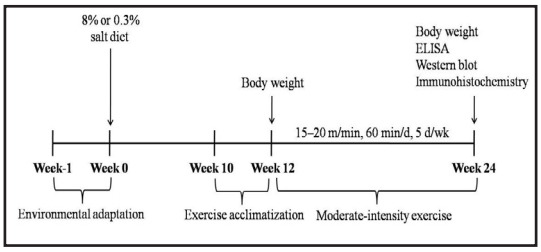

Fig. 1. Experimental Design. Six-week-old male Wistar rats were first acclimatized to the environment for 1 week before the start of the experiment. After 1 week of acclimation, the rats received a normal salt (0.3%) diet or high salt (8%) diet for 24 weeks. At the 10th week, the rats were acclimatized to exercise trained for 2 week. At the 12th week, these rats were randomly selected and received moderate-intensity exercise trained or sedentary for a period of 12 weeks. At the 24th week, animals were weighed and the parameter was measured. The animals were then euthanized and the brains were collected for Immunohistochemistry and Western blot analysis.

Exercise training protocol

Exercise training was performed on a motor treadmill (FT-200, Taimen Co., Cheng du, China) for 12 weeks (5 days per week; 60 min per day, 0° inclination), which includes an acclimation period of 2 weeks. After acclimation, training intensity was set at 55–65% of maximal aerobic velocity (MAV), which corresponds to moderate-intensity exercise (15–20 m/min). This training intensity was maintained throughout the study period. To determine the MAV, rats were submitted to an incremental exercise test as reported previously [15, 19, 20]. The rats in sedentary groups (NS + Sed and HS + Sed) were placed on a nonmoving treadmill during the training sessions.

Blood pressure measurements

Blood pressure (BP) was measured in conscious animals by a tail-cuff occlusion method [21, 22]. BP was measured at baseline and then every 2 weeks until the end of the study period. Unanesthetized rats were warmed to an ambient temperature of 30 °C by placing rats in a holding device mounted on a thermostatically controlled warming plate. Blood pressure values were averaged from at least seven consecutive cycles obtained from each rat.

Hemodynamic analyses and Renal Sympathetic Nerve Recordings

The preparation for measuring hemodynamic and renal sympathetic nerve activity (RSNA) parameters was performed as previously described [23, 24]. Briefly, rats were anaesthetized with a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture (ip). The carotid artery and jugular vein were cannulated, and the arterial line was connected to a pressure transducer (Gould P23 1D) for data recording and analysis (PowerLab, AD Instruments, Castle Hill, Australia) of systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR). Next, a retroperitoneal incision was made and the left renal sympathetic nerve was isolated. The renal nerve was placed on a platinum electrode which is connected with the recording system and immersed in warm mineral oil. The electrical signal was amplified with high- and low-frequency cutoffs of 1, 000 and 100 Hz, respectively (Grass amplifier). The rectified output from the amplifier (RC filtered, time constant: 0.5 s) was displayed using the PowerLab system (8si, AD Instruments) to record and integrate the raw renal nerve discharge. Baseline recordings of RSNA, arterial blood pressure (ABR) and HR were taken for several minutes. Maximum RSNA was detected using an intravenous bolus administration

of sodium nitroprusside (SNP, 10 μg), which lowered ABP to between 45 and 50 mmHg. Arterial baroreflex control of RSNA was determined with the response of these parameters to an injection of SNP (10 μg). The background noise defined as the signal-recorded post-mortem was subtracted from actual RSNA and expressed as percent of maximum (in response to SNP) at the end of the experiment. Because the maximum activity was similar with the SNP, we elected to use the response to SNP to determine maximum activity in this study.

Arterial baroreflex curves were constructed as we described previously [25]. In brief, several points for RSNA were taken during the fall in ABP after the administration of SNP. The logistic regression curve as described by Kent et al [26]. was fit to the data points with the following equation:

$RSNA=A_1/\{1+\exp [A_2(MAP-A_3)]\}+A_4$

Where A_1 is RSNA range, A_2 is the slope coefficient, A_3 is the pressure at the midpoint of the range, and A_4 is minimum RSNA. The peak slope (or maximum gain) was determined by taking the first derivative of the baroreflex curve described by equation 1. The first derivative is described by equation 2.

$Slope=\{ A_1 \times A_2 \times \exp [A_2(MAP-A_3)]\}/\{1+ \exp [A_2(MAP-A_3)]\}^2$

The mean value of each curve parameter was used to derive a composite curve for each group of rats before and after each intervention.

