Exercise and Proinflammatory Cytokines

Role of Proinflammatory Cytokines and Redox Homeostasis in Exercise-Induced Delayed Progression of Hypertension in Spontaneously Hypertensive Rats

Deepmala Agarwal, Masudul Haque, Srinivas Sriramula, Nithya Mariappan, Romain Pariault, Joseph Francis

Abstract—Hypertension is a well-known risk factor for various cardiovascular diseases. Recently, exercise has been recommended as a part of lifestyle modification for all hypertensive patients. However, the precise mechanisms of exercise training (ExT)–induced effects on the development of hypertension are poorly understood. Therefore, we hypothesized that chronic ExT would delay the progression of hypertension in young spontaneously hypertensive rats (SHRs). In addition, we explored whether the beneficial effects of chronic ExT were mediated by reduced proinflammatory cytokines and improved redox status. We also investigated the involvement of nuclear factor-κB in exercise-induced effects. To test our hypotheses, young normotensive (Wistar-Kyoto) and SHRs were given moderate-intensity ExT for 16 weeks. Blood pressure was determined by the tail-cuff method, and cardiac function was assessed by echocardiography. Myocardial total reactive oxygen species and superoxide production were measured by electron paramagnetic resonance spectroscopy; tumor necrosis factor-α, interleukin-1β, gp91phox, and inducible NO synthase by real-time PCR; and nuclear factor κB activity by electrophoretic mobility shift assay. Chronic ExT in hypertensive rats results in significantly reduced blood pressure, reduced concentric hypertrophy, and improved diastolic function. ExT significantly reduced proinflammatory cytokines and inducible NO synthase, attenuated total reactive oxygen species and superoxide production, and increased antioxidants in SHRs. ExT also resulted in increased NO production and decreased nuclear factor κB activity in SHRs. In summary, chronic ExT delays the progression of hypertension and improves cardiac function in young SHRs; these ExT-induced beneficial effects are mediated by reduced proinflammatory cytokines and improved redox homeostasis via downregulation of nuclear factor-κB. (Hypertension. 2009; 54:1393-1400.)

Key Words: exercise training ■ proinflammatory cytokines ■ NF-κB ■ hypertension ■ oxidative stress

Hypertension is a well-known risk factor for various cardiovascular diseases; currently, it is estimated that >72 million American adults have hypertension.¹ One of the hallmarks of hypertension is chronic low-grade inflammation. Proinflammatory cytokines (PICs), such as tumor necrosis factor-α (TNF-α),² interleukin (IL)-1β,²,³ and IL-6,³,⁴ have been reported to increase with the severity of hypertension and are of prognostic significance. In addition to PICs, free radicals, such as reactive oxygen species (ROS) and superoxide (O₂⁻), contribute to the pathogenesis of hypertension. More importantly, PICs have been found to activate ROS,⁵–⁷ which, in turn, can activate various intracellular signaling pathways, including that of nuclear factor-κB (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.

Exercise has recently been recommended as a part of lifestyle modification for all patients diagnosed with hypertension.⁸ Several previous studies investigated the effects of exercise on hypertension; however, most of the studies were performed on severely hypertensive patients or animal models by using short-term exercise protocols or exercise combined with dietary interventions.⁹–¹⁴ However, the effects of long-term exercise training (ExT) in the progression of hypertension remain unclear. More importantly, the mechanisms by which ExT exerts its effects are largely unknown. Various proposed mechanisms of the antihypertensive effects of ExT include reduced cardiac output, reduced peripheral vascular resistance,¹¹ alterations in autonomic nervous system activity,¹⁵ attenuated sympathetic modulation¹⁴,¹⁶ and hypothalamic-pituitary-adrenal axis responsiveness,¹⁷ but a complete understanding of the molecular mechanisms underlying the exercise-induced reduction of blood pressure is still lacking.
ExT ameliorates hypertension. It will help us to further understand the mechanism by which ExT ameliorates hypertension. Results from these studies and (3) chronic ExT would improve myocardial redox homeostasis and NO bioavailability. 

