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## Moderate exercise training provides left ventricular tolerance to acute pressure overload

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**Moreira-Gonçalves D, Henriques-Coelho T, Fonseca H, Ferreira RM, Amado F, Leite-Moreira A, Duarte JA.** Moderate exercise training provides left ventricular tolerance to acute pressure overload. *Am J Physiol Heart Circ Physiol* 300: H1044–H1052, 2011. First published December 24, 2010; doi:10.1152/ajpheart.01008.2010.—The present study evaluated the impact of moderate exercise training on the cardiac tolerance to acute pressure overload. Male Wistar rats were randomly submitted to exercise training or sedentary lifestyle for 14 wk. At the end of this period, the animals were anaesthetized, mechanically ventilated, and submitted to hemodynamic evaluation with biventricular tip pressure manometers. Acute pressure overload was induced by banding the descending aorta to induce a 60% increase of peak systolic left ventricular pressure during 120 min. This resulted in the following experimental groups: 1) sedentary without banding (SED + Sham), 2) sedentary with banding (SED + Band), and 3) exercise trained with banding (EX + Band). In response to aortic banding, SED + Band animals could not sustain the 60% increase of peak systolic pressure for 120 min, even with additional narrowing of the banding. This was accompanied by a reduction of  $dP/dt_{max}$  and  $dP/dt_{min}$  and a prolongation of the time constant tau, indicating impaired systolic and diastolic function. This impairment was not observed in EX + Band ( $P < 0.05$  vs. SED + Band). Additionally, compared with SED + Band, EX + Band presented less myocardial damage, exhibited attenuated protein expression of active caspase-3 and NF- $\kappa$ B ( $P < 0.016$ ), and showed less protein carbonylation and nitration ( $P < 0.05$ ). These findings support our hypothesis that exercise training has a protective role in the modulation of the early cardiac response to pressure overload.

cardioprotection; endurance training; cardiac overload; oxidative damage

THE HEART HAS A REMARKABLE adaptive ability, allowing it to continuously adjust its structure and function to variable demands and stimuli (39, 43). However, like all physiological systems (32), the heart's ability to adapt and maintain/improve its function in response to different challenges is limited by restricted boundaries, which, when surpassed, will result in a maladaptive phenotype. This can be illustrated by the elevation in workload imposed to the heart either by exercise training, which is transient and nonpathological, or by hypertension or cardiac valve disease, which is persistent and pathological. In response to increased load, the heart will develop hypertrophy to normalize wall stress and maintain cardiac output (43). Hypertrophy can be accompanied by an enhancement of cardiac performance and a shift to a compensatory cardiac phe-

notype, as in exercise training, or by chamber dilation, impaired ventricular relaxation and filling, and eventually cardiac failure when the cause has a pathological nature (14, 37, 43).

Several pharmacological options are available to improve cardiac function by reducing overload and/or modulating maladaptive remodeling. However, recent studies using exercise training in different models of chronic pressure overload suggest that the heart is capable of maintaining its function even in the presence of constant overload. These studies showed that moderate exercise training avoided heart failure (HF) development, resulting in increased survival (9, 23) and enhanced cardiac function (3, 15, 23, 35, 41). The underlying mechanisms seem to be related with a more favorable hypertrophic phenotype (9, 21–22), including reduction of myocardial fibrosis and apoptosis (15, 22–23) and increase in capillary density (15). However, it must be highlighted that these exercise-induced improvements were independent of any significant effect on the loading state of the heart. In other words, exercise training did not result in any reduction of cardiac overload, but maladaptive remodeling and functional deterioration were prevented. In light of these data, it might be suggested that it is not the presence of persistent overload per se that dictates the activation of the cellular responses involved in maladaptive remodeling and exhaustion of the heart. Rather, the maladaptation is probably related to the intolerance/inability of the heart to sustain the relative overload magnitude, with exercise training being capable of shifting cardiac performance to a level where working chronically under those conditions would be better tolerated. This hypothesis needs, however, to be supported by data showing that exercise training can increase cardiac tolerance to an acute pressure overload, which favors homeostasis and attenuates the magnitude of the cellular responses to stress. Therefore, the objective of this study was to test if moderate exercise training increases tolerance to acute pressure overload stimulus, protecting cardiac function and thus resulting in less activation of the mechanisms involved in cardiac remodeling (25, 28, 42).

### MATERIALS AND METHODS

**Animals and experimental design.** Animal experiments were performed according to the Portuguese law on animal welfare and conform to the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication No. 85–23, Revised 1996) and approved by the Ethical Committee from the University of Porto. Male Wistar rats ( $n = 24$ ; age = 5 wk; weight =  $268 \pm 13$  g at the beginning of the experiment; provided by Charles River Laboratories, Barcelona, Spain) were randomly separated into the following two groups: 1) trained ( $n = 8$ ; submitted to treadmill exercise training) and 2) sedentary ( $n = 16$ ;

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with movement confined to the cage space). Animals were maintained on a 12:12-h light-dark cycle and received food ad libitum.

**Exercise training program.** Animals assigned to the trained group were adapted to treadmill exercise for five consecutive days. This adaptation period involved a gradual increase in running time, beginning with 10 min/day and ending with 60 min/day at 15 m/min. After 2 days of rest, the animals were exercised 5 days/wk (Monday to Friday) for 14 wk. Exercise duration and treadmill speed were gradually increased over the course of the first 3 wk of training until 90 min/day at 30 m/min, 0% grade (estimated work rate of 70% maximum oxygen consumption) (24), remaining then constant until the end of the 14 wk. A similar exercise program to the one used in this study was previously shown to confer protection against doxorubicin cardiotoxicity (1). All animals from the exercise training group completed the training protocol.

**Experimental preparation for hemodynamic studies.** Twenty-four hours after the end of the 14 wk, all animals were anesthetized (pentobarbital, 6 mg/100 g ip) and placed over a heating pad (body temperature was maintained at 37°C). When a surgical plane of anesthesia was reached, animals were tracheostomized for mechanical ventilation with oxygen-enriched air (60 cpm, tidal volume set at 1 ml/100 g, model 683; Harvard Small Animal Ventilator). Anesthesia was maintained with an additional bolus of pentobarbital (2 mg/100 g) as needed. The right jugular vein was cannulated under binocular surgical microscopy (Wild M651.MS-D; Leica, Herbrugg, Switzerland) for fluid administration (prewarmed 0.9% NaCl solution) to compensate the perioperative fluid losses. The heart was exposed by a median sternotomy, and the pericardium was opened widely. Descending thoracic aorta was dissected, and a silk suture 2/0 was placed around it and passed through a plastic tube to allow aortic constriction (banding) during the experimental protocol. Finally, 2-Fr high-fidelity micromanometers (SPR-324; Millar Instruments) were inserted in the left ventricle (LV) and right ventricle (RV) to measure cardiac performance. After complete instrumentation, the animal preparation was allowed to stabilize for 15 min before the beginning of the experimental protocol.