Collection of blood and tissue samples

At the end of the study, rats were anesthetized with a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture (ip). Blood samples were collected from the abdominal aorta with heparin-moistened syringes in chilled EDTA tubes. The plasma samples were separated by centrifugation (3000x *g* for 25 min at 4 °C) and stored at -80 °C until assayed for PIC and norepinephrine (NE) levels. The brain and heart were removed and harvested quickly. The heart weight/body weight (HW/BW) ratio was measured as previously described [21]. The RVLM tissue was punched from each brain according to the methods as previously described [4]. Briefly, rats were anesthetized with a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture (ip) and the brains were quickly removed, frozen, and stored at -80 °C until use. Freshly frozen brains were cut on a cryostat to get coronal brain sections according to the rat brain atlas. The RVLM punches were made from the frozen brain sections using Stoelting brain punch (Stoelting, IL, USA).

Measurement of RVLM tissue levels of glutamate, GABA and of plasma NE

RVLM levels of glutamate and GABA were determined by High-performance liquid chromatography with electrochemical detection (ECD, Waters-2465, Waters Corporation, USA) as described previously [27]. Briefly, samples or standards were homogenized in 0.1 N perchloric acid (200 μl per sample) and centrifuged at 3, 000 rpm for 3 min. The pellets were washed and resuspended in 1 N NaOH, and the amount of total protein in each sample was determined by the Bradford method. The separation of glutamate and GABA was achieved with a Novapark C_{18} reverse-phase column (150×4.6 mm, 4 μm particle size, Waters; mobile phase: 100 mM di-sodium hydrogen phosphate anhydrous, 20% methanol, and 3.5% acetonitrile at pH 6.8) at 32.5 °C, the flow rate was 1 ml/min delivered by a Waters pump and then each neurotransmitter was measured by pre-column derivation and ECD. In brief, derivation was achieved by mixing 20 μl of working derivation reagent (6.75 mg o-phtaldialdehyde, 2.5 % methanol, 1.25 μl 2-β-mercaptoethanol and 97.5 % 0.1 M sodium tetraborate buffer) with 40 μl of filtered supernatant (Nylon-membrane/0.45 lm pore size). The detect channel potentials were set at +550 mV.

The concentration of NE in plasma was determined as described previously [22]. Briefly, plasma samples were collected after rats were decapitated and acidified with glacial acetic acid in 5 ml centrifuge tubes. NE was extracted with 300 μl of 0.2 M glacial acetic acid with 10 min of shaking and a final 40 min settlement; 20 μl of the supernatant was automatically loaded onto a Novapark C_{18} reverse-phase column (150×3.2 mm, 3 μm particle size, Waters) using a refrigerated autoinjector. The mobile phase was composed of 80 mM citric acid monohydrate, 73.4 mM citric acid trisodium salt, 0.12 mM 1-octanesulfonic acid sodium salt, and 0.1 mM EDTA adjust to pH 4.3 with phosphoric acid. The flow rate was set at 0.5 ml/min. Amino acid peaks were identified based on retention time. Extracellular amino acid concentrations were estimated by rationing peak areas of each amino acid and their respective external standard (analytical software: Empower).

Immunohistochemistry and immunofluorescence studies

Immunohistochemical labeling was performed in floating sections (18 μm) as previously described [21, 22] to identify Fra-like (Fra-LI, a marker of chronic neuronal activation), tumour necrosis factor-alpha (TNF-α) and 67-kDa isoform of glutamate decarboxylase (GAD67) (Santa Cruz Biotechnology, Santa Cruz, California) expressions. Superoxide generation was determined by fluorescent-labelled dihydroethidium (DHE, Molecular Probes) staining as previously described [22, 28]. Protein immunofluorescence staining was performed as previously described [21, 29]. The primary phosphorylated IKKβ (p-IKKβ, a marker of NF-κB activation), TNF-α and NAD(P)H oxidase subunit gp91^{phox} antibodies were from Santa Cruz Biotechnology. Conjugated secondary antibodies were used to detect the primary antibody, which included biotinylated secondary antibodies (at 1:300 dilution, ABC staining system kit, Santa Cruz, CA, USA), Alexa 488-labeled anti-rabbit secondary antibody (at 1:200 dilution, green fluorescence), or Alexa 594-labeled anti-mouse secondary antibody (at 1:200 dilution, red fluorescence) (Invitrogen, CA) in 0.1 M PBS (1 hr at room temperature). Immunohistochemistry stained sections were photographed with a conventional light microscopy (DP70, Olympus, Tokyo, Japan). Immunofluorescent staining was visualized with a confocal laser-scanning microscope (Zeiss LSM 710, Carl Zeiss, Inc). For each animal, positive immunofluorescent-staining cells within the RVLM were manually counted in three consecutive sections and an average value was reported.

