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Contribution of Heme Oxygenase 2 to Blood Pressure Regulation in Response to Swimming Exercise and Detraining in Spontaneously Hypertensive Rats

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Data Interpretation D
Manuscript Preparation E
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Background: We aimed to determine the effects of exercise followed by detraining on systolic blood pressure (SBP), heme oxygenase 2 (HO-2) expression, and carboxyhemoglobin (COHb) concentration in spontaneously hypertensive rats (SHR) to explain the role of carbon monoxide (CO) in this process.

Material/Methods: Animals were randomized into exercised and detrained groups. Corresponding sedentary rats were grouped as Time 1–2. Swimming of 60 min/5 days/week for 10 weeks was applied. Detraining rats discontinued training for an additional 5 weeks. Gene and protein expressions were determined by real-time PCR and immunohistochemistry.

Results: Aorta HO-2 histological scores (HSCORE) of hypertensive rats were lower, while SBP was higher. Swimming caused enhancement of HO-2 immunostaining in aorta endothelium and adventitia of SHR. Exercise induced elevation of blood COHb index in SHR. Synchronous BP lowering effect of exercise was observed. HO-2 mRNA expression, HSCORE, and blood COHb index were unaltered during detraining, while SBP was still low in SHR.

Conclusions: CO synthesized by HO-2 at least partly plays a role in SBP regulation in the SHR- and BP-lowering effect of exercise. Regular exercise with short-term pauses may be advised to both hypertensives and individuals who are at risk.

MeSH Keywords: **Aorta • Heme Oxygenase (Decyclizing) • Rats, Inbred SHR • Swimming**

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Background

Spontaneously hypertensive rats (SHR) represent an animal model of genetic hypertension (HT) that also develops endothelial dysfunction [1]. The endothelium plays a pivotal role in transducing and modulating stimuli, controls vascular permeability, and modulates vascular tone [2]. The conduit arteries of SHR were reported to be stiffer than in normotensives [3]. The mechanical properties of large-conduit arteries play an important role in hemodynamics through the buffering of stroke volume and propagation [4]. The aorta, besides supplying blood to the periphery, also dampens pressure oscillations [3]. SHR exhibit aortic remodeling and impaired endothelium-dependent vasorelaxation [5,6].

Carbon monoxide (CO) is an endogenously produced gas molecule causing relaxation of the vessels [7]. Heme oxygenase (HO) is a critical cytoprotective enzyme that degrades cytotoxic free heme and produces the potent vasodilator CO and the antioxidant bilirubin [7,8]. Three isoforms of HO are known. HO-1 is not constitutively present but can be induced by different stimuli, while HO-2 is normally expressed in many organs under physiologic conditions, and HO-3 shares homology with HO-2 and is thought to regulate heme homeostasis [9,10]. HO-2 is expressed in endothelial and smooth muscle layers of blood vessels, generating CO that intrinsically modulates vascular tone, while HO-1 up-regulation has been reported in pathological conditions [11–13]. Upregulation of the HO/CO system could play a beneficial role in the pathogenesis of HT.

Exercise training increases the elastic component of the aorta and preserves endothelial function in SHR [14,15], but the underlying molecular mechanism remains unclear. Recent reports indicate the role of CO in these processes [16]. Although exercise has been proven to reduce systolic blood pressure (SBP) and delay the progression of HT, the compliance with the recommended treatment is very low since "behavior change" is necessary to maintain training [17]. Detraining is defined as the loss of training-induced adaptations as a consequence of training reduction or cessation [18]. No study has demonstrated HO-2 expression levels in SHR in response to exercise and detraining. Taking into consideration the demonstrated ability of exercise training to remodel arteries, and our limited knowledge of antihypertensive therapy-induced changes in the function of aortic endothelium, we sought to determine the effects of exercise followed by detraining of 5 weeks on SBP, on the structural changes of the aortic components (mRNA expression, protein distribution, and location of HO-2 within the aortic wall). Thus, the present study was designed to determine: (1) aortic HO-2 gene and protein expression in SHR and normotensive WKY, (2) blood carboxyhemoglobin (COHb) levels, and (3) the alterations in these parameters in response to exercise and detraining. This study may provide new insights into understanding the mechanisms underlying such phenomena.

