

ORIGINAL ARTICLE

Endothelial function does not improve with high-intensity continuous exercise training in SHR: implications of eNOS uncoupling

Sylvain Battault¹, François Singh², Sandrine Gayraud¹, Joffrey Zoll², Cyril Reboul^{1,3} and Grégory Meyer^{1,3}

Exercise training is a well-recognized way to improve vascular endothelial function by increasing nitric oxide (NO) bioavailability. However, in hypertensive subjects, unlike low- and moderate-intensity exercise training, the beneficial effects of continuous high-intensity exercise on endothelial function are not clear, and the underlying mechanisms remain unknown. The aim of this study was to investigate the impact of high-intensity exercise on vascular function, especially on the NO pathway, in spontaneous hypertensive rats (SHR). These effects were studied on WKY, sedentary SHR and SHR that exercised at moderate (SHR-MOD) and high intensity (SHR-HI) on a treadmill (1 h per day; 5 days per week for 6 weeks at 55% and 80% of their maximal aerobic velocity, respectively). Endothelial function and specific NO contributions to acetylcholine-mediated relaxation were evaluated by measuring the aortic ring isometric forces. Endothelial nitric oxide synthase (eNOS) expression and phosphorylation (ser1177) were evaluated by western blotting. The total aortic and eNOS-dependent reactive oxygen species (ROS) production was assessed using electron paramagnetic resonance in aortic tissue. Although the aortas of SHR-HI had increased eNOS levels without alteration of eNOS phosphorylation, high-intensity exercise had no beneficial effect on endothelium-dependent vasorelaxation, unlike moderate exercise. This result was associated with increased eNOS-dependent ROS production in the aortas of SHR-HI. Notably, the use of the recoupling agent BH₄ or a thiol-reducing agent blunted eNOS-dependent ROS production in the aortas of SHR-HI. In conclusion, the lack of a positive effect of high-intensity exercise on endothelial function in SHR was mainly explained by redox-dependent eNOS uncoupling, resulting in a switch from NO to O₂⁻ generation.

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INTRODUCTION

Hypertension is a major public health problem, affecting approximately one billion people worldwide.¹ In humans and in experimental rodent models, essential hypertension is often associated with impaired endothelium-dependent vasorelaxation, which contributes to the progression of the pathology^{2,3} and aggravates the cardiovascular risk.^{4–7} Based on these observations, several studies have aimed to evaluate the potential beneficial effects of improving endothelial function in hypertensive subjects,^{8,9} which has been shown to confer a more favorable prognosis.⁹

Exercise training has been largely recognized as an efficient strategy in the management of endothelium-dependent vascular impairment in humans and in experimental models of essential hypertension.¹⁰ Indeed, exercise training improves endothelium-dependent vasodilatory capacity^{11–13} primarily by improving nitric

oxide (NO) bioavailability. Current evidence shows that exercise training is associated with increased eNOS expression,^{14,15} phosphorylation of its main activation site (ser1177)¹⁶ and stabilization of its dimerized state.^{17,18} In addition, exercise training increases antioxidative enzyme activity, which blunts reactive oxygen species (ROS) production¹⁹ and contributes to prevent the reaction between ROS and NO,^{20,21} rendering NO more available.

However, the benefits of exercise on endothelial function depend on the exercise intensity. Although high-intensity (≥80% of the maximal aerobic velocity) exercise training has been consistently reported to improve endothelium, and more specifically NO-dependent vasorelaxation, in normotensive subjects,^{22–24} this positive effect has not been reported in hypertensive models.²⁵ Moreover, the underlying mechanisms remain unknown and whether the endothelial nitric

¹Laboratoire de Pharm-Ecologie Cardiovasculaire (EA4278), Faculty of Sciences, Avignon University, Avignon, France and ²E.A. 3072, Fédération de Médecine Translationnelle, Faculty of Medicine, University of Strasbourg, Strasbourg, France

³These two authors are senior co-authors.

Correspondence: Dr C Reboul, Laboratoire de Pharm-Ecologie Cardiovasculaire (EA4278), Faculty of Sciences, Avignon University, 33 rue Louis Pasteur, Avignon 84000, France. E-mail: cyril.reboul@univ-avignon.fr

or Dr G Meyer, Laboratoire de Pharm-Ecologie Cardiovasculaire (EA4278), Faculty of Sciences, Avignon University, 33 rue Louis Pasteur, Avignon 84000, France.

E-mail: gregory.meyer@univ-avignon.fr

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oxide synthase (eNOS)–NO pathway could be negatively affected remains to be investigated.

The aim of this study was to evaluate the consequence of continuous high-intensity exercise training (80% of the maximal aerobic velocity, 1 h per day for 6 weeks) on the endothelial function and eNOS functional state in spontaneous hypertensive rats (SHR). As expected, unlike moderate-intensity exercise, continuous high-intensity exercise training did not enhance endothelial function in this model. This lack of a beneficial effect of continuous high-intensity exercise seems to be mainly explained by a redox-dependent uncoupling of eNOS, reinforcing ROS production in the aortic tissue of SHR.²⁶