Western blotting

Measurement of RVLM protein was performed as previously described [28, 30]. Briefly, protein extracted from the RVLM was used for measurements of TNF-α, the 67-kDa isoform of glutamate decarboxylase (GAD67), atrial natriuretic peptide (ANP), beta-myosin heavy chain (β-MHC), NAD(P)H oxidase subunit NOX 2 (gp91^{phox}) and NOX 4 (Santa Cruz Biotechnology Inc, Santa Cruz, California) expressions by western blot. Protein loading was controlled by probing all blots with β-actin antibody (Thermo Scientific, USA) and normalizing TNF-α, GAD67, ANP, β-MHC, NOX 2 and NOX4 protein intensities to that of β-actin. The bands were analyzed using NIH ImageJ software.

ELISA studies

The levels of TNF-α, IL-6 and IL-1β in plasma and tissues were quantified using commercially available rat ELISA kits (Biosource International Inc, Camarillo, California) according to manufacturer's instructions. The NF-κB p65 active ELISA (Active Motif, USA) kit was used to measure the binding activity of free NF-κB p65 in nuclear extracts, as described previously [21, 31].

Statistical analysis

All data are presented as mean ± SE and analyzed using a two-way ANOVA with a Bonferroni post-hoc test. Blood pressure data were analyzed by repeated measures ANOVA to examine with-in group changes over time. Statistical comparison was performed using Prism 5 (GraphPad Software). A probability of $P<0.05$ was considered statistically significant.

Results

Mean arterial pressure (MAP)

As shown in Fig. 2, high salt diet in sedentary rats caused significant increase in MAP starting at week 8 when compared to NS + Sed and remained increased for the duration of the study. At the end of the study, the results of acute experiment also indicated that HS + Sed rats had higher levels of systolic, diastolic, and mean

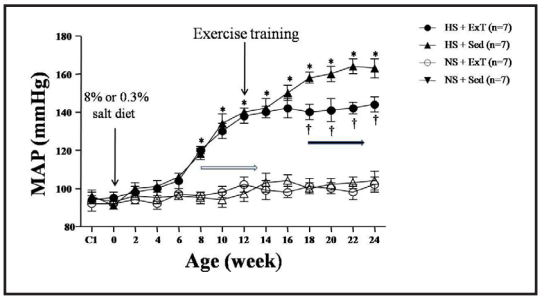

Fig. 2. Time course of mean arterial pressure (MAP) in NS rats and HS rats. MAP was significantly increased in HS + Sed compared with NS + Sed rat from week 8 of high salt diet (empty arrow). MAP was significantly reduced in HS + ExT compared with HS + Sed rat from week 18 of high salt diet (filled arrow). Values are mean ± SE; *P<0.05 vs. NS groups; †P<0.05 HS + Ex vs. HS + Sed.

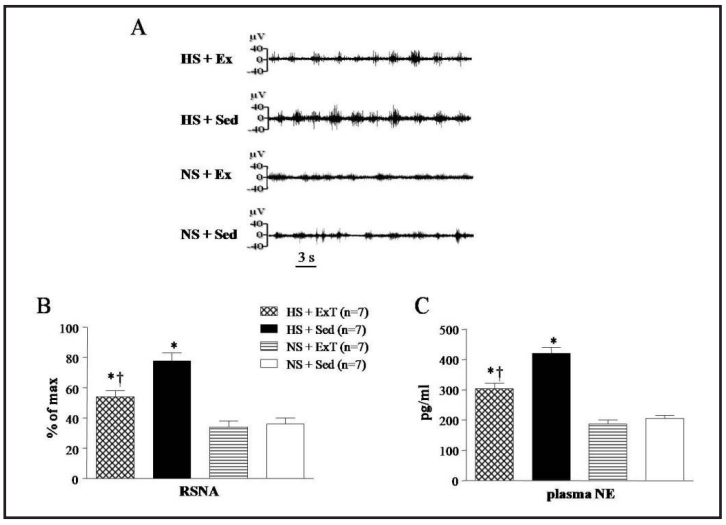

Fig. 3. Effects of exercise training on renal sympathetic nerve activity (RSNA) and the plasma levels of norepinephrine (NE) of NS rats and HS rats. (A) Example of recording RSNA of a rat in each group as indicated. (B) RSNA was increased in HS rats compared with NS rats. Exercise training attenuated RSNA of HS rats. (C) Plasma levels of NE in HS rats were higher than NS rats. Exercise training decreased plasma NE of HS rats. Values are mean ± SE; *P<0.05 vs. NS groups; †P<0.05 HS + Ex vs. HS + Sed.

arterial blood pressure (SBP, DBP, and MAP, respectively) when compared to NS + Sed rats (Table 1). Exercise training for 12 weeks prevented high salt-induced increase in MAP, SBP and DBP in comparison with HS + Sed, the MAP was found to be significantly lower in HS + ExT rats from week 18 of exercise training when compared to HS + Sed rats (Fig. 2, Table 1).