**Experimental protocol.** To document the effectiveness of our chronic exercise training protocol, three animals from the sedentary group (SED) and three animals from the exercise group (EX) were killed at this time to evaluate cardiomyocyte hypertrophy and quantify MnSOD. The remaining animals (5 exercised and 13 sedentary) were distributed by the following three groups: sedentary without banding (SED + Sham,  $n = 6$ ), sedentary submitted to banding (SED + Band,  $n = 7$ ), and exercise-trained submitted to banding (EX + Band,  $n = 5$ ). Sustained and selective acute pressure overload to the LV was obtained by controlled banding of the thoracic descending aorta, just above the diaphragm, during 120 min. Briefly, this was performed by gently pulling a silk suture, previously placed around the descending thoracic aorta, against a plastic tube, until an elevation of 60% of left ventricular peak systolic pressure ( $LVP_{max}$ ) was obtained. At that time, the constriction was fixed with the help of a clamp, and the imposed overload was monitored continuously. Adjustment of the constriction was provided to maintain the same cardiac overload during the entire protocol. Hemodynamic measurements were made in baseline steady-state conditions (immediately before banding) and at 30, 60, 90, and 120 min of banding. All recordings were made with the ventilation suspended at end-expiration. Parameters were automatically recorded and converted on-line to digital data with a sampling frequency of 1,000 Hz. RV and LV pressures were measured at end-diastole and peak systole. Peak rates of RV and LV pressure rise ( $dP/dt_{max}$ ) and pressure decline ( $dP/dt_{min}$ ) were measured as well. The relaxation rate was estimated with the time constant  $\tau$  by fitting the isovolumetric pressure fall to a monoexponential function. All animals completed the 120 min of the protocol and were considered for statistical analysis.

**Tissue collection and homogenization.** Once hemodynamic data collection was completed, animals were killed with an anesthetic

overdose (pentobarbital, 10 mg/100 g ip), and the heart and right gastrocnemius muscle were excised and weighed. Under binocular magnification ( $\times 3.5$ ), the LV free wall was dissected from the RV and weighed separately. Heart weight was normalized to body weight. Samples from RV and LV free wall were fixed and prepared for light microscopy and for transmission electron microscopy (TEM) following routine procedures. Samples from RV and LV were also collected for biochemical studies. Briefly, a portion ( $\sim 20$ – $25$  mg) of LV and RV muscle was separated and homogenized in homogenization buffer (0.25 M sucrose, 1 mM EDTA, 20 mM HEPES, pH 7.6; 100 mg of tissue/ml of buffer) using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0–4°C (3–5 times for 5 s at low speed, with a final burst at a higher speed). Homogenates were centrifuged (2 min at 2,000 g, 4°C) to eliminate cellular debris, and the resulting supernatant was stored at  $-80^{\circ}\text{C}$  for later determination of protein carbonyl derivatives, nitrotyrosine formation, and MnSOD protein expression. The protein content of the cardiac muscle homogenate was assayed with the Bio-Rad RC-DC method, following the instructions of the manufacturer.

**TEM analysis.** Small sections from RV and LV were processed for semiquantitative TEM analysis (model EM10A; Zeiss) at 60 kV as described previously (1). The severity of tissue damage was scored per specimen section from zero to three as previously described (2) taking into account the amount of cardiomyocytes exhibiting intracellular edema and/or mitochondrial swelling: *grade 0* = no change from normal; *grade 1* = a limited number of isolated cells (until 5% of the total cell number); *grade 2* = groups of cells (5–30% of cell total number); and *grade 3* = diffuse cell damage (30% of total cell number). The overall level of tissue damage for each heart was achieved calculating the mean score of the respective analyzed grids. An examiner blinded to each tissue sample code scored all grids independently.

**Light microscopy and immunohistochemistry.** Cubic pieces from RV and LV were fixed [4% (vol/vol) buffered paraformaldehyde] by diffusion during 24 h and subsequently dehydrated with graded ethanol and included in paraffin blocks. Xylene was used in the transition between dehydration and impregnation. Serial sections (5  $\mu\text{m}$  of thickness) of paraffin blocks were cut by a microtome and mounted on silane-coated slides. The slides were dewaxed in xylene and hydrated through graded alcohols finishing in PBS solution prepared by dissolving 1.44 g  $\text{Na}_2\text{HPO}_4$ , 0.24 g  $\text{KH}_2\text{PO}_4$ , 8 g NaCl, and 0.2 g KCl and adjusting pH to 7.2. Deparaffinized sections were stained for hematoxylin-eosin and for immunohistochemical staining of NF- $\kappa\text{B}$  (p50) and the active form of caspase-3 (p12 and p17). Hematoxylin-eosin staining was performed by immersing slides in Mayer's hematoxylin solution for 3–4 min followed by immersion in 1% eosin solution for 7 min, dehydration with graded alcohols through xylene, and mounting with DPX. Cardiomyocyte surface area (CSA) was measured, and only round to ovoid nucleated myocytes were considered for analysis. Regarding immunohistochemistry, after deparaffinization and rehydration of the slides, endogenous peroxidase activity was blocked with 3%  $\text{H}_2\text{O}_2$  in Tris-buffered saline (TBS) for 15 min, followed by  $2 \times 5$  min washes with 0.1% TBS with Tween 20 (TTBS). To reduce nonspecific binding, slides were incubated with blocking solution (3% BSA in TTBS) for 2 h. Next, sections were incubated overnight at 4°C with an antibody directed to active caspase-3 (rabbit polyclonal antibody; 1:200, ab13847; Abcam) or to NF- $\kappa\text{B}$  (rabbit polyclonal antibody; 1:200, sc-114; Santa Cruz Biotechnology). Sections were then washed  $2 \times 5$  min with TTBS and incubated with secondary antibody (goat anti-rabbit IgG-horseradish peroxidase; 1:200, sc-2004; Santa Cruz) for 2 h at room temperature. The Peroxidase Antiperoxidase Method was used for active caspase-3 (Rabbit Peroxidase Anti-Peroxidase Soluble Complex antibody; 1:200; 2 h at room temperature, P1291; Sigma, St. Louis, MO). 3, 3'-Diaminobenzidine was used as the precipitating substrate for the localization of peroxidase activity (Fast DAB tablets; 45 s incubation RT, D4193; Sigma). RV and LV samples from 3–5 animals/group

were further sectioned in three pieces coming from the basal, intermediate, and apical cardiac regions of each animal. For quantitative comparisons, random microscopic fields (magnification of  $\times 400$ ) from each region were considered, totaling a mean of  $3,272 \pm 743$  nuclei counted/group. Analysis was performed by taking into account the percentage of positive nuclei from the total number of cardiomyocytes or from the total number of extracellular space nuclei. Negative controls included staining tissue sections with omission of the primary antibody.

