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Exercise improves endothelial function: A local analysis of production of nitric oxide and reactive oxygen species

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ABSTRACT

This study aimed at investigating the acute effects of aerobic exercise on endothelium-dependent vasomotor function of rat aorta, as well as mechanisms involved in endothelial nitric oxide (NO) bioactivity.

Wistar rats were assigned to either a resting control (C, n = 21) or acutely exercised (E, n = 21) groups (60 min, 55–60% of maximum speed). After exercise, thoracic aorta was excised and cut into rings. Two rings were promptly applied to evaluate vasomotor function and the rest of aorta was used for additional measurements.

Acute exercise significantly improved maximum ACh-induced relaxation (C, 91.6 ± 1.2 vs. E, 102.4 ± 1.7%, $p < 0.001$) and sensitivity to ACh (C, −7.3 ± 0.06 vs. E, −7.3 ± 0.02 log M, $p < 0.01$), and was accompanied by significantly increases on serine1177 eNOS phosphorylation, reflecting its enhanced activation. However, acute exercise also enhanced both superoxide and hydrogen peroxide production, as assayed by dihydroethidium oxidation, lucigenin chemiluminescence and Amplex Red assays. We also provided evidence for Nox2 NADPH oxidase (Nox) activation through gp91dstat-mediated inhibition of superoxide signals. Enhanced arterial relaxations associated with acute exercise were nearly-completely prevented by catalase, suggesting a role for paracrine hydrogen peroxide. Despite increased detectable oxidant generation, cellular oxidative stress was not evident, as suggested by unaltered GSH:GSSG ratio and lipid hydroperoxides.

Collectively, these results demonstrate that one bout of moderate aerobic exercise improves endothelial function by increasing NO bioavailability, while superoxide and hydrogen peroxide are generated in a controlled fashion.

1. Introduction

Endothelial dysfunction is considered an independent predictor of cardiovascular risk, playing important roles in the pathophysiology of atherosclerosis [1]. Among non-pharmacological therapies, aerobic exercise training has been shown to improve endothelium-dependent vasodilatation in both experimental models [2] and humans [3], with or without cardiovascular disease. Salutary effects of aerobic exercise on endothelial function are mainly related to improved NO bioavailability due to its increased production and/or to its decreased inactivation by superoxide [4]. In contrast to moderate-intensity exercise, high-intensity exercise was reported to accentuate cardiovascular risk, frequently associated with oxidative stress induction [5]. Although uncontrolled ROS production has been related to endothelial dysfunction and vascular pathophysiology, redox-mediated signaling regulates several physiological functions, including vascular adaptations to exercise [6,7]. Chronic adaptation to physical activity results from cumulative responses to acute bouts of exercise. Thus, such immediate adjustments during and after exercise must be elucidated to better understand the mechanisms involved in chronic adaptation to regular moderate exercise. Indeed, acute improvements on endothelial function

Abbreviations: ACh, acetylcholine; C, rested control group; C-R, concentration–response; DHE, dihydrodroethidium; DETC, diethylcarbamate; DPI, diphenyleneiodonium; E, exercise group; eNOS, endothelial nitric oxide synthase; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; FOX, ferrous oxidation-xylenol orange; GSH, reduced glutathione; GSSG, oxidized glutathione; L-NAME, Nω-nitro-L-arginine methyl ester; NADPH, nicotinamide adenine dinucleotide phosphate; NE, norepinephrine; NO, nitric oxide; PEG, polyethylene glycol; PBS, phosphate buffered saline; ROS, reactive oxygen species; Scr, scrambled; SNP, sodium nitroprusside; SOD, superoxide dismutase.

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triggered by acute exercise have presented controversial results. Positive effects have been associated with improved NO bioavailability [8–10] and absent or negative effects with higher levels of systemic oxidative stress markers [5,9]. Therefore, the relationship between vascular ROS production during and immediately after a moderate exercise bout with overall mechanisms controlling NO bioavailability and endothelial function needs to be better investigated. Indeed, based on the potential increases of ROS during acute exercise concomitantly to NO, we hypothesized that exercise-induced vascular ROS may contribute to support the salutary effects of moderate acute exercise on improvement of endothelial function. Here, we analyzed the effects of acute aerobic exercise on endothelium-dependent and independent vasomotor function, endothelial nitric oxide synthase activation, local ROS generation, NADPH oxidase activation, and vascular oxidative stress markers.

2. Methods

2.1. Animal models

Forty-two male Wistar rats (4–5 months of age) were housed in the Animal Unit of the Faculty of Medicine, University of São Paulo in a temperature-controlled (22–23 °C) room with a 12:12 h light–dark cycle. Rats were maintained on a standard rodent chow diet and water ad libitum. The Ethics Committee in Research of the School of Physical Education and Sport, University of São Paulo, Brazil approved this study (protocol # 803).

2.2. Exercise capacity test protocol

All rats were initially acclimated to running on a motor-driven treadmill at 5–10 m/min, 0% grade for 5–10 min/day for 1 week. Rats were individually submitted to a progressive exercise test in order to determine their exercise capacity. The exercise capacity test protocol consisted of an incremental speed (5m/min every 5 min, 0% grade) until exhaustion [11]. Exhaustion was defined when the rat could no longer keep pace with the treadmill speed, and the maximal speed velocity (Vmax) was determined.