Renal sympathetic nerve activity (RSNA)

Conscious RSNA was measured 5 h after rats recovered from anesthesia. HS + Sed rats exhibited higher RSNA (% of max) when compared with NS + Sed rats. Exercise training attenuated RSNA in HS rats (Fig. 3A and B). Plasma NE, a marker of sympathetic activity, was also higher in HS + Sed rats than in NS + Sed rats. Exercise training prevented the increase in plasma NE of HS rats (Fig. 3C).

Table 1. Haemodynamic and anatomical measurements. Data were obtained at conclusion of study. Data are presented as mean ± SEM. NS, normal salt diet; HS, high salt diet; BW, body weight; HW, heart weight; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; HR, heart rate. Values are mean ± SE. *P<0.05 NS + Ex vs. NS + Sed. #P<0.05 HS + Sed vs. NS groups. †P<0.05 HS + Ex vs. HS + Sed

Parameters	NS + Sed	NS + Ex	HS + Sed	HS + Ex
n	21	21	21	21
Initial BW, g	292 ± 2	290 ± 3	294 ± 4	293 ± 2
Final BW, g	465 ± 6	425 ± 4*	472 ± 9	418 ± 4†
BW gain, g	173 ± 4	135 ± 3*	178 ± 6	125 ± 3†
HW, mg	1851 ± 18	1901 ± 20	2451 ± 26#	1889 ± 20†
HW/BW, mg/g	3.98 ± 0.03	4.47 ± 0.05*	5.19 ± 0.1#	4.51 ± 0.07†
SBP, mmHg	146 ± 15	142 ± 13	188 ± 18#	172 ± 17#†
DBP, mmHg	88 ± 11	84 ± 10	152 ± 15#	113 ± 10#†
MAP, mmHg	107 ± 3	103 ± 2	164 ± 14#	132 ± 13#†
HR, bpm	372 ± 12	330 ± 9*	412 ± 10#	366 ± 8†

TNF-α expression and NF-κB activation in the RVLM

HS + Sed rats showed increases in the level of TNF-α (Fig. 4A and C), p-IKKβ (Fig. 4B and D) and NF-κB p65 activity (Fig. 4E) in the RVLM compared with NS + Sed rats. Exercise training attenuated the increases in RVLM TNF-α expression, NF-κB p65 activity and p-IKKβ in HS rats (Fig. 4).

Fra-LI and GAD67 expression in the RVLM

To determine whether exercise training influences sympathoexcitation in the RVLM, we examined the expression of Fra-like (Fra-LI, fos family gene; indicating chronic neuronal excitation) by immunohistochemistry staining. To further determine whether exercise training induced effects are mediated by alterations in neurotransmitter in the RVLM, we determined the level of 67-kDa isoform of glutamate decarboxylase (GAD67) in the RVLM.

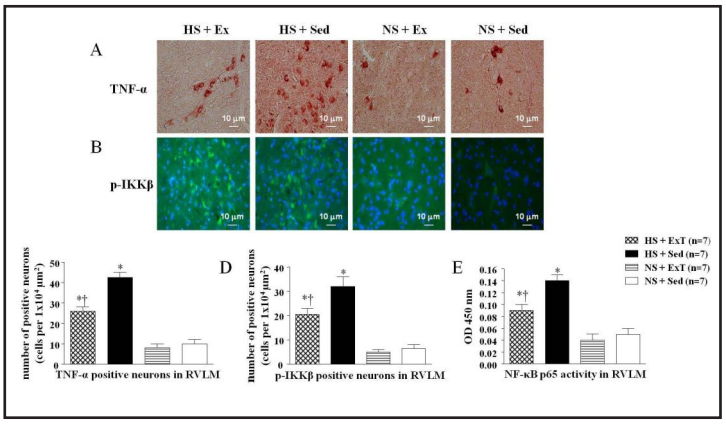

Fig. 4. Effects of exercise training on TNF-α expression and NF-κB activation in the RVLM of NS rats and HS rats. (A and C) A representative immunohistochemistry image and the column diagram showing the effects of exercise training on the positive neurons of TNF-α in the RVLM of NS rats and HS rats. (B and D) A representative immunofluorescence image and the column diagram showing the effects of exercise training on the positive neurons of p-IKKβ in the RVLM of NS rats and HS rats. (E) NF-κB activity assay showing increased NF-κB p65 activity in RVLM of HS + Sed rats when compared to NS + Sed rats, whereas, exercise training attenuated the increases in RVLM NF-κB p65 activity in HS rats. Values are mean ± SE. *P<0.05 vs. NS groups; †P<0.05 HS + Ex vs. HS + Sed.

