Voluntary physical exercise-induced vascular effects in spontaneously hypertensive rats

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ABSTRACT

Forced training has been shown to have beneficial vascular effects in various animal exercise models. In the present study, we explored possible physiological and molecular effects of voluntary physical exercise on various vascular beds. SHR (spontaneously hypertensive rats) performed voluntary exercise for 5 weeks in a computerized wheel cage facility. Ex vivo myograph studies revealed an increased sensitivity of the ACh (acetylcholine)-mediated vasodilation in resistance arteries of the exercised animals (ED50 = 15.0 ± 3.5 nmol/l) compared with the controls (ED50 = 37.0 ± 8.8 nmol/l; P = 0.05). The exercise/control difference was abolished after scavenging reactive oxygen radicals. In conduit arteries, ACh induced a similar vasodilatory response in both groups. The in vivo aortic wall stiffness, assessed by means of Doppler tissue echography, was significantly lower in the exercising animals than in controls. This was demonstrated by significantly increased peak systolic aortic wall velocity (P = 0.03) and the velocity time integral (P = 0.01) in exercising animals compared with controls. The relative gene expression of eNOS (endothelial nitric oxide synthase) was similar in both groups of animals, whereas Cu/ZnSOD (copper/zinc superoxide dismutase) gene expression was significantly increased (+111%; P = 0.0007) in the exercising animal compared with controls. In conclusion, voluntary physical exercise differentially improves vascular function in various vascular beds. Increased vascular compliance and antioxidative capacity may contribute to the atheroprotective effects associated with physical exercise in conduit vessels.

INTRODUCTION

Epidemiological data suggest that physical exercise has beneficial effects on the outcome of many cardiovascular diseases [1]. In addition to positive metabolic effects, exercise training has been demonstrated to exert various direct vascular effects in both animals and humans [2,3]. However, the molecular mechanisms underlying exercise-induced vascular effects are still not fully understood.

It is well established that the availability of NO (nitric oxide) in the vascular wall is crucial for the atheroprotective properties of the vessel wall [4]. Production of this molecule can be regulated from the level of de novo synthesis of eNOS (endothelial NO synthase) to enzyme activity. Furthermore, high oxidative stress in terms of...
increased production of oxygen free radicals has been shown to abate the biological effects of NO [5]. Exercise training appears to enhance free radical (superoxide) production as well as the synthesis of SOD (superoxide dimutase) [6]. Increases in SOD expression following chronic exercise have been suggested to be atheroprotective [7]. Interestingly, Meilhac et al. [8] showed that exercise training reduced atherosclerosis in mice through an increased antioxidant defence mechanism. In SHR (spontaneously hypertensive rats), short-term exercise for 5–12 weeks has shown beneficial effects on vascular reactivity [9,10] and eNOS protein levels [10]. Cu/ZnSOD activity and protein levels have been shown to be increased in Fisher rats after 8 weeks of exercise [11].

However, most of the previous data has been based on forced training protocols, which may not necessarily reflect the situation in human exercise. It is conceivable that animals exposed to forced training might demonstrate different neurohumoral patterns due to environmental stress. In order to mimic the human exercise situation, a voluntary running rat model has been established [12].

In this model, SHR perform voluntary physical exercise in a computerized wheel cage system. Adult SHR of 9 weeks of age were used in the study, since animals of this age are considered to have established hypertension. Using this voluntary exercise model, effects of exercise on central haemodynamics in adult SHR have been studied extensively [13,14]. It has also been shown that plasma level of nitrate, the stable metabolite of NO, was increased after 35 days of voluntary exercise in SHR [15]. However, plasma nitrate levels may not necessarily reflect NO synthesized in the vascular wall and could potentially be influenced by dietary intake as well as kidney metabolism [16,17]. Furthermore, the possible impact of voluntary exercise on specific vascular function in this model has not been fully investigated.

The aim of the present study was to investigate the effects of exercise on large and small vessel function using the voluntary running exercise model. Possible involvement of the NO pathway was explored. An ex vivo approach was used to assess the endothelial function in resistance and conduit vessel segments to dissect out the specific underlying mechanisms. In vivo conduit vessel stiffness was studied non-invasively using Doppler tissue echography. The physiological findings were verified further at the protein and gene expression levels.

**MATERIALS AND METHODS**

**Animals**
The experiments were performed in 30 female SHR (M&B, Ejby, Denmark). Animals arrived at the age of 8 weeks and underwent acclimatization for 1 week before the onset of the experiment. All animals were housed under standard conditions. The experimental protocol was approved by the Regional Animal Ethic Committee, Göteborg University, Göteborg, Sweden.

