British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.02224.x
www.brjpharmacol.org

RESEARCH PAPER

Aerobic exercise reduces oxidative stress and improves vascular changes of small mesenteric and coronary arteries in hypertension

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Keywords
aerobic exercise; hypertension; vascular alterations; oxidative stress

Received
5 March 2012
Revised
29 June 2012
Accepted
12 August 2012

BACKGROUND AND PURPOSE

Regular physical activity is an effective non-pharmacological therapy for prevention and control of hypertension. We investigated the effects of aerobic exercise training in vascular remodelling and in the mechanical and functional alterations of coronary and small mesenteric arteries from spontaneously hypertensive rats (SHR).

EXPERIMENTAL APPROACH

Normotensive Wistar Kyoto (WKY), SHR and SHR trained on a treadmill for 12 weeks were used to evaluate vascular structural, mechanical and functional properties.

KEY RESULTS

Exercise did not affect lumen diameter, wall thickness and wall/lumen ratio but reduced vascular stiffness of coronary and mesenteric arteries from SHR. Exercise also reduced collagen deposition and normalized altered internal elastic lamina organization and expression of MMP-9 in mesenteric arteries from SHR. Exercise did not affect contractile responses of coronary arteries but improved the endothelium-dependent relaxation in SHR. In mesenteric arteries, training normalized the increased contractile responses induced by U46619 and by high concentrations of acetylcholine. In vessels from SHR, exercise normalized the effects of the NADPH oxidase inhibitor apocynin and the NOS inhibitor L-NAME in vasodilator or vasoconstrictor responses, normalized the increased O_2^- production and the reduced Cu/Zn superoxide dismutase expression and increased NO production.

CONCLUSIONS AND IMPLICATIONS

Exercise training of SHR improves endothelial function and vascular stiffness in coronary and small mesenteric arteries. This might be related to the concomitant decrease of oxidative stress and increase of NO bioavailability. Such effects demonstrate the beneficial effects of exercise on the vascular system and could contribute to a reduction in blood pressure.

Abbreviations

CSA, cross-sectional area; dAUC, differences of area under the concentration–response curves; DEA-NO, diethylamine NONOate; ECM, extracellular matrix; E_{inc}, incremental elastic modulus; E_{max}, maximal response; eNOS, endothelial NOS; KHS, Krebs–Henseleit solution; PFA, paraformaldehyde; ROS, reactive oxygen species; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase; WKY, Wistar–Kyoto; WT, wall thickness

Introduction

Hypertension is associated with vascular structural, mechanical and functional alterations such as increased wall-to-lumen ratio and vascular stiffness, impairment of endothelium-dependent vasodilator responses and enhancement of vasoconstrictor responses to different agonists. Reactive oxygen species (ROS) seem to play a major role in these alterations through their effects on cellular function such as inactivation of NO, regulation of cell growth and differentiation, modulation of synthesis and degradation of extracellular matrix (ECM) proteins and activation of many kinases and of pro-inflammatory genes (Lee and Griendling, 2008; Briones and Touyz, 2010; Drummond *et al.*, 2011).

A sedentary lifestyle has been identified as a risk factor for development of cardiovascular disease, and aerobic activity is considered to be an effective component of prevention of cardiovascular events (Mitchell *et al.*, 2010). In terms of hypertension, aerobic exercise is a well-recommended non-pharmacological measure that is effective for prevention and control of high blood pressure levels (Fagard and Cornelissen, 2007). Exercise training is known to change the morphology of vessels from spontaneously hypertensive rats (SHR) (Amaral *et al.*, 2000; Melo *et al.*, 2003; Horta *et al.*, 2005; Moraes-Teixeira *et al.*, 2010) and improve vascular stiffness in hypertensive humans (Collier *et al.*, 2008; Guimarães *et al.*, 2010) and SHR aorta (Hägg *et al.*, 2004). In addition, improved endothelium-dependent responses after exercise training have been demonstrated in hypertensive humans (Higashi and Yoshizumi, 2004; Yung *et al.*, 2009) and animal models (Chen and Chiang, 1996; Yung *et al.*, 2009) mainly through a significant increase in NO production and/or decrease in NO inactivation (Higashi and Yoshizumi, 2004).

Hypertension appears to be responsible for increased coronary resistance, impaired autoregulation and left ventricular dysfunction (Harrison *et al.*, 1988). On the other hand, resistance arteries are mainly involved in the regulation of blood pressure. Most of the studies exploring the beneficial effects of exercise on vascular alterations associated with hypertension have been performed in large arteries and there is a lack of information on the beneficial effects of exercise in coronary and/or resistance arteries. In addition, the exact mechanisms whereby exercise contributes to improve cardiovascular health need to be better understood and amelioration of oxidative stress is emerging as a strong candidate. Growing evidence indicates that exercise increases antioxidant capacity (Gomez-Cabrera *et al.*, 2008; Kimura *et al.*, 2010) and prevents oxidative damage by reducing oxidative stress observed in hypertension (Rush *et al.*, 2003; Agarwal *et al.*, 2009; 2012; Yung *et al.*, 2009; Kimura *et al.*, 2010).

The purpose of the present study was to investigate the effects of aerobic exercise training in the vascular remodelling and in the mechanical and functional alterations of coronary and small mesenteric arteries from SHR and to elucidate the underlying mechanisms. We would propose that aerobic exercise training improves the hypertension-associated vascular alterations by modulating oxidative stress and NO bioavailability, and this in turn could contribute to the observed effects of exercise on blood pressure.

Methods

Animals

All animal care and experimental procedures were approved by the Animal Care and Use Committee of the Universidad Autónoma de Madrid, according to the guidelines for ethical care of experimental animals of the Spanish and European Community laws (RD 223/88 MAPA and 609/86). The results are reported in accordance with the ARRIVE guidelines for experiments involving animals (McGrath *et al.*, 2010). A total of 40 (SHR 16, SHR Trained 16, WKY 8) animals were used in the experiments described here.

Three-month-old male rats were obtained from the colonies of SHR and Wistar–Kyoto (WKY) rats inbred at the Animal House of Facultad de Medicina, Universidad Autónoma de Madrid. Rats were housed at a constant room temperature, humidity and light cycle (12:12 h light–dark) with free access to tap water and fed with standard rat chow *ad libitum*. A group of normotensive WKY rats (n = 8) were used as controls and the SHR separated into two groups, SHR (no exercise training, sedentary n = 16) and SHR with exercise training (see below; referred to as SHR-Trained n = 16).

Body weight was measured weekly. Systolic blood pressure (SBP) and heart rate were measured in awake animals, before the beginning of the exercise training protocol and every 15 days until the end of the protocol using tail–cuff plethysmography.

Exercise training protocol

Exercise training was performed on a motor treadmill (Motor-driven Treadmill LI8706, Letica Scientific Instruments, Barcelona, Spain) for 12 weeks, five times per week for 60 min, gradually progressing towards 55–65% (15–20 m min^{-1}) of maximal running speed. To determine the maximal exercise capacity, rats were submitted to a progressive exercise test using an incremental speed protocol of 5 m min^{-1} every 3 min and no grade until exhaustion. The treadmill exercise test was repeated after 6 weeks in order to adjust training intensity. Rats were considered to be exhausted when they could no longer run at the treadmill speed. The sedentary rats were handled at least twice a week for habituation to the experimental protocols.

Plasma and tissue samples

Twenty-four hours after the last session of exercise training, rats were killed by decapitation; and blood, heart, vascular

mesenteric bed and soleus muscle samples were immediately removed. Blood samples were collected in tubes containing EDTA as anticoagulant, placed in ice and centrifuged at 1500× *g* for 10 min at 4°C. The plasma was frozen and kept at –80°C until used for nitrite analysis. The soleus muscle was removed and immediately stored at –80°C until analysis of citrate synthase activity. The heart and mesenteric vascular arcade were removed and maintained in cold (4°C) Krebs–Henseleit solution (KHS) (115 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11.1 mM glucose and 0.01 mM Na_2EDTA) bubbled with a 95% O_2–5% CO_2 mixture, pH 7.4. Second- and third-order branches of the mesenteric artery were dissected free of fat and connective tissue, and segments of the left descending coronary artery and septal artery were gently isolated and cleaned from surrounding cardiac tissue under a dissecting microscope. Segments of third-order branches of the mesenteric artery and septal coronary artery were used for the study of structural and mechanical properties. Moreover, small mesenteric arteries and left descending coronary artery were used for reactivity experiments. In addition, second- and third-order branches of mesenteric arteries were stored at –80°C for Western blot analysis.

Citrate synthase activity

Citrate synthase activity was determined in mixed right soleus and used as a marker of muscle oxidative activity (Alp *et al.*, 1976). The enzyme activity was measured in whole muscle homogenates, and the complex resulting from acetyl-CoA and oxaloacetate was determined at 412 nm and 25°C, after a 10 min incubation. Citrate synthase activity was expressed as nmol min^{-1} mg^{-1} of protein.

Pressure myography

The structural and mechanical properties of small mesenteric arteries and coronary arteries were studied with a pressure myograph (Danish Myo Tech, Model P100, J.P. Tranding I/S, Aahurs, Denmark), as previously described (Briones *et al.*, 2003). Briefly, the vessel was placed on two glass microcannulae and secured with surgical nylon sutures, and vessel length was adjusted so that the vessel walls were parallel without stretching. Intraluminal pressure was then raised to 140 mmHg, and the artery was unbuckled by adjusting the cannulae. The segment was set to a pressure of 70 mmHg and allowed to equilibrate for 60 min at 37°C in calcium-free KHS ($0Ca^{2+}$; omitting calcium and adding 10 mM EGTA) gassed with a mixture of 95% O_2 and 5% CO_2. Intraluminal pressure was then reduced to 3 mmHg. A pressure–diameter curve was obtained by increasing intraluminal pressure in 20 mmHg steps between 20 and 140 mmHg. Internal and external diameters (D_{i0Ca} and D_{e0Ca}) were measured. Finally, the artery was set to 70 mmHg in $0Ca^{2+}$, pressure-fixed with 4% paraformaldehyde (PFA, in 0.2 M phosphate buffer, pH 7.2–7.4) at 37°C for 60 min and kept in 4% PFA at 4°C for confocal microscopy studies.