The present study was to investigate the effects of regular long-term, moderate-intensity ExT in young spontaneously hypertensive rats (SHR) and to elucidate the mechanisms behind these effects. We hypothesized the following: (1) regular chronic, moderate-intensity ExT would delay the progression of hypertension in young SHRs; (2) the beneficial effects of chronic ExT in young SHRs would be mediated by reduced myocardial PICs and NF-κB activity; and (3) chronic ExT would improve myocardial redox homeostasis and NO bioavailability. Results from these studies will help us to further understand the mechanism by which ExT ameliorates hypertension.

Materials and Methods
For an expanded Materials and Methods section, please see the online Data Supplement at http://hyper.ahajournals.org.

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.

Animals and Experimental Design
Seven-week-old male normotensive Wistar-Kyoto (WK) and spontaneously hypertensive (SHR) rats were randomly assigned either to the sedentary group (SHRsed, n=10; WKsed, n=10) or to the exercise group (SHRex, n=10; WKex, n=10). Exercise groups were subjected to moderate-intensity exercise on a motor-driven treadmill continuously for a period of 16 weeks. Animals were euthanized 24 hours after the last exercise session at the age of 23 weeks, and left ventricle (LV) tissues were collected for later analyses. We performed the following experimental procedures: blood pressure measurements, echocardiographic analysis, real time RT-PCR, Western blot analysis, electron paramagnetic resonance studies, antioxidant assays, electrophoretic mobility shift assay, reverse-phase high-performance liquid chromatography, ELISA, and statistical analysis.

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 the WKsed and SHRsed groups. Chronic ExT resulted in reduction in BW in both WK and SHR rats (Table 1).

ExT 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 1). At the end of the study, chronic ExT was found to have significantly reduced SBP, DBP, and MAP in trained SHRs compared with SHRsed rats (Table 1). Interestingly, BP in the 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 1).

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 with WK rats (Table 1). Echocardiographic studies (Table 2) revealed that SHRsed rats had significantly higher interventricular septal thickness, posterior wall thickness, relative wall thickness, and left ventricular mass index, without modification of LV chamber size, compared with WKsed rats. These echocardiographic changes suggest the presence of concentric hypertrophy in SHRsed rats. Chronic ExT significantly reduced interventricular septal thickness, posterior wall thickness, and relative wall thickness in hypertensive rats when compared with their sedentary controls, indicating reduced concentric hypertrophy with ExT. In addition, moderate ExT increased LV chamber size and decreased left ventricular mass index in these animals, although values did not reach statistical significance. However, 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 with WKsed rats.

ExT Improves LV Diastolic Function in SHRs
We evaluated the cardiac performance of all of the rats using M-mode and Doppler echocardiography (Table 2). LV systolic function was not altered in hypertensive rats, as indicated by the lack of significant changes in LV ejection fraction and fractional shortening in SHRsed compared with WKsed rats. However, diastolic function was severely impaired in SHRs, as indicated by significantly increased Tei index and increased isovolumic relaxation time (an indicator of impaired LV relaxation) in SHRsed compared with WKsed rats. Chronic moderate-intensity ExT significantly reduced Tei index and isovolumic relaxation time in SHRs, indicative of improved diastolic function. ExT did not alter systolic function in SHRs or in WK rats.

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 and plasma. SHRsed rats exhibited marked increases in expression of TNF-α and IL-1β in the LV as compared with WKsed rats (Figures 2 and S1, available in the online Data Supplement). 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.

Table 1. Weights and Blood Pressure Parameters at the End of the Training Period

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WKsed</th>
<th>WKex</th>
<th>SHRsed</th>
<th>SHRex</th>
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<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>BW, g</td>
<td>365.7±8.9</td>
<td>327.6±9.7 †</td>
<td>387.9±3.4</td>
<td>327.2±9.7 †</td>
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<tr>
<td>HW, mg</td>
<td>1.01±0.04</td>
<td>1.21±0.04 †</td>
<td>1.44±0.02*</td>
<td>1.24±0.05†</td>
</tr>
<tr>
<td>HW/BW, mg/kg</td>
<td>2.88±0.04</td>
<td>3.18±0.1†</td>
<td>3.45±0.06*</td>
<td>3.68±0.02</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>134±3</td>
<td>140±7</td>
<td>233±2*</td>
<td>202±0.5†</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>89±2</td>
<td>98±6</td>
<td>176±2*</td>
<td>152±5†</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>101±2</td>
<td>112±6</td>
<td>191±0.5*</td>
<td>168±3†</td>
</tr>
</tbody>
</table>

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.
We also examined the mRNA and protein levels of gp91phox by RT-PCR and Western blotting. Sedentary SHRs had significantly increased levels of total ROS and O$_2^-$ (Figure 3B) production compared with WKsed rats. Chronic ExT significantly attenuated total ROS and O$_2^-$ production in SHRs. ExT did not affect free radical production in WK rats. Furthermore, gp91phox expression was markedly higher in SHRsed rats when compared with WKsed rats; this expression was significantly reduced by chronic ExT (Figure 4A through 4C).