**Slot-blot analysis.** For protein carbonyl derivatives and nitrotyrosine assay, a given volume of sample containing 20  $\mu\text{g}$  of protein was slot-blotted on a nitrocellulose membrane. Regarding protein carbonylation, samples were first derivatized with 2,4-dinitrophenylhydrazine (DNPH). Briefly, samples were mixed with 1 vol of 12% SDS plus 2 vol of 20 mM DNPH/10% TFA, followed by 30 min of incubation in the dark, after which 1.5 vol of 2 M Tris base/18.3% of  $\beta$ -mercaptoethanol was added for neutralization. After diluting the derivatized proteins in TBS to obtain a final concentration of 0.001  $\mu\text{g}/\mu\text{l}$ , a 100- $\mu\text{l}$  volume was slot blotted. After slot blot, nonspecific binding to membranes was blocked with 5% (wt/vol) nonfat dry milk in TTBS, and membranes were incubated with primary antibody diluted 1:1,000 in 5% (wt/vol) nonfat dry milk in TTBS [anti-nitrotyrosine (Chemicon clone 2A8.2) or anti-DNPH (Novus Biologicals)] for 2 h at room temperature, washed, and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit (GE Healthcare). The blots were developed by using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions, followed by exposure to X-ray films (Kodak Biomax Light Film; Sigma). The films were analyzed with QuantityOne Software (version 4.6.3; Bio-Rad, Hercules, CA). All three groups were included in each assay membrane (3 membranes). The "Volume Rectangle Tool" option was used to measure the total signal intensity inside a boundary drawn around the bands detected in the films. Background was subtracted from each band volume by using local background subtraction. Intensities of bands acquired from each sample were compared, and the results were normalized to SED + Sham.

**Western blot analysis.** Equivalent amounts of proteins were electrophoresed on a 12.5% SDS-PAGE, which were prepared in duplicate. The proteins from one of the gels were blotted on a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech), and the other gel was stained with colloidal Coomassie blue for protein visualization. To verify an equal amount of sample loaded, membranes containing transferred proteins were reversibly stained with Ponceau S. Nonspecific binding to the membrane was blocked with 5% (wt/vol) nonfat dry milk in TTBS, followed by incubation with anti-GAPDH (sc-47724; Santa Cruz), anti-MnSOD (ALX-804-25; Alexis), anti-DNPH (Novus Biologicals), or anti-nitrotyrosine (Chemicon clone 2A8.2) for 2 h at room temperature. Next, membranes were washed and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit (GE Healthcare). For carbonyl assay, the sample was first derivatized as described before. Blots containing animals from the three groups were further developed, and the results were normalized to SED + Sham. Films and gels were scanned with the Gel Doc XR System (Bio-Rad). Band detection, quantification, and matching between gels and corresponding films were performed using QuantityOne software (version 4.6.3; Bio-Rad), as described above.

**Identification of SDS-PAGE bands by mass spectrometry.** Protein bands, matching to labeled bands for carbonylation and nitration, were excised manually from 12.5% SDS-PAGE stained with Colloidal Coomassie Blue. The gel pieces were washed three times with 25 mM ammonium bicarbonate/50% acetonitrile and further dried in a Speed-Vac (Thermo Savant). Next, 25  $\mu\text{l}$  of 10  $\mu\text{g}/\text{ml}$  sequence grade-modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate were added to the dried gel pieces, and the samples were incubated overnight at 37°C. Extraction of tryptic peptides was performed by

addition of 10% formic acid/50% acetonitrile three times and then lyophilization in a Speed-Vac (Thermo Savant). Tryptic peptides were resuspended in acetonitrile/formic acid solution and mixed (1:1) with a matrix consisting of  $\alpha$ -cyano-4-hydroxycinnamic acid. Aliquots of samples were spotted on the MALDI sample target plate. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer; Applied Biosystems, Foster City, CA) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 4,500 Da with ca. 1,500 laser shots. For each sample spot, a data-dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a routine mass accuracy of better than 20 ppm. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal Mascot (Matrix Science) software for searching the peptide mass fingerprints and MS/MS data. Searches were performed against the SwissProt (02/03/10) under rodentia as the taxonomic category and the following parameters: 1) two missed cleavages by trypsin; 2) mass tolerance of precursor ions 25 ppm and product ions 0.3 Da; 3) carboxymethylated cysteines fixed modification; and 4) oxidation of methionine as variable modification.

**Statistical analysis.** The Shapiro-Wilk test was performed to check normality of the data. For comparisons within and between groups (hemodynamic data), a repeated-measures two-way ANOVA (time  $\times$  group) was performed. Between-group comparisons of slot-blot and Western blot data were performed with a one-way ANOVA. Significance level was set at  $P < 0.05$ . Significant differences were evaluated with Tukey's post hoc analysis. Data from CSA, immunohistochemistry, and TEM were analyzed with nonparametric tests, namely the Kruskal-Wallis Test with the Mann-Whitney Test used for post hoc comparisons, and the significance level was set at  $P < 0.016$ , which resulted from dividing 0.05 by the number of comparisons done among groups. Data are expressed as means  $\pm$  SD.

## RESULTS

**Chronic effects of exercise training protocol.** Exercise training did not result in any significant alteration concerning body, heart, LV, RV or gastrocnemius weight (data not shown). However, histological analysis revealed that exercise training induced an increase in CSA from both RV ( $209 \pm 79$  vs.  $319 \pm 116$   $\mu\text{m}^2$ , SED and EX group, respectively,  $P = 0.000$ ) and LV ( $338 \pm 164$  vs.  $390 \pm 194$   $\mu\text{m}^2$ , SED and EX group, respectively,  $P = 0.000$ ), suggesting that important structural adaptations occurred in cardiac muscle (Fig. 1A). Regarding the hemodynamic profile at baseline, despite a tendency to enhanced relaxation and contractile function, none of the parameters was significantly altered by training ( $P < 0.05$ ). Our exercise training protocol, however, resulted in a significant 30% increase in the EX group of MnSOD protein expression ( $P = 0.002$  vs. SED), an important antioxidant enzyme (Fig. 1B).