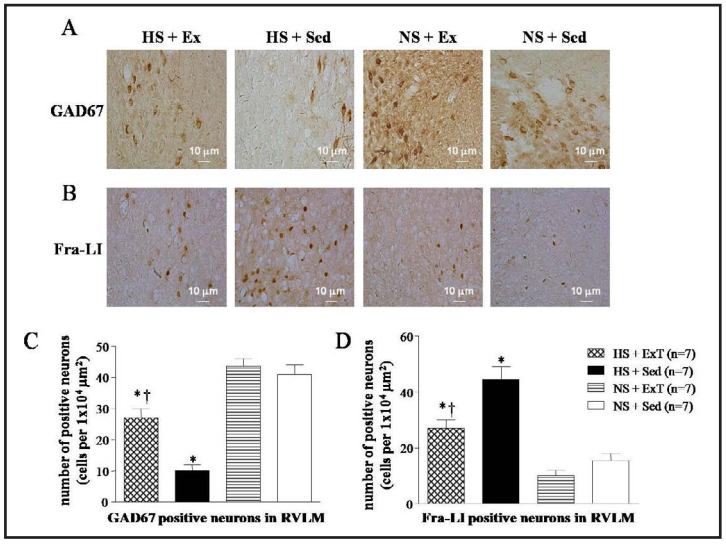

Fig. 5. Effects of exercise training on the 67-kDa isoform of glutamate decarboxylase (GAD67) immunoreactivity and neuronal activity in the RVLM of NS rats and HS rats. (A and C) A representative immunohistochemistry image and the column diagram showing the effects of exercise training on the positive neurons of GAD67 (brown yellow) in the RVLM of NS rats and HS rats. (B and D) A representative immunohistochemistry image and the column diagram showing the effects of exercise training on the Fra-LI (brown dots, an indicator of chronic neuronal excitation) expression in the RVLM of NS rats and HS rats. Values are mean ± SE. *P<0.05 vs. NS groups; †P<0.05 HS + Ex vs. HS + Sed.

We observed that HS + Sed rats exhibited lower level of GAD67 in the RVLM compared to NS + Sed rats. Interestingly, exercise training significantly upregulated GAD67 level in HS rats compared to NS rats (Fig. 5A and C).

Table 2. The RVLM and plasma levels of pro-inflammatory cytokines (n = 7). Values are mean ± SE. *P<0.05 HS + Sed vs. NS groups. †P<0.05 HS + Ex vs. HS + Sed

Group	RVLM (pg/mg protein, n = 7)			Plasma (pg/mL, n = 7)		
	TNF-α	IL-1β	IL-6	TNF-α	IL-1β	IL-6
NS + Ex	2.1 ± 0.2	17.4 ± 1.8	17.9 ± 1.7	11.2 ± 1.5	58.3 ± 5.4	40.2 ± 2.9
NS + Sed	3.0 ± 0.3	19.1 ± 2.2	22.5 ± 2.1	12.4 ± 1.5	60.5 ± 5.7	49.6 ± 3.7
HS + Ex	4.2 ± 0.4*†	34.6 ± 3.2*†	39.6 ± 4.2*†	13.9 ± 1.8†	91.6 ± 8.9*†	85.7 ± 8.2*†
HS + Sed	6.2 ± 0.7*	55.3 ± 4.8*	69.7 ± 6.6*	28.1 ± 3.2*	125.6 ± 12.4*	114.6 ± 10.2*

Furthermore, we found that HS + Sed rats exhibited increased Fra-LI activity in the RVLM neurons compared to NS + Sed rats. Notably, this upregulation of Fra-LI activity was significantly attenuated by exercise training in HS rats. However, exercise training did not change Fra-LI activity in NS rats (Fig. 5B and D).

PICs in the RVLM and plasma

To investigate the influence of exercise training on PICs within the RVLM and plasma of hypertensive rats, we examined the levels of TNF-α, IL-6 and IL-1β in the RVLM and plasma. We observed that the levels of TNF-α, IL-6 and IL-1β exhibited marked increases in the RVLM and plasma in HS rats compared to NS rats (Table 2). This upregulation of TNF-α, IL-6 and IL-1β was significantly attenuated by exercise training in HS rats (Table 2).

Superoxide and NAD(P)H oxidase in the RVLM

HS + Sed rats had more superoxide in the RVLM, as determined by fluorescent labeled dihydroethidium (DHE) and the NAD(P)H oxidase subunit NOX 2 (gp91^{phox}) and NOX 4, when compared with NS + Sed rats (Fig. 6). Exercise training decreased gp91^{phox}, NOX 4 and DHE in the RVLM of HS rats (Fig. 6).