Because decreased local antioxidant protection is one of the potential sources of ROS formation, we analyzed various enzymatic and nonenzymatic antioxidant levels in LV tissue. We observed that SHRsed rats had no changes in myocardial O$_2^-$ dismutase (Figure S2) and reduced glutathione (Figure S3) concentrations when compared with WKsed rats. In addition, SHRsed exhibited significantly increased oxidized disulfide glutathione (Figure S4) concentration and reduced glutathione/oxidized disulfide glutathione ratio (an important marker of cellular redox balance) (Figure S5) in comparison with WKsed rats. Chronic ExT in SHRs resulted in significantly increased reduced glutathione/oxidized disulfide glutathione ratio, decreased oxidized disulfide glutathione levels, and increased O$_2^-$ dismutase activity, indicative of improvements in antioxidant defense by ExT. ExT did not affect antioxidant levels in WK rats.

**ExT Reduces Myocardial NO Synthase and Peroxynitrite in SHRs**

Hypertensive rats showed significantly higher mRNA and protein expression of myocardial inducible NO synthase (iNOS) when compared with WKsed rats. Chronic ExT resulted in significantly decreased iNOS expression in SHRs.

### Table 2. Echocardiographic Analysis of Cardiac Hypertrophy and Function

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>WKex</th>
<th>SHRsed</th>
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<td></td>
<td>N</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>LVd, mm</td>
<td>8.050±0.14</td>
<td>8.320±0.40</td>
<td>7.543±0.20</td>
<td>8.009±0.08</td>
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<td>LVd, mm</td>
<td>4.933±0.18</td>
<td>5.150±0.38</td>
<td>5.143±0.26</td>
<td>5.609±0.09</td>
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<td>IVS, mm</td>
<td>1.550±0.05</td>
<td>1.7±0.05</td>
<td>2.071±0.08*</td>
<td>1.745±0.06†</td>
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<tr>
<td>IVS, mm</td>
<td>2.367±0.12</td>
<td>2.540±0.04</td>
<td>2.843±0.13*</td>
<td>2.480±0.05†</td>
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<td>PWT, mm</td>
<td>1.525±0.06</td>
<td>1.7±0.07</td>
<td>1.925±0.07*</td>
<td>1.627±0.04†</td>
</tr>
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<td>PWT, mm</td>
<td>2.225±0.105</td>
<td>2.3±0.13</td>
<td>2.683±0.11*</td>
<td>2.273±0.05†</td>
</tr>
<tr>
<td>FS, %</td>
<td>36.85±3.0</td>
<td>36±2.8</td>
<td>29.80±1.2</td>
<td>28.34±0.4</td>
</tr>
<tr>
<td>EF, %</td>
<td>73.23±4.9</td>
<td>73.10±4.1</td>
<td>65.12±2.2</td>
<td>61±1.5</td>
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<tr>
<td>Tel index</td>
<td>0.280±0.04</td>
<td>0.345±0.04</td>
<td>0.699±0.04*</td>
<td>0.534±0.04†</td>
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<tr>
<td>LVMi, g</td>
<td>2.00±0.17</td>
<td>2.31±0.25</td>
<td>2.67±0.08*</td>
<td>2.34±0.16</td>
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<tr>
<td>RWT, ms</td>
<td>0.380±0.02</td>
<td>0.450±0.02</td>
<td>0.530±0.03*</td>
<td>0.407±0.01†</td>
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<tr>
<td>NRT, ms</td>
<td>18.75±1.4</td>
<td>19.27±1.0</td>
<td>30.55±4.5*</td>
<td>23.96±3.5†</td>
</tr>
</tbody>
</table>

Values are mean±SE. LVd and LVd indicate left ventricular internal diameter at diastole and systole, respectively; IVS and IVS, interventricular septal thickness at diastole and systole, respectively; PWT and PWTs, posterior wall thickness at diastole and systole, respectively; FS, fractional shortening; EF, ejection fraction; LVMi, left ventricular mass index; RWT, relative wall thickness; NRT, isovolumic relaxation time.