METHODS

Experimental protocol

All investigations complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with the regulations of the French Ministry of Agriculture. All experiments were approved by the ethics committee of Marseille (CNREEA 14). Male SHR ($n=24$) and Wistar Kyoto (WKY) rats ($n=10$) were obtained from Janvier (Laval, France) at 10 weeks of age. Four rats were housed per cage under controlled conditions of temperature ($21 \pm 1^\circ\text{C}$), humidity ($60 \pm 10\%$) and lighting (12 h a day). They had free access to tap water and standard food. After a period of acclimation, the maximal aerobic velocity of SHR was evaluated using a driven treadmill during a continuous and progressive maximal exercise test. The driven treadmill was set at a speed of 10 m min^{-1} for 3 min, after which the speed was increased by 3 m min^{-1} every 90 s until 85–90 % of the expected maximal aerobic velocity was reached. The speed was then increased by 1 m min^{-1} every 60 s until maximal aerobic velocity. Next, SHR were randomly assigned to sedentary (SHR), moderate (SHR-MOD) or high-intensity exercise (SHR-HI) groups. Exercise sessions consisted of continuous running for 1 h, 5 days a week for 6 weeks at 55% of maximal aerobic velocity for SHR-MOD and 80% of maximal aerobic velocity for SHR-HI.

Blood pressure and body mass

The mean (MBP), systolic (SBP) and diastolic (DBP) blood pressures were assessed in conscious rats by tail-cuff method using the CODA tail-cuff system (Kent Scientific, Torrington, CT, USA). The rats were warmed for 15 min before measurements. The rats underwent 3 days of habituation to blood pressure measurements before the baseline blood pressure measurements were assessed. MBP, SBP and DBP were measured at weeks 2, 4 and 6 of the protocol. The MBP, SBP and DBP values were calculated as the average of 10 measurements.

Isolation of aortic rings

Under anesthesia (sodium pentobarbital, 100 mg kg^{-1} , intraperitoneal), the thoracic aorta was quickly removed and placed in cold Krebs–Henseleit bicarbonate buffer (composition in mM: NaCl 118, NaHCO_3 25, KCl 4.8, KH_2PO_4 1.2, CaCl_2 1.25, glucose 11). After the removal of adherent tissue, the vessels were cut into 2–3-mm long rings. The aortic rings were mounted onto stainless steel supports and suspended in the tissue bath containing Krebs–Henseleit buffer at 37°C , continuously bubbled with O_2 – CO_2 (95%–5%) gas mixture. The rings were connected to an isometric force transducer (EMKA Technologies, EMKA Paris, France), linked to an amplifier (EMKA Technologies) and a computerized acquisition system to record changes in the isometric force. The resting tension was adjusted to 2.0 g. After 60 min of stabilization, the muscular and endothelial integrity were tested with a single dose of phenylephrine (10^{-6} M) and acetylcholine (ACh, 10^{-6} M). Then, each vessel ring was precontracted with phenylephrine (10^{-6} M). After the precontraction reached a plateau, endothelium-dependent relaxation was examined by challenging the aortic rings with cumulative concentrations of ACh (10^{-9} – 3.10^{-5} M). Considering that eNOS inhibition with L-nitro arginine methyl ester (L-NAME) totally abolishes ACh-dependent vasorelaxation in aortic tissue,²⁷ which clearly shows that eNOS is the main pathway involved in the endothelium response to ACh in the aorta, we chose to investigate

the eNOS-dependent vasorelaxation in response to ACh by inhibiting the other possible interacting pathways. The eNOS–NO pathway vasorelaxant contribution was therefore evaluated in the presence of a combination of indomethacin (10^{-5} M), a cyclooxygenase (COX) inhibitor to blunt the production of PGI_2 , and apamin (10^{-7} M) plus TRAM 34 (10^{-6} M), which are SK_{Ca} and IK_{Ca} inhibitors, respectively, to blunt the pro-relaxation contribution of endothelium-derived hyperpolarizing factor. All inhibitory drugs were added to the bath 30 min before the ACh dose–response assay was performed. To evaluate the role of eNOS uncoupling, the contribution of NO pathway in the response of endothelium to ACh was also investigated in the presence of NOS coupling agent BH_4 ($100 \mu\text{M}$). Finally, endothelium-independent relaxation was assessed on precontracted aortic rings challenged with a maximal dose of sodium nitro-prusside (10^{-5} M). The vessel rings were rinsed to stabilize the resting tension levels between each drug intervention.

Western blotting analysis

Proteins from aorta homogenates were separated on polyacrylamide-sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies at 4°C in 10% milk or 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween-20 overnight. The primary antibodies used in this study were anti-mouse eNOS (1:1000; BD transduction Laboratories, Lexington, KY, USA), anti-mouse eNOS-p^{Ser1177} (1:1,000; BD Transduction Laboratory), anti-rabbit GAPDH (glyceraldehyde 3-phosphate dehydrogenase; 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-rabbit nitrotyrosine (1:15 000; Millipore Corporation, Billerica, MA, USA). Immunodetection was carried out using an ECL or ECL Plus system (SuperSignal West Pico Chemiluminescence Substrate, Thermo Scientific (Villebon-sur-Yvette, France) or Luminata Forte Western HRP substrate, Millipore Corporation, respectively), and the membranes were then exposed to X-ray films for visualization. The eNOS protein content was expressed relative to the GAPDH content. The eNOS-p^{Ser1177} protein content was expressed relative to the eNOS content.