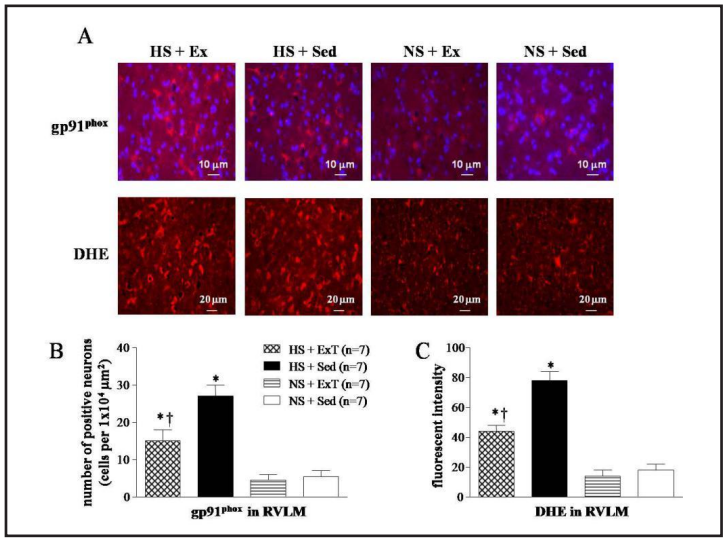

Fig. 6. Superoxide and NAD(P)H oxidase in the RVLM of NS rats and HS rats. (A) Immunofluorescence for the NAD(P)H oxidase subunit gp91^{phox} (red) and superoxide as determined by fluorescent-labeled dihydroethidium (DHE) in the RVLM in different groups. (B) Comparison of gp91^{phox} positive neurons in the RVLM in different groups. (C) Immunofluorescent intensity of DHE in the RVLM of different groups of rats. Values are mean ± SE. *P<0.05 vs. NS groups; †P<0.05 HS + Ex vs. HS + Sed.

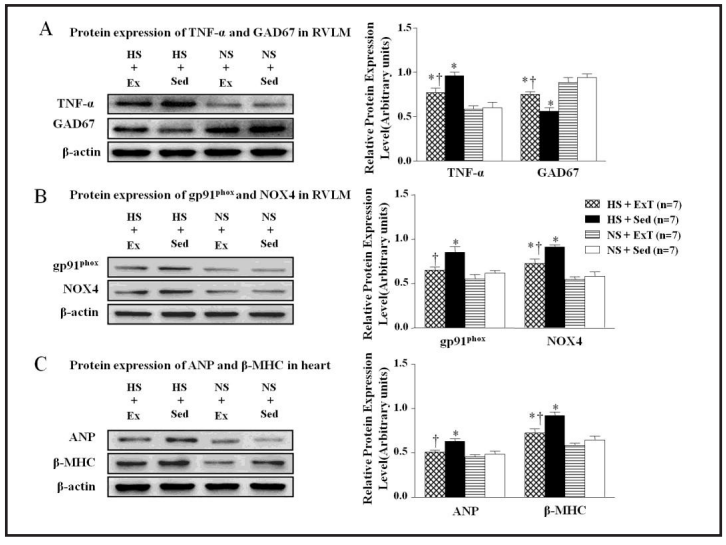

Fig. 7. Protein expression of TNF-α, GAD67, gp91^{phox} and NOX4 in the RVLM and ANP and β-MHC in the heart of NS rats and HS rats. (A) HS rats had higher levels of TNF-α and lower levels of GAD67 in the RVLM when compared with NS rats. Exercise training decreased the protein expression of TNF-α and increased the protein express of GAD67 in the RVLM of HS rats. (B) HS rats had higher levels of gp91^{phox} and NOX4 in the RVLM when compared with NS rats. Exercise training decreased the protein expression of gp91^{phox} and NOX4 in the RVLM when compared with NS rats. (C) Western blot for ANP and β-MHC expressions in the heart were lower in exercise training-treated HS rats than in sedentary HS rats. Values are mean ± SE. *P<0.05 vs. NS groups; †P<0.05 HS + Ex vs. HS + Sed.

