Contribution of Nitric Oxide, Angiotensin II and Superoxide Dismutase to Exercise-induced Attenuation of Blood Pressure Elevation in Spontaneously Hypertensive Rats

Hiroshi KOHNO,† MPE, Satoshi FURUKAWA,¶ MPE, Hisashi NAITO,† PhD, Kazutoshi MINAMITANI,† PhD, Daijiro OHMORI,‡ PhD, and Fumiyuki YAMAKURA,‡ PhD

SUMMARY

Moderate chronic exercise attenuates the elevation of blood pressure in young spontaneously hypertensive rats. In order to elucidate the physiological process of the effects of exercise, we examined the involvement of nitric oxide, angiotensin II, and superoxide dismutase in this process. Rats were exercised by voluntary running in a wheel-cage for 10 weeks. Systolic blood pressure in the exercised rats (195±4 mmHg, n=27) was significantly (p<0.05) lower than in the post-control rats (212±3 mmHg, n=28). The concentration of total plasma nitrite was significantly higher in exercised rats (14.9±1.5 µmol l⁻¹) than in the post-control rats (9.9±0.7 µmol l⁻¹, p<0.05). Superoxide dismutase activity in the exercised rats was significantly higher (p<0.05) than in the post-control rats (thoracic aorta: 4.6±0.3 U mg protein⁻¹ vs 3.6±0.3 U mg protein⁻¹, heart: 12.7±0.6 U mg protein⁻¹ vs 10.2±0.6 U mg protein⁻¹, p<0.05). The plasma angiotensin II concentration was higher in the post-control rats (74.4±14.0 pg mL⁻¹) than in the exercised rats (45.0±6.4 pg mL⁻¹, p<0.05), and in the pre-control rats (47.2±6.0 pg mL⁻¹). The results suggest that exercise acts to decrease the level of superoxide by increasing superoxide dismutase activity in the aorta and heart and to decrease levels of angiotensin II, both of which, in turn, increase the effective concentration of nitric oxide. We conclude that the combination of these effects with the increased NO formation resulted in the low blood pressure seen in the exercised rats. (Jpn Heart J 2002; 43: 25-34)

Key words: Blood pressure, Exercise training, Nitric oxide, Superoxide dismutase, Spontaneously hypertensive rat, Angiotensin II

Experimental Studies

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VASCULAR tone and contractile behavior of vascular smooth muscle cells are regulated by many autacoids and hormones. Endothelium-derived NO is one factor that regulates these processes. When the endogenous production of NO is prevented by inhibition of NO synthase (NOS), hypertension is induced.\textsuperscript{1,2} NO produces vasodilation by increasing the levels of cyclic guanosine 3', 5'-monophosphate (cGMP), thereby activating cGMP-dependent protein kinase, in smooth muscle cells.\textsuperscript{3} Another hormone, angiotensin II (ANG II) also has effects on vascular cells and is known to participate in the development of many cardiovascular diseases, including hypertension.\textsuperscript{4} A recent report showed that ANG II can decrease levels of NO through the promotion of oxygen radical production, particularly superoxide anion, in the endothelium.\textsuperscript{5} These oxygen radicals in turn react rapidly with NO to form peroxynitrite, a known reactive oxidant.\textsuperscript{6} Therefore, the amount of ANG II and production of superoxide anion may be important factors in the regulation of NO-induced vasodilation.\textsuperscript{7}

The spontaneously hypertensive rat (SHR), which is used as an animal model for essential hypertension, has been found to have impaired endothelium-dependent vasodilation. This impairment can be explained by several different mechanisms: diminished production of NO,\textsuperscript{8} an impaired ability of the basilar artery to respond to NO,\textsuperscript{9} or an increased release of endothelium-derived hyperpolarizing factor.\textsuperscript{10} Furthermore, increased levels of ANG II have been found in SHR compared with those in Wistar-Kyoto rats, the normotensive reference strain.\textsuperscript{11}

It is well known that endurance training in patients with borderline or moderate hypertension generally reduces blood pressure.\textsuperscript{12} It has been proposed that the reduction in blood pressure is due to stimulation of endothelial NO formation in rats\textsuperscript{13} and humans.\textsuperscript{14} The recent finding of a shear stress-responsive element in the NOS gene promoter from human endothelial cells indicates that the expression of NOS in endothelial cells may be transcriptionally regulated by changes in the dynamics of blood flow which are increased during exercise.\textsuperscript{15} This finding further supports the concept that regular exercise stimulates endogenous NO formation.