* P<0.05 WKsed vs SHRsed rats.
† P<0.05 SHRsed vs SHRex rats.

Figure 1. Time course of blood pressure (in millimeters of mercury) in WK rats and SHRs. A. SBP; B. DBP; C. MAP. Blood pressure was significantly reduced in SHRsed compared with SHRex 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.

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$_2^-$ production in the LV by electron paramagnetic resonance. We also examined the mRNA and protein levels of gp91phox by RT-PCR and Western blotting. Sedentary SHRs had significantly increased levels of total ROS and O$_2^-$ (Figure 3A) and O$_2^-$ (Figure 3B) production compared with WKsed rats. Chronic ExT significantly attenuated total ROS and O$_2^-$ production in SHRs. ExT did not affect free radical production in WK rats. Furthermore, gp91phox expression was markedly higher in SHRsed rats when compared with WKsed rats; this expression was significantly reduced by chronic ExT (Figure 4A through 4C).

Because decreased local antioxidant protection is one of the potential sources of ROS formation, we analyzed various enzymatic and nonenzymatic antioxidant levels in LV tissue. We observed that SHRsed rats had no changes in myocardial O$_2^-$ dismutase (Figure S2) and reduced glutathione (Figure S3) concentrations when compared with WKsed rats. In addition, SHRsed exhibited significantly increased oxidized disulfide glutathione (Figure S4) concentration and reduced glutathione/oxidized disulfide glutathione ratio (an important marker of cellular redox balance) (Figure S5) in comparison with WKsed rats. Chronic ExT in SHRs resulted in significantly increased reduced glutathione/oxidized disulfide glutathione ratio, decreased oxidized disulfide glutathione levels, and increased O$_2^-$ dismutase activity, indicative of improvements in antioxidant defense by ExT. ExT did not affect antioxidant levels in WK rats.
ExT did not affect iNOS expression in WK rats (Figure 4B through 4D). Direct measurement and quantification of peroxynitrite (OONO−) by electron paramagnetic resonance studies revealed that SHRsed rats had significantly increased myocardial production of OONO− in comparison with WKsed rats. Interestingly, chronic ExT in SHRs resulted in significantly decreased OONO− production (Figure 3C).

**ExT Normalizes Myocardial NO Level in SHRs**
Myocardial total nitrate/nitrite concentration, 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 S6).

**ExT Attenuates NF-κB Binding Activity in SHRs**
SHRsed had significantly higher myocardial NF-κB binding activity than WKsed rats. Chronic ExT resulted in a significant decrease in NF-κB binding activity in SHRs. NF-κB binding activity was unaltered by ExT in WK rats (Figure 5).

**ExT Decreases Plasma Norepinephrine Levels in SHRs**
At the end of the study, plasma norepinephrine (NE) levels were found to be significantly higher in SHRsed compared with WKsed rats. Chronic ExT resulted in significantly decreased plasma NE concentrations in SHRs but did not change plasma NE level in WK rats (Figure S7).

**Discussion**
In this study, we investigated the effects of chronic moderate-intensity ExT and possible mechanisms of these effects on young SHRs, a genetic model of hypertension that shares many common features of human essential hypertension. The salient findings of this study are as follows: (1) regular long-term, moderate-intensity ExT delays the progression of hypertension, reduces cardiac hypertrophy, and improves diastolic cardiac function in young SHRs with developing hypertension; (2) training-induced beneficial effects in SHRs 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 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. Previous reports suggest that ExT did not reduce BP in severely hypertensive patients and rats.9,13 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.9,13,14 Nonetheless, results of our study suggest that regular moderate-intensity ExT delays the progression of hypertension.

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.12,21 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 in-

Figure 4. Effects of exercise on gp91phox and iNOS expression in the LV of WK rats and SHRs. A, mRNA expression of gp91phox; B, A representative Western blot; C, Densitometric analysis of protein expression; D, mRNA expression of iNOS. Values are mean±SE; n=6 in each group. *P<0.05 WKsed vs SHRsed rats; †P<0.05 SHRsed vs SHRex rats.