Table 1. The experimental design.

WKY rats	WKY sedentary	Time 1
		Time 2
	WKY exercised	Exercise
		Detrained
SHR	SHR sedentary	Time 1
		Time 2
	SHR exercised	Exercise
		Detrained

Material and Methods

Animal care

Male, 8-week-old normotensive Wistar-Kyoto (WKY) rats and age-matched SHR (Harlan Laboratories, USA) were used in the study. The animals were housed in groups of 4–5 per cage (42×26×15 cm) in a room with controlled temperature (23±2°C) and relative humidity (60±5%) under a 12-h light cycle (07: 00 to 19: 00 h) with free access to water and food. At the beginning of the experimental period, SHR and WKY rats were mainly assigned as either sedentary or exercised. SHR sedentary and WKY sedentary rats were further divided into 2 groups as "Time 1" and "Time 2", while SHR exercised and WKY exercised rats were divided as exercise and detrained groups. Exercise was applied for 10 weeks and detraining for 5 weeks as described below. The rats in the sedentary groups lived in their cages for the same duration. Blood samples of animals in "Time 1" groups were obtained 10 weeks after the beginning of experiments, simultaneously with the exercise groups and "Time 2" groups 15 weeks after the start, simultaneously with the detrained animals. A total of 8 groups were established. Experimental groups are demonstrated in Table 1. The project was approved by Pamukkale University Ethics Committee of Animal Care and Usage (PAUHADEK-2012/025 and PAUHDEK-2013/001).

Aerobic exercise training and detraining protocol

The exercise protocol conformed to the American Physiological Society's Resource Book for the Design of Animal Exercise Protocols [19]. Swimming training was performed in a container called the Morris water maze filled with tap water (45-cm deep) maintained at 31.0±2.0°C by a feedback-controlled electric heating coil. For adaptation, swimming training was limited to 10 min on the first day and increased by 10 min each day, until 60 min was reached. Rats swam for a total period of 10 weeks, 60 min/day, and 5 days/week [20]. This protocol is defined as an aerobic endurance training and moderate intensity exercise and corresponds to the intensity below the anaerobic threshold in rats [21]. Swimming rats were individually observed. One of the WKY rats drowned while swimming. Rats in sedentary and detraining groups swam once a week for 10 min. The rats in the detraining groups underwent the same training protocol and then discontinued training during the next 5 weeks (detraining groups) [22].

Tissue collection and analysis of blood carboxyhemoglobin (COHb) concentrations

Twenty-four hours after the last exercise session in the training groups and on the 15th week for the detrained and corresponding sedentary groups, the rats were anaesthetized with xylazine (10 mg/kg, i.p.) and ketamine (90 mg/kg, i.p.). Blood samples were collected from the abdominal aorta of the animals into standard tubes containing heparin (15 IU/ml) to determine blood COHb concentrations (n=10). The air in the injector was thoroughly removed and the tubes were sent to a private laboratory in dry ice on the same day for COHb index measurements by blood gas analysis. The aortas of the animals were divided into pieces. The upper portion was placed into cryotubes containing RNAlater solution (Life Tech., USA) for the determination of HO-2 mRNA expression. It was frozen in liquid nitrogen and stored at –80°C until analyzed. Abdominal aortas were immersed in 10% formaldehyde without excess cleaning and were used for immunohistochemistry.

SBP measurements

SBP of the animals was measured at the beginning of the experiment and repeated every 2 weeks thereafter. Measurement of SBP (n=10) was evaluated in the conscious state using a computerized indirect tail cuff method (Commat may nibp 200-A). Rats were kept up until calm in animal holders at 34°C. All animals were placed in a restrainer for 15 min, a cuff was attached to their tail, and SBP was then recorded [23]. Three readings were taken for each rat and averaged.