Calculation of structural and mechanical parameters

From internal and external diameters measurements in passive conditions, the following structural parameters were calculated: wall thickness = $(D_{e0Ca} - D_{i0Ca})/2$; cross-sectional area (CSA) = $(\pi /4) \times (D_{e0Ca}^2 - D_{i0Ca}^2)$; and wall to lumen ratio = $(D_{e0Ca} - D_{i0Ca})/2D_{i0Ca}$.

Incremental distensibility represents the percentage of change of the arterial internal diameter for each mmHg change in intraluminal pressure and was calculated according to the formula: Incremental distensibility = $\Delta D_{i0Ca} /(D_{i0Ca} \times \Delta P) \times 100$.

Circumferential wall strain (ε) = $(D_{i0Ca} - D_{00Ca})/D_{00Ca}$, where D_{00Ca} is the internal diameter at 3 mmHg, and D_{i0Ca} is the observed internal diameter for a given intravascular pressure both measured in $0Ca^{2+}$ medium; and circumferential wall stress (σ) = $(P \times D_{i0Ca})/2WT$, where P is the intraluminal pressure (1 mmHg = 133.4 N m^{-2}), and WT is wall thickness at each intraluminal pressure in $0Ca^{2+}$ medium.

Arterial stiffness-independent of geometry is determined by Young's elastic modulus (E = stress/strain). The stress–strain relationship is nonlinear; therefore, it is more appropriate to obtain a tangential or incremental elastic modulus (E_{inc}) by determining the slope of the stress–strain curve ($E_{inc} = \delta\sigma/\delta\varepsilon$). E_{inc} was obtained by fitting stress–strain data from each animal to an exponential curve by using the equation $\sigma = \sigma_{orig}e^{\beta\varepsilon}$, where σ_{orig} is the stress at the original diameter (3 mmHg). Taking derivatives of this equation, we see that $E_{inc} = \beta\sigma$. For a given σ-value, E_{inc} is directly proportional to β. An increase in β implies an increase in E_{inc}, which means an increase in stiffness.

Confocal microscopic study of nuclei distribution

Pressure fixed intact arteries were incubated with the nuclear dye Hoechst 33342 (0.01 mg mL^{-1}) for 15 min. After washing, the arteries were mounted on slides with a well made of silicon spacers to avoid artery deformation. They were viewed using a Leica TCS SP2 confocal system fitted with an inverted microscope and Argon and Helium-Neon laser sources with oil immersion lens (×40) (excitation, 351–364 nm and emission 400–500 nm). At least two serial optical sections (stacks of images) of 0.5 µm thick serial optical slices were taken from the adventitia to the lumen in different regions along the artery length. Individual images of the endothelial layer were also captured. Metamorph image analysis software (Molecular Devices Corp. Downingtown, PA) was used for quantification. The nuclei numbers were measured in *Z*-axis as previously described (Arribas *et al.*, 1997) with minor modifications.

To allow comparison among the different groups, the following calculations were performed on segments 1 mm long: artery volume (in mm^3) (volume = wall CSA (mm^2) × 1 mm); total number of adventitial and smooth muscle cells (cell n = n of nuclei per stack × n of stacks per artery volume); total number of endothelial cells (EC) was calculated per luminal surface of 1-mm-long artery; luminal surface area = $2\pi \times$ diameter/2.

Organization of internal elastic lamina

The elastin organization within the internal elastic lamina was studied in segments of small mesenteric arteries, using fluorescence confocal microscopy based on the auto fluorescent properties of elastin (excitation wavelength 488 nm and

emission wavelength 500–560 nm) as previously described (Briones *et al.*, 2003). Briefly, the experiments were performed in intact pressure-fixed (70 mmHg, PFA 4%) segments with a Leica TCS SP2 confocal system. Serial optical sections from the adventitia to the lumen (*z* step = 0.5 μm) were captured with a ×63 oil objective using the 488 nm line of the confocal microscope. A minimum of two stacks of images of different regions was captured in each arterial segment. Quantitative analysis of the internal elastic lamina was performed with Metamorph Image Analysis Software, as previously described (Briones *et al.*, 2003). From each stack of serial images, individual projections of the internal elastic lamina were reconstructed, and total fenestrae number and fenestra area were measured.

NO production

The NO production was determined in intact segments of small mesenteric arteries using a fluorescent indicator, diaminofluorescein diacetate (DAF-2-DA) (excitation wavelength 488 nm and emission wavelength 510–560 nm). Segments were equilibrated under identical conditions for 60 min at 37°C in KHS buffer gassed with a mixture of 95% O_2 and 5% CO_2, and then DAF-2-DA (10 μM) was added to the buffer for 30 min. Intact segments were mounted in glass slides with KHS buffer, coverslipped and visualized with a Leica TCS SP2 confocal system. Serial optical sections from the adventitia to the lumen were captured with a ×40 objective. For comparison, images from sedentary and trained SHR were taken the same day with the same imaging settings of the microscope. The specificity of the method was assessed by incubation of the segments with the NOS inhibitor L-NAME (100 μM). This drug inhibited the fluorescent signal. For quantification the Metamorph Image Analysis Software was used to analyse fluorescence intensity.

Superoxide anion production

The oxidative fluorescent dye dihydroethidium was used to evaluate O_2^- production *in situ*. This dye freely permeates the cell; and, in the presence of O_2^-, is oxidized to ethidium bromide (excited at 546 nm and emission spectrum of 610 nm), which is trapped by intercalation with DNA. Frozen tissue segments of small mesenteric and coronary arteries were cut into 10-μm-thick sections and placed on a glass slide. Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs–HEPES buffer (130 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 0.24 mM $MgCl_2$, 8.3 mM HEPES, 11 mM glucose, pH 7.4). Afterwards, fresh buffer containing dihydroethidium (2 μM) was applied topically onto each tissue section, coverslipped and incubated for 30 min in a light-protected humidified chamber at 37°C and then viewed in a fluorescent laser scanning confocal microscope (Leica TCS SP2, ×40 objective) using the same image settings for each experimental condition. Fluorescence was detected with a 568-nm-long pass filter. For quantification, three rings per animal were analysed with a Metamorph Image Analysis Software, and the mean fluorescence densities in the target region were calculated.

Collagen determination

Segments of small mesenteric arteries were removed from the mesenteric bed and immediately fixed in 4% PFA in phosphate buffer for 1 h, transferred to a cryomold containing OCT embedding medium (Tissue Tek, Sakura) and frozen in liquid nitrogen. Frozen transverse sections (10 μm) were incubated with picrosirius red [0.1% (wt/vol) Sirius red 3FB in saturated aqueous picric acid] for 30 min with gentle agitation for collagen staining. Colour images were captured and analysed using a morphometric computer system (Leica Quantimet 500, ×40 objective). Collagen content was estimated by quantity of collagen stained in the vessel area and normalized by total vessel area.

Measurements of plasma nitrite

Plasma nitrite levels were measured by a modified Griess method. Experiments were performed at a room temperature. After loading the plate with samples, conversion of nitrate to nitrite was performed by addition of vanadium (III) chloride (8 mg mL^{-1}) to each well. Thereafter, Griess reagents, sulfanilamide (2%) and *N*-(1-naphthyl) ethylenediamine dihydrochloride (0.1%) were rapidly added. Absorbance at 540 nm was measured using a plate reader following 30 min of incubation.

Vascular reactivity

Segments of small mesenteric arteries and coronary arteries, 2 mm in length, were mounted in a small-vessel chamber myograph for measurement of isometric tension. Two tungsten wires (40 μm diameter) were introduced through the lumen of the segments and mounted as described (Beltrán *et al.*, 2004). After a 30 min equilibration period in oxygenated KHS at 37°C and pH 7.4, segments were stretched to their optimal lumen diameter for active tension development. Segments were washed with KHS and left to equilibrate for 30 min; contractility of segments was then tested by an initial exposure to a high-K^+ solution (120 mM K^+-KHS), which was identical to KHS, except that NaCl was replaced by KCl on an equimolar basis. The presence of endothelium was determined by the ability of 10 μM ACh to induce relaxation in small mesenteric arteries pre-contracted with phenylephrine and in coronary arteries pre-contracted with 5-HT at a concentration that produce approximately 50% of the contraction induced by K^+-KHS in each case. All experiments were performed in segments with endothelium.

Concentration–response curves to ACh (1 nM–10 μM) or to diethylamine NONOate (DEA-NO 1 nM–10 μM) were studied in segments pre-contracted with phenylephrine or with the TXA_2 analogue U46619 for small mesenteric arteries and with 5-HT for coronary arteries. Concentration–response curves to U46619 (0.1 nM–10 μM) or to 5-HT (10 nM–30 μM), were also constructed by its cumulative addition to segments under basal tone. The modulation by NO and O_2^- on the contraction induced by U46619 or the relaxation induced by ACh was analysed by pre-incubation of the arteries with the non-selective NOS inhibitor L-NAME (100 μM) or with the antioxidant NADPH oxidase inhibitor apocynin (300 μM), alone or in combination for 30 min before the corresponding concentration-response curves.