Measurement of ROS

ROS production was measured by electron paramagnetic resonance in freshly frozen aorta homogenates, as previously described.¹⁷ Briefly, the homogenates were treated with a solution of CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine) at a concentration of $400 \mu\text{M}$ (1:1 v/v), placed into electron paramagnetic resonance glass capillary tubes (Noxygen Science Transfer and Diagnostics, Elzach, Germany) and then placed inside the e-scan spectrometer (Bruker, Ettlingen, Germany) for data acquisition. The procedure was then repeated on the same samples but in the presence of L-NIO ($10 \mu\text{M}$), a specific eNOS inhibitor. The difference between the first and second results was considered to be the eNOS-dependent ROS production. Alternatively, the procedure was also repeated in the presence of N-acetylcysteine (2 mM) or of BH_4 ($100 \mu\text{M}$). The ROS production was normalized to the protein content of each sample and then expressed in $\mu\text{mol min}^{-1} \text{ mg}^{-1}$.

Statistical analysis

The data are expressed as the mean \pm s.e.m. For comparison of multiple experimental conditions, Student's *t*-test, analysis of variance or repeated-measures analysis of variance followed by the Bonferroni adjusted *t*-test were used. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Exercise training does not correct hypertension in SHR

SHR presented higher SBP, DBP and MBP at the beginning of and throughout the protocol (Figure 1a). At the end of the exercise training, the SBP, DBP and MBP tended to be lower in SHR trained at moderate intensity than in their sedentary counterparts (5.4, 8.9 and 4.6%, respectively), but the values did not reach significance (Figure 1a). By contrast, high-intensity exercise training did not lead to any changes in SBP, DBP and MBP in SHR (Figure 1a). WKY and SHR had no difference in resting heart rates throughout the protocol

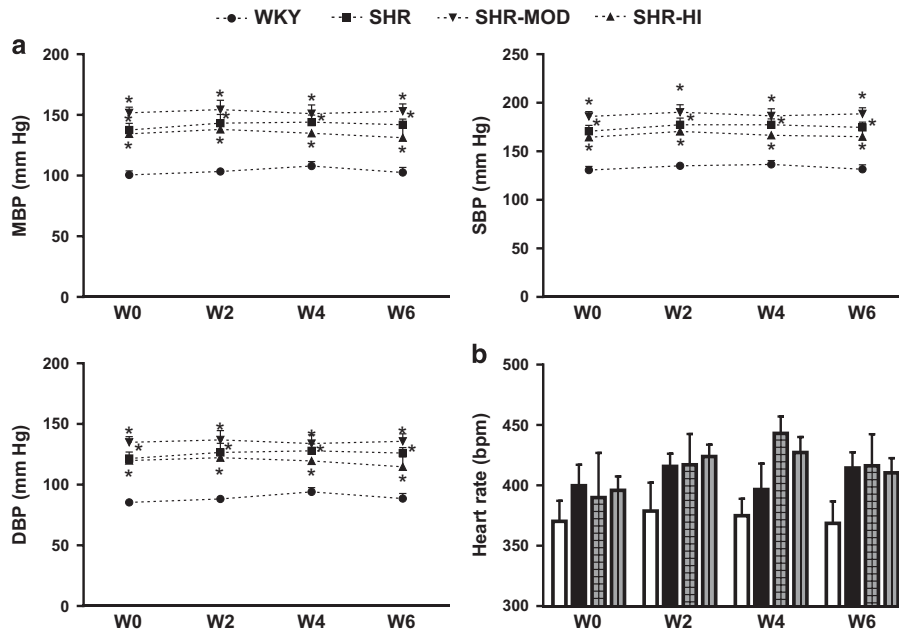


Figure 1 High-intensity exercise training does not correct hypertension in SHR. (a) Evaluation of the mean (MBP), systolic (SBP) and diastolic (DBP) blood pressures at baseline (W0) and in week 2 (W2), week 4 (W4) and week 6 (W6) of the protocol. (b) Evaluation of heart rates during the protocol. Values are the mean \pm s.e.m. * $P < 0.05$ vs. WKY.

(Figure 1b). Both moderate- and high-intensity exercise had no effect on this parameter (Figure 1b).

Effect of continuous moderate- or high-intensity exercise training on endothelium-dependent vasorelaxation

The vasorelaxation response to ACh was altered in SHR compared with their normotensive counterparts (Figure 2a). This alteration was characterized by a lower maximal response to ACh (Figure 2c), with no alteration of the sensitivity (EC_{50}) (Figure 2b). Considering that no difference was observed regarding the response to sodium nitroprusside (SNP) (WKY, $98.2 \pm 0.3\%$; SHR, $98.0 \pm 1.2\%$; NS), these results highlight the endothelial dysfunction in the SHR aortas compared with the control rats. Thus we next evaluated whether exercise training was able to modulate endothelium-dependent relaxation in SHR. As expected, in SHR, continuous moderate-intensity exercise training improved ACh-dependent vasorelaxation with regard to aortic sensitivity to ACh (Figure 2b) but had no effect on the maximal response (Figure 2c). By contrast, high-intensity exercise training had no significant effect on the maximal response (Figures 2a and c) or the sensitivity (Figure 2b) to ACh but tended to worsen both parameters. No difference in the maximal response to SNP was observed between the WKY, SHR, SHR-MOD and SHR-HI (SHR-MOD, $97.5 \pm 0.6\%$, SHR-HI, $98.4 \pm 0.7\%$) groups (Figure 2d).