**Computerized wheel cage model for spontaneous exercise**
This model has been described previously in detail [12]. In brief, the rats were randomly divided into an exercising group and a control group. All animals were allocated individually into separate cages of the same size for 5 weeks. The specially designed wheel cages provide the experimental animals with free access to the exercise wheel at any time. Wheel revolutions were registered by a microprocessor every 30 min during the whole experiment.

**In vivo telemetry**
The in vivo telemetry technique has been reported elsewhere [18]. Five animals in each group were implanted with radiotelemetry transmitters for blood pressure recordings.

**Corticosterone measurement**
Corticosterone levels in blood serum were measured by radioimmunolabelling assay (Coat-A-Count Rat Corticosterone; DPC TKRC1; Diagnostic Products, Los Angeles, CA, U.S.A.).

**Experimental protocol**
After the 5-week training period, animals were injected with an overdose of sodium pentobarbital and then killed. Blood samples were taken immediately after opening the right atrium. First, the intestinal compartment was isolated and placed in ice-cold PSS [physiological salt solution: 119 mmol/l NaCl, 4.7 mmol/l KCl, 5.5 mmol/l glucose, 25 mmol/l NaHCO₃, 1.18 mmol/l KH₂PO₄, 0.026 mmol/l EDTA, 2.5 mmol/l CaCl₂, 1.17 mmol/l MgSO₄ (pH 7.4) and equilibrated with 5% CO₂]. Secondly, the rats were perfused with 50 ml of ice-cold PSS. The aorta and heart were dissected and placed in ice-cold PSS. The right and left ventricles were weighed.

**Ex vivo vascular function**

**Mounting procedure**
The second or third branch [normalized internal diameter: controls, 179 ± 6 µm; exercising animals, 167 ± 7 µm; P = NS (not significant)] of the mesenteric artery was dissected free from adjacent connective tissue. Sections of approx. 2 mm were mounted on to two stainless-steel wires (diameter, 40 µm) in a MultiMyograph System (610M; Danish Myo Technology, Aarhus, Denmark)
for recordings of their isometric wall tension at constant temperature (37 °C) and pH (7.4) in PSS. The resting vessel segments were then normalized via passive stretching to 90% of the relaxed in vivo diameter at a transmural pressure of 100 mmHg [19].

Thoracic aortic rings of approx. 3 mm length were mounted on two stainless-steel hooks (diameter, 400 µm), one of which was connected to a force transducer. Vessel preparations were immersed in PSS-containing vessel chambers (40 ml). Chambers were gassed continuously and a constant temperature was achieved by means of an external circulatory heating system. Isometric tension forces were recorded and amplified through a Grass system, and the data were collected by a digital data acquisition system (PharmLab; AstraZeneca, Mölndal, Sweden). Paired vessel segments from control and exercising rats were investigated in the same vessel chamber. After the mounting procedure, each preparation was stretched to 5 mN (resting tension) and equilibrated for 30 min. Subsequently, segments were stretched further to 10 mN and stabilized for 5 min.

Pharmacological protocol
First, the NE (noradrenaline) dose–response relationship was studied in mesenteric arteries to verify NE sensitivity. This was not done in the aortic segments. Similar protocols were then used in the following investigation of aortic and mesenteric vessels. Before the start of the experiment, vessel strips were treated with KCl (100 mmol/l) and NE (0.1 µmol/l; aritrenol, Sigma, St. Louis, MO, U.S.A.) to cause maximum contraction. After subsequent equilibration, the endothelium-dependent vaso-dilatory responses were studied by ACh (acetylcholine, 1 mmol/l–1 µmol/l) following NE contraction (0.1 µmol/l). Sodium nitroprusside (1–10 µmol/l; Sigma) was used to validate the endothelium-independent relaxation. The same protocol was carried out after incubation with MnTBAP [Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; 1 µmol/l; Calbiochem, San Diego, CA, U.S.A.], MnTBAP + l-NNA (N⁰-nitro-l-arginine; 10 µmol/l; Sigma) or MnTBAP + l-NNA + indomethacin (1 µmol/l; Confortid; Dumex-Alpharma, Copenhagen, Denmark). The three consecutive pharmacological interventions were used to eliminate: (i) oxidative stress (MnTBAP); (ii) NO-dependent relaxation (l-NNA + MnTBAP); and (iii) prostanoid-dependent relaxation (indomethacin + l-NNA + MnTBAP).