It has been reported that moderate exercise can normalize the resting systolic blood pressure in male SHR,\textsuperscript{16} however, little is known about the mechanism of this effect. A recent study by Jonsdottir, et al\textsuperscript{17} showed that exercise training of nine week old SHRs for 35 days resulted in enhanced NO formation. However, since NO formation can be increased by the development of hypertension in SHR,\textsuperscript{18,19} experiments using control SHRs are required to clarify the cause of the increased NO formation.

In order to determine the cause of enhanced NO formation in SHRs following exercise, we have used age-matched control groups in this study. We mea-
sured total nitrite concentration in plasma as an index of NO levels and systolic blood pressure in SHRs after 10 weeks of voluntary running and in SHRs that had not undergone exercise as controls. Furthermore, since oxygen radicals may influence systolic blood pressure, we felt it would be useful to know if the levels of some of the factors associated with the generation of oxygen radicals, such as superoxide dismutase (SOD) and ANG II, are altered in SHR by voluntary exercise training. We measured SOD activity in thoracic aortas and hearts, and ANG II levels in plasma samples from both groups of rats, in order to determine whether these factors may have a role in the attenuation of blood pressure elevation in young SHRs following chronic exercise. A preliminary account of the present work has been presented elsewhere.

**MATERIALS AND METHODS**

**Animals:** Seventy-five three week old male spontaneously hypertensive rats (SHR/Izm: Disease Model Cooperative Research Association, Kyoto, Japan) were obtained from Funabashi Farm (Funabashi, Chiba), housed separately, and fed standard laboratory chow (CE4, Clea Japan) and tap water ad libitum in a climate-controlled laboratory animal facility (22±1°C, 50±5% relative humidity, and a 12 hour dark-light cycle).

**Experimental procedures:** Protocols were approved by the Institutional Animal Care and Use Committee of Juntendo University, Chiba, and were performed according to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, the Physiological Society of Japan. After two weeks of acclimatization, the body weights and systolic blood pressures of the SHRs were measured. Rats were assigned randomly into pre-control SHR (n=15, 91±8 g, 135±2 mmHg), post-control SHR (n=30, 94±6 g, 133±3 mmHg), or exercise groups (n=30, 96±7 g, 129±2 mmHg). There were no statistical differences in the body weights or systolic blood pressures of rats between the three groups before the training period.

Rats in the pre-control SHR group were sacrificed at the start of the experiment in order to obtain blood and organs. In the exercised group, each rat had free access to a wheel with a running volume measure counter. The exercised SHRs were allowed to run spontaneously for 10 weeks, and running volumes were recorded simultaneous every two days. The whole average running distance during this period was 4980±442 m day⁻¹. The exercised and post-control SHRs were handled at least three times per week to become accustomed to the experimental protocols. Systolic blood pressures and body weights were recorded every two weeks at the same time. Blood pressure was measured using a tail-cuff method (BP98A: Softron Co. Ltd., Tokyo, Japan). At the end of the 10 week voluntary
exercise period, body weights and blood pressures were recorded. Twelve hours before blood sampling, food was removed, drinking water was replaced with distilled water, and the running wheel was removed. Blood samples were collected by decapitation, centrifuged for 10 minutes at 3000 x g, and the plasma kept at −80°C until analysis. Plantaris muscle, heart and thoracic aorta were sampled from each rat, frozen with liquid nitrogen, and kept at −80°C.

**Biochemical Assay:** Plasma nitric oxide was estimated by measuring the total amount of nitrate and nitrite present in the plasma. Briefly, after plasma samples were filtered through an Ultrafree-MC microcentrifuge filter (Millipore, Bedford, USA), plasma nitrate was converted to nitrite by nitrate reductase (Sigma, St. Louis, USA). The resulting total nitrite was measured following the addition of 2, 3-diaminonaphthalene triazole (Dojindo Laboratory Co. Ltd., Tokyo) which reacts with nitrates to form 1(H)-naphtho triazole, a fluorescent product. Additional amounts of nitrate and nitrite in the plasma are represented as total nitrite in this text. Citrate synthase activity was measured in plantaris muscle by the method of Srere. SOD activity in thoracic aorta and heart was measured using a modified method of McCord & Fridovich. We improved the sensitivity of the measurement of the SOD by 15 times compared to the standard method by reducing the volume of the assay mixture from 3.0 mL to 0.2 mL and by using a Hitachi U-3000 spectrophotometer equipped with a micro-cuvette holder. With this modification, it was possible to measure SOD activity in individual thoracic aortas and hearts. Tissue protein concentration was estimated by the method of Lowry, et al. ANG II was measured by radio immunoassay, which was carried out on ethanol extracted plasma (Angiotensin II kit, SRL Inc. Tokyo). The lower limit of detection of ANG II is 3 pg mL⁻¹.