Effect of continuous high-intensity exercise training on eNOS-mediated vasorelaxation

Exercise training is especially known to modulate the eNOS–NO pathway.^{16,17,19} Thus, to better understand the lack of a beneficial effect of high-intensity exercise training on endothelium-dependent vasorelaxation in our model, we next focused our study on the contribution of the eNOS–NO pathway to the endothelium-dependent response to ACh in SHR-HI aortic tissue. Endothelium-dependent vasorelaxation is mainly dependent on (i) NO production by eNOS, (ii) PGI₂ synthesis under the control of COX activity and

(iii) endothelium-derived hyperpolarizing factor-mediated opening of the SK_{Ca} and IK_{Ca} channels. To identify the contribution of eNOS to endothelium-dependent vasorelaxation, we performed dose–response experiments with ACh in the presence of indomethacin, a COX inhibitor, to blunt the production of PGI₂, and apamin plus TRAM 34 (SK_{Ca} and IK_{Ca} inhibitors, respectively) to blunt the hyperpolarizing contribution to vasorelaxation. We observed that the eNOS-dependent response to ACh in the aorta was not decreased in SHR compared with control rats. Indeed, no alteration of the maximal response to ACh was observed (Figures 3a and c), but a higher sensitivity to ACh was observed in SHR compared with WKY rats (Figure 3b). High-intensity exercise training significantly impaired the eNOS-dependent maximal response to ACh in SHR-HI compared with SHR. In addition, ACh sensitivity, which was increased in SHR, was normalized in SHR-HI to the level observed in the WKY aortas (Figure 3b), suggesting some potential deleterious effect of high-intensity exercise training on the eNOS–NO pathway.

Continuous high-intensity exercise training alters the eNOS functional state in SHR aortas

Next, to understand these results, we evaluated the effects of our experimental conditions on eNOS and eNOS-^{pSer1177} by western blotting. The expression of eNOS was increased in SHR aortic tissue with no supplemental effect of exercise training (Figure 4). Indeed, no difference in eNOS expression was observed between SHR and SHR-HI. The eNOS-^{pSer1177}/eNOS ratio was not modified in SHR compared with WKY aortas, and no effect of high-intensity exercise training on this parameter was observed in SHR (Figure 4). Because eNOS produces NO in its coupled state, whereas eNOS uncoupling results in a switch from NO to O₂⁻ generation,^{28,29} we next evaluated ROS production in the aortic tissue in the presence or absence of a specific inhibitor of eNOS (L-NIO, 10 μ M). Notably, the total ROS production was increased in both SHR and SHR-HI aortas compared

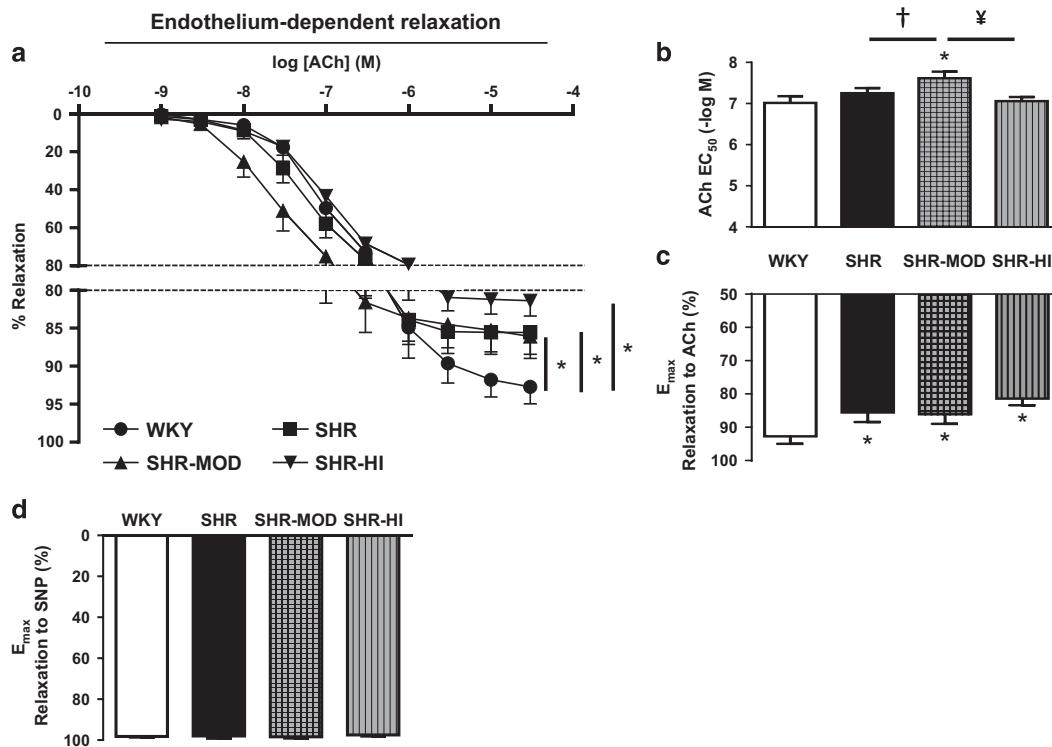


Figure 2 High-intensity exercise training does not correct endothelium dysfunction in SHR. (a) Dose-dependent response of aortic rings to cumulative doses of ACh. The data are expressed as the percentage of relaxation relative to the precontraction level. (b) ACh concentration inducing 50% of the maximal response to ACh in aortic rings. (c) Maximal relaxation of aortic rings in response to ACh expressed as the percentage of relaxation relative to the precontraction level. (d) Maximal relaxation of aortic rings in response to SNP, expressed as the percentage of relaxation relative to the precontraction level. The values are the mean \pm s.e.m. * $P < 0.05$ vs. WKY group. † $P < 0.05$ vs. SHR group. ‡ $P < 0.05$ vs. SHR-MOD.