In vivo measurement of thoracic aorta stiffness using Doppler tissue echography
At the end of the experimental period, five animals in each group with the implanted telemetry underwent ultrasound examination of the thoracic aortic stiffness using a human Doppler tissue echography protocol validated previously [20]. Systolic and diastolic blood pressure were recorded online. The animals were anaesthetized with isoflurane gas (Forene isofluran; Abbott Scandinavia, Solna, Sweden), and the anterior chest wall was shaved mechanically. The animals were maintained in a lightly anaesthetized state with an isoflurane dose of 1.5–2.0% in air, resulting in a heart rate of approx. 300 beats/min.

A Philips HDI 5000 sonograph (ATL HDI 5000; ATL Ultrasound, Bothell, WA, U.S.A.) was used with a 15 MHz linear transducer equipped for Doppler tissue echography technology. A three-point ECG was connected to the right upper and both lower extremities of the rat. A parasternal transducer position was used to obtain a longitudinal view of the thoracic aorta caudally of the cardiac apex. Colour Doppler mode was used to verify the flow velocity pattern in the aorta. Adequate image quality was obtained in all the animals. Doppler tissue echography was used thereafter in the pulse wave Doppler mode with a velocity range between ±1 cm/s and a gate size of approx. 1.5 mm. Doppler gain setting was adjusted for an optimal Doppler tissue echography signal. Maximum systolic and diastolic Doppler tissue echography velocity and velocity time integral were averaged from three consecutive cardiac cycles. One operator performed all the ultrasound measurements and was blinded to the identity of the animals.

There was no difference between systolic and diastolic blood pressure during measurement. Diastolic diameter of the thoracic aorta was similar in both groups (results not shown). Aortic stiffness was studied by comparing the maximum wall motion velocity and the velocity time integral between the control and experiment group after correction of diastolic vessel diameter and blood pressure. Reproducibility was validated by repeated two-dimensional localization of the thoracic aorta and measurement of the Doppler tissue echography signals. Intra-observer variability was < 5%.

Immunohistochemistry
Thoracic aortic segments (3 mm) were fixed in 4% buffered formaldehyde, embedded in O.C.T.™ Compound (Sakura, Tokyo, Japan) and frozen in ice-cold isopentane. Sections of 6–10 µm were cut on a cryostat and slides were stored at −20 °C. Following several standard pretreatment steps, sections were incubated with the specific primary antibody [anti-eNOS antibody diluted 1:100 (#N30020-050; Transduction Laboratories, Erembodegem, Belgium), or anti-Cu/ZnSOD antibody diluted 1:100 (#sc-11407; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 24 h. Thereafter slides were incubated with biotinylated secondary antibodies (diluted 1:400; #BA-2001 and #BA-1000; Vector Laboratories, Burlingame, CA, U.S.A.) and, finally, stained using Vectastain ABC and DAB kits (Vector Laboratories).
Quantification of gene expression in aortic tissue

RNA extraction and cDNA synthesis

The aortic tissue was homogenized and total RNA was extracted using Trizol® Reagent (Invitrogen, Paisley, Renfrewshire, Scotland, U.K.). cDNA synthesis was carried out by RT (reverse transcription) using ThermoScript™ RT-PCR system (Invitrogen) according to the manufacturer’s protocol.

Quantitative real-time PCR

Relative quantification of mRNA expression was performed on a LightCycler (Roche Diagnostics, Mannheim, Germany), using SYBR® green chemistry. For amplification, 2 µl of diluted cDNA (1:8) was added to a reaction mixture consisting of PCR buffer [100 mmol/l Tris/HCl (pH 8.3) and 500 mmol/l KCl; Sigma], 5 mmol/l MgCl₂ (Sigma), 0.2 mmol/l dNTP, 0.1 g/l BSA (MBI Fermentas, Hanover, MD), 1 unit of Taq DNA polymerase (Sigma), 0.5 × SYBR® green I (Sigma) and forward and reverse primers [GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 0.5 µmol/l forward and reverse primers; eNOS, 0.7 µmol/l forward and reverse primers; Cu/ZnSOD, 0.5 µmol/l forward primer and 0.7 µmol/l reverse primer] in a final volume of 20 µl.