Figure 5. Effects of exercise on LV NF-κB binding activity in WK rats and SHRs. A, Representative electrophoretic mobility shift assay results of NF-κB binding activity; B, Densitometric analysis of NF-κB binding intensity (mean±SE). Values are mean±SE; n=6 in each group. *P<0.05 WKsed vs SHRsed rats; †P<0.05 SHRsed vs SHRex rats.
increased, although changes did not reach statistical significance. This observation is in accordance with several previous studies. Recently, Pluim et al. 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.

In the present study, chronic ExT also resulted in reduced cardiomyopathy in SHRs. This finding is significant from a clinical perspective, because pathological cardiomyopathy is known to lead to cardiac failure. Conversely, ExT in WK rats resulted in eccentric hypertrophy. Eccentric hypertrophy, also known as physiological hypertrophy, is mainly related to training-induced volume overload and is considered as a cardiac adaptation of ExT. These results were in accordance with previous studies.

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. Few studies have documented the reduction in inflammatory markers by ExT on obese individuals or diabetic patients. 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 and coronary heart disease, 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. The success of β-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. This finding was in agreement with recently published data showing significantly reduced cardiac NE concentrations in trained SHRs. These findings, together with previous findings from our laboratory that TNF-α blockade attenuates sympathoexcitation in heart failure, 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 suggested 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. We reported previously that cytokines and their transcription factor, NF-κB, contribute to the induction of oxidative stress in heart failure and hypertension. 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$_2^-$ production rates were significantly higher in SHRs compared with WKs rats; 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$_2^-$ 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 and our finding that decreased oxidative stress is associated with decreased PICs in SHRs may further support the idea that ExT is responsible for the exercise-induced decrease in oxidative stress in SHRs. In addition, we observed that ExT resulted in attenuation of increased expression of gp91phox (a subunit of NADPH oxidase, a major source of ROS) in SHRs. Angiotensin II is a major regulator of NADPH oxidase activity; therefore, the possible contribution of the renin-angiotensin system to exercise-mediated effects cannot be ignored. Nonetheless, our data lead us to conclude that pressure-lowering and antihypertrophic 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. The evidence that iNOS is predominantly induced by cytokines and our finding that ExT reduces PICs in SHRs led us to explore whether ExT results in decreased myocardial iNOS expression. We observed that mRNA and protein expression of myocardial iNOS were markedly higher in SHRs compared with WKs rats; these levels were significantly decreased by chronic ExT in SHRs. Furthermore, reduced myocardial total nitrate/nitrite levels in SHRs compared with WKs rats were normalized in SHRs, 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$_2^-$ in SHRs seems responsible for attenuated O$_2^-$-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. Recent work from our laboratory suggests that NF-κB blockade reduces cytosolic and mitochondrial oxidative stress and attenuates hypertension in SHRs. 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 downregulation of NF-κB activity. Our present observation that NF-κB activity was increased in SHRs compared with WKs rats further
supports this hypothesis. More importantly, chronic ExT resulted in downregulation of NF-κB activity in SHR. 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 the detrimental positive feedback cycle involved in the progression of hypertension.

Perspectives

The findings of this study indicate that chronic 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 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/effects relationship could not be established in this study, we can attribute the beneficial effects of ExT on hypertension to an altered interplay among sympathetic activity, PICs, and oxidative stress via NF-κB-mediated signaling pathways. Future 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 elude the mechanisms of the beneficial effects of ExT in SHR. However, the comparison of different intensities of ExT with regard to parameter studies could certainly be an important perspective of this study.

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Disclosures

None.

References


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Role of Proinflammatory Cytokines and Redox Homeostasis in Exercise-Induced Delayed Progression of Hypertension in Spontaneously Hypertensive Rats

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EXPERIMENTAL PROCEDURES

Animals and Experimental Design

Seven week old male normotensive Wistar-Kyoto (WK) and spontaneously hypertensive rats (SHR) were used in this study. All animals were housed in temperature (23 ± 2°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). 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 analyses.

