Original Article

Effects of Different Levels of Exercise Volume on Endothelium-Dependent Vasodilation: Roles of Nitric Oxide Synthase and Heme Oxygenase

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The objective of this study was to examine the effects of moderate and high levels of exercise volume on endothelium-dependent vasodilation and associated changes in vascular endothelial/inducible nitric oxide synthase (eNOS and iNOS) and heme oxygenase (HO). Male Sprague-Dawley rats were assigned to sedentary control, acute (2 weeks), or chronic (6 weeks) treadmill running at moderate intensity (50% maximal aerobic velocity) with different durations of exercise episodes: 2 h/d (endurance training, moderate volume) and 3 h/d (intense training, high volume). Endothelium-dependent vascular function was examined in isolated thoracic aorta. Co-localization and contents of aortic eNOS/iNOS and HO-1/HO-2 were determined with immunofluorescence and Western blotting. Compared with sedentary controls, rats subjected to acute and chronic endurance training showed enhanced endothelium-dependent relaxation ($p < 0.01$). Whereas acetylcholine-induced dilation was inhibited completely by NOS inhibitor *N*^ω-nitro-L-arginine methyl ester (L-NAME) in sedentary controls, the dilation in the training groups was only partly blocked by L-NAME (inhibition was $98 \pm 3\%$, $79 \pm 6\%$, and $77 \pm 5\%$ in sedentary control, acute, and chronic training groups, respectively, $p < 0.01$). The remnant dilation in the training groups was further inhibited by HO inhibitor protoporphyrin IX zinc, with concomitant elevation in aortic eNOS as well as HO-1 and HO-2. In contrast to endurance exercise, high-volume intense training resulted in mild hypertension with significant impairment in endothelium-dependent vasodilation and profuse increases in aortic iNOS and eNOS ($p < 0.01$). In conclusion, endothelium-dependent vasodilation is improved by endurance exercise but impaired by chronic intense training. Elevations of vascular eNOS and HO-1/HO-2 may contribute to enhanced vasodilation, which can be offset by intense training and elevation in vascular iNOS. (*Hypertens Res* 2008; 31: 805–816)

Key Words: exercise, endothelium-dependent vascular relaxation, heme oxygenase, nitric oxide synthase

Introduction

It is well established that regular physical activity reduces the

risk of cardiovascular diseases (1–3). Some authors have reported that intense physical activity provides added benefits compared with mild to moderate activity (2, 4). However, intensive exercise can over-activate the sympathetic

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adrenomedullary system, resulting in cardiac injury and dysfunction (5, 6). Most guidelines for hypertension and atherosclerosis management, supported by several lines of evidence, recommend moderate-intensity aerobic activity for ≥ 30 min on most days of the week (7–10). Despite the large number of reports on exercise-related changes in the cardiovascular system, there are limited dose-response studies comparing the effects of moderate- vs. high-volume physical activity on vascular function, and the related mechanisms remain unclear.

Heme oxygenase (HO) is an enzyme that, when degrading heme, creates carbon dioxide (CO) (11, 12), another endogenous gas known to dilate blood vessels in a manner similar to nitric oxide (NO). It is well known that during exercise, increased blood flow and sheer stress augment endothelium-dependent vasodilation through the upregulation of endothelial nitric oxide synthase (eNOS) (13, 14). However, it is unclear whether or not HO adds to exercise-induced enhancement in endothelium-dependent vascular function beyond the eNOS pathway. HO-1 is readily inducible by different stimuli, including NO and hypoxia, while HO-2 is the constitutive isoform of the enzyme. It was reported that exercise could induce HO-1 in myocardium and skeletal muscle as well as in circulating neutrophils or lymphocytes (15, 16). Thus far there has been no report on exercise-induced vascular HO and HO-associated endothelial function.

Therefore, the present study was designed to examine effects of moderate to high levels of exercise on endothelium-dependent and HO-associated vascular relaxation in normal rats, as well as on corresponding changes in vascular HO-1/HO-2 and eNOS. We also examined whether or not endurance and intense exercise would lead to changes in inducible NOS (iNOS) that can induce free radical damage to tissues.