Real-time quantitative PCR

Total RNA extraction and cDNA synthesis

The samples were homogenized in a microtube using Tissue Lyser (rotor-stator homogenizer Heidolph, RZR 2021) and total RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA concentrations were measured with a NanoDrop spectrophotometer (Thermo Scientific) and about 1 µg RNA was used for the synthesis of complementary DNA (cDNA) using a cDNA Synthesis Kit (QuantiTect Reverse Transcription Kit, Qiagen) according to the manufacturer's specifications. Reverse transcription was carried out at 42°C for 15 min, followed by incubation at 95°C for 3 min. The cDNAs were stored at –20°C until they were used as a template in real-time RT-PCR.

Table 2. Primers and UPL probes used for real-time gene expression analysis (5'→3').

Primer sequences	UPL number	References
HO-2		
TTT TAA GCT TGC CAC CAC TG (Forward)	90013523	Abraham et al. 2012
CCT GGT TCT CCC AGT CTT CA (Reverse)		
β-actin		
Single assay	ID: 500 153	Ye et al. 2012

Real-time PCR analysis

Real-time PCR analysis (n=3–5) was performed using a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). The list of PCR primer and UPL probes for target gene and reference gene (β-actin) are given in Table 2. Final reaction volume for the analysis of expression for the target gene was performed in 20 µL: 0.5 µl of primer, 0.2 µl of UPL probe, 10 µl of 2×LightCycler 480 Probes Master, 4 µl of cDNA sample, and 4.8 µl of PCR-grade water. For the reference gene, each reaction tube contained 1 µl of signal assay, 10 µl of 2×LightCycler 480 Probes Master, 4 µL of template cDNA, and 5 µL of PCR-grade H_2O in a total of 20 µL PCR mixture. The cycling conditions were 95°C for 10 min, followed by 45 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s. All runs included 1 negative cDNA control consisting of DNase- and RNase-free water. Expression of each gene was normalized using housekeeping β-actin gene as control and final results were produced using LightCycler 480 software. All samples were run in triplicate. The expression of each gene was calculated using the ΔΔCT method and compared with the expression in the control group. Each value is represented as the mean fold-change of RNA expression compared with the controls.

Immunohistochemistry

Fixation and tissue preparation

Aortas (n=3–5) were removed and put in 10% neutral buffered formalin for 72 h and then embedded in paraffin. Paraffin sections (5 µm) were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions. Three sections from each animal were processed for HO-2 immunohistochemistry. Negative controls were performed by omitting the primary antibody.

Antibodies and staining procedure

Endogenous peroxidase activity was blocked in 3% hydrogen peroxidase for 10 min, and the sections were incubated with saponin to facilitate binding of the primary antibody to the antigenic areas. Epitopes were stabilized by application of serum blocking solution (goat serum; Invitrogen, Catalog number 859043) for 60 min at room temperature. Sections were incubated in phosphate-buffered saline (PBS) at room temperature for 60 min using the following primary antibody: HO-2 (1: 100, Santa Cruz Biotechnology, sc-7697). In the next step, secondary antibody was applied to tissue slides: anti-goat IgG and avidin-biotin-complex-peroxidase (ABC; Catalog number 32020; Invitrogen, Carlsbad, CA, USA). Diaminobenzidine (DAB; D 3939; Sigma-Aldrich, St. Louis, MO, USA) was used as the chromogen. In the following step, the slides were counterstained with hematoxylin for 1 min, dehydrated in graded ethanol, and mounted in conventional medium.

The intensity of immunoperoxidase reaction was classified as follows: negative (–) when the cells were devoid of any detectable HO-2 expressions, slightly positive (+), moderately positive (++), and strongly positive (+++). Negative controls were performed by omitting the primary antibody resulting in no staining. The findings were observed and photographed under an OLYMPUS BX51 microscope. For each tissue, a histological score (HSCORE) value was derived by summing the percentages of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining, using the formula HSCORE=ΣPi (i+l), where i represents the intensity scores and Pi is the corresponding percentage of cells. In each slide, 5 randomly selected areas were evaluated under a microscope (20× magnification) and the percentage of the cells for each intensity within these areas was determined at different times by blinded investigators.