Western blot analysis

Segments of mesenteric arteries were homogenized in ice-cold Tris–EDTA buffer. Homogenates (20 μg protein per lane)

were separated by 10% SDS-PAGE and then transferred to a nitrocellulose membrane. The blot membrane was then incubated in blocking buffer [5% non-fat dry milk, 10 mM Tris–HCl (pH 7.6), 150 mM NaCl and 0.1% Tween 20] for 2 h at room temperature and then incubated overnight at 4°C with sheep polyclonal antibody for Cu/Zn-superoxide dismutase (Cu/Zn-SOD) (1:1500, Calbiochem, Germany), rabbit polyclonal antibody for Mn-SOD (0.033 μg mL^{-1}, StressGen Bioreagents Corp, Victoria, Canada) and EC-SOD (Enzo Life Sciences, Farmingdale, NY, USA), mouse monoclonal antibodies for eNOS (1:500, Transduction Laboratories, Lexington, UK) and goat polyclonal antibodies for MMP-2 and -9 (1:1000, Santa Cruz Biotechnology, CA, USA). Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (anti-sheep, anti-goat and anti-mouse IgG antibodies, Amersham Biosciences, Piscataway, NJ, USA). Enhanced chemiluminescence reagents (Amersham Biosciences) were used to visualize the autoradiogram, which was later exposed to photographic film. The film was developed, and the bands were analysed using Scion Image software (Scion Corporation based on NIH image). GAPDH expression levels were used as loading control to normalize the protein levels.

Data analysis and statistics

In coronary arteries, contractile responses to U46619 and 5-HT were expressed in mN mm^{-1} due to changes in the K^+-KHS contraction in the different experimental groups. In small mesenteric arteries, contractile responses to U46619 were expressed as a percentage of contraction to K^+-KHS that was similar in WKY and SHR. The maximal response (E_{max}) values for contractions were calculated by a nonlinear regression analysis of each individual concentration–response curve using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). To compare the effect of some inhibitors on the U46619 responses in segments from the different experimental groups, some results were expressed as 'differences of area under the concentration–response curves' (dAUC) in control and experimental situations. AUCs were calculated from the individual concentration–response curve plots; the differences were expressed as a percentage of the AUC of the corresponding control situation. Vasodilator responses were expressed as a percentage of the previous tone.

Results are expressed as means ± SEM of the number of experiments indicated in each case. Statistical analysis was performed using one-way ANOVA (followed by a Tukey *post hoc* test) for comparisons among three groups of different parameters; two-way ANOVA for repeated measurements (followed by a Bonferroni's *post hoc* test) was used for concentration–responses curves and for pressure-structural parameters curves. Student *t*-test was used for comparisons between two groups. Differences were considered statistically significant when $P < 0.05$.

Materials

ACh, apocynin, DEA-NO, HEPES, L-NAME, phenylephrine, U46619, 5-HT were obtained from Sigma Chemical Co. (St Louis, MO, USA). Stock solutions of U46619 were made in ethanol and further dilutions in distilled water. All others stock solutions were made in distilled water.

Results

Effect of exercise training on systolic blood pressure and heart rate

Table 1 shows that 12 weeks of exercise training decreased but did not normalize SBP and heart rate in SHR ($P < 0.01$). There was no significant difference in body weight among all three groups. Soleus citrate synthase activity, a good metabolic marker for exercise training efficiency, was greater in trained rats compared with sedentary, non-exercised animals (Table 1).

Effect of exercise training on structural and mechanical properties of coronary and small mesenteric arteries from hypertensive rats

Lumen diameter and cross sectional area of coronary arteries were similar in SHR and WKY; however, wall thickness and wall-to-lumen ratio were larger in SHR (Figure 1). In small mesenteric arteries from SHR, lumen diameter was diminished, wall thickness and wall-to-lumen ratio were greater and cross-sectional area was similar compared with WKY (Figure 1). Exercise training did not change the altered structural parameters observed in coronary and mesenteric arteries from SHR (Figure 1).

Table 1

Effects of hypertension and exercise training on body weight, systolic blood pressure, heart rate and citrate synthase activity

	WKY (5)	SHR (6)	SHR Trained (8)
Body weight (g)	368 ± 7	370 ± 11	355 ± 5
Systolic blood pressure (mmHg)	124 ± 3	216 ± 5*	194 ± 2*†
Heart rate (beats min^{-1})	306 ± 1	381 ± 4*	343 ± 6*†
Citrate synthase (nmol min^{-1} mg^{-1} protein)	99 ± 5	89 ± 4	124 ± 4*†

Data are means ± SEM. Number of animals is indicated in parenthesis. *$P < 0.05$ versus WKY; †$P < 0.05$ versus SHR; one-way ANOVA.

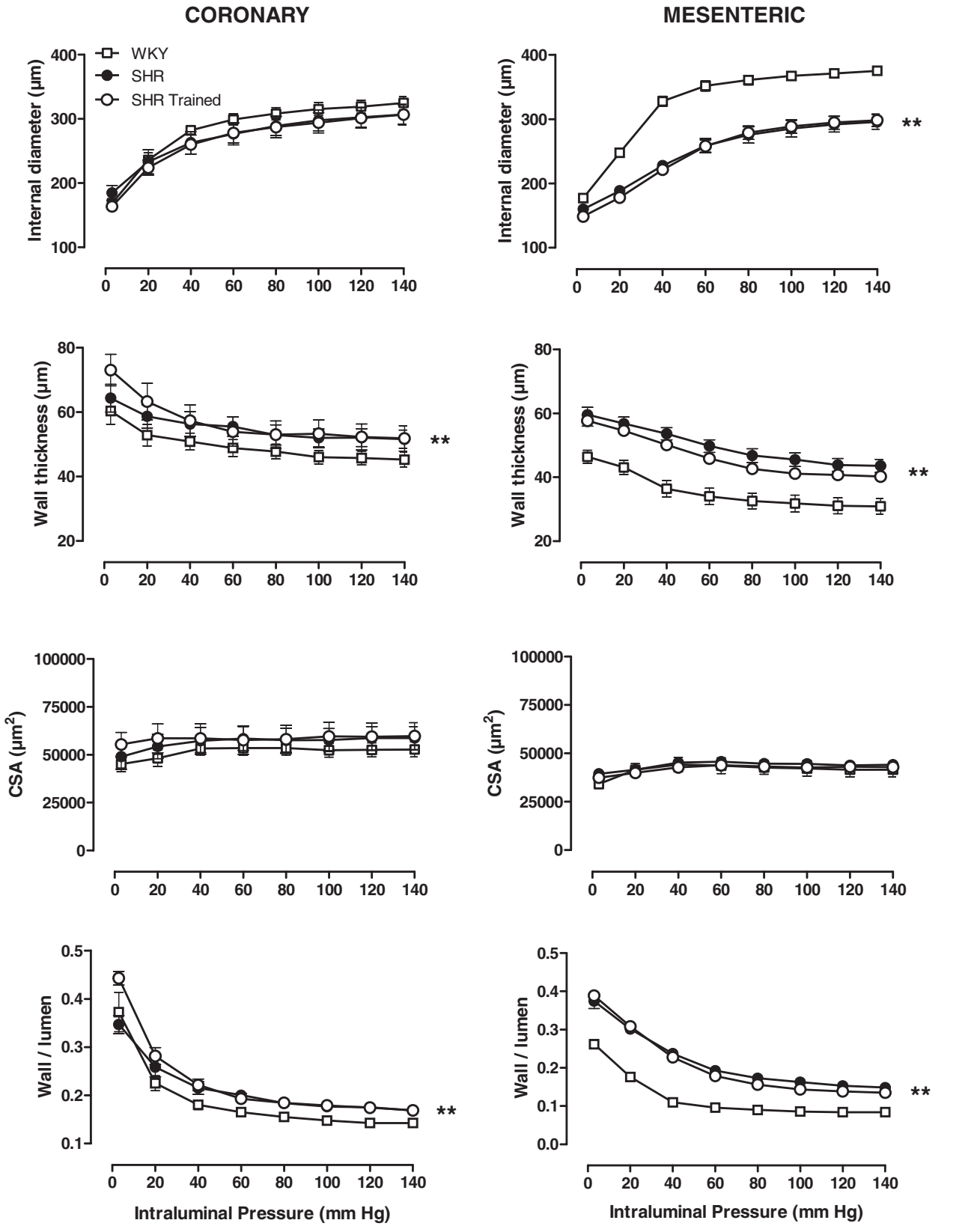

Figure 1

Exercise training does not affect hypertensive vascular remodelling. Relationships between internal diameter, wall thickness, CSA and wall-to-lumen ratio and intraluminal pressure curves in coronary and small mesenteric arteries from WKY, SHR and SHR trained incubated in $0Ca^{2+}$-KHS. Coronary arteries, $n = 4–7$; mesenteric arteries, $n = 5–7$. Data are means ± SEM. $^{**}P < 0.01$ versus WKY.