Methods

Animals and Exercise Training

All experimental procedures were performed under protocols approved by the Animal Care Committee of the Animal Center at the Chinese Academy of Science in Shanghai and by the Committee on the Care and Use of Animals in Research at Shanghai Jiao Tong University School of Medicine.

Male Sprague-Dawley rats at 2 months of age, obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Science, were housed individually in a temperature-controlled room with a 12:12-h light–dark cycle. Water and rat chow were provided *ad libitum*.

The maximal aerobic velocity (MAV) was evaluated for each rat according to Reboul *et al.* (17) with modifications. Briefly, after 1 week of acclimation to treadmill running, the rats were allowed to run on a treadmill at a beginning speed of 10 m/min for 30 s. Then the speed was increased by 4 m/min every 30 s until 85–90% of the expected MAV was reached. Thereafter the speed increment was adjusted to 2 m/min every

30 s. The highest velocity obtained before the animal was unable or unwilling to continue was recorded as MAV. The whole evaluation process took about 4 to 6 min, depending on the running potential of individual animals.

After MAV determination, rats were assigned either to a sedentary control group or to treadmill running at 50% MAV in three groups: acute endurance exercise (2 h/d, 5 d/week for 2 weeks), chronic endurance exercise (2 h/d, 5 d/week for 6 weeks), or chronic intense exercise (3 h/d, 5 d/week for 6 weeks) ($n=10$ per group). In a preliminary study, we found that rats were unable to run for 3 h after a short acclimation training period. Therefore, no group of acute intense training was included in the present study.

During acclimation training, an electric shock was applied on the first day to make the rats start running, after which they would run spontaneously. During the first week of endurance training, all rats ran at 50% MAV for about 1 h per day on the first day, increasing gradually to 2 h (endurance training) or 3 h (intense training) per day by the end of the week. During the 2 h running, there were two occasions when the running speed was lowered gradually to a full stop within 2 to 3 min, allowing the rats to rest for about 4 to 5 min. This was done in order to achieve the prescribed exercise volume without stimulation. In the intense running group, it was necessary to make the rats run by prodding them with sticks during exercise training until they reached the prescribed volume. The running intensity was kept to this initial MAV to maintain relatively constant levels of exercise volume throughout the experiment. The experiment was carried out in a quiet, well-ventilated room with low humidity, at room temperature around $18\pm 2^\circ\text{C}$. Age-matched sedentary rats were handled the same as the exercised rats except that they did not engage in regular running.

Blood pressure and heart rate were measured in the conscious state by the tail-cuff method before exercise and every 2 weeks after the beginning of exercise. At the end of the exercise protocol, the thoracic aorta was isolated and vascular function was examined *in vitro*. Separated segments of the aorta were examined with Western blotting or immunohistochemistry for the contents or co-localization of HO-1/HO-2 and eNOS/iNOS.

Assessment of Vasomotor Function *In Vitro*

Vascular responses were determined 24 h after the last exercise session. The method was modified slightly from our previous description (18). Briefly, after anesthesia, the thoracic aorta was removed carefully, cleaned of fat and adherent connective tissues, cut into segments 2 to 3 mm in length, and mounted on two stainless-steel stirrups in a 10 mL organ chamber with Krebs-Henseleit buffer containing (in mmol/L): NaCl 118.0, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 25.0, glucose 11.0, and $\text{Na}_2\text{-EDTA}$ 0.5, at 37°C . One stirrup was connected to an isometric force transducer for tension measurement. The rings were stretched to a resting

Table 1. Basic Data of Exercise Training

	Prescribed intensity, %MAV	Prescribed volume, km/week	MAV at the beginning of training, m/min	Actual running distance, m/d	Actual running duration, min/d	Actual volume, km/week
Acute exercise-endurance	50	10	35.8±5.9	2,160±522	125±21	10.5±0.3
Chronic exercise-endurance	50	10	36.0±7.2	2,205±180	133±7	10.8±0.1
Chronic exercise-intense	50	20	36.5±7.1	3,905±297**	205±12**	19.5±0.1**

MAV, maximal aerobic velocity. Data are mean±SD, $n=10$. ** $p<0.01$ vs. endurance exercise.

tension of 2.0 g and allowed to equilibrate for 60 min, with the buffer changed every 10 min. Contraction response was evaluated with 60 mmol/L of KCl twice followed by 1 μ mol/L of phenylephrine. Endothelium-dependent vascular relaxation was assessed by 1 μ mol/L of acetylcholine when a contractile plateau was reached after 1 μ mol/L phenylephrine. Then, dose-response relaxation to cumulated acetylcholine and sodium nitroprusside was examined.