Statistical analyses

Statistical analyses were performed using the computer software SPSS version 21.0 (Statistical Package for Social Sciences). Continuous variables were defined by the mean ± standard error (SE). Kruskal-Wallis variance analysis was used for comparing independent groups. For post hoc analysis, the Mann-Whitney U test with Bonferroni correction method was used when the Kruskal-Wallis variance analysis determined a significant difference. Friedman test was used for comparing dependent groups and post hoc Wilcoxon signed rank test with Bonferroni correction method. p<0.05 was accepted as statistically significant.

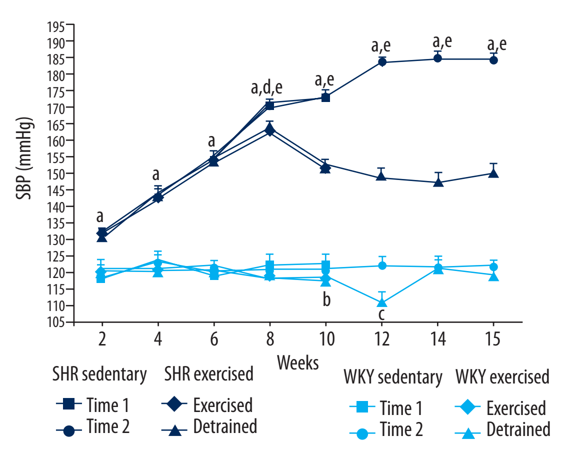

Figure 1. Systolic blood pressure (SBP) measurements of the rats. n=10. Values are expressed as mean ±SE. SBP measurements of the SHR groups. SBP measurements of the WKY groups. a) *p*<0.05, difference of SHR groups from corresponding WKY groups; b) *p*<0.05, difference of WKY sedentary rats Time 1 group from WKY exercise group; c) *p*<0.05, difference of WKY sedentary rats Time 2 group from WKY detrained group; d) *p*<0.05, difference of SHR sedentary rats Time 1 group from SHR exercise group; e) *p*<0.05, difference of SHR sedentary rats Time 2 group from SHR detrained group.

Results

Baseline SBP values inside WKY and SHR groups were not different from each other, but the basal SBP of SHR was higher compared to corresponding WKY (p<0.05) groups. This difference continued throughout the experimental period (Figure 1). The BP-lowering effect of swimming exercise was more prominent in hypertensive rats. Although exercise-induced reduction of SBP began on the 8th week, statistically significant decrements were observed on the 10th week in both WKY and SHR rats (p<0.05). The favorable effect of swimming on SBP was preserved at the 1st measurement of detraining in the WKY group, but it persisted until the 15th week in SHR rats (p<0.05).

No statistically significant alteration was observed in aorta HO-2 relative mRNA expression (Figure 2). Aortas isolated from exercise SHR and detrained SHR groups showed enhanced immunostaining for HO-2 in endothelium and adventitia compared to Time 1 and 2 of SHR. Positive HO-2 expression was observed in endothelium and adventitia of WKY exercise group. Brown staining appeared poor within the endothelium and tunica media but was more evident in tunica adventitia at Time 1 and 2 of WKY rats (Figure 3). The exercise protocol used resulted in statistically insignificant enhancement of aorta HO-2 HSCORE in hypertensive rats (Figure 4). Aorta HO-2 HSCORE was higher in aortas of Time 1 WKY than in aortas of

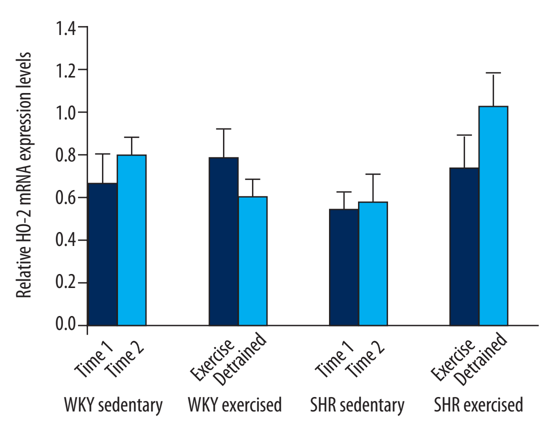

Figure 2. The mRNA expression level of HO-2 in the aortas of rats. n=3-5. Real-time PCR showing the relative RNA expression level of HO-2 in the aortas of rats. β-actin was used as control to calculate the relative expression of HO-2 by the 2–ΔΔCT method. Data presented are the mean ±SE of 3 independent experiments.