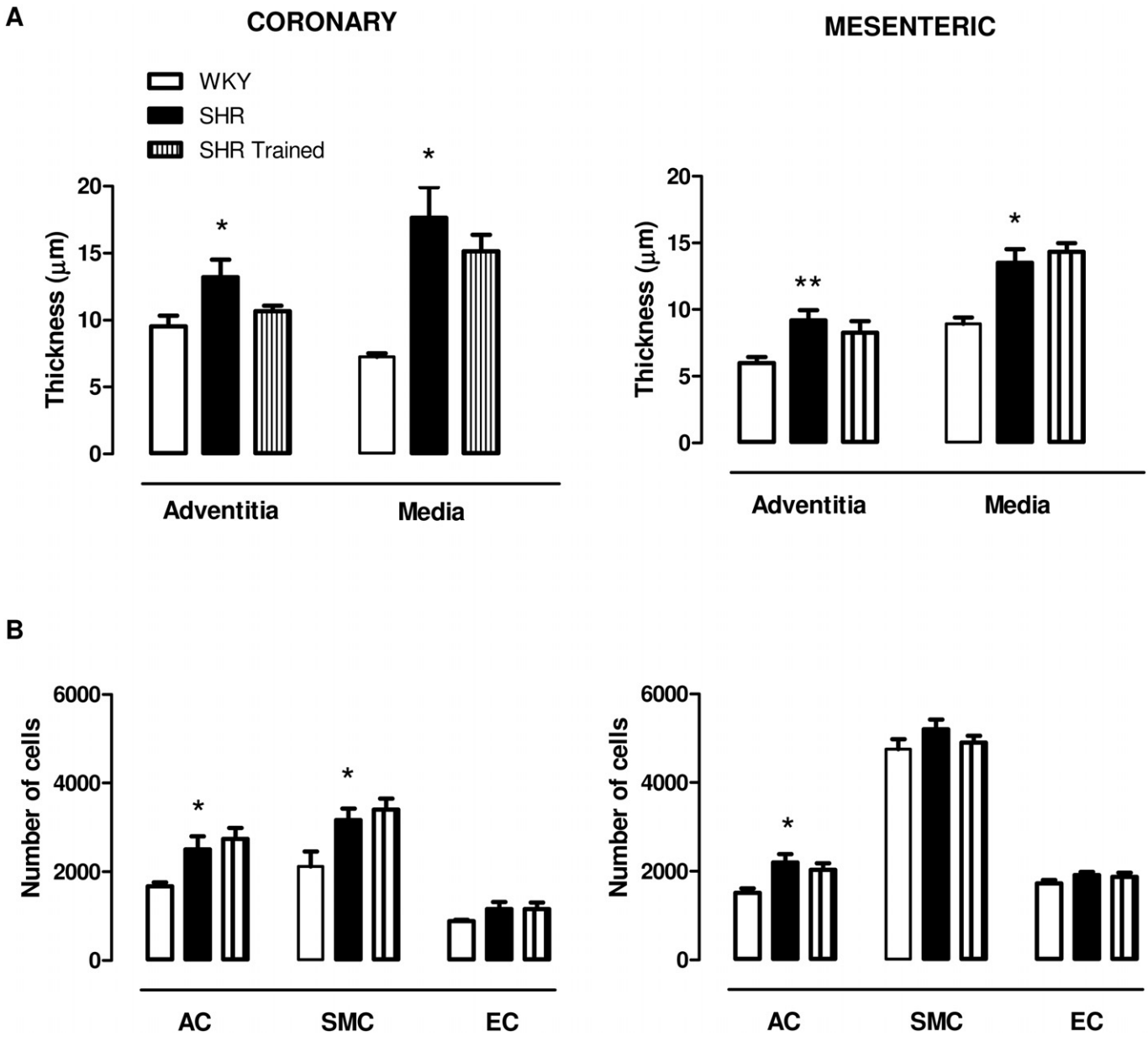

Figure 2

Exercise training does not affect layer thickness and cell number in hypertensive arteries. (A) Adventitia and media thickness and (B) total number of adventitial (AC), smooth muscle (SMC) and endothelial (EC) cells in coronary and small mesenteric arteries from WKY, SHR and SHR trained. Data expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus WKY. $n = 3–8$.

Adventitia and media thickness was higher in both coronary and mesenteric arteries from SHR compared with WKY (Figure 2A). In coronary arteries, the total number of adventitial and smooth muscle cells but not endothelial cells was increased in SHR compared with WKY. In small mesenteric arteries, the total number of adventitial but not of smooth muscle or endothelial cells was increased in SHR compared with WKY (Figure 2B). Exercise training did not affect adventitia and media thickness and cell number either in small mesenteric arteries or in coronary arteries (Figure 2).

The mechanical parameters of coronary arteries and small mesenteric arteries are shown in Figure 3. Incremental distensibility of coronary and small mesenteric arteries was significantly smaller in SHR than in WKY at low pressure, and this was improved by exercise training (Figure 3A). Coronary arteries from SHR also showed an increased stiffness when compared with vessels from WKY rats as shown by the leftward shift of the stress–strain relationship (Figure 3B) and the significantly larger value of β (WKY: 5.23 ± 0.45, $n = 4$, SHR: 12.73 ± 0.74, $n = 5$, $P < 0.01$). Exercise training of SHR restored the increased stiffness observed (β = 5.46 ± 0.34,

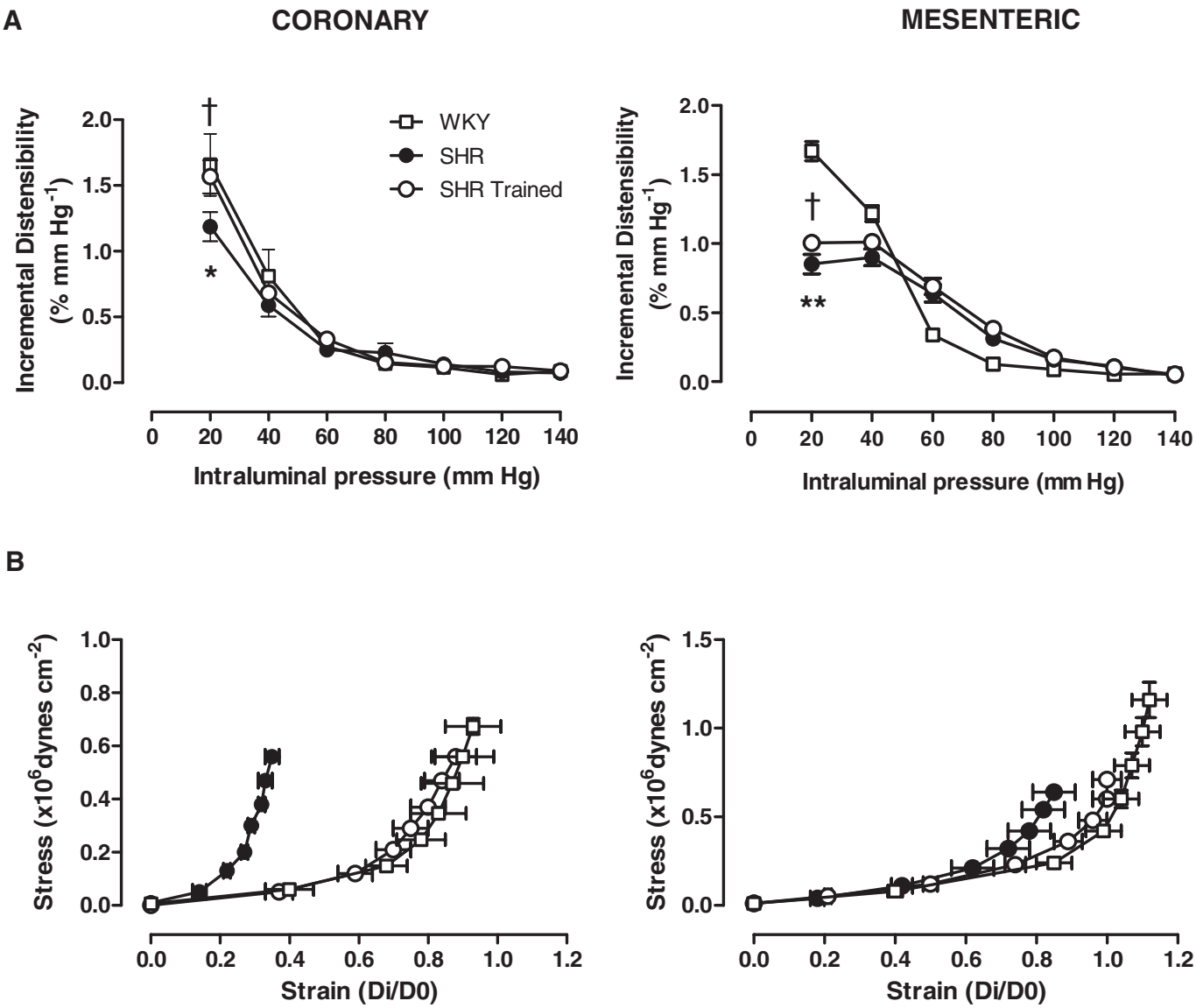

Figure 3

Exercise training improves vascular mechanical properties in hypertension. Incremental distensibility–intraluminal pressure and stress–strain relationships in coronary and small mesenteric resistance arteries from WKY, SHR and SHR trained incubated in $0Ca^{2+}$-KHS. Coronary arteries, n = 4–7; mesenteric arteries, n = 5–8. Data are means ± SEM. *P < 0.05, **P < 0.01 versus WKY; †P < 0.05 versus SHR.

n = 6, P < 0.01 vs. SHR). As with coronary arteries, mesenteric arteries from SHR showed an increased stiffness when compared with vessels from WKY rats (Figure 3; β-values: WKY: 4.39 ± 0.15, n = 5, SHR: 5.75 ± 0.33, n = 7, P < 0.05). Exercise training of SHR normalized the increased stiffness observed in mesenteric arteries (β = 4.86 ± 0.19, n = 8, P < 0.05 vs. SHR).

Small mesenteric arteries from SHR showed significantly increased collagen content (Figure 4A) and decreased area and number of fenestrae in the internal elastic lamina (Figure 4B) compared with WKY rats. Exercise training reversed the increased total collagen content and restored the decreased the area of fenestrae in SHR animals but did not affect the number of fenestrae (Figure 4A and B).

MMP-2 protein expression was similar in mesenteric arteries from the three experimental groups (Figure 4C). However, a reduced MMP-9 protein expression was observed in mesenteric arteries of SHR compared with WKY rats that was normalized by exercise training (Figure 4C).