**Effects of LV acute pressure overload on cardiac hemodynamics.** Regarding the SED + Sham group, all parameters remained unaltered throughout the experimental protocol, which documents the stability of the experimental preparation and is in accordance with previous reports from our group using the same approach (10, 16, 27, 42). Pressure overload by descending thoracic aortic banding resulted in several alterations in LV function from the SED + Band group that were prevented by exercise (Fig. 2). Importantly, there were no alterations in RV hemodynamics among banded groups (Fig. 2, E and F), sug-

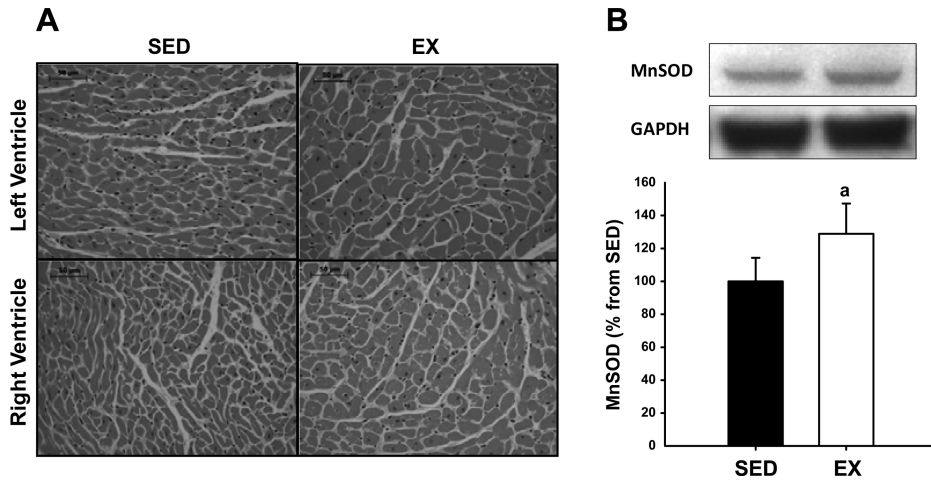


Fig. 1. Cardiac adaptations to exercise training. *A*: representative examples of light micrographs stained for hematoxylin-eosin from left ventricle (LV) and right ventricle (RV) of sedentary (SED) and exercise (EX) groups. *B*: Western blots with graphic representation of MnSOD protein expression. Error bars are means  $\pm$  SD. <sup>a</sup> $P = 0.002$  vs. SED.

gesting that this overload was selective for LV. After aortic constriction, a 60% increase in cardiac overload was achieved, as represented by the rise in  $LVP_{max}$  (Fig. 2A). This increase in  $LVP_{max}$  was successfully sustained until 60 min by both groups. After that, SED + Band animals showed a progressive decline, despite a progressive additional narrowing of the descending aorta, reaching a minimum at 120 min that was significantly lower than at baseline ( $70.6 \pm 21.7$  vs.  $92.6 \pm 24.4$  mmHg;  $P = 0.002$ ) and than in EX + Band ( $70.6 \pm 21.7$  vs.  $154.6 \pm 22.1$  mmHg;  $P = 0.000$ ). Note that EX + Band remained stable along the entire protocol, with no apparent decompensatory response, indicating that these hearts were able to better sustain the increased afterload. Regarding  $dP/dt_{max}$ ,

we observed a similar response in both groups until 60 min of banding. After this point, while EX + Band showed a nonsignificant increase in contractility as illustrated by the slight rise in  $dP/dt_{max}$  at 90 min ( $P = 0.107$  vs. 0 min) and 120 min ( $P = 0.09$  vs. 0 min), SED + Band tended to deteriorate until the end of the protocol (Fig. 2B). This deterioration only reached significance at 120 min compared with their baseline values ( $2,144 \pm 1,164$  vs.  $4,677 \pm 1,313$  mmHg/s;  $P = 0.01$ ) and with EX + Band ( $2,144 \pm 1,164$  vs.  $8,610 \pm 2,880$  mmHg/s;  $P = 0.002$ ).

Regarding diastolic function, acute pressure overload induced important disturbances in SED + Band. Figure 2C shows that, while EX + Band did not suffer any alteration in