Time 1 SHR group. The latter alteration was statistically significant (p=0.0001). In line with these results, aortas isolated from Time 1 and 2 WKY groups showed enhanced immunostaining for HO-2 compared to Time 1 and 2 of SHR (Figure 3).

Blood COHb levels of the groups were measured as a representative of blood CO concentrations and are presented in Figure 5. Blood COHb indexes of both SHR and WKY rats were increased in response to exercise, but the augmentation was statistically significant only in the SHR groups (p<0.0001). No significant alteration was observed between blood CO concentrations of control and hypertensive animals. Although detraining of 5 weeks resulted in decrement of blood COHb indexes, the decline was not statistically significant. Blood COHb levels of the SHR detraining group were higher than in the WKY detraining group (p<0.0001).

Discussion

Aerobic exercise protocols with moderate intensity have been recommended for BP regulation [24]. However, non-compliance with exercise has been reported to be very high [25]. Clinical research and experimental animal model studies have indicated that HT is associated with endothelial dysfunction [26]. An impairment of endothelium-dependent relaxations has been observed in different vessels from SHR [27]. Nevertheless, the effects of exercise training and detraining on the aorta HO-2 mRNA and protein levels of SHR remain unclear. According to the results of the present study, aorta HO-2 HSCORE of hypertensive rats was lower and SBP was higher compared to normotensive rats. Ten weeks of swimming resulted in enhancement of HO-2 immunostaining in aorta endothelium and adventitia of SHR. The exercise protocol resulted in increased blood COHb index in SHR. A synchronous BP-lowering effect of swimming exercise was observed. Detraining of 5 weeks was not enough to revert the studied parameters.

SBP of SHR was found to be higher than WKY in the first measurement and continued to increase during the subsequent weeks. This finding is compatible with previous results demonstrating the rise of SBP during ageing [25,28,29]. The reducing effect of swimming exercise on SBP of both normotensive animals and SHR was observed in the 10th week. The training-induced hypotensive effect is known to be established by physiological adaptations in the heart and vascular system, such as augmentation in sympathetic baroreflex sensitivity, increased in capillary density, and enhanced endothelial vasodilatation [30]. Previous reports have shown that, while exercise of 10 weeks reduces BP in SHR [23,25,29], 1 and 2 weeks of detraining are not sufficient to revert cardiorespiratory benefits induced by 10 weeks of training [23]. This is why we extended detraining up to 5 weeks in the present study.

The endothelium produces various substances collectively termed endothelium-derived relaxing factors (EDRFs) and endothelium-derived constricting factors (EDCFs) [31]. The initial mechanism of endothelial dysfunction itself may be associated with a lack of endothelium-derived relaxing factors and/or accentuation of various endothelium-derived constricting factors. The involvement and role of endothelium-derived factors in the development of endothelial dysfunction in individual experimental models of HT may vary, depending on the triggering stimulus, strain, age, and vascular bed investigated [31].

Both vascular endothelium and smooth muscle cells express HO [32] and produce CO that elicits vasorelaxation [33]. HO-1 is an inducible isoform in response to oxidative stress, hypoxia, heavy metals, and cytokines, while, HO-2 is the constitutive isoform of the enzyme [16,34–37]. HO-1 and HO-2 are expressed in many vascular tissues, such as mesenteric artery, tail artery, aorta, and pulmonary artery [7]. The stimulation of HO seems to induce an improvement of endothelial dysfunction in SHR. However, studies investigating HO in HT revealed conflicting results [12,34–36] and thus the importance of HO-2 in vascular tissues in regulating BP became questionable [35,36]. Although it appears that HO-2 plays an important role in maintaining the basal contractile force in certain vascular tissues, some authors suggest that expression of HO-2 cannot be induced [38]. We have examined HO-2 immunostaining in aorta of SHR and observed decreased HO-2 HSCORE and increased SBP in SHR compared to control rats. The coexistence of decrement of an enzyme synthesizing a vasodilator mediator (CO) with increased BP is reasonable. On the other hand, aorta HO-2 relative mRNA expression as well as blood