Effect of exercise training on vasoconstrictor responses: role of NO and O_2^-

Coronary arteries. The maximal response induced by K^+-KHS (120 mM) was greater in WKY than in SHR and exercise training did not affect the impaired K^+-KHS response (Table 2). The TXA_2 mimetic U46619 (0.1 nM–10 μM) also induced a greater concentration-dependent vasoconstriction in WKY than in SHR when the results were expressed as active wall tension and exercise training did not modify such responses (Table 2). However, U46619-induced responses were similar in all groups when the results were expressed as

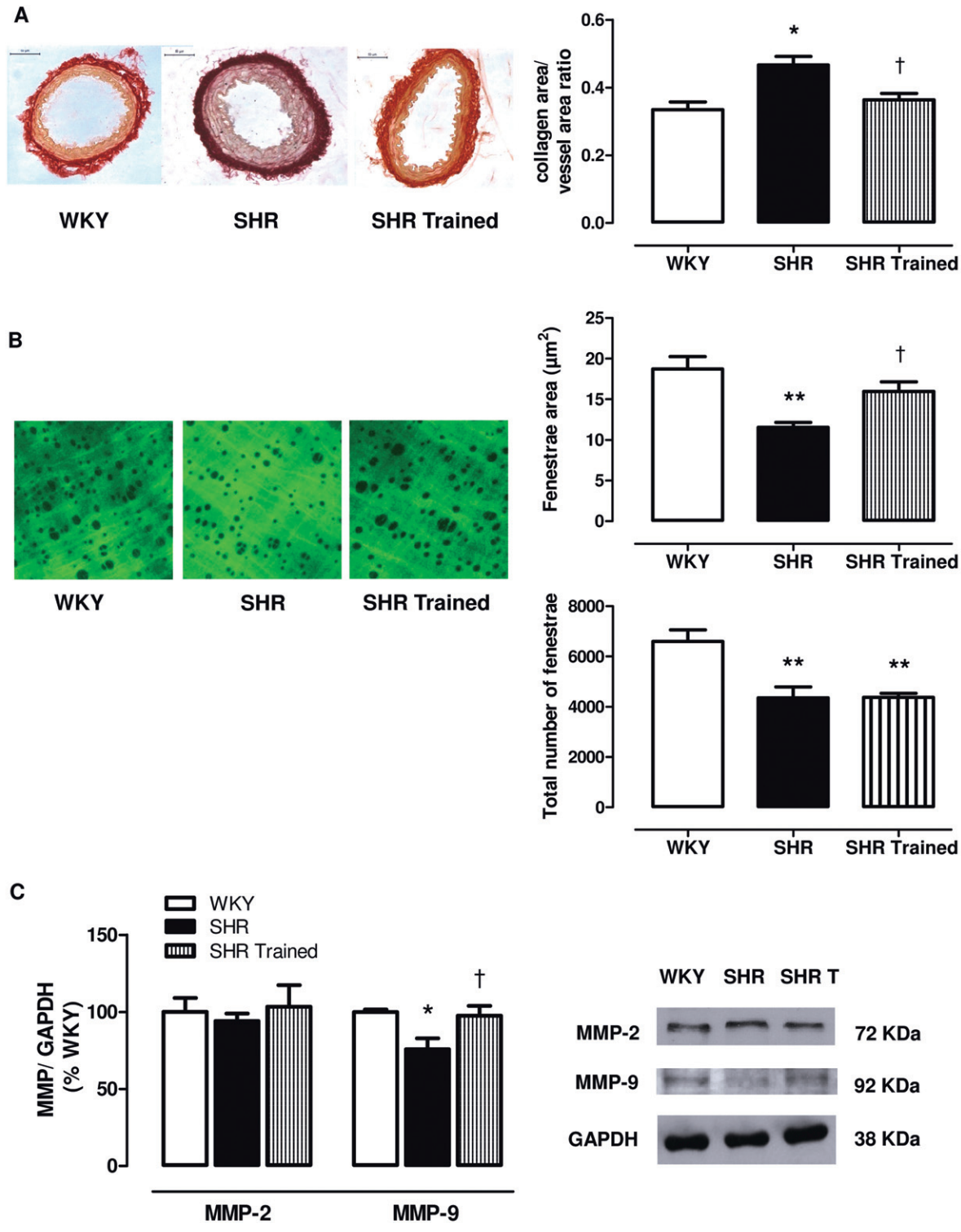

Figure 4

Exercise training normalizes ECM proteins in mesenteric arteries from hypertensive rats. (A) Representative images and respective quantification of collagen staining (Picrosirius Red) of transverse sections obtained from small mesenteric arteries from WKY, SHR and SHR Trained ($n = 4–6$). Bars indicates 50 μm. (B) Representative confocal projections of the internal elastic lamina and quantitative analysis of the area of fenestrae and total number of fenestrae in the internal elastic lamina from small mesenteric arteries of WKY, SHR and SHR Trained ($n = 5–7$). Projections were obtained from serial optical sections captured with a fluorescence confocal microscope (×63, oil immersion objective; zoom, ×2). Image size 119 × 119 μm. (C) Densitometric analysis and representative Western blots of MMP-2, MMP-9 and GAPDH protein expression in homogenates of small mesenteric arteries from WKY, SHR and SHR Trained ($n = 5–6$). Data are means ± SEM. *$P < 0.05$, **$P < 0.01$ versus WKY; †$P < 0.05$ versus SHR.

Table 2

Maximal vasoconstrictor responses (mN mm^{-1}) induced by K$^+$-KHS (120 mM), U46619 or 5-HT in arteries from WKY, SHR and SHR Trained groups

	WKY	SHR	SHR Trained
K$^+$-KHS (8–16)			
Coronary	1.6 ± 0.1	0.5 ± 0.07**	0.6 ± 0.06**
Mesenteric	3.3 ± 0.2	3.7 ± 0.2	3.7 ± 0.3
U46619 (E_{max}) (5–15)			
Coronary	2.39 ± 0.7	0.69 ± 0.2*	0.55 ± 0.1*
Mesenteric	1.68 ± 0.4	3.48 ± 0.3**	2.47 ± 0.28†
5-HT (E_{max}) (6–7)			
Coronary	1.59 ± 0.4	0.61 ± 0.1**	0.62 ± 0.1**

Data are means ± SEM. Number of animals is indicated in parenthesis. *P < 0.05 versus WKY; **P < 0.01 versus WKY; $^{\dagger}P$ < 0.05 versus SHR; one-way ANOVA.

a percentage of K$^+$-KHS contraction (results not shown). Similar differences between WKY, SHR and SHR Trained groups were observed in the vasoconstrictor responses induced by 5-HT (10 nM–30 μM, Table 2).

Small mesenteric arteries. The maximal response induced by K$^+$-KHS was similar in WKY and SHR and exercise training did not modify such responses (Table 2). However, the contractile response induced by U46619 was greater in arteries from SHR than WKY, and exercise training inhibited this response (Table 2; Figure 5A).

In order to analyse whether exercise training altered the participation of NO in small mesenteric arteries in the response to U46619, some arteries were pre-incubated with the NOS inhibitor L-NAME (100 μM). The contraction induced by U46619 was enhanced by L-NAME in all groups (Figure 5B). The effect of L-NAME was similar in arteries from WKY than SHR as shown by the dAUC values [WKY 135 ± 61 (n = 5), SHR 122 ± 21 (n = 4), n.s.]. Exercise training increased the effect of L-NAME on U46619 contractile responses [dAUC SHR Trained 441 ± 89 (n = 7), P < 0.05 vs. SHR], suggesting an increased NO bioavailability after exercise. To confirm this issue, we measured NO levels. Unaltered vascular NO production (WKY: 25.4 ± 11, SHR: 10.8 ± 6 arbitrary units, n = 5) and plasma nitrites concentration was detected in SHR when compared with WKY (Figure 5D). Exercise training increased NO production in small mesenteric arteries (Figure 5C) and plasma nitrite concentration (Figure 5D). eNOS expression remained unaltered by hypertension or exercise training (Figure 5E). All together, these results suggest that exercise training increased NO production and/or NO bioavailability in SHR.

To analyse whether exercise training altered the participation of O_2^- in U46619 responses of small mesenteric arteries, some arteries were pre-incubated with apocynin (300 μM). This drug reduced the contraction induced by U46619 in arteries from SHR but not from WKY (Figure 6A). However, the combination of apocynin and L-NAME did not modify U46619 responses in SHR (Emax control: 83.96 ± 13.9, n = 4; apocynin + L-NAME: 90.8 ± 6.6%, n = 5). Exercise training abolished the inhibitory effect of apocynin in U46619 response (Figure 6A), suggesting that exercise training is decreasing oxidative stress. In agreement, O_2^- production was higher in mesenteric arteries from SHR than WKY, and exercise training decreased vascular O_2^- (Figure 6B). In addition, apocynin pre-incubation decreased vascular O_2^- production in SHR (Figure 6C). To determine whether reduced antioxidant defences participates in the increased O_2^- levels, we measured Cu/Zn-SOD, EC-SOD and Mn-SOD protein in homogenates of mesenteric arteries of SHR compared with WKY rats. We found that all SOD isoforms were reduced in arteries from SHR. Exercise training normalized the expression of Cu/Zn-SOD but not of EC-SOD and Mn-SOD (Figure 6D).

Effect of exercise training on endothelium dependent and independent relaxation: role of NO and O_2^-

Coronary arteries. The endothelium-dependent relaxation induced by ACh in 5-HT-contracted coronary arteries was reduced in SHR compared with WKY and exercise training improved this relaxation (Figure 7A). The endothelium-independent relaxation induced by DEA-NO was similar among groups (Figure 7A). L-NAME incubation inhibited ACh relaxation in coronary arteries from all experimental groups (Figure 7). In addition, apocynin pre-incubation improved the impaired relaxation induced by ACh in coronary arteries from SHR animals without affecting to the relaxation in arteries from WKY (Figure 7B and C). Apocynin also decreased O_2^- production in coronary arteries from SHR (Figure 7C). Exercise training abolished the effect of apocynin on ACh-induced relaxation (Figure 7D).