**Statistics:** Results from the different groups were compared using one-way ANOVA. If ANOVA indicated a significant difference among groups, pair wise differences between groups were compared using the Duncan multiple-comparison procedure. SOD and citrate synthase activities were compared using the Mann-Whitney U-test. Results are reported as mean ± SE with p<0.05 considered statistically significant.

**RESULTS**

**Effect of exercise on body weight and systolic blood pressure:** After the training period, the average body weights of the exercised SHR in both series of experiments were significantly lower than that of the post-control group (Table I). To quantitate the effect of the training, citrate synthase activity in plantaris muscle was measured. Citrate synthase activity was significantly higher in the exercise group than in the post-control group (Table I). At the end of the exercise period,
the systolic blood pressures of the exercise SHR (195±4 mmHg) were lower than those of the post-control SHRs (212±3 mmHg, p<0.05, Figure 1).

**Total nitrite and angiotensin II levels in plasma:** Plasma total nitrite in the pre-exercise SHRs at the beginning of the exercise training period was 11.4±0.8 µmol l⁻¹ (Figure 2). At the end of the training period, the average amount of plasma total nitrite in the exercise group (14.9±1.5 µmol l⁻¹) was significantly higher than that of the post-control group (9.9±0.7 µmol l⁻¹) (Figure 2). Therefore, endogenous formation of NO increased in the exercise group compared with the post-control group. The average plasma ANG II value in the exercise group was similar to that of the pre-control group, but was significantly lower than that

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**Table I.** Body Weight, ANG II And Citrate Synthase Activity of Plantaris Muscle before (Pre-control) and after (Exercise & Post-control) 10 Week Exercise Training

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Body Weight (g)</th>
<th>Angiotensin II (pg ml⁻¹)</th>
<th>Citrate Synthase Activity (µmol min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-control</td>
<td>15</td>
<td>120±7</td>
<td>47.3±6.0</td>
<td>–</td>
</tr>
<tr>
<td>Post-control</td>
<td>28</td>
<td>314±3#</td>
<td>74.4±14.0</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td>Exercise</td>
<td>27</td>
<td>300±5#*</td>
<td>45.0±6.4*</td>
<td>0.41±0.08*</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE. # Significantly different from pre-control group. * Significantly different from post-control group.

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**Figure 1.** Differences in the average systolic blood pressure among three groups. #: Significantly different from pre-control group. *: Significantly different from post-control group.
of the post-control group (Table I).

**Superoxide dismutase activity in thoracic aorta and heart:** To elucidate the potential role of superoxide in NO metabolism during exercise, we measured SOD

![Figure 2](image-url)  
**Figure 2.** Differences in the average NO metabolite concentrations among three groups. #: Significantly different from pre-control group. *: Significantly different from post-control group.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Thoracic aorta (U mg protein⁻¹)</th>
<th>Heart (U mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-control</td>
<td>3.6±0.3</td>
<td>10.2±0.6</td>
</tr>
<tr>
<td>Exercise</td>
<td>4.6±0.3*</td>
<td>12.7±0.6*</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE. * Significantly different from post-control group.

**Table II.** SOD Activity in Thoracic Aorta and Heart.
activity in thoracic aortas and hearts from rats in the exercise group and post-control group. The average SOD activity in thoracic aortas and hearts from the exercise group was significantly higher than that of the post-control group (Table II).

**DISCUSSION**

**Blood pressure:** We found that in SHRs, exercise for 10 weeks reduces the elevation of systolic blood pressure with aging. It has been reported elsewhere that the resting systolic blood pressure in male SHR is normalized by moderate chronic exercise. In our study, citrate synthase activity in the plantaris muscle of the exercised rats was significantly higher than in the post-control rats (Table I). Since citrate synthase activity is a marker of energy metabolism, this result suggests that the exercise training we used had a substantial effect on the muscle. Therefore we can conclude that voluntary wheel running may have a significant effect on the attenuation of systolic blood pressure elevation in age matched SHRs.