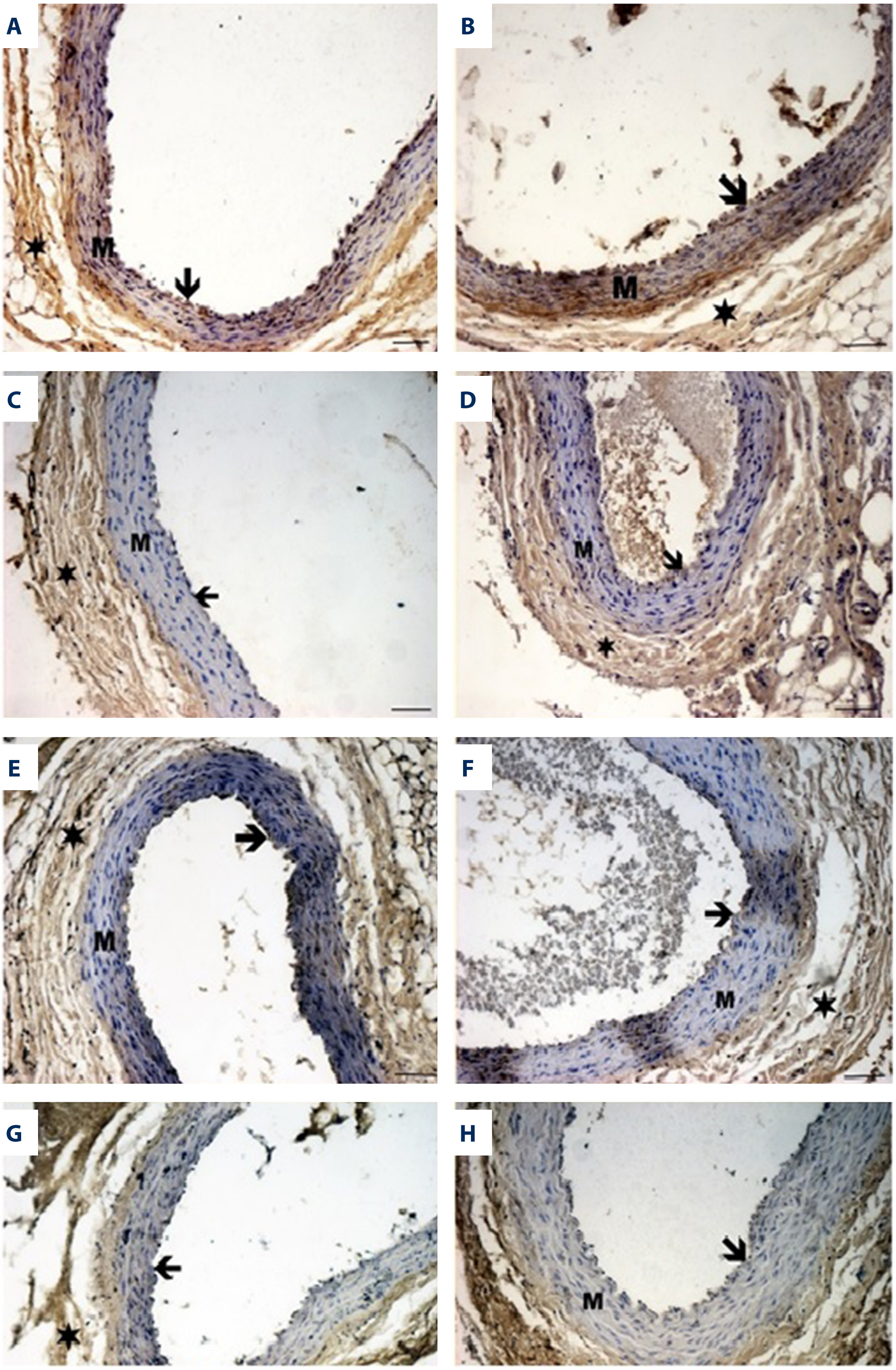

Figure 3. Immunohistochemical staining for HO-2 (brown) in the aortas of rats. n=3–5. (**A**) WKY sedentary rats Time 1 group; (**B**) WKY sedentary rats Time 2 group; (**C**) WKY exercise group, (**D**) WKY detrained group; (**E**) SHR sedentary rats Time 1 group, (**F**) SHR sedentary rats Time 2 group; (**G**) SHR exercise group; (**H**) SHR detrained group. Endotelium (arrow). Tunica media with negative staining for HO-2 (M). Positive immunreactivity is localized in Tunica adventitia (asterix). Immunoperoxidase hematoxyline, bar: 150 µm.

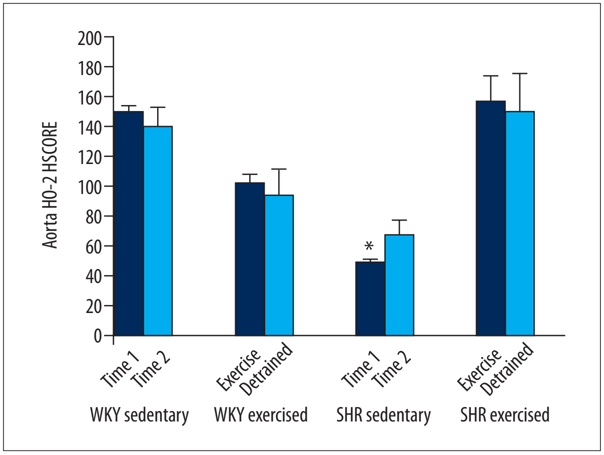

Figure 4. HSCORE analysis for expression of HO-2 in the aortas of rats. n=3-5. Values are expressed as mean ±SE. * $p<0.05$, difference from WKY sedentary rats Time 1 group.

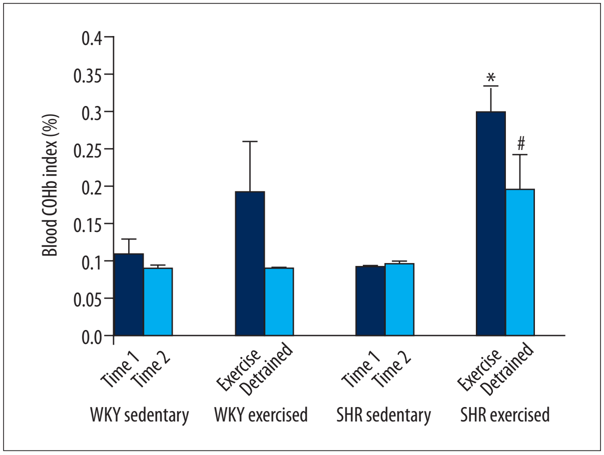

Figure 5. Blood COHb indexes (%). n=10. Values are expressed as mean ±SE. * $p<0.05$, difference from WKY sedentary rats Time 1 group; $^{\#}p < 0.05$, difference from WKY detrained group.

COHb index did not accompany the decrement observed in immunostaining data. Ndisang et al. previously found that blood COHb levels may be used as an index to evaluate the status of the endogenous CO system. Similar to our results, blood COHb levels did not differ between SHR and control WKY in their experiments [12]. The incompatibility between the results may be explained by the difference in specificity of the antibodies used [39]. Additionally, aortas used for mRNA expressions were cleaned before analysis, but the aorta portions used for immunohistochemical analysis were immersed in formaldehyde without excess cleaning, and thus included more adventitial tissue. This may provide another explanation for the diversity of the results. Recent studies, however, showed that the adventitia plays an important role in various vascular processes, including HT [40]. Moreover, the contribution of adventitia to passive mechanical properties of the artery has been paid more attention and it has been shown that adventitia contributes to vascular biomechanics in terms of shear, circumferential, and axial modulus [41]. A series of landmark studies have also shown that the adventitia is a useful platform for expression of tissue-permeant hormones. The resulting focus on the adventitia raises an important question as to its physiological role as a paracrine mediator of vascular function and a potential therapeutic target.