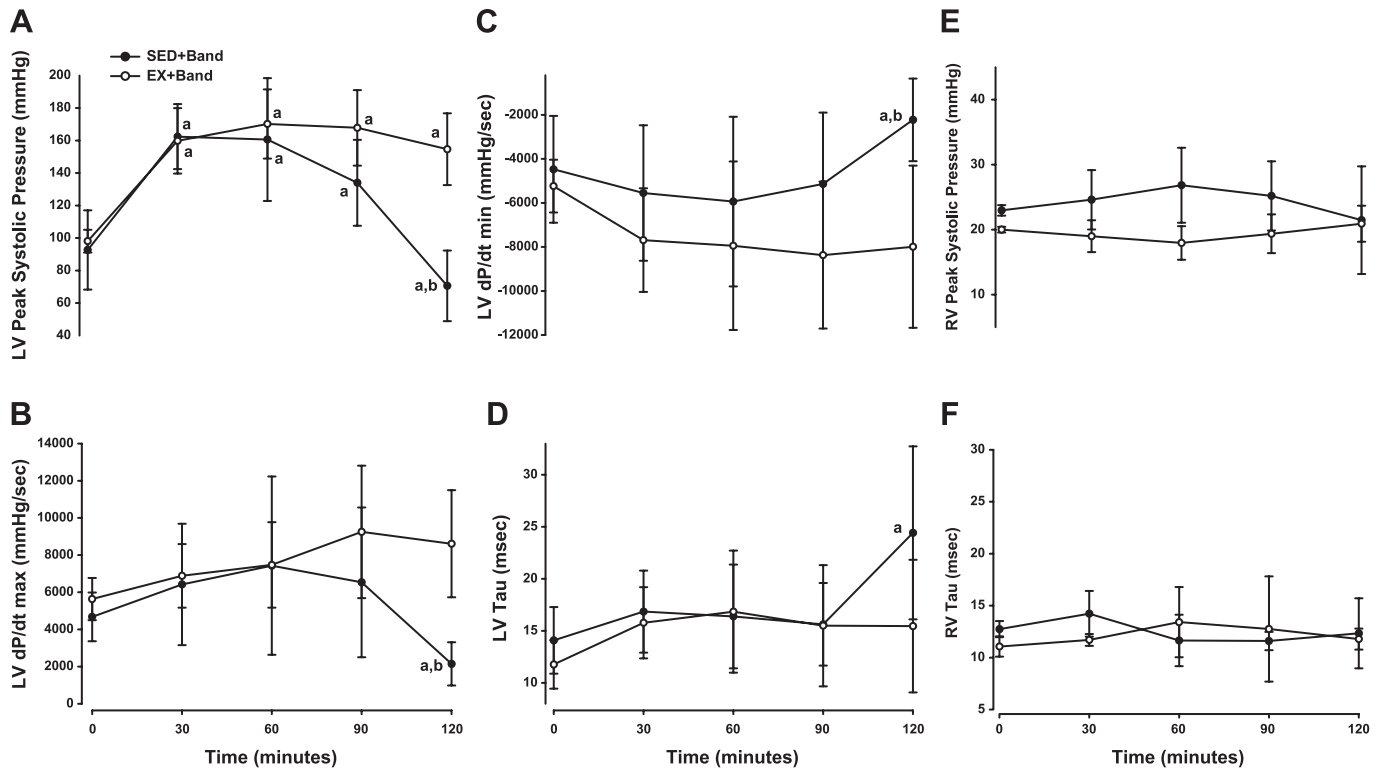


Fig. 2. Effects of 120 min of acute pressure overload on systolic and diastolic function of LV (A-D) and RV (E-F).  $dP/dt_{max}$ , peak pressure rise;  $dP/dt_{min}$ , peak pressure fall; Tau, time constant. SED + Band, sedentary submitted to aortic banding; EX + Band, exercised submitted to aortic banding. Error bars are means  $\pm$  SD.  $P < 0.05$  vs. 0 min (a) and vs. EX + Band (b).

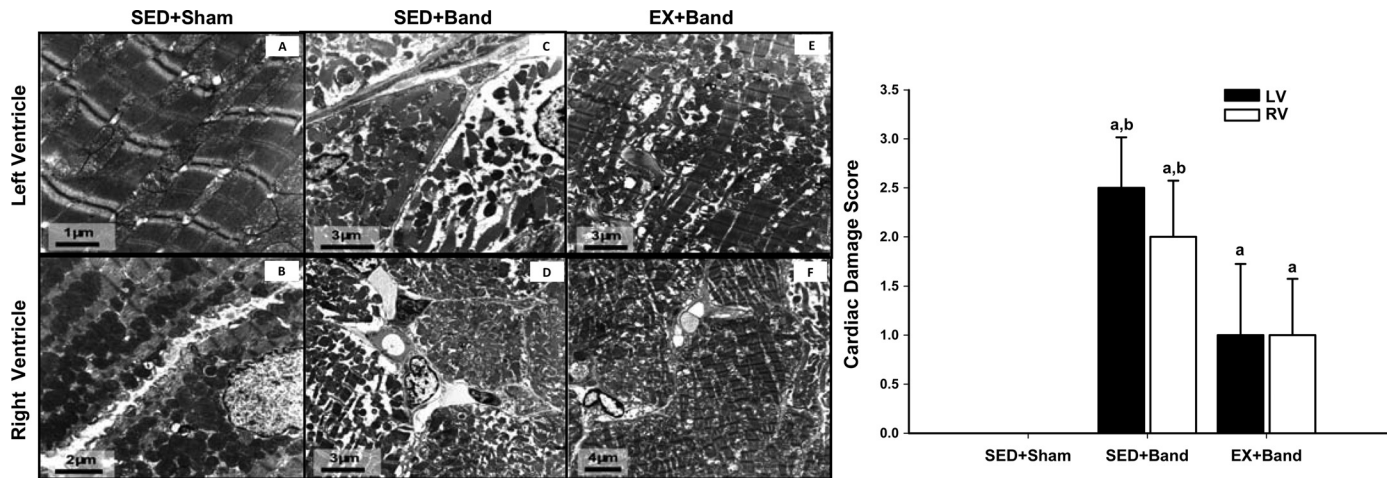


Fig. 3. Representative electron micrographs from LV and RV of sedentary animals submitted to sham surgery (SED + Sham) (A and B, respectively), SED + Band (C and D, respectively), and EX + Band (E and F, respectively). Results are depicted in the graph. Error bars are means  $\pm$  SD.  $P < 0.016$  vs. SED + Sham (a) and vs. EX + Band (b).

$dp/dt_{min}$ , SED + Band saw their velocity of pressure fall, being significantly impaired at 120 min compared with their baseline values ( $-2,224 \pm 1,877$  vs.  $-4,470 \pm 2,425$  mmHg/s;  $P = 0.049$ ) and with EX + Band ( $-2,223.9 \pm 1,877.3$  vs.  $-7,989.2 \pm 3,682.7$  mmHg/s;  $P = 0.003$ ). Compared with baseline values, SED + Band presented a significant increase in tau at 120 min, indicating a slower relaxation ( $14.1 \pm 3.2$  vs.  $24.4 \pm 8.3$ ;  $P = 0.027$ ), whereas in EX + Band no significant alterations were registered ( $11.8 \pm 2.3$  vs.  $15.4 \pm 6.4$ ;  $P = 0.332$ ) (Fig. 2D).

No significant alterations were observed in heart rate, end-diastolic pressure, or end-systolic pressure during the protocol (data not shown).

*Effects of LV acute pressure overload on cardiac ultrastructure.* Myocardial damage is shown in Fig. 3. The foremost altera-

tions observed were the intracellular edema and mitochondrial swelling, with the SED + Band group being the most affected ( $P < 0.016$ ). Significant alterations were detected in both RV and LV from both overloaded groups, although more severely in SED + Band ( $P < 0.016$ ). The SED + Sham group exhibited normal structure, with preserved mitochondrial morphology and without any evidence of cellular or interstitial edema.