Oligonucleotides for LightCycler PCR assay

Oligonucleotide primers were designed using Primer Express version 1.0 (PerkinElmer Applied Biosystems, Warrington, Cheshire, U.K.) for GAPDH and eNOS and LightCycler Probe Design Software version 1.0 (Roche Diagnostics, Mannheim, Germany) for Cu/ZnSOD, based on sequences from the GenBank® database (Table 1). GAPDH was selected as endogenous control.

Principles of relative quantification

A standard curve was obtained by performing amplifications of the cDNA of the three genes in a series of 2-fold serial template dilutions of cDNA of total RNA from intact aortic vessel segments from 1:1 to 1:16 dilutions. The initial amount (IA) was determined by the following formula: IA = C₀ − b/m, where C₀ is the crossing point, b is the y-axis intercept and m is the slope of the individual standard curve [21]. The relative gene expression level of a target gene is expressed as an arbitrary unit, representing the ratio between the dilution factor of the target and the reference gene.

Validation of PCR amplification

Standard curves for the three genes (GAPDH, eNOS and Cu/ZnSOD) were obtained by plotting log-dilution (x-axis) against C₀ values (y-axis). The correlation factor for linear regression analysis (R²) of the three genes studied was 0.99, 0.98 and 0.99 respectively. Amplification efficiencies for the three genes, expressed as the slopes of the standard curves, were similar (−3.5, −3.2 and −3.3 respectively). The standard curves were subsequently used for calculations of the relative dilution value of each unknown sample. Specificity of the PCR product was validated by melting curve analyses.

Validation of endogenous control gene

The calculated relative dilution values for GAPDH, obtained by the second derivative maximum method [22], were similar in experimental and control groups (2.38 ± 0.34 and 2.48 ± 0.31; P = NS).

Statistics

All data are expressed as means ± S.E.M., unless otherwise indicated. For analysis of vascular reactivity, Prism™ 3.0 (GraphPad, San Diego, CA, U.S.A.) was used. Vascular relaxation was expressed as the percentage relaxation of NE preconstruction, and ED₅₀ values were obtained after non-linear regression analysis to represent ACh sensitivity. Due to the complete blockade of vasodilatation after NO-blockade in aortic strips, AUC (area under the curve) analysis was used to compare the vascular response.

Student’s t test was used to compare the following data between the exercising and control groups: body and heart weights, running distance, blood pressure, corticosterone levels, ED₅₀ and AUC values, Doppler tissue echography data and finally relative gene expression ratios.

For all statistical analyses, a P value of less than 0.05 was regarded as significant.

RESULTS

Body and heart weights

BWTs (body weights) were slightly higher (P = 0.01) in the exercising animals (228 ± 4 g) compared with the control group (216 ± 3 g). Also, both left and right ventricle weights, normalized to BWT, were higher in the exercising animals (3.6 ± 0.07 mg/g of BWT and 0.78 ± 0.02 mg/g of BWT) than in controls (3.3 ±
0.05 mg/g of BWT and 0.66 ± 0.02 mg/g of BWT; $P < 0.01$ and $P < 0.001$ respectively).

**Running activity**
The daily average running distance was increased significantly ($P < 0.05$) from 5.9 ± 0.7 km in the first week of running to 13.8 ± 1.5 km the second week. Thereafter the daily running distance reached a plateau at 15.8 ± 1.1 km from week 3 to week 5 (Figure 1).

**Corticosterone levels**
No significant difference in corticosterone levels was detected between the exercising (756 ± 59 ng/ml) and control (749 ± 74 ng/ml) groups.

**In vivo telemetry data**
Average blood pressure levels were 119 ± 5 and 116 ± 4 mmHg in the exercising and control groups respectively ($P = NS$) prior to the experiments. No significant changes of blood pressure were detected throughout the exercise period in either group (results not shown).

**Mesenteric vascular function**
NE induced similar constriction in mesenteric resistance arteries from exercising and control animals (ED$_{50}$ values were 5.4 ± 0.7 and 5.1 ± 0.8 µmol/l respectively; $P = NS$), and the maximal tension development was 3.3 ± 0.2 mN/mm in the exercising animals and in the controls. Preconstriction with 0.1 µmol/l NE during each protocol caused similar responses in both groups. Maximum dilation responses to ACh in mesenteric resistance arteries were similar (approx. 90 %) in both groups of animals regardless of pharmacological treatment. Sodium nitroprusside evoked a similar maximum dilation of approx. 95 % in both groups. However, the vasodilator response to ACh had a significantly lower ED$_{50}$ ($P = 0.05$) in the exercising animals (15.0 ± 3.5 nmol/l) compared with the controls (37.0 ± 8.8 nmol/l). There was a significant reduction in ED$_{50}$ values in both groups after treatment with MnTBAP; however, there was no distinguishable difference between the groups. ED$_{50}$ values were 5.8 ± 1.6 nmol/l and 11.8 ± 4.7 nmol/l ($P = NS$) for exercising animals and controls respectively. Incubation with both MnTBAP and l-NNA had no further effect on the vasodilatory response in either group (ED$_{50}$ values were 9.6 ± 3.6 nmol/l in the exercising animals compared with 8.7 ± 3.1 nmol/l in controls; $P = NS$). In addition, incubation with MnTBAP, l-NNA and indomethacin did not affect vasodilation in any of the groups. ED$_{50}$ values were 4.8 ± 1.5 nmol/l for exercising animals and 5.5 ± 1.0 nmol/l for control animals ($P = NS$).