Regular exercise is one of the most important nonpharmacological tools in reducing overall cardiometabolic risk [42]. The mechanism(s) underlying BP reduction is (are) still controversial. The adaptations that accompany exercise training are dependent on factors such as modality, intensity, duration, and frequency of the physical activity as well as the age of onset. Previous experiments demonstrated that the intensity of exercise was positively correlated with HO activity in rat aortic smooth muscle. Nine weeks of moderate intensity swimming exercise was shown to enhance HO-1 mRNA expression in aorta of SHR. Additionally, long-term aerobic exercise significantly raised HO activity and serum CO content of cardiac and vascular smooth muscle in SHR models, indicating that motion can increase HO activity and serum CO content and may act to prevent and alleviate HT [16]. The mechanisms may be listed as: (1) exercise induces HO-1 activity and CO generation, improving cGMP to regulate BP; (2) the endogenous CO generated by the action of the hypothalamus can be adjusted to reduce BP; (3) endogenous CO can also inhibit the reaction of peripheral baroreceptor sensitivity to lower BP; and (4) HO-1/CO and inducible nitric oxide synthase (iNOS)/NO system can lower BP through the compensatory action [43,44]. In the present study we demonstrate for the first time that 10 weeks of swimming exercise increases HO-2 immunostaining in aortas of SHR. This result is accompanied by slight, statistically non-significant post-exercise enhancements in relative mRNA HO-2 expression. We also observed increased blood COHb index in exercised SHR. It was demonstrated recently that, although thoracic aorta HO-2 expression increases in L-NAME-induced hypertensive rats in response to swimming, gastrocnemius and mesenteric resistance arteries HO-2 expression are unaffected [45]. To the best of our knowledge, this is the only study reporting the HO-2 response to exercise in HT. In addition to HO-1, our results indicate the contribution of HO-2 synthesized CO to the exercise-induced SBP regulation in SHR. A limitation of the present study is that we examined only the aortic response, and other resistance vessels may respond differently to various levels of exercise.

Sun et al. provided evidence of exercise-induced elevation of vascular HO-1 and HO-2 as well as enhanced HO-related dilation in normotensive rats. These authors demonstrated that: 1) endurance exercise training (treadmill running at moderate intensity) enhances HO-related acetylcholine-induced vasorelaxation and 2) HO-1 and HO-2 contents increase in the aorta after endurance training [46]. The training-induced HO-1 was reported to be mainly in endothelial and perivascular layers, while HO-2 was induced across the 3 layers of the aortic wall of normotensive rats [46]. In agreement with these results, we demonstrated positive HO-2 expression in endothelium and adventitia of WKY exercise group.

Detraining was reported to be closely associated with poor outcomes of HT [18]. An understanding of the interaction of endothelial function and BP in response to detraining through describing a “safe” exercise breakout time might be relevant for developing potential future exercise regimens for the treatment of HT. SBP of normotensive rats began increasing after the cessation of swimming. Although the detraining for 5 weeks applied herein was not enough to reverse the BP-lowering effect of exercise, the favorable effect of exercise might be more resistant to cessation in hypertensives [25,29]. Similarly, 5 weeks of detraining did not result in any statistically significant alteration in HO-2 expression and blood COHb index, indicating that cessation of exercise for this time period was not enough to revert the exercise-induced physiological adaptations in either SBP or CO released by HO-2.

Conclusions

Results of the present study indicate the participation of HO-2 in HT and cardiovascular adaptation to swimming exercise. Abnormal CO metabolism and function may contribute to the development of HT, but do not seem to be the only factor involved. The interaction of CO and other homeostatic mechanisms, as well as other gasotransmitters and the effect of CO on other systems, should be taken into consideration when the role of CO in the development and maintenance of HT is being explored. Another important novelty of our study is that cessation of exercise for 5 weeks may be “safe” in terms of BP and endothelial function determined by only CO. The effects of different durations of exercise and detraining and the physiological mechanisms involved in these processes in HT remain unknown.

Conflict of interest

None.

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