*Effects of LV acute pressure overload on protein expression of the active form of caspase-3 and NF- $\kappa$ B.* Immunohistochemistry analysis revealed positive staining for both active caspase-3 and NF- $\kappa$ B after only 120 min of acute pressure overload in the SED + Band group. As shown in Fig. 4, exercise training prevented activation of caspase-3 ( $P < 0.016$ ). Although no signs of active caspase-3 were found in

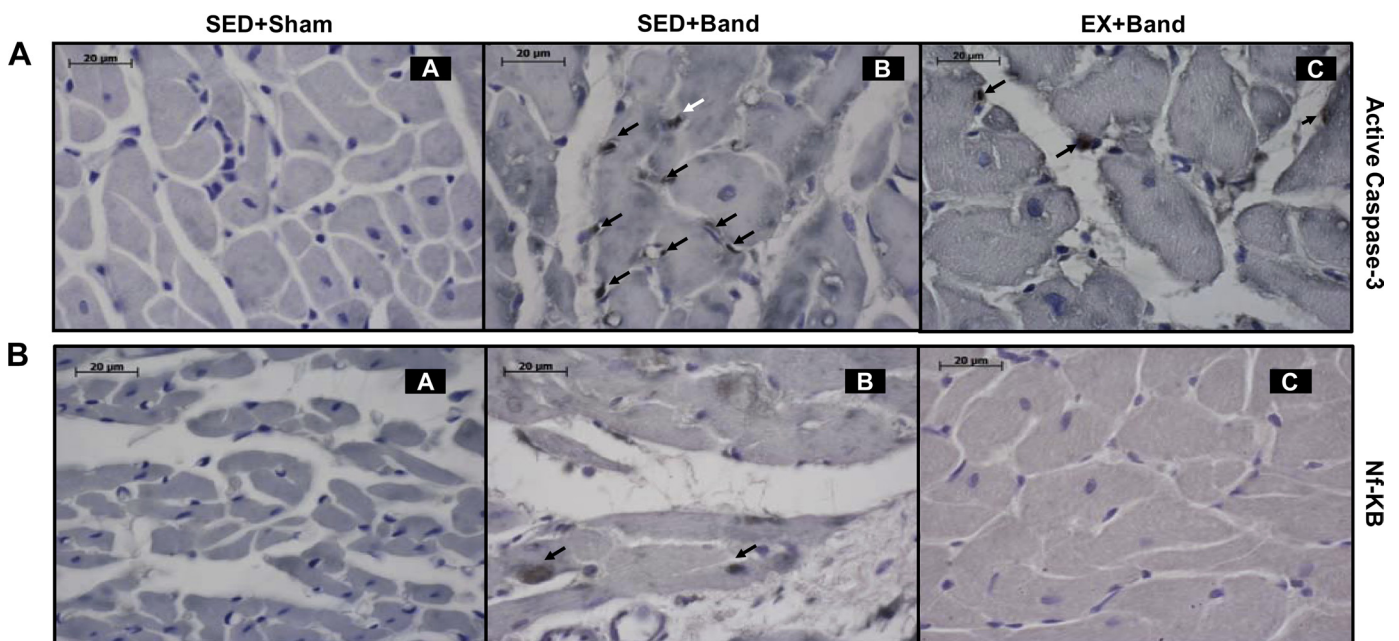


Fig. 4. Representative light micrographs from immunohistochemistry ( $\times 1,000$ ). Top: no positive nuclei (A); positive cardiomyocyte nuclei (white arrow; B and C) and noncardiomyocyte nuclei (black arrows; B) for active caspase-3. Bottom: positive cardiomyocyte nuclei for NF- $\kappa$ B (black arrows).

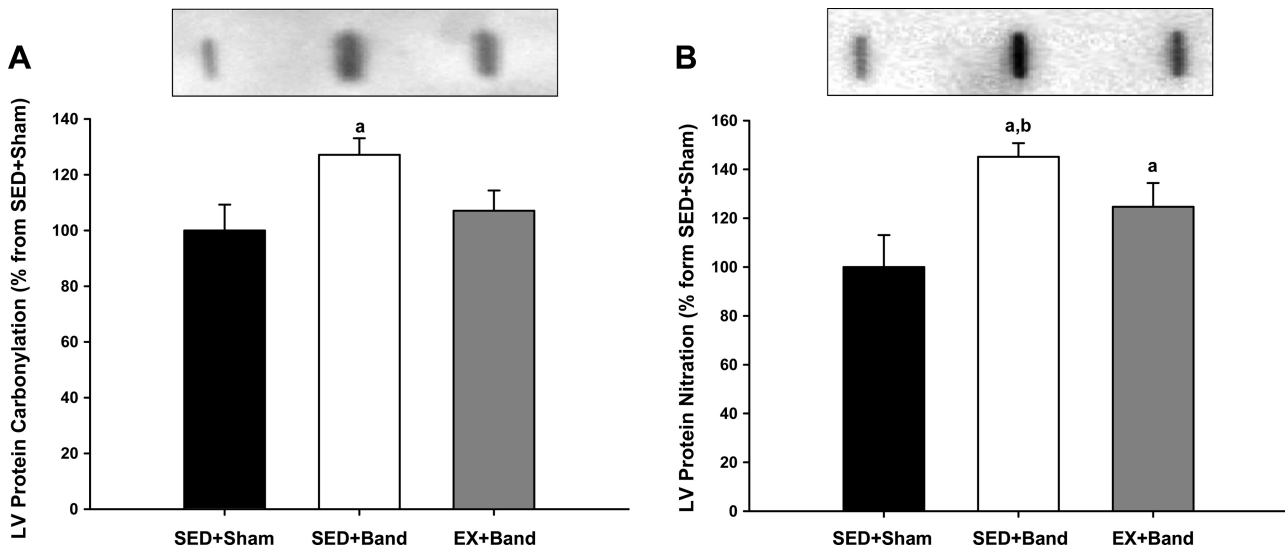


Fig. 5. Oxidative modification of cardiac proteins. *A*: LV total carbonyl formation. *B*: LV total tyrosine nitration. Error bars are means  $\pm$  SD.  $P < 0.05$  vs. SED + Sham (a) and vs. EX + Band (b).

SED + Sham, 4% positive cardiomyocyte nuclei were observed in SED + Band and only 0.5% in EX + Band. Significance was only found in SED + Band compared with SED + Sham ( $P = 0.000$ ) and EX + Band ( $P = 0.000$ ). Regarding noncardiomyocytes, 0.2% positive nuclei were found in SED + Sham, 10% in SED + Band, and 5% in EX + Band. Although significance was obtained in SED + Band and EX + Band compared with SED + Sham ( $P < 0.016$ ), protein expression of active caspase-3 observed in LV from EX + Band was significantly lower in relationship to SED + Band ( $P = 0.007$ ). No differences were found in RV in any of the groups. Concerning NF- $\kappa$ B staining, differences were only detected in LV cardiomyocytes from SED + Band compared with SED + Sham ( $P = 0.009$ ) and EX + Band ( $P = 0.000$ ), with 6.9, 0, and 0.1% of positive cardiomyocyte nuclei, respectively.