**Aortic vascular function**
In the aorta, preconstriction with 0.1 µmol/l NE caused similar vasoconstriction in both groups without and with MnTBAP incubation. Following MnTBAP and l-NNA incubation, NE caused a significantly greater constrictor response in the exercising group compared with controls (the maximum tension development was 13.8 ± 1.3 and 16.3 ± 1.6 mN in the control and exercising animals respectively; $P = 0.02$). Sodium nitroprusside evoked a similar vasodilation of approx. 90 % in both groups during all incubation protocols.

The ACh-induced vasodilatory responses were similar in both groups of animals (ED$_{50}$ values: 47.9 ± 16.4 nmol/l in the exercising group compared with 79 ± 56.9 in controls; $P = NS$). The maximum vasodilation was 39 ± 3 and 42 ± 3 % in the exercising and control groups respectively ($P = NS$). Incubation with MnTBAP did not alter the ACh-induced response in either of the groups (ED$_{50}$ values: 171.2 ± 92.4 nmol/l in the exercising group compared with 139.8 ± 62.5 nmol/l in controls; $P = NS$). After incubation with MnTBAP and l-NNA, the ACh-induced vasodilatory response was completely abolished in both groups ($P = NS$, as determined by AUC analysis). Finally, incubation with MnTBAP, l-NNA and indomethacin restored the ACh-mediated vasodilation slightly in both of the groups ($P = NS$, as determined by AUC analysis). The maximum ACh-induced vasodilatory response was 11 ± 6 and 18 ± 7 % in the control and exercising groups respectively ($P = NS$).

**In vivo aortic vessel stiffness**
A typical Doppler tissue echography signal obtained from the anterior thoracic aortic wall (Figure 2A) and a two-dimensional longitudinal view of the thoracic aorta (Figure 2B) are shown. There was no difference between systolic and diastolic blood pressure between the groups during the ultrasound study. Diastolic diameter of the thoracic aorta was similar in both of the groups (results not shown). Peak systolic wall velocity was significantly higher ($P = 0.03$) in the exercising group compared with controls (Figure 2C). Peak diastolic wall
Two-dimensional guided Doppler tissue echography of the thoracic aortic wall

(A) Typical Doppler tissue echography signal obtained from the anterior thoracic aortic wall. y-Axis represents the wall motion velocity in cm/s, whereas the x-axis represents time. Maximum systolic and diastolic wall motion velocities are represented by the peak values, whereas velocity time integrals are obtained by delineation of the outer contour of the Doppler tissue echography signals.

(B) Typical two-dimensional image showing a longitudinal view of the thoracic aorta, which is approx. 2.2 mm in diameter during diastole.

(C) Comparison of the maximum wall motion velocities in the exercising group (solid bars) and controls (open bars) during systole (s), while the diastolic (d) maximum wall motion velocities were similar in both of the groups.

(D) Comparison of the velocity time integral in the exercising group (solid bars) and controls (open bars) during both systole (s) and diastole (d).

velocity was similar ($P = NS$) between the groups (Figure 2C). Both the systolic and diastolic velocity time integrals, representing the amplitude of vessel movement, were significantly greater ($P = 0.01$) in the exercising group compared with controls (Figure 2D).

**Immunohistochemical protein localization of eNOS and Cu/ZnSOD**

Immunohistochemical staining of aortic vascular sections revealed distinct endothelial localization of eNOS in both control (Figure 3A) and exercising (Figure 3B) animals, whereas Cu/ZnSOD expression had a more transmural distribution pattern in control (Figure 3C) and exercising (Figure 3D) animals.