Small mesenteric arteries. ACh (1 nM–10 μM) induced a concentration-dependent relaxation in arteries from WKY pre-contracted with phenylephrine. However, in SHR, ACh induced a biphasic response characterized by a relaxant response at concentrations 1 nM–100 nM followed by a contraction at higher concentrations (Figure 8A). Exercise training did not affect the relaxant response but decreased the contractile response induced by ACh observed in small

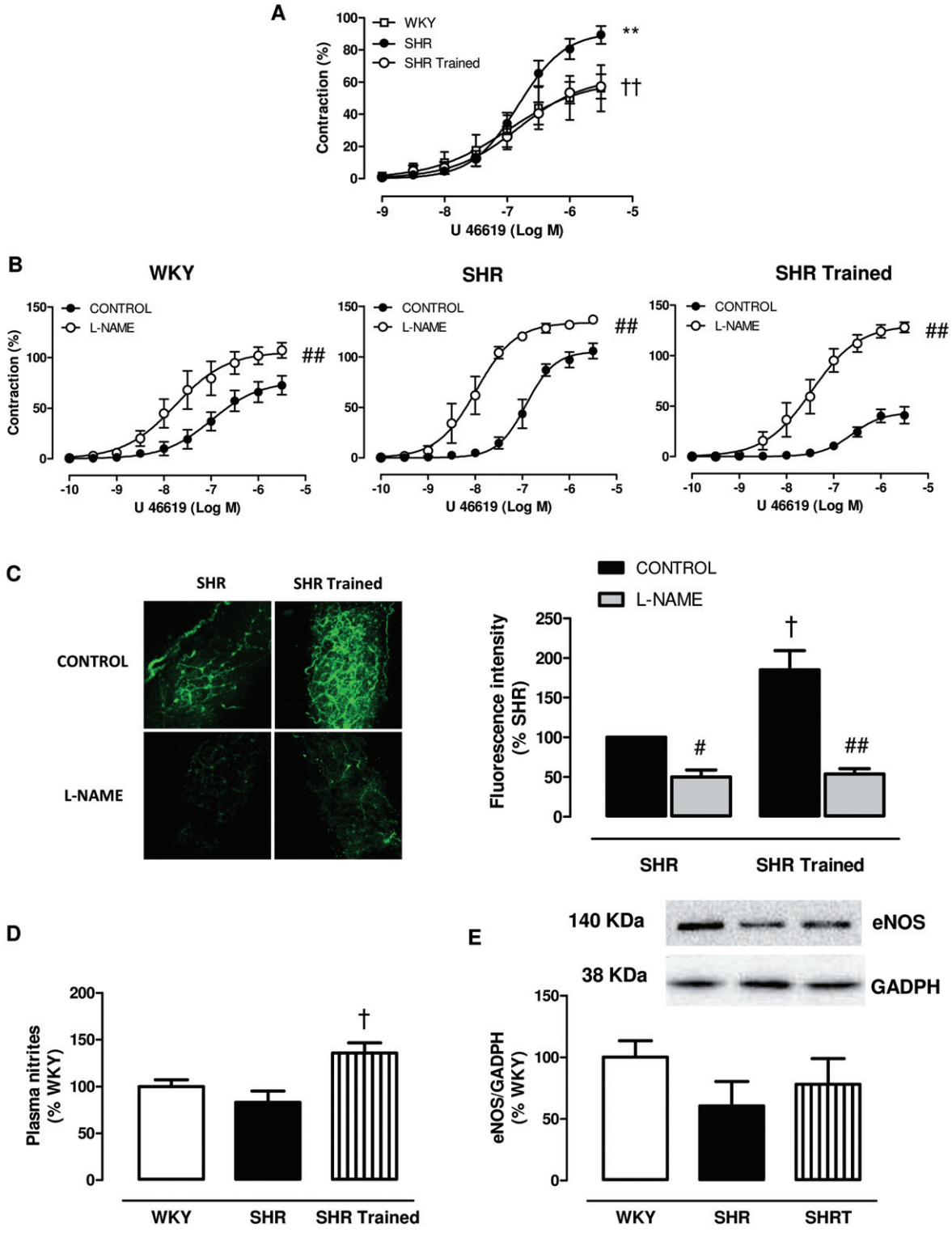

Figure 5

Exercise training normalizes vascular contraction by increasing NO production and/or bioavailability. (A) Concentration–response curve to U46619 and (B) effect of L-NAME (100 μM) on the concentration–response curve to U46619 in small mesenteric arteries from WKY, SHR and SHR Trained (n = 4–15). (C) Representative fluorescent microphotographs of confocal microscopy images of NO production in the absence or in the presence of L-NAME of small mesenteric arteries from SHR and SHR Trained. Projections were obtained from serial optical sections captured with a fluorescence confocal microscope (×40, oil immersion objective; zoom, ×1). Image size 375 × 375 μm. Quantitative analysis of NO production is also shown (n = 8). (D) Plasma nitrite levels from WKY, SHR and SHR Trained (n = 5–9). (E) Densitometric analysis and representative blots of eNOS protein expression in mesenteric arteries from WKY, SHR and SHR Trained. GAPDH is also shown (n = 7–9). Data are means ± SEM. $^{**}P < 0.01$ versus WKY. †$P < 0.05$, ††$P < 0.01$ versus SHR. #$P < 0.05$, ##$P < 0.01$ versus Control.

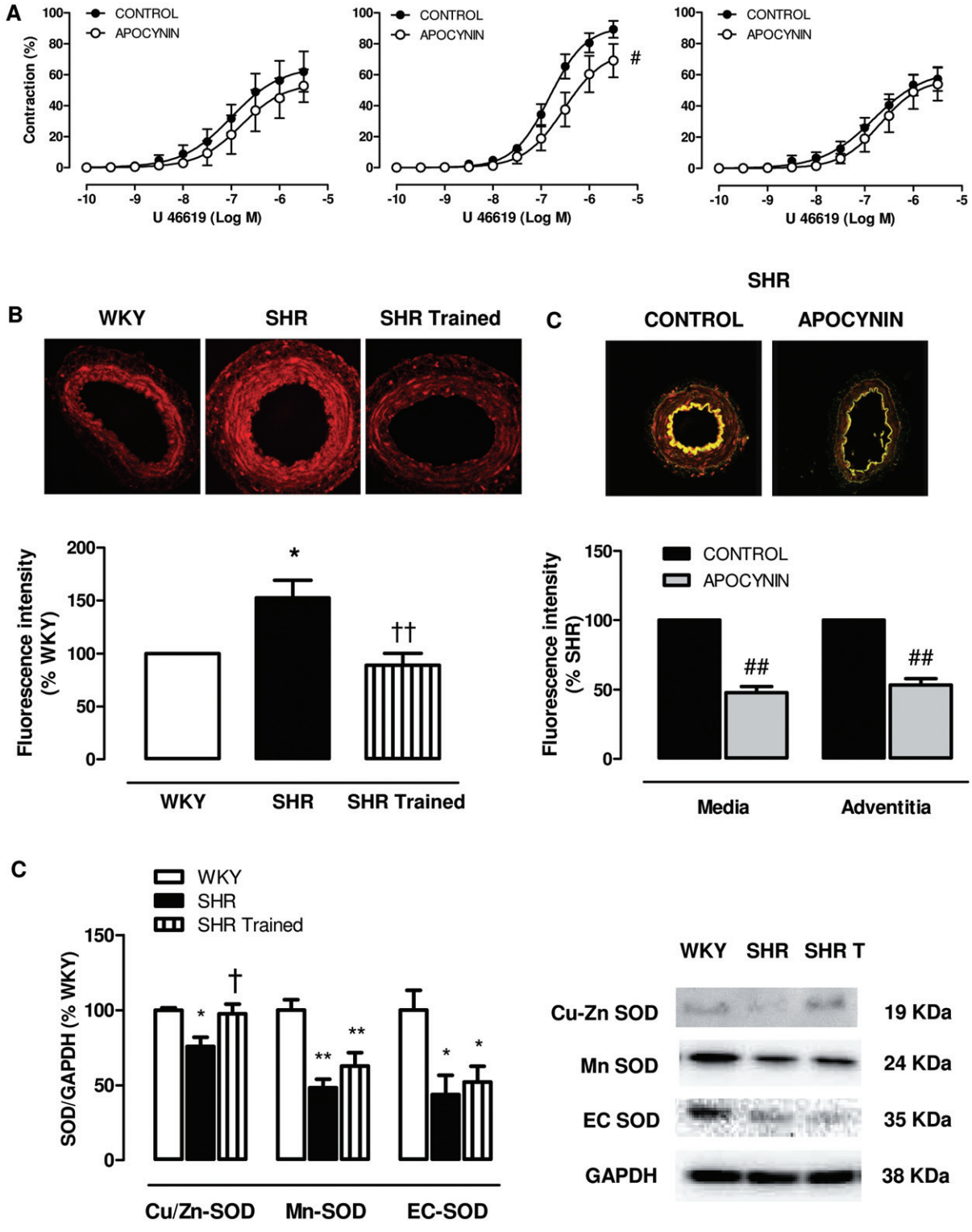

Figure 6

Exercise training normalizes vascular contraction by decreasing production of O_2^- and/or bioavailability. (A) Effect of apocynin (300 μM) on the concentration–response curve to U46619 in small mesenteric arteries from WKY, SHR and SHR Trained (n = 6–15). (B) Representative fluorescent microphotographs of confocal microscopy images and quantitative analysis of O_2^- production from small mesenteric arteries from WKY, SHR and SHR Trained. Image size 238 × 238 μm (n = 5–8). (C) Effect of apocynin on O_2^- production in small mesenteric arteries from SHR. Image size 375 × 375 μm (n = 5). (D) Densitometric analysis and representative Western blot of Cu/Zn-SOD, Mn-SOD and EC-SOD protein expression in homogenates of small mesenteric arteries from WKY, SHR and SHR Trained. The expression of GAPDH is shown as the loading control (n = 5–7). Data are means ± SEM. *P < 0.05, **P < 0.01 versus WKY. †P < 0.05, ††P < 0.01 versus SHR. #P < 0.05, ##P < 0.01 versus Control.