**Effects of LV acute pressure overload on total protein carbonylation and tyrosine nitration.** Total protein carbonyl and 3-nitrotyrosine contents were analyzed and are shown in Fig. 5. Acute pressure overload resulted in increased levels of protein carbonylation (Fig. 5, *A* and *B*) in both ventricles of SED + Band, although only significant in LV, where a 30% increase was observed ( $P = 0.036$  vs. SED + Sham). No differences were noted between EX + Band and SED + Sham groups ( $P = 0.747$ ). Regarding 3-nitrotyrosine protein formation, the overloaded groups presented increased levels in the LV (Fig. 5C). SED + Band was the most affected group, presenting 40% more total protein nitration than SED + Sham ( $P = 0.000$ ). Exercise training did not completely prevent 3-nitrotyrosine protein formation (25% increase,  $P = 0.017$  vs. SED + Sham), but its levels were attenuated compared with SED + Band ( $P = 0.042$  vs. SED + Sham).

**Identification of proteins more susceptible to oxidative stress by SDS-PAGE-MS/MS.** As shown in Fig. 6, *B* and *C*, two DNP-positive bands and one 3-nitrotyrosine-positive band at different molecular weights were detected by Western blot, suggesting that there are proteins more prone to oxidation while others to nitration. The results also suggest that there are some basal levels of protein oxidation and nitration in proteins

of the normal healthy heart. To identify proteins more susceptible to oxidative/nitrative damage, MALDI-TOF/TOF analysis was performed (Table 1). Aconitate hydratase and ATP synthase subunit  $\beta$  were identified in the two DNP-positive bands and ATP synthase subunit  $\alpha$  in the 3-nitrotyrosine-positive band.

## DISCUSSION

The present study provides unequivocal evidence that an acute LV pressure overload results in severe functional disturbances in the heart of healthy sedentary animals, paralleled by important histological and biochemical changes. Importantly, our results show that exercise training increases cardiac tolerance to loading conditions, with no systolic or diastolic dysfunction and no early activation of important signaling pathways involved in maladaptive remodeling.

Several reports have consistently shown that exercised hearts have some degree of protection against several cardiac insults like myocardial infarction (11), ischemia-reperfusion (12), and doxorubicin-induced cardiac toxicity (1). Moreover, exercise training also seems to modulate important features of maladaptive cardiac remodeling induced by chronic pressure overload, resulting in an improved cardiac phenotype (3, 9, 15, 23, 35, 41). Our study adds the novelty that exercise-trained hearts are more protected against sudden acute increases in cardiac overload. Indeed, as evidenced by our hemodynamic results, exercised animals responded very well to the acute cardiac overload, presenting normal values of tau and  $dP/dt_{min}$ , which is suggestive of a preserved calcium handling function, since the time constant tau reflects the efficiency of calcium transport to the sarcoplasmic reticulum (SR) (36) and  $dP/dt_{min}$  is correlated with SR calcium-ATPase activity (SERCA2a) (7). On the contrary, tau and  $dP/dt_{min}$  were altered by acute pressure overload in sedentary animals, which might indicate an impaired calcium homeostasis (12–13). Apart from this hypothetical calcium overload, we cannot exclude the possibility that changes in the myofilament's sensitivity to calcium may have also contributed to the altered  $dP/dt_{min}$  and tau in SED +

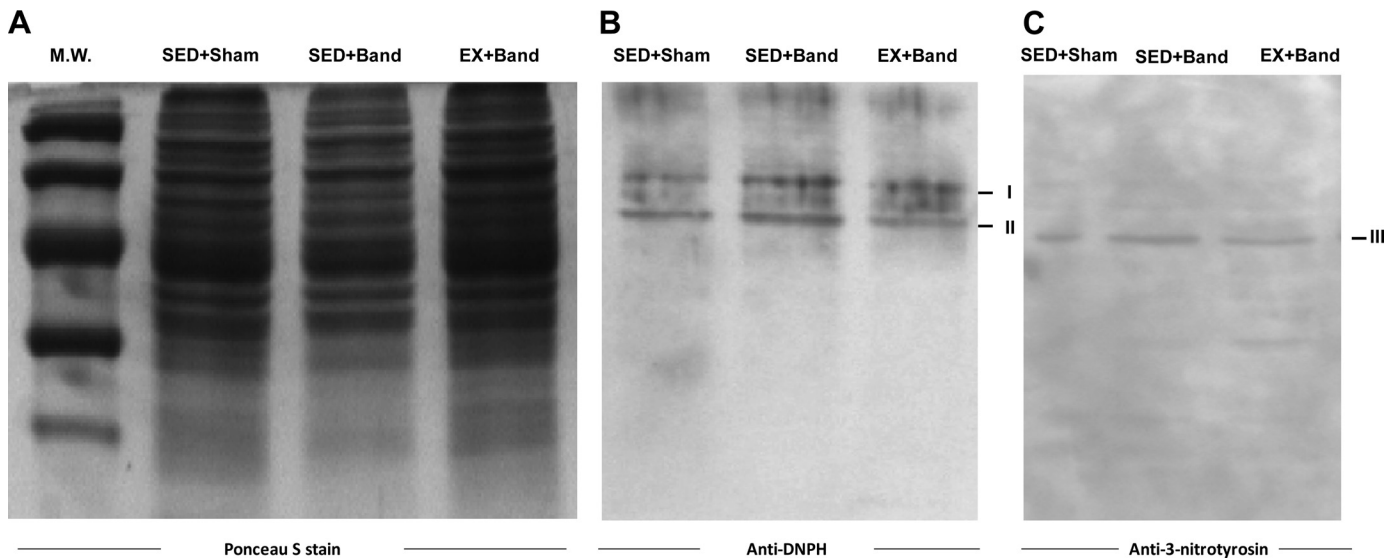


Fig. 6. Localization of proteins more susceptible to oxidative damage: A: Ponceau S staining of cardiac proteins from LV showing equal protein loading. B: representative Western blots from the 2,4-dinitrophenylhydrazine (DNPH)-positive bands. C: representative Western blots from the 3-nitrotyrosine-positive bands. MW, mol wt; I and II, DNPH-positive bands; III, 3-nitrotyrosine-positive band.