To investigate possible mechanisms responsible for exercise-induced relaxation, the rings were further incubated with nitric oxide synthase (NOS) inhibitor N^G -nitro-L-arginine methyl ester (L-NAME, 10 μ mol/L, 30 min) and/or HO inhibitor protoporphyrin IX zinc (II) (ZnPPIX, 1 μ mol/L, 30 min) before phenylephrine administration. Acetylcholine-induced vascular relaxation was re-examined after NOS and HO blockade.

Immunohistochemistry Study

Aortic segments not used in the *in vitro* experiment were immersed in 4% polyformaldehyde and embedded in paraffin. Histological sections 5 μ m thick, prepared from paraffin-embedded tissue samples, were used for immunohistochemical analysis. Immunohistochemical localization of eNOS, iNOS, HO-1, and HO-2 was performed using antibodies against eNOS, iNOS, HO-1, or HO-2. For double immunofluorescence reactions, sections were incubated in a mixture of primary antibodies that included a monoclonal anti-eNOS antibody (1:1,000 dilution, Sigma, St. Louis, USA) and a polyclonal anti-iNOS antibody (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, USA), or in a mixture of primary antibodies that included a Stressgen monoclonal anti-HO-1 antibody (1:100 dilution; Assay Designs, Ann Arbor, USA) and a monoclonal anti-HO-2 antibody (1:400 dilution; Assay Designs). After incubation with the primary antibodies overnight at 4°C, sections were rinsed three times, followed by incubation with streptavidin-fluorescein isothiocyanate (FITC)-conjugated and tetramethylrhodamine isothiocyanate (TMRITC)-conjugated secondary antibodies for 30 min in a light-protected humidified chamber. The sections were then washed with PBS and the fluorescent co-localization of eNOS and iNOS, or that of HO-1 and HO-2, was observed under a confocal microscope (Leica TCS SP2 Microsystems, Heidelberg, Germany). Digital images were captured and imported

Fig. 1. Blood pressure and heart rate after different levels of exercise. Acute Ex-Endurance, acute endurance exercise; Chronic Ex-Endurance, chronic endurance exercise; Chronic Ex-Intense, chronic intense exercise. * $p<0.05$, ** $p<0.01$ vs. Sedentary Control.

into Adobe JPEG files, and the areas of red and green and respective fluorescence intensities (scale units ranging from 0 to 255, absolute red or green to absolute black) were analyzed individually using Image-Pro Plus 5.0 software (Media Cybernetics, Bethesda, USA). The obtained mean intensity for each image was inverted, with values of brighter color being higher, and divided by the respective area. The mean values in different groups were then compared with the control value as reference. Histological analysis was performed on aortic segments from 6 to 8 rats from each group.

Fig. 2. Acetylcholine-induced and nitroprusside-induced vasorelaxation after exercise at different training levels. Acute and chronic endurance training enhanced endothelium-dependent vascular dilation, but chronic intense training impaired endothelium-dependent dilation (A, $p < 0.01$). There were no significant changes in endothelium-independent vascular dilation (B, $p > 0.05$). Sed Control, sedentary control; Acute Ex, acute endurance exercise; Chronic Ex-E, chronic endurance exercise; Chronic Ex-I, chronic intense exercise.