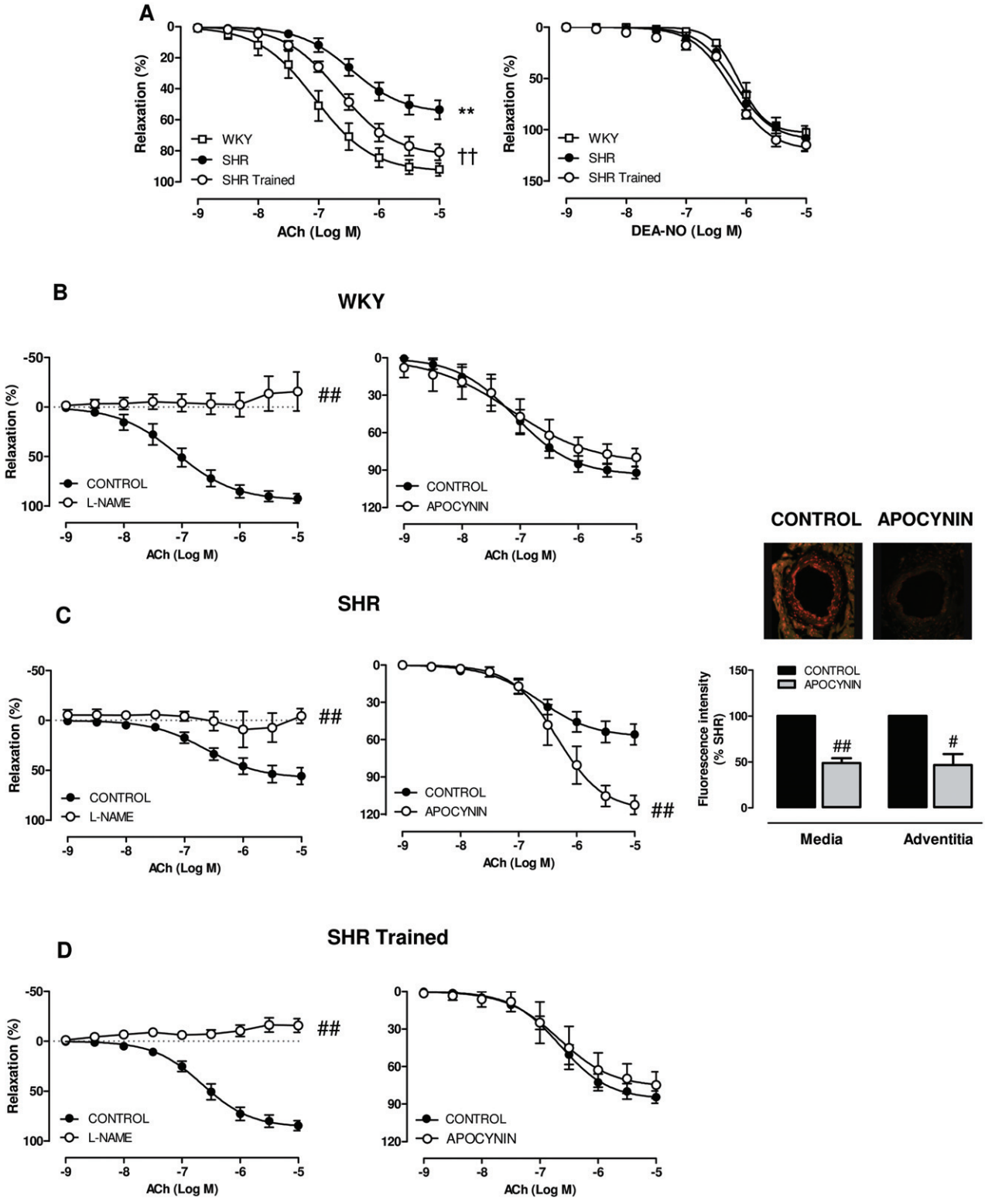

Figure 7

Exercise training reduces endothelial dysfunction by increasing NO bioavailability. (A) Concentration–response curves to ACh and diethylamine NONOate (DEA-NO) in coronary arteries from WKY, SHR and SHR Trained. Effect of L-NAME (100 μM) and apocynin (300 μM) on the concentration–response curve of ACh in coronary arteries from WKY (B), SHR (C) and SHR Trained (D) (n = 4–14). Representative fluorescent microphotographs of confocal microscopy images and quantitative analysis of O_2^- production in coronary arteries from SHR in the absence (Control) and in the presence of apocynin in also shown in panel B. Image size 375 × 375 μm (n = 4). **P < 0.01 versus WKY. ††P < 0.01 versus SHR. # P < 0.05, ##P < 0.01 versus control.

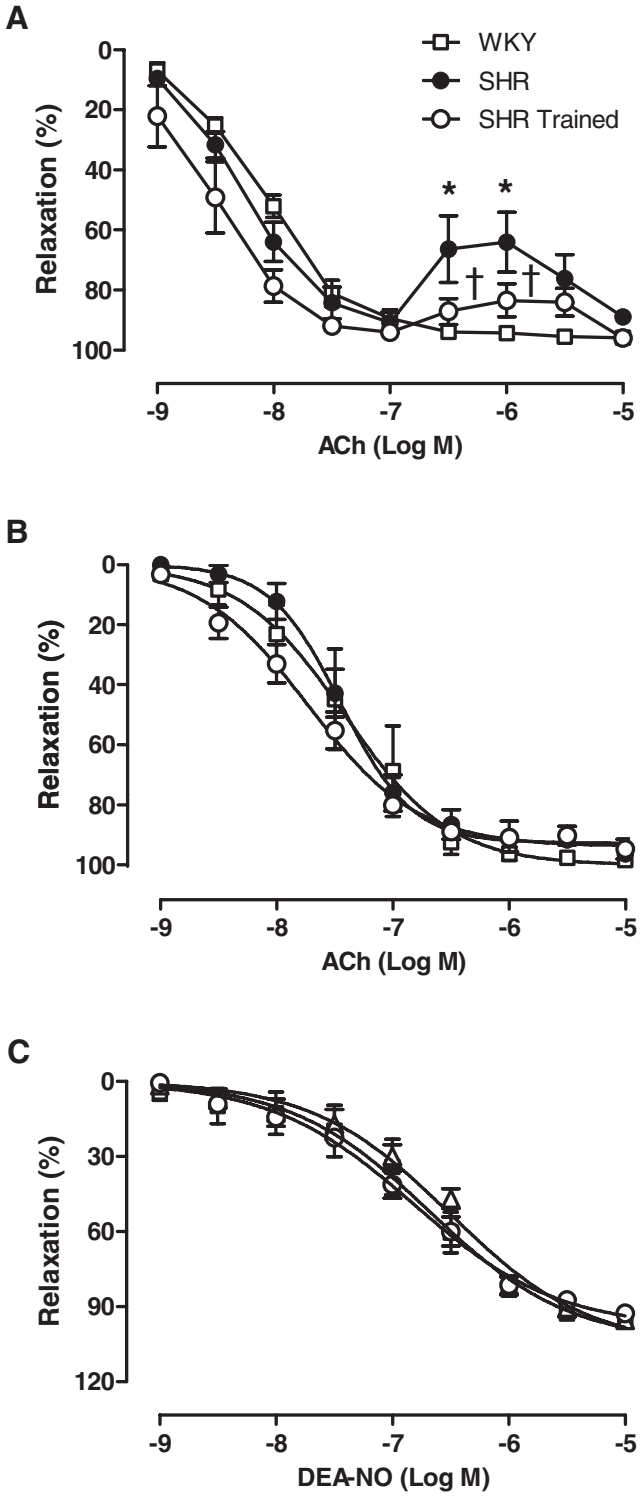

Figure 8

Exercise training decreases the participation of endothelium-dependent vasoconstrictor factors on ACh responses. Concentration–response curves to ACh in small mesenteric arteries pre-contracted with phenylephrine (A) or U46619 (B) from WKY, SHR and SHR Trained. (C) Concentration–response curve to diethylamine NONOate (DEA-NO) in small mesenteric arteries pre-contracted with U46619 from WKY, SHR and SHR Trained. *$P < 0.05$ versus WKY. †$P < 0.05$ versus SHR. $n = 4–8$.

mesenteric arteries from SHR (Figure 8A). It has been described that vasoconstrictor responses induced by ACh in vessels from hypertensive animals are due to the release of vasoconstrictor prostanoids acting on TP prostanoid receptors (Félétou *et al.*, 2011). Thus, when the concentration–response curves to ACh were performed in arteries pre-contracted with the TP receptor agonist U46619, the contractile response induced by ACh was abolished, and only relaxation responses were observed in all groups with no differences between them (Figure 8B). Furthermore, the endothelium-independent relaxation induced by the NO donor DEA-NO in U46619-contracted small mesenteric arteries was similar among groups (Figure 8C).

Discussion

It is well recognised that regular physical activity is associated with a number of health benefits including a reduction in cardiovascular disease and mortality (Mitchell *et al.*, 2010). The present study demonstrates that low-intensity aerobic exercise improved mechanical and functional alterations of the coronary and small mesenteric arteries from SHR and decreased blood pressure. Exercise also reduced oxidative stress and increased NO bioavailability.