**Plasma total nitrite:** Since NO is mainly metabolized to nitrate and nitrite in blood in vivo, total nitrite can be used as a measure of the endogenous formation of NO. It has been shown previously that the nitrate level in rats after 12 hours of fasting does not differ from the level measured in rats on a low nitrate diet. Since the food was removed and the drinking water was replaced with distilled water twelve hours before blood sampling, the measured amounts of total nitrite in plasma samples reflect the level of endogenous formation of NO in this study. In our study, plasma total nitrite levels were elevated after 10 weeks of voluntary exercise. Furthermore, the total nitrite level in the post-control group was not statistically different compared to the pre-control group (Figure 2). The recent study by Jonsdottir, et al also showed that exercise training of SHR for 35 days increased the formation of NO. However, they did not use an age-matched post-control group to distinguish between two possible causes of enhanced NO formation in their experiments; exercise and the development of hypertension. In our study, we have confirmed that exercise is the cause of enhanced NO formation. Therefore, we conclude that increased formation of NO by chronic exercise is one of the factors that attenuates the elevation of systolic blood pressure with aging in SHR.

On the other hand, Kelm, et al reported enhanced production of NO in the hearts of SHRs and Nava, et al reported increased nitric oxide synthase activity in the hearts of SHRs. These results support the concept that high blood pressure upregulates NO formation. In our study, we did not find increased levels of NO in the plasma of rats from the post-control group. The cause of this apparent discrepancy is not clear at present, however, one possible explanation is that plasma
NO concentration could be mostly controlled by nitric oxide synthase in aortas rather than that of heart. In order to examine this possibility, measurements of the activity and amount of nitric oxide synthase in aortas and heart of exercised and non-exercised SHRs are now underway in our laboratory. The other possibility is that they used SHRs between 16 weeks and 25 weeks of age, which are older than our sample (15 weeks). Therefore, the elevation of blood pressure in SHRs might be more extensive in their case. The extensive elevation of blood pressure might cause an elevation in NO synthase activity as a compensatory mechanism.

**Angiotensin II and superoxide dismutase:** In this study, we found that the elevation of plasma levels of ANG II with aging was prevented by chronic exercise in SHR. Recent evidence suggests that hypertension is associated with increased vascular oxidant stress through an increase in levels of ANG II. Furthermore, Laursen, et al. and Rajagopalan, et al. reported that ANG II-induced hypertension was associated with increased vascular superoxide production and that SOD played a role in restraining the elevation in blood pressure caused by angiotensin II. Therefore, we speculate that the diminished production of superoxide by low levels of ANG II in the plasma of the exercised rats may result in increased levels of NO, since superoxide is known to react with NO. This mechanism may contribute to the lower blood pressure found in these rats.

Three potential enzymatic sources of reactive oxygen species in vascular tissue have been identified. These are NADH/NADPH oxidase, xanthine oxidase, and NO synthase. NADH/NADPH oxidase is present in endothelial and vascular smooth muscle cells and its activity is increased following stimulation with ANG II, via activation of p22phox, to produce superoxide. The other two enzymes are also possible sources for superoxide production, but regulation of these enzymes is poorly understood at present.

Endothelial superoxide dismutase has been proposed as a factor involved in the regulation of levels of NO. In this study, we observed a slightly negative correlation between the specific activity of superoxide dismutase and the systolic blood pressure among individuals from both exercised ($r=-0.43$) and post-control SHR groups ($r=-0.66$, data not shown). In addition, the average SOD activity of the exercise group was significantly higher than that of the post-control group. We conclude that in SHRs that have undergone chronic exercise, the increase in SOD activity could be another important factor that contributes to the normalization of blood pressure. In agreement with our conclusion, recent studies have shown that endogenously added HB-SOD, which is a genetically engineered protein having a C-terminal basic peptide with high affinity for heparin sulfate on endothelial cells, decreases the blood pressure of SHR but not that of normotensive rats.
In summary, chronic exercise in SHRs resulted in increased production of NO and increased SOD activity in the thoracic aorta and heart, and prevented elevation of ANG II concentrations in the plasma. We speculate that the latter two findings could contribute to a decrease in the steady state concentration of vascular superoxide and in turn upregulate the concentration of vascular NO. In order to confirm this, we are now comparing the activities of NADH/NADPH oxidase and xanthine oxidase, which are major sources of superoxide formation in the vascular system, in aortas of exercised and non-exercised SHRs.

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