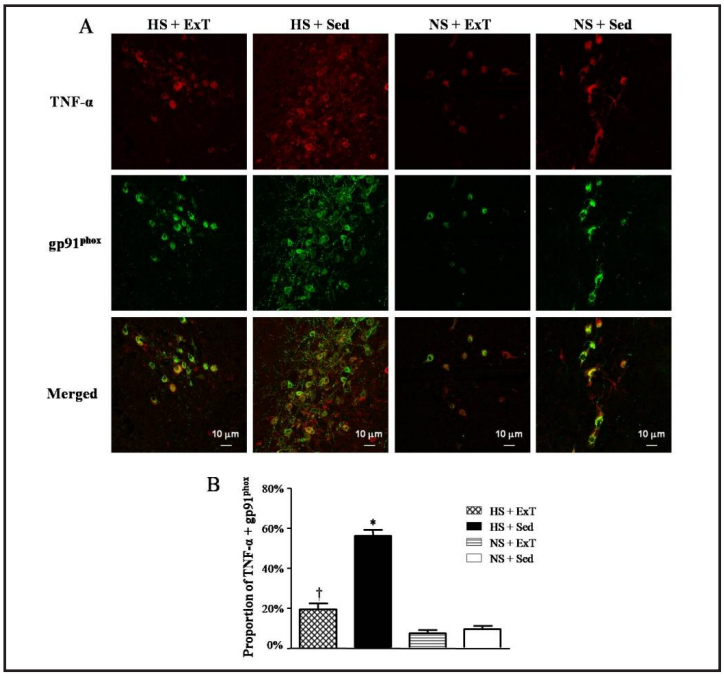

Fig. 8. Laser confocal images (×40) showing co-expression of TNF-α (red) and gp91^{phox} (green) in the RVLM of NS rats and HS rats. (A) Representative images of co-expression of TNF-α (red) and gp91^{phox} (green) in the RVLM of NS rats and HS rats. (B) Corresponding quantification (n=5 per group). Values are mean ± SE. *P<0.05 vs. NS groups; †P<0.05 HS + Ex vs. HS + Sed.

The protein expression of TNF-α, GAD67, gp91^{phox} and NOX 4 in the RVLM

HS + Sed rats had higher levels of TNF-α, gp91^{phox} and NOX 4, and lower level of GAD67 in the RVLM than those of NS + Sed rats (Fig. 7A, B). Exercise training attenuated the increases TNF-α, gp91^{phox} and NOX 4, and a decrease in GAD67 in the RVLM of HS rats (Fig. 7A, B).

Table 3. Neurotransmitters in the RVLM (n = 7). Values are mean ± SE. *P<0.05 HS + Sed vs. NS groups. †P<0.05 HS + Ex vs. HS + Sed

Group	NS + Ex	NS + Sed	HS + Ex	HS + Sed
glutamate (ng/mg)	287 ± 52	301 ± 58	391 ± 72*†	490 ± 80*
GABA (ng/mg)	297 ± 49	314 ± 52	205 ± 48*†	128 ± 43*

Cardiac hypertrophy

The heart weight/body weight (HW/BW) ratio was measured as indicators of cardiac hypertrophy. HS rats had increased cardiac hypertrophy, as assessed by the ratio of heart weight to body weight (HW/BW) (Table 1), which were reduced following exercise training. The protein expressions of markers of cardiac hypertrophy, atrial natriuretic peptide (ANP) and beta-myosin heavy chain (β-MHC) were measured in the left ventricular tissue of the heart using western blot. HS rats resulted in increased protein expression of ANP and β-MHC in the left ventricular tissue of the heart, which were decreased by exercise training (Fig. 7C).

Neurotransmitters in the RVLM

HS rats had elevated level of glutamate and lower level of GABA in the RVLM. Exercise training prevented the decrease in RVLM GABA and the increase in RVLM glutamate in HS rats (Table 3).

Co-expression of TNF-α and gp91^{phox} in the RVLM

To check the co-localization of TNF-α-positive neurons and gp91^{phox}-positive neurons in the RVLM, we performed double labeling studies. Double labeling results also revealed that 56.2% of the TNF-α-positive neurons are also positive for gp91^{phox} in HS rats (Fig. 8). Only 19.8% of TNF-α-positive neurons were positive for gp91^{phox} in the RVLM of HS rats following exercise training (Fig. 8).

Discussion

In the present study, we evaluated the impact of exercise training of 12 weeks on blood pressure and cardiac hypertrophy in salt-induced hypertension. The novel findings of this study are that: (1) exercise training may attenuate hypertension induced cardiac hypertrophy and sympathoexcitation by restoring the balances between the excitatory and inhibitory neurotransmitters in RVLM; (2) NF-κB activity, oxidative stress and PIC in the RVLM may be involved in the exercise training induced effects.

Exercise training (ExT), a part of lifestyle modification, has been recognized as an important strategy for antihypertension [9, 20]. In this work, 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 shown in Fig. 2, high salt diet resulted in significant increase in MAP in sedentary rats beginning from week 8 and remained increased for the duration of the study. Regular exercise training resulted in significant reduction in MAP beginning from week 18 of training and remained significantly lower until the end of the study. Our study also showed that exercise training resulted in reduced cardiac hypertrophy in HS rats, as indicated by decreased whole heart weight/bodyweight ratio, protein expressions of atrial natriuretic peptide and beta-myosin heavy chain. These results of the present study suggest that regular exercise delays the progression of hypertension. This finding is significant from a clinical perspective, because evidence suggest that a reduction of BP by only 5 mmHg can significantly reduces the risk of stroke, heart failure, and mortality from cardiovascular diseases [32].