Band, since it is known that alterations in the activation pattern of kinases and phosphatases induced by oxidative stress modify the phosphorylation status of myofilament proteins altering its affinity to calcium (46). However, because the real contribution of myofilament calcium sensitivity to the relaxation rate is very controversial (8, 48), it seems more reasonable to assume the failure of SERCA2a as mainly responsible for the altered  $dP/dt_{min}$  and tau observed in SED + Band animals (7, 36). The resulting calcium overload would also explain the increased mitochondrial swelling, as well as the expression of active caspase-3 found in sedentary overloaded animals. In fact, as suggested by our immunohistochemical results, the decreased hemodynamic profile of sedentary overloaded animals was also accompanied by significant expression of active caspase-3 and NF- $\kappa$ B. Importantly, this response was prevented in EX + Band animals, highlighting the positive role of exercise training in modulating the expression of both caspase-3 and NF- $\kappa$ B, which are known to play an essential role in the transition between compensatory hypertrophy and HF. Indeed, the inhibition of both apoptosis (17, 44, 49) and NF- $\kappa$ B (20, 38, 45) has been shown to prevent cardiac remodeling and dysfunction.

An interesting point highlighted by our morphological data was the fact that cardiomyocytes do not seem to respond

equally to acute pressure overload. There was a great intra- and intergroup heterogeneity in terms of cellular response. In fact, while some cells presented serious ultrastructural damage, apoptosis, and NF- $\kappa$ B activation, others had a normal structure and ultrastructure. This heterogeneity was even more evident in SED + Band animals than in EX + Band animals. A potential explanation for the homogeneous cellular phenotype observed in EX + Band might result from the functional demand imposed by exercise training, favoring the autophagic response and/or death of the most dysfunctional and susceptible cells to injury. This idea, although speculative, is supported by reports showing that endurance exercise training is accompanied by terminal deoxynucleotidyl-dUTP nick end labeling-positive nuclei and activation of apoptotic mediators (Bax, cytochrome *c*, cleaved caspase-3, and cleaved PARP) in LV cardiomyocytes (18) as well as enhanced abundance of cardiac progenitor cells (21). In addition, cardiac autophagy has been recently associated with exercise training, with some data pointing to its involvement in exercise-induced cardioprotection (4, 40). However, the role of apoptosis and autophagy in the exercised heart is still under debate, and further data are needed to clarify this issue.

The heterogeneous susceptibility of cells to damage can also be linked with the different levels of reactive oxygen and

Table 1. Proteins identified in the DNPH and 3-nitrotyrosine-positive bands by SDS-PAGE-MS/MS

Band No.	Protein Name	Accession No.	Mol Wt, kDa	pI	Peptide Count	Protein Score	Score CI, %	Coverage, %	Biological Function
I	Aconitate hydratase, mitochondrial	ACON_RAT	85.38	7,87	11	191	100	14	Metabolism
II	ATP synthase subunit $\alpha$ , mitochondrial	ATPA_RAT	59.72	9,22	17	451	100	41	Oxidative phosphorylation
III	ATP synthase subunit $\beta$ , mitochondrial	ATPB_RAT	56.32	5,19	14	197	100	36	Oxidative phosphorylation

Identification of cardiac proteins more susceptible to carbonylation and nitration, identified by SDS-PAGE-MS/MS. The following information is presented: no. of identified protein, protein accession no. and name, protein mol wt, isoelectric point (pI) of the protein, peptide count, protein score, protein score confidence index (CI, %), % of protein coverage, and protein biological function. DNPH, 2,4-dinitrophenylhydrazine.



nitrogen species (RONS) production and scavenging efficiency. We found that 2 h of LV pressure overload were enough to result in increased protein oxidation and tyrosine nitration in sedentary-overloaded animals, whereas, in exercise-overloaded animals these changes were attenuated (Fig. 5). Importantly, it seems that, while some proteins were more prone to oxidative damage, others seem to be more susceptible to nitrative damage. This is in agreement with previous findings following ischemia-reperfusion, where different susceptibility of proteins to oxidation and tyrosine nitration was also described (31). Our results also suggest that mitochondria are a preferential target of acute pressure overload-induced oxidative damage. We found increased levels of oxidative damage in sedentary-overloaded animals with the  $\alpha$ -subunit of ATP synthase the more prone to nitration and the  $\beta$ -subunit of ATP synthase and aconitate hydratase the most susceptible to carbonylation (Fig. 6). Oxidative damage to proteins is generally related to alterations in their biochemical characteristics, such as enzymatic activities, structural functions, or susceptibility to proteolysis (5–6). Thus the increased oxidation of aconitate hydratase, which is a rate-limiting enzyme of the tricarboxylic acid cycle (29, 33), and ATP synthase, responsible for the use of electrochemical  $H^+$  gradient energy from the mitochondrial respiratory chain to produce ATP (30), might impair energy production in sedentary-overloaded animals. For instance, the limited availability of ATP can interfere with myosin detachment from actin, calcium dissociation from Tn-C, and active sequestration of calcium by the SR (26) and, consequently, affect systolic and diastolic function. In addition, it is important to note that the enhanced oxidative stress induced by acute pressure overload can further directly damage calcium-handling proteins, favoring the accumulation of this ion in the cytosol (12), with a consequent interference in relaxation rate, as suggested by our hemodynamic results. All together, these data suggest that mitochondrial dysfunction might be involved in the early deterioration of cardiac function. Attenuation of oxidative damage in cardiac proteins from the exercise-trained overloaded heart might be partially justified by the increased expression of MnSOD and other mitochondrial adaptations induced by training. Indeed, recent evidence suggests that improvements in both antioxidant defense mechanisms and mitochondria might at least partially underlie the exercise-induced cardiac protection (1, 12, 19). MnSOD was shown to protect against oxidative damage in ischemia-reperfusion (12) and pressure overload-induced heart failure (47). Our exercise training protocol resulted in a 30% increase in MnSOD protein (Fig. 1B), and our laboratory has previously shown increased activity of this antioxidant enzyme using a similar exercise training protocol (1). In addition, exercise training seems to promote the development of an improved mitochondrial phenotype that is resistant to reactive oxygen species-induced cytochrome *c* release and doxorubicin cardiac toxicity (1, 19). Altogether, these data suggest that acute pressure overload-induced RONS can lead to mitochondrial dysfunction, and exercise training can prevent it.

In conclusion, our results highlight the vulnerability of the normal healthy heart to severe acute pressure overload that might occur in pathological conditions and act as an initial trigger of early cardiac damage. Exercise training seems to induce a cardioprotective phenotype that is clearly advantageous in increasing the tolerance to acute cardiac overload.

Protection against this acute stimulus might modulate cumulative deleterious adaptations in sedentary hearts that will be manifested later in life (34).

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#### DISCLOSURES

None.

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