Tissue preparations incubated with medium lacking primary antibody served as negative controls. No immunoreactivity was detected in these slides, which verified the specificity of the immunostaining (results not shown).

**Quantification of gene expression**

**eNOS gene expression**

No significant difference was detected in eNOS gene expression between the exercising and control groups. Relative gene expression levels (normalized to GAPDH) were $0.17 \pm 0.02$ in the exercising group and $0.20 \pm 0.03$ in controls ($P = NS$).

**Cu/ZnSOD gene expression**

After 5 weeks of exercise training, Cu/ZnSOD gene expression in the exercising group was up-regulated by $+111\%$ compared with controls. Relative gene expression levels (normalized to GAPDH) were $0.34 \pm 0.04$ in the exercising group and $0.16 \pm 0.02$ in controls ($P = 0.0007$).

**DISCUSSION**

In the present study, we characterized vascular effects of chronic physical exercise in SHR. Voluntary exercise exerted highly differential vessel-type-specific effects on the vasodilatory machinery. In resistance arteries, ACh sensitivity was significantly improved in the trained animals compared with the controls. This difference between the groups was abolished after scavenging of oxygen free radicals. NO blockade did not change the vasodilator responses in either group. In conduit vessels, NE-induced vasoconstriction was significantly greater in the exercising group than controls following NO inhibition; however, ACh caused similar vasodilatory responses in both groups. The animals in the exercising group exhibited significantly improved in vivo aortic compliance compared with the non-exercising group. Furthermore, voluntary exercise caused significantly increased Cu/ZnSOD gene expression in the aortic tissue, whereas gene expression of eNOS was unaffected in exercising animals. The exercised rats also had significantly increased left ventricle weight compared with non-exercising controls. In the moderately hypertensive female SHR, no blood pressure reduction was seen after 5 weeks of exercise.

In mesenteric arteries, we observed an improved ACh sensitivity in the exercising compared with non-exercising animals. This difference between the groups was diminished after treatment with MnTBAP and was
completely abolished after treatment with both MnTBAP and l-NNA. We conclude that the distinct ACh sensitivity is due mainly to a differential production of superoxide and, to a lesser extent, to available NO. Surprisingly, l-NNA had no effect on ACh-mediated vasodilation. However, in a previous study using a forced training protocol, Yen et al. [9] showed a 20% inhibitory effect of l-NNA on ACh-mediated vasodilation in mesenteric arteries in trained male SHR, but only insignificant effects in untrained controls. Furthermore, in the same study [9], the maximum ACh-mediated vasodilation was approx. 55% compared with the present study showing approx. 95% vasodilation. This discrepancy could be due to the difference in age between the two study groups, 16 weeks compared with 13 weeks. It has been found that NO-dependent vasodilation decreases with age [23], and that the ACh sensitivity also deteriorates with increasing age [24,25]. This may explain the higher ACh- and lower NO-dependent vasodilation in the present study. However, these discrepancies may also be gender-dependent. In contrast with Yen et al. [9], we found similar NE sensitivity and NE-induced precontraction in both resistance and conduit arteries. A possible reason for this difference could be differing stress levels caused by forced or voluntary training programmes. In the present study, measurement of corticosterone levels revealed a similar stress level in the exercising group compared with the control group. Furthermore, it seems that forced exercise may induce greater production of NO in the mesenteric arteries than voluntary exercise, as shown by the l-NNA inhibition protocol in the present study.

Despite improved ACh sensitivity, we failed to show any effects of physical exercise on blood pressure reduction. Several investigators have shown previously blood-pressure-lowering effects following physical activity in this voluntary exercise model [3,14]. However, only SHR with a high enough initial blood pressure were used in these studies. SHR were used in the present study due to their nature of exercising voluntarily in contrast with Wistar–Kyoto rats, which have been widely used both as experimental and control animals in previous studies using mainly forced training protocols. The average blood pressure level was normal or only modestly high in the SHR at the beginning of the study, and further substantial blood pressure reduction would be unphysiological. Using a similar experimental protocol, Chen et al. [26], as well as Graham et al. [10], failed to show any blood-pressure-lowering effect of exercise. This is in accordance with our present finding. It is conceivable that the improved resistance vessel function may impact on other physiological parameters rather than the resting blood pressure. Other functional tests, e.g. maximum exercise blood pressure, may be able to address this issue.