Although the peripheral mechanisms underlying the cardiovascular changes evoked by exercise training have been widely described [11-13], the central mechanisms involved in cardiovascular control that can be changed by ExT are not well understood. It is well known that the RVLM is a key region for central control of sympathetic outflow and plays a crucial role in maintaining resting BP and sympathetic tone [33, 34]. The tonic activity of the RVLM is related to the interplay of a number of neurotransmitters and neuromodulators in these sympathetic neurons. The sympathetic outflow from the RVLM depends upon the balance of these excitatory and inhibitory neuromodulators, including glutamate, GABA and angiotensin II [19, 35]. In this study, we observed that HS rats exhibited an increase in the circulating plasma NE (an indirect marker of sympathetic activity) and elevated RSNA, and increased the level of glutamate as well as increased the expression of Fra-LI activity in RVLM neurons (indicative of increased neuronal activity) when compared to NS rats. Concomitantly, when compared to NS rats, HS rats had significantly reduced the levels of GABA and GAD67, a marker to recognize GABAergic neurons in the RVLM. More importantly, exercise training attenuated sympathetic excitation, caused reduction in Fra-LI staining and prevented the increase in glutamate and decreases in GABA and GAD67 in the RVLM of HS rats. These results provide sufficient evidence that exercise training may restore the balance between excitatory and inhibitory neurotransmitter in the RVLM and play an important role in the pathogenesis of salt-induced hypertension.

Increased proinflammatory cytokines (PIC) expression in autonomic control areas, as the RVLM, has been identified as a major central molecular mechanism to decrease increase pressure variability and renal sympathetic nerve activity, thus contributing to the development/maintenance of hypertension [5, 36]. Nuclear factor-kappa B (NF-κB) is one of the most important downstream transcription factors responsible for the transcription of PIC, and thereby regulates BP [20, 21]. It has been demonstrated that increased PIC activate NF-κB signaling pathway and activation of NF-κB in turn further upregulates PIC [20]. A grow body of evidence has shown that ExT decreases the pro-inflammatory profile in autonomic brain areas, increases baroreflex function, and attenuates sympathetic activity in angiotensin II infused rats and heart failure rabbits [16, 17, 37, 38]. In the spontaneously hypertensive rats it was also shown that sinoaortic denervation blocked ExT–induced resting bradycardia, pressure fall and reduced sympathetic vasomotor variability [10, 39, 40]. In this study, we also observed that exercise training reduced PIC expression, p-IKKβ

and NF-κB p65 activity in the RVLM of HS rats, but not in NS rats. Meanwhile, the significant reductions in circulating plasma levels of PIC (TNF-α, IL-1β, and IL-6) and NE in HS + ExT rats were observed when compared to HS + Sed rats. There are no comparable changes in normotensive rats receiving ExT. These results suggest that some of the beneficial effects of ExT in salt-induced hypertension are mediated by attenuated the level of PIC in the RVLM.

Previous studies have shown that oxidative stress, particularly the superoxide anion ($O_2^{\bullet-}$), plays a key role in the development of hypertension and cardiac hypertrophy. NAD(P)H oxidase, a complex enzyme consisting of two membrane-bound components ($gp91^{phox}$ and $p22^{phox}$) and three cytosolic components ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$) [41], can be activated by PIC and NF-κB in peripheral tissues. Interestingly, cytoplasmic and membrane-associated NAD(P)H oxidase proteins have been found throughout the neuraxis, indicating that NAD(P)H oxidase-dependent oxidative stress are also generated within the nervous system . Our recent studies also indicate that cytokines and their transcription factor, NF-κB, contribute to the induction of NADPH oxidase (NOX) derived reactive oxygen species (ROS) in heart failure [42] and hypertension [31]. There is growing evidence from studies on animals that exercise training prevents cellular damage by reducing oxidative stress in hypertension [20, 43, 44]. One of the mechanisms underlying the effect of exercise training on oxidative damage could be decreased protein expression of NAD(P)H oxidases and/or inactivation of these proteins, as evidenced in various animal models of hypertension [41, 45]. In the present study, we also found that exercise training resulted in attenuation of increased expression of the NAD(P)H oxidase subunit $gp91^{phox}$ and ROS (DHE staining) in the RVLM of HS rats. More importantly, exercise training resulted in downregulation of NF-κB activity in HS rats. Therefore, one possible mechanism by which exercise training exerts its beneficial effects could be *via* downregulation of NF-κB activity, thereby attenuating oxidative stress in the RVLM of salt-induced hypertension.

Given the results from that report and our current results, it is plausible to suggest that ExT may be contributed to reductions of PIC and ROS production and of an imbalance between excitatory and inhibitory neurotransmitters within the RVLM, and thus, lead to disruption of the positive feedback cycle involved with hypertension and cardiac hypertrophy. Our findings provide further evidence and insight for the beneficial effect of exercise training on hypertension and cardiac hypertrophy.

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Disclosure Statement

The authors declare to have no conflict of interests.

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