Western Blotting

Fresh aortic segments were frozen in liquid nitrogen and stored at -80°C until assay. After pulverization, frozen aortic tissue was homogenized with a Polytron homogenizer (PT 10-35; Brinkman, San Francisco, USA) in lysis buffer (50 mmol/L β -glycerophosphate, 100 mmol/L Na_3VO_4 , 2 mmol/L MgCl_2 , 1 mmol/L EGTA, 0.5% Triton X-100, and 1 mmol/L DTT) containing protease inhibitors (20 mmol/L pepstatin, 20 mmol/L leupeptin, 1,000 U/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). Protein concentration was determined using a protein assay (Bio-Rad Laboratories; Hercules, USA). The soluble lysates (20 μg per lane) were subjected to 10% (w/v) SDS-PAGE; proteins were then transferred to nitrocellulose membranes and blocked with 5% (w/v) nonfat milk/Tris-buffered saline Tween 20 (TBST) for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies directed against tubulin (1:5,000); eNOS (1:1,000); iNOS (1:1,000); HO-1 (1:1,000), and HO-2 (1:1,000) in 5% (w/v) milk/TBST. The membranes were washed three times with TBST and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using electrochemoluminescence (Roche, Mannheim, Germany) and quantified by scanning densitometry (Model 670 scanning densitometer, Bio-Rad Laboratories). Because of a limited supply of tissue for blotting, two segments from different individual rats in the same group were pooled and treated as one sample. Therefore, there were 5 samples in each group.

Plasma Nitrite and Nitrate Examination

Rats were sacrificed and blood samples collected for examination of plasma nitrite and nitrate (NOx) with an assay kit (Jian-Cheng Biomedical Engineering, Nanjing, China) according to the manufacturer's protocol.

Statistical Analysis

Data are presented as means $\pm$ SD. Differences in blood pressure, heart rate, running distance, and duration among groups were analyzed with ANOVA followed by Student-Newman-Keuls post hoc analysis. Repeated measurement ANOVA was used for concentration-response relation or time-dependent vascular relaxation. Significance was defined as $p < 0.05$.

Results

Basic Data

Table 1 shows prescribed exercise intensity and volume with actual exercise levels in different groups. Rats in the chronic intense exercise group had higher weekly exercise volume with longer duration of exercise episodes compared with the acute group. Running velocity, which represents exercise intensity, was 50% MAV in all training groups.

At the end of exercise training, no significant change in blood pressure was found in the acute group. Although rats in the chronic endurance group showed a trend of lower blood pressure, the difference from control rats was not significant ($p > 0.05$, Fig. 1A). However, rats run intensely for 6 weeks

Fig. 3. Representative tracing (A) of vascular contraction and relaxation to phenylephrine (PE) and acetylcholine (ACh) and time-dependent responses to submaximal ACh (B, C, D) with or without inhibition of nitric oxide synthase (NOS) and/or heme oxygenase (HO). L-NAME, *N*^ω-nitro-L-arginine methyl ester (NOS inhibitor, 10 $\mu\text{mol/L}$); ZnPPiX, protoporphyrin IX zinc (II) (HO inhibitor, 1 $\mu\text{mol/L}$). Sed Control, sedentary control; Acute Ex, acute endurance exercise; Chronic Ex-E, chronic endurance exercise; Chronic Ex-I, chronic intense exercise.

had significantly higher blood pressure than the controls ($p < 0.01$, Fig. 1A). Chronic endurance training induced a small reduction in heart rate ($p < 0.05$, Fig. 1B).

Exercise Training and Vascular Relaxation

Acute and chronic endurance exercise caused similar levels of endothelium-dependent vascular relaxation in response to cumulated acetylcholine (Fig. 2A) and sodium nitroprusside

Fig. 4. Maximal relaxation in response to submaximal acetylcholine after nitric oxide synthase (NOS) and heme oxygenase (HO) blockade in rats with exercise training at different levels. L-NAME, *N*^ω-nitro-L-arginine methyl ester (NOS inhibitor, 10 $\mu\text{mol/L}$); ZnPPIX, protoporphyrin IX zinc (II) (HO inhibitor, 1 $\mu\text{mol/L}$). Sed Control, sedentary control; Acute Ex, acute endurance exercise; Chronic Ex-E, chronic endurance exercise; Chronic Ex-I, chronic intense exercise. ** $p < 0.01$ vs. Sed Control.

(Fig. 2B). Chronic intense running impaired acetylcholine-induced relaxation ($p < 0.01$), but did not change sodium nitroprusside-induced vascular relaxation (Fig. 2).