The aortic strips demonstrated different vasodilatory patterns compared with the mesenteric arteries. No difference was seen between the exercising animals and the controls in the vasodilatory response to ACh. Similarly,
there was no detectable difference between the two groups after incubation with the oxygen free radical scavenger MnTBAP. Inhibition of NO completely abolished the ACh-induced vasodilation, whereas further prostaglandin blockade restored the response slightly in both groups. Interestingly, following NO inhibition, the NE-induced vasoconstriction was significantly greater in aortic strips of the exercising group compared with controls. This may suggest a greater NO dependency in the exercised aortae. Indeed, Yen et al. [33] showed greater NE sensitivity in denuded aortic and mesenteric rings in the exercising group compared with controls, which is partially in line with our current data. This suggests a more important role of the endothelium for the NE-induced vascular activity in the exercising group than controls. Recently, Graham et al. [10] showed significantly increased ACh-mediated vasodilation in thoracic aortic strips from exercised SHR compared with control rats. Although Graham and co-workers [10] used a forced training low-intensity exercise protocol (945 m/day compared with 15 800 m/day used in the present study), the differences are apparent. It is conceivable that controlled low-intensive training could be more beneficial than voluntary exhausting exercise, a finding supported by, among others, Ji [27]. The dose–response relationship between exercise intensity and vascular effects justifies further investigation.

Using a real-time PCR approach, we found increased gene expression of Cu/ZnSOD in the aortic tissue, whereas eNOS expression remained unaffected in the exercising group compared with controls. Our findings of elevated Cu/ZnSOD levels in aortic tissue are supported by previous reports of training-induced increases in this enzyme [28,29]. The activation of SOD in exercise is proposed to result from increased superoxide production during exercise [6,30]. Three isoforms have been identified: (i) the cytosolic Cu/ZnSOD, (ii) mitochondrial MnSOD (manganese SOD), and (iii) extracellular (ec)SOD. ecSOD has been shown to be up-regulated in aortic tissue in mice following forced training [28]. However, in rat vascular tissues, Cu/ZnSOD is thought to be the dominant SOD isoform [31], which is confirmed by the transmural expression pattern in our immunohistochemical study. Vascular dysfunction with increased vascular oxidative stress has been shown in Cu/ZnSOD-deficient mice [31]. Indeed, ecSOD gene expression was also significantly up-regulated in the exercising rats (results not shown). However, the physiological significance of ecSOD in rats remains uncertain due to the tetramerization of the enzyme [32]. Interestingly, gene transfer of ecSOD with its heparin-binding domain does improve vasorelaxation and reduces arterial blood pressure in SHR [33].

In contrast with several previous studies, in which an up-regulated eNOS protein expression [10,28,34] or systemic NO formation [15] was reported, we failed to show any differences in expression level of this enzyme. Indolfi et al. [35] showed that eNOS protein expression was increased following only 7 days of exercise in rat carotid arteries after balloon angioplasty. However, these effects could be due to improved re-endothelialization following physical training. Recently, Laufs et al. [36] have shown that endothelial progenitor cells increased after short-term physical training. However, besides the data from Graham et al. [10], who showed marginally increased eNOS protein expression after 6 weeks forced training, few other investigators succeeded in showing any positive effects of eNOS regulation by exercise in SHR. Indeed, it has been shown that SHR have higher endogenous eNOS expression than Wistar–Kyoto rats [10,37]. This important difference in NO metabolism between species may explain the lack of eNOS effect in the present study. Furthermore, it is conceivable that the rate-limiting factor in the NO-pathway is the breakdown of the active NO by oxidative stress in SHR. Thus improved antioxidant defence should be a more efficient way to improve NO availability rather than via eNOS expression.