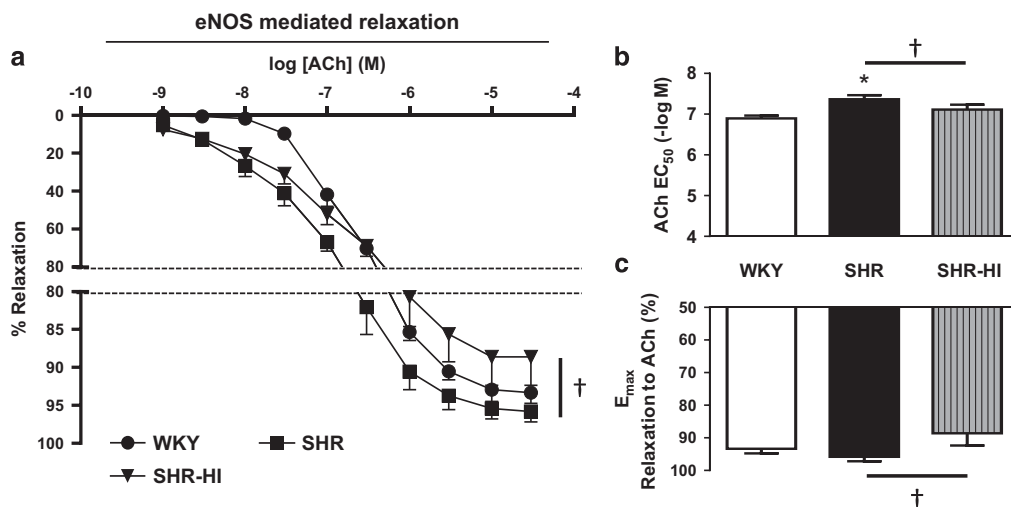


Figure 3 High-intensity exercise decreases eNOS-dependent vasorelaxation in SHR. (a) Dose-dependent response to a cumulative dose of ACh in aortic rings pretreated with indomethacin+TRAM 34+apamin. The data are expressed as the percentage of relaxation relative to the precontraction level. (b) ACh concentration inducing 50% of the maximal response to ACh in aortic rings pretreated with indomethacin+TRAM 34+apamin and expressed as the percentage of relaxation relative to the precontraction level. The values are the mean \pm s.e.m. * $P < 0.05$ vs. WKY group. † $P < 0.05$ vs. SHR group.

with WKY aortas (Figure 5a). Although no difference in the total aortic ROS production was observed between the SHR and SHR-HI groups, the specific inhibition of eNOS only reduced ROS production in the SHR-HI aortas and had no effect on WKY or SHR (Figure 5a). Thus eNOS-dependent ROS production was higher in the SHR-HI

group than in the two other groups (Figure 5b). This result strongly suggests eNOS uncoupling in the SHR-HI aortas. In pro-oxidant conditions, NO can react with O_2^- to produce peroxynitrite, which results in a protein modification called nitrotyrosination. Thus, as previously described,^{17,26} we measured aortic nitrotyrosination by

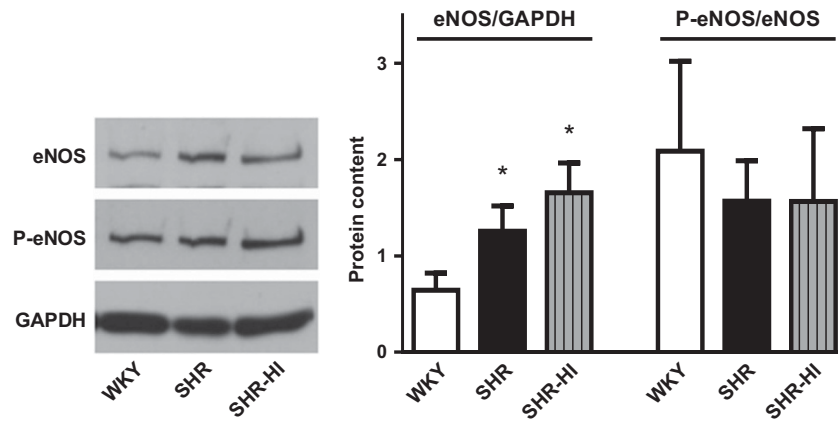


Figure 4 High-intensity exercise does not affect eNOS expression and eNOS phosphorylation in SHR. eNOS expression and phosphorylation at Ser 1177 (P-eNOS) measured by western immunoblotting. The eNOS and P-eNOS levels are relative to the expression values of GAPDH and total eNOS, respectively. The values are the mean \pm s.e.m. * $P < 0.05$ vs. WKY group.