2.3. Acute aerobic exercise protocol

Rats were randomly assigned to either a rested control (C, n = 14) or acutely exercised (E, n = 14) groups. Forty-eight hours after the exercise capacity test, E-group rats were individually submitted to an acute bout of aerobic exercise (60 min, 55–60% Vmax, 0% grade). In order to minimize the influence of manipulation stress, C-group rats were placed in a box on the top of the treadmill without running. Immediately after exercise or resting period, a pair of rats (from both C and E groups) was killed for experimental measurements.

2.4. Preparation of vessel segments

Rats were euthanized by asphyxia in a carbon dioxide chamber. Thoracic aorta was quickly excised, cleaned of adhering tissue and cut into rings (4 mm long). Two rings were promptly applied to evaluate *in vitro* vasomotor responses and the rest of aorta was used for other measurements: ROS, nitrate/nitrite, oxidative stress markers, SOD activity and eNOS/Akt phosphorylation. Every vasomotor protocol and biochemistry analysis was simultaneously performed on C and E rats.

2.4.1. Vascular reactivity studies

In order to investigate the effects of acute aerobic exercise bout on vasomotor response, two fresh aortic rings of each rat from both C and E groups were utilized to determine *in vitro* vascular responsiveness. Rings were carefully incubated into a four-chamber organ bath containing oxygenated (95% O_2 and 5% CO_2) Krebs solution (mM): NaCl 115, KCL 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.5, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11.1, mM), at 37 °C, pH 7.4. Rings were mounted on a force transducer (BIOPAC, Goleta, CA, USA), using an initial passive tension of 2.0 g, corresponding to the previously determined maximal contractile response evoked by norepinephrine.

After 60 min of stabilization with Krebs solution being changed every 20 min, a single dose of norepinephrine (NE 0.1 μM) was applied to induce vasoconstriction and four different vascular reactivity protocols on endothelium-intact aortic ring were designed:

(1) endothelium-dependent vasodilation, through an acetylcholine (ACh) cumulative concentration–response (C-R) curve (ACh: 10^{-10} to 10^{-4} M);
(2) endothelium-independent vasodilatation, through sodium nitroprusside C-R curve (SNP: 10^{-10} to 10^{-4} M) and;
(3) role of eNOS on vasodilatation, by 30-min incubations with Nω-nitro-L-arginine methyl ester (L-NAME, 0.1 mM), an eNOS inhibitor, before ACh C-R curve (ACh: 10^{-10} to 10^{-4} M);
(4) role of hydrogen peroxide on endothelium-dependent vasodilation as in "1", assessed through 5-min pre-incubations with catalase (1200 U/ml).

In some cases, the endothelium of one aortic ring was removed by rubbing them gently with a stainless needle before ACh C-R curve.

2.5. eNOS/Akt phosphorylation

Fifty micrograms of proteins from aorta homogenate were separated by SDS-PAGE in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated with 5% nonfat milk for 2 hours and overnight at 4 °C with primary antibodies against total eNOS (1:1000, Upstate), phosphorylated eNOS (Serine1177, 1:1000, Santa Cruz), total Akt (1:1000, Calbiochem), phosphorylated Akt (Serine473, 1:1000, Calbiochem) and β-actin (1:5000, Sigma-Aldrich), and subsequently with peroxidase-conjugated secondary antibodies for 2 h at room temperature. The antibody-protein complex was measured through enhanced chemiluminescence system (Amersham Corp.) and detected by autoradiography. Densitometric analysis was performed by Scion Image software and β-actin expression was used as a loading control.

2.6. Superoxide production

Two different methods were applied to measure vascular superoxide production: dihydrodroethidium (DHE) oxidation and lucigenin chemiluminescence.

The oxidation products of fluorescent probe DHE (3 μM) were analyzed in slices of aorta (30 μm) by fluorescence microscopy with 488 nm excitation and emission spectrum of 610 nm. Samples incubated with polyethylene glycol superoxide dismutase (PEG-SOD, 500 U/ml) were used as negative controls.

Alternatively, fresh intact aortic rings were used to measure superoxide production using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence technique [12]. After lucigenin background count, the aortic ring was placed into photomultiplier tubes and light emissions were detected using a luminometer (Biolumat LB 9505; Berthold, Bad Wildbad, Germany).

Specific sources of superoxide were addressed through pre-incubations with known inhibitors: global NADPH oxidase activity by diphenyliodonium (DPI, 20 μM, 5 minutes); Nox2 by the specific cell-permeable tat-peptide against Nox2/Gp91^{phox} Gps91ds-tat: YGRKKRRQRRRCSTRIRRQL-NH2 vs. scrambled sequence: YGRKKRRQR RRCLRITRQSR-NH2, 50 μM, 30 minutes); mitochondrial superoxide production by carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 500 nM, 30 minutes). Superoxide dismutase was