Functional evidence of increased NO availability was assessed in vivo in the present study using novel high-resolution Doppler ultrasound to measure aortic wall movement. Fibre Doppler travels along the aorta in rats and mice [38]. The ultrasound equipment used in the present study, with a frame rate of 300/s, facilitates real-time imaging of the vessel motion. However, the resolution of a 15 MHz ultrasound probe is typically 120 µm, which, in comparison with the amplitude of the aortic wall motion of approx. 160 µm, may introduce substantial inaccuracy when attempting to measure any diameter changes during the cardiac cycles. By adapting a newly established human vascular imaging protocol using Doppler tissue echography, wall motion velocities can be accurately measured [20]. The distinct tissue Doppler signals also facilitate reproducible measurements of velocity time integral, which represents motion amplitude. After correction of vessel diameter and blood pressure, these parameters are shown to be useful in assessing vascular stiffness [39]. Vessel stiffness has been shown to be an independent predictor for coronary artery atherosclerosis [40] and is closely linked to local NO-related endothelial function [41]. By studying this parameter using the high-resolution ultrasound technique in vivo, we showed improved vessel compliance in exercised rats. We consider it important evidence supporting an increased resting NO availability in vivo. Interestingly, using an ex vivo approach, Kingwell and co-workers [42] reported increased arterial compliance but unaffected ACh-mediated vasodilation in aortic strips from spontaneously exercised Wistar–Kyoto rats after 15 weeks of moderate exercise training.
There is increasing evidence that the endothelium senses and responds to fluid mechanical forces such as blood pressure and shear stress [43]. In addition to numerous in vitro cell-culture-based studies, we have shown previously [44] that a series of endothelial factors are differentially regulated by shear stress and pressure in intact human conduit vessels. Recently, by means of magnetic resonance imaging, it has been shown [45] that shear stress is increased along the descending aorta during exercise when taking into consideration vessel dimension, flow velocity and flow pulsatility. Interestingly, shear stress has been shown previously [46] to up-regulate Cu/ZnSOD mRNA levels in human aortic endothelial cells. Thus it is conceivable that increased shear stress levels during exercise may regulate expression of several atheroprotective mediators and thereby account for the positive vascular effects of exercise training.

During exercise, the mesenteric arteries, as opposed to the aortic bed, are considered to be an inactive bed with decreased blood flow as a consequence in rats [26]. However, the decrease in blood flow does not necessarily imply a decrease in shear stress. Several investigators have shown that flow in the mesenteric arteries is increased or unaffected [47] in humans and may be slightly decreased in SHR [26] during exercise. In contrast with the observation by Eriksen and Waaler [47], Perko et al. [48] showed that diameter and flow in the superior mesenteric artery were decreased during exercise in man, which may lead to slightly decreased or unchanged shear stress levels. However, in flow-regulating arterial segments such as resistance arteries, as studied in the present study, the vessel lumen could be substantially decreased during exercise, which, in the light of the fact that vessel radius impacts shear stress by a power of four, could cause locally increased shear stress instead. This may suggest that the improved ACh-mediated relaxation in mesenteric resistance arteries observed in our present study could also be a response to elevated shear stress levels. Chen et al. [26] showed in an in vivo setting that ACh responsiveness was improved in mesenteric arteries following voluntary exercise. This was, however, not linked to the NO pathway. These in vivo data confirm further our present data. In addition, we have shown in the present study that improved antioxidant defence could be the mechanism underlying the enhanced ACh responsiveness in the mesenteric arteries. In active muscle beds, such as rat gracilis skeletal muscle arterioles, it has been shown that ACh-mediated vasodilation is increased after exercise [49] due to an enhanced NO pathway. Furthermore, Sun et al. [50] have also shown that arterioles of plantaris skeletal muscle, but not mesenteric arteries, demonstrated functional adaptation to exercise activity. Apparently, exercise appears to improve ACh responsiveness in various vascular beds through distinct mechanisms. The magnitude of shear stress increase in different vasculature could be one of the possible explanations for this finding. Furthermore, the native function of a certain vascular bed as well as the vessel size, which may impact on the ratio between the regulated endothelium and the underlying amount of smooth muscle cell layers, could be important properties determining the physiological phenotype as well as the specific biological pathway to be regulated.

Besides the fluid mechanical effects, exercise is also known to exert systemic neurohumoral effects on whole body physiology [51]. It has been shown that increased free radical production during exercise could be a systemic factor triggering up-regulation of several antioxidant enzymes [27].

Endothelial function and vascular stiffness are two of the widely used surrogate markers associated with clinical outcomes of cardiovascular diseases [39]. ACh-mediated vasodilation is widely used to test endothelial function both in vivo and ex vivo [9,42,52,53]. However, as illustrated in the present study, the vasodilatory mechanism is highly complex and involves numerous vasoactive substances. Due to the complex vasomotor regulating pathways and many compensatory mechanisms, the simple approach of using ACh-mediated vasodilation alone as a vascular marker for endothelial function is unsatisfactory in dissecting possible underlying mechanisms relevant for endothelial dysfunction and progression of atherosclerosis. Distinct molecular pathways may be responsible for control of vessel stiffness and vascular reactivity.

In summary, voluntary physical exercise in SHR improved ACh-mediated vasodilation through decreased oxidative stress in mesenteric arteries, but not in conduit vessels. Increased vascular compliance and antioxidative capacity may contribute to the atheroprotective effects associated with physical exercise in conduit vessels.

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