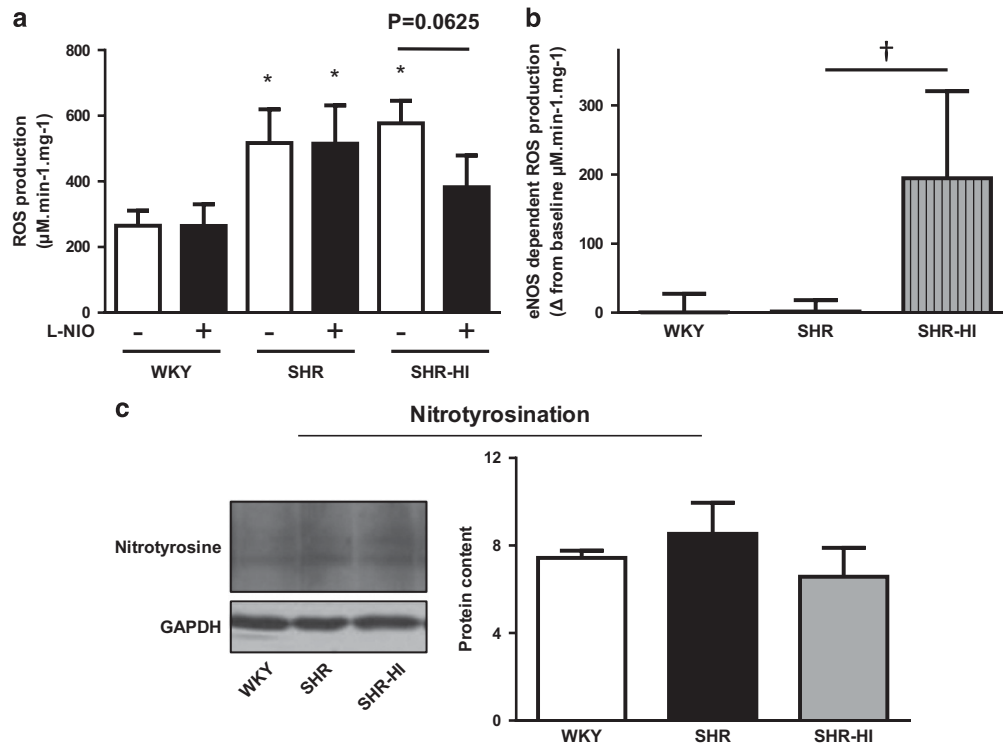


Figure 5 High-intensity exercise increases eNOS uncoupling in SHR. (a) Total aortic ROS production measured by electron paramagnetic resonance (EPR) in fresh frozen aorta homogenates either pretreated with L-NIO or left untreated. (b) eNOS-dependent ROS production measured by EPR in fresh frozen aorta homogenates. The values correspond to the difference between ROS measurements obtained in the presence or absence of L-NIO. (c) Protein nitrotyrosination measured by western immunoblotting. The nitrotyrosination level is relative to the expression values of GAPDH. The values are the mean \pm s.e.m. * $P < 0.05$ vs. WKY group. † $P < 0.05$ vs. SHR group.

western blotting as an index of peroxynitrite formation. Protein nitrotyrosination tended to be higher in the aortas of SHR, without reaching significance, whereas those from the SHR group that exercised at high intensity tended to be less nitrotyrosinated than their sedentary counterparts (Figure 5c). Considering that eNOS uncoupling is explained in the literature by either the redox modulation of its S-glutathionylation^{28,30} or the oxidation of its co-factor tetrahydrobiopterin (BH₄),^{31–33} we next evaluated whether the use of a reducing agent (NAC, 2 mM) or BH₄ (100 µM) could modulate ROS production in the SHR-HI aortas. Notably, although

NAC had no significant effect on ROS production in the SHR aortas (Figure 6a), the use of this reducing agent significantly blunted ROS production in the SHR-HI aortas (Figure 6a). In addition, the use of the recoupling agent BH₄ revealed the same pattern of results; BH₄ did not alter ROS production in the aortas of SHR, but it decreased this parameter in the SHR-HI group (Figure 6b). Altogether, these results suggest that high-intensity exercise training is associated with an altered endothelium redox state that subsequently increases the level of eNOS uncoupling in the aortas of SHR. Finally, we measured eNOS-dependent vasorelaxation in both the SHR and SHR-HI aortas