Exercise training and vascular remodelling in SHR

Hypertension is associated with vascular structural and mechanical changes including vascular remodelling and increased stiffness (Intengan and Schiffrin, 2000; Rizzoni and Agabiti-Rosei, 2012; Mulvany, 2012). The main feature of hypertensive vascular remodelling is an increase in the wall-to-lumen ratio, and this parameter has a prognostic value for cardiovascular events (Rizzoni *et al.*, 2003; Mathiassen *et al.*, 2007). In healthy subjects, exercise training induces structural vascular adaptations both in conductance and resistance coronary arteries (Laughlin *et al.*, 2012). However, very little information is available on the effects of exercise on coronary and mesenteric resistance arteries of hypertensive patients and/or animal models of hypertension. Our results show that compared with WKY, both small mesenteric arteries and coronary arteries from SHR show eutrophic remodelling with unaltered cross-sectional area and increased wall-to-lumen ratio. Vessels from SHR also showed increased wall thickness that was due to increased adventitia and media thickness. This was associated with increased adventitial cells number in both arteries and with increased smooth muscle cells only in coronary arteries. In addition, SHR small mesenteric and coronary arteries display smaller incremental distensibility and enhanced vascular stiffness, which is consistent with previous studies performed mainly in mesenteric arteries (Mulvany, 1993; Intengan *et al.*, 1999; Briones *et al.*, 2003). Exercise training completely normalized the increased vascular stiffness and improved the decreased vascular distensibility observed at low pressure in small mesenteric and coronary arteries. Reduction of arterial stiffness by exercise training was observed in large arteries from hypertensive patients (Collier *et al.*, 2008; Guimarães *et al.*, 2010) and SHR (Hägg *et al.*, 2004). However, to date, no studies have dem-

onstrated the beneficial effects of exercise training in the increased vascular stiffness of coronary and mesenteric resistance arteries in hypertension. Of note, vascular stiffness is considered to be an indicator of subclinical organ damage in hypertension (Mancia *et al.*, 2007). Exercise training did not modify the vascular remodelling observed in coronary arteries or mesenteric arteries from SHR. Improvement (Amaral *et al.*, 2000; Melo *et al.*, 2003; Rossoni *et al.*, 2011) or no effects (Melo *et al.*, 2003) of exercise training on vascular structure have been observed by other authors in other vascular beds from hypertensive animals. The questions of whether more prolonged or more intense training programmes or training started before the development of structural alterations would restore abnormal vessel structure remains as an attractive hypothesis that needs further consideration.

We previously demonstrated that in addition to collagen, elastin alterations are important determinants of small mesenteric arteries vascular stiffness (Briones *et al.*, 2003). In fact, differences in elastin organisation particularly at the internal elastic lamina are central elements in small artery remodelling and increased stiffness in hypertension (Briones *et al.*, 2003). Thus, there is a correlation between β-values (slope of the stress–strain relationship) and the area of fenestrae (González *et al.*, 2005; 2006), demonstrating that the smaller the size of the fenestra the higher the stiffness of the vessel. Here, we observed that exercise training normalized the increased collagen deposition and improved the diminished fenestra size in the internal elastic lamina of small mesenteric arteries from SHR, providing an explanation of the beneficial effects of exercise in vascular stiffness and on vascular distensibility, particularly at low pressures, where elastin protein is operative. Recent studies performed in aorta from SHR also demonstrated that exercise training normalized alterations in the deposition of elastic components (Moraes-Teixeira *et al.*, 2010; Jordão *et al.*, 2011). Imbalance between the synthesis and degradation of ECM proteins might affect vascular stiffness. Our results demonstrate that exercise training differently affects expression of MMPs. Whereas exercise did not affect MMP-2 expression, it significantly normalized the decreased expression of the elastin degrading protein MMP-9, observed in the small mesenteric arteries from SHR.

Under pathological conditions, the production of ROS increases ECM proteins such as collagen and fibronectin (Briones and Touyz, 2010). In addition, we have previously demonstrated that in hypertension, the decrease of oxidative stress correlates with the normalization of the increased vascular stiffness, the altered internal elastic lamina structure and collagen deposition observed in small mesenteric arteries (Briones *et al.*, 2009). Here, we have observed that exercise training decreased local oxidative stress (see below). Therefore, it is highly possible that there is a direct relationship between the improved NO–ROS status and the decreased vascular stiffness and alterations in ECM deposition observed after low intensity exercise.

Exercise training and vascular function in SHR

Endothelial dysfunction in hypertension is a multifactorial process resulting mainly from impaired NO availability due to a decrease in NO production and/or an increase in NO degradation associated to the increase of O_2^- production in this pathology (Briones and Touyz, 2010; Tang and Vanhoutte, 2010). The final consequence in general is increased contractile responses and/or decreased vasodilator responses.

The data presented here show that hypertension differently affects contractile responses in small mesenteric arteries and coronary arteries. The responses of coronary arteries to U46619, 5-HT and K^+-KHS were lower in SHR than WKY, in agreement with other studies (Vazquez-Pérez *et al.*, 2001), and suggest that contractile machinery and/or excitation-contraction coupling mechanisms may be impaired in the hypertensive coronary vasculature. In contrast, U46619-induced responses in mesenteric arteries were greater in SHR than WKY without changes to K^+-KHS, as described previously (Beltrán *et al.*, 2004). Interestingly, exercise training did not affect the altered contractile responses in SHR coronary arteries but it normalized the enhanced responses in mesenteric arteries.

Previous studies have demonstrated that aerobic exercise training of low intensity decreases oxidative stress in different tissues, including arteries of SHR by increasing the efficiency of the antioxidant system (Rush *et al.*, 2003; Bertagnolli *et al.*, 2008; Agarwal *et al.*, 2009; 2012), thus increasing NO bioavailability (Hägg *et al.*, 2004; Higashi and Yoshizumi, 2004). Our results further provide evidence that low intensity aerobic exercise of SHR decreases oxidative stress and increases NO bioavailability, allowing a complete reversal of the augmented contractile response observed in small mesenteric arteries. This is based on the following evidence: apocynin decreased U46619-induced contractile responses in SHR but not in WKY, and exercise training abolished this effect; O_2^- production was greater in SHR than WKY and exercise training abolished this increase; Cu/Zn SOD expression was decreased in SHR compared with WKY and exercise training also normalized this down-regulation; exercise training increased L-NAME effect on U46619-induced responses and vascular and plasma NO production.

Endothelium-dependent vasodilatation was impaired in coronary arteries from SHR, as previously described in coronary arteries from humans and hypertensive animal models (Crabos *et al.*, 1997; Treasure *et al.*, 1993; Vazquez-Pérez *et al.*, 2001). Exercise training improved the impaired ACh vasodilatation by a mechanism dependent, at least in part, on the antioxidant effects of exercise. This is based on the fact that apocynin, which decreased O_2^- production in SHR, also improved the impaired ACh-induced relaxation in SHR but not in WKY, and exercise training abolished this effect. This is in agreement with other studies suggesting that regular physical activity was efficient in improving endothelial function of patients with coronary artery disease (Hambrecht *et al.*, 2003) by improving NO bioavailability. However, to our knowledge, this is the first study demonstrating that the reduction of oxidative stress induced by exercise is responsible for the improvement in coronary artery endothelial dysfunction in hypertension. Other authors have demonstrated that exercise training also improves the participation of EDHF-dependent mechanisms in endothelium dependent relaxations (Gündüz *et al.*, 2011; Yen *et al.*, 1995).

Enhanced production of endothelium-derived contracting factors, such as prostanoids, can also antagonize relaxing

actions and participate in endothelial dysfunction in hypertension (Félétou *et al.*, 2011). Our results show that in phenylephrine-contracted SHR small mesenteric arteries, high concentrations of ACh induce endothelium-dependent contractions. This contractile response was abolished when the arteries were pre-contracted with U46619 instead of phenylephrine suggesting the involvement of TP receptors in this response. Importantly, this effect was attenuated in trained animals, suggesting that exercise might also affect the COX–TP receptor pathway. In support of this hypothesis, we also observed that exercise training normalized the increased U46619 responses in these arteries.

Aerobic training decreases blood pressure in hypertensive humans and animals (Bertagnolli *et al.*, 2008; Agarwal *et al.*, 2009; 2012; Lamina, 2010; Fernandes *et al.*, 2012) mainly by reducing vascular resistance (Fagard and Cornelissen, 2007; Yung *et al.*, 2009). In the present study, we observed that low intensity exercise attenuated the high blood pressure. This might be due, at least in part, to the beneficial effects observed here in the function, the mechanical properties and in the deposition of ECM proteins in the mesenteric and/or coronary vasculature. Alternatively, this haemodynamic effect of exercise training can contribute to the normalization of the altered properties of the arteries, and this poses the unresolved question of whether vascular abnormalities are a cause or a consequence of high blood pressure.

Here, we have observed that hypertension differently affected mesenteric and coronary arteries at the structural, mechanical and functional level. For example, coronary arteries from SHR showed a much higher increase in vascular stiffness than mesenteric arteries when compared with their normotensive controls. In addition, coronary arteries from SHR showed decreased vasoconstrictor induced responses to different stimuli, whereas hypertensive mesenteric arteries showed an increase or no alteration of these responses. It is possible that these differences might be related to the physiological roles of these vessels in the whole cardiovascular system. What is important is that independently of these differences, exercise training had a beneficial effect in both vascular beds pointing to the idea that these beneficial effects are not only limited to organs affected by exercise (muscle and heart mainly) but also to other peripheral organs that in the end can participate in blood pressure control.

In conclusion, the results presented here demonstrated that aerobic exercise training improved NO bioavailability and decreased the participation of ROS and prostanoids in vascular responses of coronary arteries and/or small mesenteric arteries from SHR. These changes contribute to the normalization of the altered vascular function by reducing contractile responses and by increasing endothelium-dependent relaxations. The reduction of oxidative stress might also beneficially modulate some of the structural and mechanical alterations such as collagen deposition and internal elastic lamina organization, which might be associated with the reduced vascular stiffness. All these beneficial changes in the resistance vasculature might partially decrease blood pressure and they also constitute strong evidence that low-intensity aerobic exercise is beneficial to the vascular system and could be considered as co-adjutant or as an alternative therapy, when appropriate, to pharmacological treatments for hypertension.

Acknowledgements

This study was supported by Ministerio de Economía y Competitividad (SAF2009-07201), Instituto de Salud Carlos III (Red RECAVA, RD06/0014/0011 and RD06/0007), Fundación Mutua Madrileña and Fundación Mapfre. AMB is supported through the Ramon y Cajal program (RYC-2010–06473). FR Roque was the recipient of a CAPES-Brazil scholarship. EM Oliveira and DV Vassallo hold scholarships from CNPq, Brazil. We thank Dr Rafael Manso for providing treadmills.

Conflict of interest

No conflicts of interest to declare.

Author contributions

FRR, AMB and MS conceived and designed the experiments. FRR, ABGR, MG, MSA SMR and TF collected, analysed and interpreted the data. FRR, AMB, VC, DVV, EMO and MS drafted and revised the article.

All authors approved the final version of the manuscript.

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