Figure 3A demonstrates a typical tracing of a time-dependent response of aortic segments to submaximal acetylcholine (1 $\mu\text{mol/L}$) in the absence or presence of nonselective NOS inhibitor L-NAME and HO inhibitor ZnPPIX. Acute and chronic endurance training induced an increase in relaxation to submaximal acetylcholine compared with sedentary control. However, chronic intense running decreased relaxation to submaximal acetylcholine (maximal relaxation to 1 $\mu\text{mol/L}$ acetylcholine in sedentary control, acute endurance training, chronic endurance training, and chronic intense training was $84.0 \pm 1.3\%$, $91.8 \pm 1.6\%$, 93.7 ± 1.7 and $68.4 \pm 2.5\%$, respectively) ($p < 0.01$, Figs. 3B and 4A). Incubation with NOS inhibitor L-NAME showed a complete blockade of endothelium-dependent vascular relaxation in sedentary and chronic intense-training animals (Figs. 3C and 4B). In acute and chronic endurance-training animals, however, endothelium-dependent vascular relaxation was not completely blocked by L-NAME alone (Figs. 3C and 4B). The remaining effect of relaxation after NOS inhibition was removed by blockade of HO inhibitor, ZnPPIX (Figs. 3D and 4C).

Effects of Exercise Training on Aortic NOS and HO Contents

Figure 5 shows enhanced eNOS (upper lane, red) and iNOS (lower lane, green) along the endothelial (left) and perivascular (right) areas in exercise-trained aortic segments. However, chronic intense-training rats demonstrated markedly diffuse increases in both eNOS and iNOS in three layers across the aortic wall (Fig. 5). Both constitutive (HO-2, upper lane, green) and inducible (HO-1, lower lane, red) forms of HO were elevated in the aorta of exercised rats. HO-1 elevation was especially evident along the endothelium-lined side (left side) and perivascular area (right side) of the aorta (Fig. 6), coincident with exercise-induced eNOS in the same aortic layers. HO-2 was induced relatively evenly across the three aortic layers.

Changes in total contents of aortic NOS and HO by Western blotting (Fig. 7) showed results similar to those by immunofluorescence assay. However, iNOS content was slightly increased in the chronic endurance training group (Fig. 7A). HO-1 and HO-2 were increased in all exercise groups (Fig. 7B).

In accordance with NOS elevation in the aorta of exercised rats, plasma NOx levels were also significantly increased after exercise. Plasma NOx was significantly higher in acute endurance exercise rats (269 ± 55 $\mu\text{mol/mL}$, $p < 0.05$) and chronic endurance exercise rats (322 ± 94 $\mu\text{mol/mL}$, $p < 0.05$) compared with sedentary control rats (186 ± 35 $\mu\text{mol/mL}$). Chronic intensive exercised rats had the highest level of plasma NOx (829 ± 137 $\mu\text{mol/mL}$, $p < 0.01$ vs. control), which was significantly higher than the acute and chronic endurance

Fig. 5. Immunofluorescent co-localization of endothelial nitric oxide synthase (eNOS, red) and inducible NOS (iNOS, green) (A) and relative fluorescence intensities (B, C) in aorta of rats after exercise. Both eNOS and iNOS were increased after endurance training along the endothelium side (left) and perivascular area (right). Intense-training rats showed marked and diffuse increases in eNOS and iNOS in three aortic layers. Sed Control, sedentary control; Acute Ex-E, acute endurance exercise; Chronic Ex-E, chronic endurance exercise; Chronic Ex-I, chronic intense exercise. ** $p < 0.01$ vs. Sed Control.

exercise groups ($p < 0.01$).

Discussion

The main findings of the present study are: 1) endurance exercise training enhanced HO-related acetylcholine-induced vasorelaxation, 2) HO-1 and HO-2 contents were significantly increased in the aorta after acute and chronic endurance training, and 3) endurance training induction of HO-1 was mainly in endothelial and perivascular layers, coincident with distribution of eNOS, while HO-2 was induced across the three layers of the aortic wall. Although elevation of HO

mRNA and protein were reported in the rat cardiovascular system after exercise (15, 16), to the best of our knowledge, the present study is the first report demonstrating exercise-induced increases in vascular HO-1 and HO-2 with enhancement in HO-related vasorelaxation. We also found that high volume intense exercise led to hypertension and impairment of endothelium-dependent vascular relaxation, with marked and diffuse increases in eNOS and iNOS across three aortic layers.