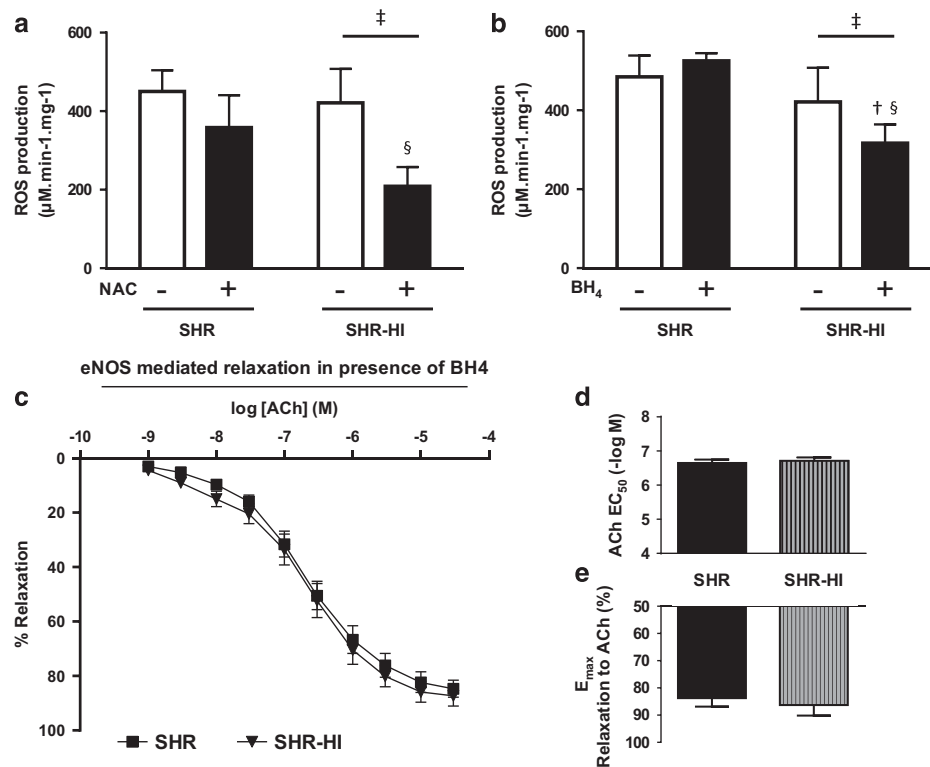


Figure 6 Reducing agent (*N*-acetylcysteine (NAC)) and BH₄ reverse eNOS uncoupling SHR-HI. (a–c) Total aortic ROS production measured by electron paramagnetic resonance (EPR) in freshly frozen aorta homogenates pretreated with NAC (2 mM) or left untreated. (d) Total aortic ROS production measured by EPR in freshly frozen aorta homogenates pretreated with BH₄ (100 µM) or left untreated. (e) Dose-dependent response to the cumulative addition of ACh on aortic rings pretreated with indomethacin+TRAM 34+apamin and BH₄. The data are expressed as the percentage of relaxation relative to the precontraction level. (f) Half-maximal effective concentration of ACh on aortic rings pretreated with ITA and BH₄. (g) Maximal relaxation to ACh in aortic rings pretreated with ITA and BH₄. The values are the mean ± s.e.m. **P*<0.05 vs. WKY group. †*P*<0.05 vs. SHR group. ‡*P*<0.05 vs. SHR-HI group. §*P*<0.05 vs. SHR treated with NAC or BH₄.

in the presence of BH₄. Notably, in the presence of this recoupling agent, no difference between these two populations was observed (Figures 6c–e).

DISCUSSION

The main finding of this study was that high-intensity exercise training is associated with an altered eNOS functional state in the aortas of SHR, which could explain why this strategy was not able to correct endothelial dysfunction in this population. There is evidence to suggest that the effects of exercise training on the vascular endothelium depend on its intensity.³⁴ Indeed, high-intensity exercise may have no positive^{35–37} or deleterious effects^{38,39} on endothelial function. Although high-intensity exercise has been consistently reported to have beneficial effects^{22–24} on hypertension in normotensive rats, a previous study by Sun *et al.*²⁵ showed that, in a rat model, low-intensity (30% of maximal aerobic velocity) rather than moderate-intensity (60% of maximal aerobic velocity) exercise conferred beneficial effects on blood pressure and vessel function. Consistent with these results, the present study provides new insight to explain why continuous high-intensity aerobic exercise training is not effective at improving endothelial function in SHR.

The effects of exercise training on endothelial function are well-known consequences of an improved eNOS–NO pathway function.^{14,40,41} However, although the effects of moderate exercise training on this pathway are well described,^{42–44} those of high-intensity exercise training are less known and seem controversial. Indeed, several studies have demonstrated that moderate- or

low-intensity exercise is effective at increasing NO bioavailability and endothelium-dependent relaxation in both animal models of hypertension^{11,25,45,46} and hypertensive humans^{38,47,48}; by contrast, there are only a few studies that have investigated the effect of continuous high-intensity exercise training on endothelial function. Goto *et al.*⁴³ reported that long-term moderate-intensity (50% V_{O₂max}) exercise, but not high-intensity (75% V_{O₂max}) exercise, augmented endothelium-dependent vasodilation through increased NO production in healthy subjects. To explain this difference, Goto *et al.*⁴³ suggested that increased oxidative stress (as evidenced by increased plasma concentrations of 8-OHdG (8-hydroxy-2'-deoxyguanosine) and serum concentrations of MDA-LDL (malondialdehyde-modified low-density lipoprotein)) may have resulted in decreased NO bioavailability in subjects exercising at high intensity. Similar mechanisms are likely to occur in hypertensive subjects. Indeed, Sun *et al.*²⁵ compared the effects of low- and high-intensity exercise training in SHR and reported that low-, but not high-intensity, exercise improves endothelium-dependent vasorelaxation. We report here that, although moderate exercise training is well known to increase the level of eNOS phosphorylation at its main activation site in healthy rodents,^{49,50} high-intensity exercise training has no effect on eNOS-Pser1177 in the aortas of SHR. These results could partially explain why no specific effect of high-intensity exercise training was observed on endothelial function, but they are not sufficient to explain why eNOS-dependent vasorelaxation was reduced in SHR-HI compared with their sedentary counterparts.