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.1, 2 Animals ran an average distance of 1093 m/day. A similar exercise protocol has been used by previous investigators.3, 4

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), which utilizes a tail-cuff occlusion method and volume pressure recording (VPR) sensor technology. VPR measures six parameters simultaneously: systolic blood pressure, diastolic blood pressure, mean blood pressure, heart pulse rate, tail blood volume, and tail blood flow. In this system, unanesthetized rats from each group were warmed to an ambient temperature of 30°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, in oxygen) 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
Cardiac hypertrophy was evaluated by left ventricular mass index (LVMI) and relative wall thickness (RWT). The LV mass (LVM) was calculated according to the conventions of the American Society of Echocardiography\textsuperscript{5} using the following equation: \[ \text{LVM} = 0.80 \times (1.04 \times (\text{PWTd} + \text{IVSd} + \text{LVIDd})^3 - (\text{LVIDd})^3) + 0.6. \] 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 \[ 2 \times \text{PWTd} / \text{LVIDd}. \] LV systolic function was estimated by LV fractional shortening (FS\%) using equation, \[ \text{FS (\%)} = \left( \frac{(\text{LVIDd} - \text{LVIDs})}{\text{LVIDd}} \right) \times 100. \] Tei index (an indicator of systolic and diastolic dysfunction) was determined from Doppler recordings of LV inflow and outflow as described previously.\textsuperscript{6} Tei index was calculated using the equation, \[ \text{Tei index} = \frac{(a - b)}{b}, \] where \( a \) is the interval between cessation and onset of mitral inflow, and \( b \) is the ejection time of LV outflow. Altered Tei index with unchanged systolic function parameters (FS and EF) was considered indicative of diastolic dysfunction. To provide more direct evidence of diastolic dysfunction, isovolumic relaxation time (IVRT) was further determined by Doppler echocardiography.

**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-\( \alpha \)), and interleukin (IL)-1\( \beta \), and gp91\textsuperscript{phox}, and iNOS genes by using specific primers. Rat primers used are listed in the Supplementary Table. Total RNA isolation, cDNA synthesis and real-time RT-PCR were performed as previously described.\textsuperscript{7} 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 2\( \mu \)g 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, Foster City, CA). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 3 min, followed for 45 cycles (15 s at 95°C, and 1 min at 60°C). To confirm the specific PCR product, a dissociation step (15 s at 95°C, 15 s at 60°C, and 15 s at 95°C) was added to check the melting temperature. 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.

**Western Blot Analysis**

Protein expression in LV tissue was analyzed by western blot analysis as described previously.\textsuperscript{7} We used antibodies against TNF-\( \alpha \), IL-1\( \beta \), gp91\textsuperscript{phox}, and iNOS (Santa Cruz Biotechnology). Protein extracts (25 \( \mu \)g) 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°C.
with the primary antibodies TNF-α (1:1,000 dilution), IL-1β (1:1,000 dilution), gp91phox (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-κB activity in the LV tissue. Nuclear extracts were prepared using a 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/cm² 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 (O2•−), 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.8
Antioxidant Assays

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.

Myocardial Total Nitrate/Nitrite Concentration

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

Reverse-Phase High-Performance Liquid Chromatography

Plasma norepinephrine (NE) level 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. 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.

ELISA

Plasma circulating levels of IL-1β were measured using a commercially available rat IL-1β ELISA kit (Invitrogen, Carlsbad, 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 within-group changes over time. Results were considered significant when \( p < 0.05 \).
References:


Table S1. Rat primers used for real-time RT-PCR

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Figure S1. Effects of exercise training on plasma circulating level 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.

Figure S2. Effects of exercise training on LV superoxide dismutase level (SOD) level 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.
Figure S3. Effects of exercise training on LV reduced glutathione level (GSH) levels 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.

Figure S4. Effects of exercise training on LV oxidized glutathione disulfide (GSSG) levels 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.
Figure S5. Effects of exercise training on LV GSH to GSSG ratios 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.

Figure S6. Effect of exercise training on plasma norepinephrine levels in normotensive WK and SHR rats. N=10 in each group. *p<0.05 WKsed vs SHRsed; †p<0.05 SHRsed vs SHRex.
Figure S7. Effect of exercise training on myocardial total nitrate/nitrite concentration in normotensive WK and SHR rats. N=6 in each group. *p<0.05 WKsed vs SHRsed; †p<0.05 SHRsed vs SHReX.

Figure S7. Effect of exercise training on myocardial total nitrate/nitrite concentration in normotensive WK and SHR rats. N=6 in each group. *p<0.05 WKsed vs SHRsed; †p<0.05 SHRsed vs SHReX.