It is well recognized that NO participates in exercise-induced enhancement of vascular function and remodeling (13, 14, 19–21). In the present study, we also found a significant increase in aortic eNOS content after acute and chronic

Fig. 6. Immunofluorescent co-localization of aortic constitutive heme oxygenase (HO-2, green) and inducible heme oxygenase (HO-1, red) (A) and relative fluorescence intensities (B, C). Both HO-1 and HO-2 isoforms were enhanced after exercise at different training levels. While HO-2 was distributed in three layers of the vessel, HO-1 was relatively abundant in endothelial (left) and perivascular (right) layers. Sed Control, sedentary control; Acute Ex-E, acute endurance exercise; Chronic Ex-E, chronic endurance exercise; Chronic Ex-I, chronic intense exercise. * $p < 0.05$, ** $p < 0.01$ vs. Sed Control.

endurance training, in accordance with increased acetylcholine-induced vascular relaxation. However, eNOS is not the only signal that enhances the exercise-induced increase in endothelium-dependent relaxation. Blockade of NOS with L-NAME partly reversed the acetylcholine-induced vasorelaxation in the endurance training groups (Figs. 3 and 4). Further inhibition of HO removed the remnant relaxation completely, indicating the involvement of HO in exercise-induced vasodilation. The significant increase in aortic HO-1 in the endothelial layer further supported the role of endothelium-associated HO-1 in endurance training-related vasorelaxation. Aortic content of HO-2 was also elevated, demonstrating direct par-

ticipation of the HO system in cardiovascular adaptation to endurance training. The present findings provide direct evidence of exercise-induced elevation of vascular HO and enhanced HO-related dilation, which is in agreement with a previous finding that exercise increased vascular response to the vasorelaxing effect of CO-releasing molecules in hypertensive rats (22).

The mechanisms underlying the exercise-induced increase in HO remain unclear. Heme oxygenase-1 is an antioxidant stress protein that is mainly induced by reactive oxygen species, inflammatory cytokines, NO, and hyperthermia (12, 23). Peake and Suzuki suggested that neutrophil activation during

Fig. 7. Western blot analysis of aortic nitric oxide synthase (NOS, A) and heme oxygenase (HO, B). Sed Control, sedentary control; Acute Ex-E, acute endurance exercise; Chronic Ex-E, chronic endurance exercise; Chronic Ex-I, chronic intensive exercise. eNOS, endothelial NOS; iNOS, inducible NOS. * $p < 0.05$, ** $p < 0.01$ vs. Sed Control.

exercise may challenge endogenous antioxidant defense mechanisms (23). As an important part of the defense system, HO may be upregulated due to muscle damage and thermal stress during exercise, and this in turn may play a role in tissue protection and vascular dilation. In a recent report, Steensberg *et al.* found that NO synthase inhibition markedly reduced exercise-induced expression of HO-1 mRNA in

human skeletal muscle, and infusion of NO donor nitroglycerin augmented expression of the HO-1 mRNA attenuated by NO synthase blockade (24). Therefore, they suggested NO to be a proximal signal that controls exercise-induced HO-1 mRNA expression in human skeletal muscle. Others also found that NO was required for CO-induced cerebrovascular dilation (25). Therefore, exercise-induced NO may be a

potential inducer of HO. In the present study, exercise-induced HO-1 localization and elevation were coincident with eNOS in the endothelium and perivascular area in endurance-trained rat aorta (Figs. 5 and 6), demonstrating close interaction between these two systems. In addition to their antioxidant effects, the NOS and HO systems may exert synergistic action in regulating vascular relaxation.