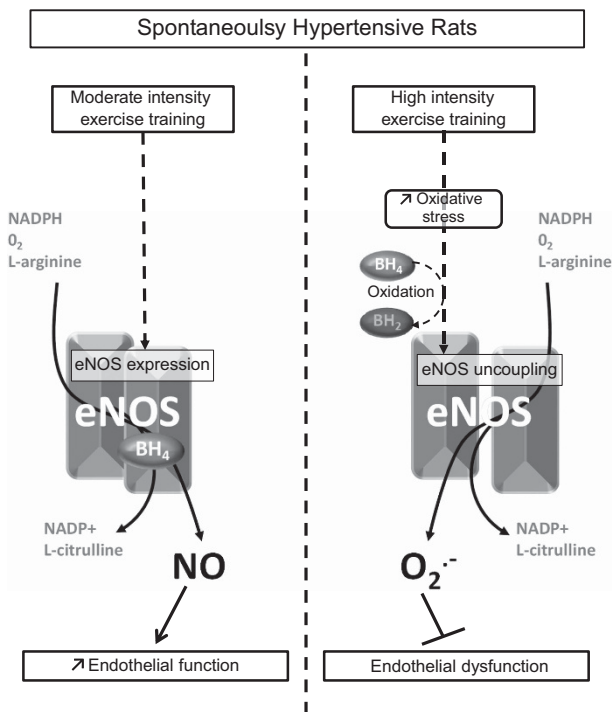


Figure 7 High-intensity exercise does not improve endothelial function in SHR. Moderate-intensity exercise training increases NO bioavailability by increasing the eNOS protein level and therefore improves endothelial function.^{11,45,46} By contrast, high-intensity exercise training that is responsible for increased oxidative stress results in redox-dependent eNOS uncoupling, diminished eNOS/NO function and did not enhance endothelial function.

In pro-oxidant conditions, NO can react with superoxide anion (O_2^-) and produce peroxynitrite, a potent cytotoxic molecule. This biochemical reaction is further associated with the reduced bioavailability of NO^{51,52} and thus could help to explain our results. However, here, protein nitrotyrosination, an index of peroxynitrite formation, tended to be higher in SHR and was reduced by high-intensity exercise training. Because higher eNOS function is associated with higher NO bioavailability and has been reported to be associated with an increased level of protein nitrotyrosination in pro-oxidant conditions,¹⁷ these results may suggest diminished NO formation in SHR that exercised at high intensity compared with sedentary rats. In addition, high-intensity exercise is well known to be associated with increased oxidative stress when compared with moderate-intensity exercise.⁵³ However, in our model, ROS production was increased in SHR, as previously described,^{54–56} and no additional effect of high-intensity exercise training was observed. Consequently, the worsening effect of high-intensity exercise on endothelial function could not be directly attributed to oxidative stress.

eNOS is also well known as a critical homodimeric enzyme. In physiological conditions, the dimerized state of the enzyme has a key role in coupling electron transfer in the reductase domain with NO synthesis in the heme domain via the delivery of electrons required for oxygen activation at the catalytic heme site. However, in some pathological conditions or stress, the enzyme can be uncoupled, resulting in a switch from NO to O_2^- synthesis,^{57–60} which is particularly true in hypertension.^{5,28} In our study, although the total ROS production was not different between the SHR and SHR-HI aortas, eNOS-dependent ROS production was increased in the

exercised animals, suggesting that, in SHR, high-intensity exercise training was primarily associated with the functional uncoupling of the enzyme. Such uncoupling is mainly explained in the literature by the depletion/oxidation of the cofactor BH_4 ^{5,33,61–63} or by redox-dependent glutathionylation of the enzyme.^{28,64} In the present study, the use of NAC, an agent with thiol-reducing properties,^{65–67} or BH_4 , a recoupling agent,^{68,69} reduced ROS production in only SHR-HI aortas, strongly suggesting that this increase in eNOS-dependent ROS production was mainly related to redox-dependent eNOS uncoupling.²⁸ Because our data do not suggest increased vascular oxidative stress at the steady state, we can speculate that the eNOS uncoupling observed here is due to the particularly high oxidative stress generated during high-intensity exercise training,⁵³ which could result in the oxidation of BH_4 and/or the direct redox-dependent glutathionylation of eNOS. Indeed, mitochondrial ROS production increased with regard to oxygen consumption.⁷⁰ However, the ability of chronic treatment with BH_4 to prevent the deleterious effects of high-intensity training should be tested to better evaluate this point. Finally, because no difference was reported in eNOS-dependent vasorelaxation between the SHR and SHR-HI aortic rings in the presence of BH_4 , we can conclude that the eNOS uncoupling consequence of continuous high-intensity exercise training mainly helps to explain why this strategy has no beneficial effect on arterial endothelial function in SHR.

Study limitation

In the present study, data obtained on the functional state of eNOS are mainly focused on the impact of high-intensity exercise training, and no data are provided on how moderate exercise training could affect this pathway. However, even if this could constitute a limitation of the present work, the effect of moderate exercise training on the eNOS–NO pathway has already been studied not only in healthy^{37,71} but also in SHR.^{11,45,46} Those studies reported that exercise training at moderate intensity improve the eNOS–NO pathway function.

CONCLUSION

In conclusion, we report here that continuous high-intensity exercise training is associated with the dysfunction of the eNOS–NO pathway, characterized mainly by a lack of increase in eNOS phosphorylation of the enzyme at its activation site and by its functional uncoupling, thus increasing eNOS-dependent ROS production (Figure 7). These effects of continuous high-intensity exercise training on the functional state of eNOS could help to explain why this exercise training strategy cannot improve endothelial function in SHR and therefore cannot be considered a good protective strategy in this population.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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