Another important finding is that chronic intense exercise resulted in mild hypertension and endothelial dysfunction. The vasorelaxation impairment was associated with marked and diffuse increases in iNOS and eNOS throughout the aortic wall, as well as a marked elevation in plasma NOx. Although several lines of evidence indicate that exercise intensity is positively related to cardiovascular risk reduction, excessive exercise stress can result in myocardial injury, DNA damage, and endothelium-dependent vasorelaxation impairment (2, 4–6, 26–28). Isometric exercise can also increase total peripheral resistance, thus contributing to increased blood pressure (29). In this study, chronic intense isotonic exercise resulted in blood pressure elevation and impaired endothelium-dependent relaxation, along with a significant increase of vascular iNOS, leading to significant amounts of free radicals that could be detrimental to endothelial function. The elevation of plasma NOx, especially chronic intense exercise, reveals significant increases in systemic reactive oxygen species products that may add to local injury of endothelium and its function. Although eNOS and HO were also induced after intense training, the marked elevation of iNOS may put excessive oxidative stress on vessels and offset the beneficial effects of eNOS as well as HO, leading to overt hypertension.

It is also noted that there was a shorter duration of relaxation after acetylcholine in the presence of L-NAME in the chronic endurance group than in the acute endurance group (Fig. 3C). As the beneficial effects of exercise may differ in response to a graded increase in exercise volume, this lower relaxation response may indicate that the chronic exercise volume we chose was somewhat higher than what we expected to yield the best benefit. It was also found that chronic endurance exercise also increased iNOS content in the aorta (Figs. 5 and 7), which may actually undergo vascular damage following an adaptation period during chronic endurance exercise. This is one of the limitations of the present study. To clarify this issue, more detailed data on exercise intensity and volume are needed in future studies.

Jennings *et al.* found that activity 3 or 7 times per week of 45 min of bicycling at 60–70% maximum work capacity decreased blood pressure, and the increased work capacity was linearly related to the amount of exercise performed each week. However, with 7 exercise sessions per week, blood pressure was only slightly lower than that with 3 sessions per week, and the same exercise 7 times per week enhanced physical performance with little further reduction in cardiovascular risk factors (30, 31). These results also suggest the value of moderate instead of intense exercise in cardiovascular protec-

tion. Furthermore, the study by Lee *et al.* showed that although physical activity is associated with decreased cardiovascular risk, longer sessions of exercise did not have a different effect on risk than shorter sessions of physical activity, as long as the total energy expended was similar (32). These results lend some support to recommendations that allow for the accumulation of shorter exercise sessions to reduce cardiovascular risk (33), as opposed to requiring one longer, continuous session. We also found that mild exercise intensity (30% MAV), 20 min per day, 7 d per week improved insulin resistance and endothelium-dependent vascular relaxation in spontaneously hypertensive rats (34). Therefore, the proper choice of exercise intensity and duration of exercise episodes is important for cardiovascular risk reduction.

It is well accepted that a cumulated volume of exercise or chronic exercise provides a better cardiovascular benefit than a single session of exercise. However, some studies also found that as short as 2 h (16, 24, 35) of exercise or a half-marathon run (36) could induce significant increases in HO-1 and NOS genes or proteins. Exercise lasting 1 to 6 weeks and even a single session of moderate-intensity exercise were found to induce NO-related vasorelaxation (37–39). In accordance with these results, acute endurance training for 2 weeks as well as that for 6 weeks in the present study also demonstrated an improvement in acetylcholine-induced vasodilation, demonstrating the body's quick response to exercise and the favorable effects that also ensue.

In conclusion, the present data indicate that acute and chronic endurance exercise was associated with not only elevated eNOS but also elevated vascular HO-1/HO-2 and enhanced HO-related vasodilation. Furthermore, chronic intense exercise could lead to endothelial dysfunction and hypertension. Excessive induction of iNOS may confer oxidative stress on vessels, thus offsetting the benefits of exercise-induced HO and eNOS production. Nonetheless, a limitation of the present study is that we examined only the aortic response to exercise. Resistance vessels such as mesenteric or femoral arteries may respond differently to various levels of exercise. Although the role of soluble guanylate cyclase (sGC)–CO–mediated vasodilation in hypertension has been reported to be similar in the aorta and resistance arteries (40–43), the responses of resistance vessels to different levels of exercise and the relevance of the present findings to clinical settings need further investigation.

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