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Original article

The benefits of endurance training in cardiomyocyte function in hypertensive rats are reversed within four weeks of detraining

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ABSTRACT

The aim of the present study was to verify the effects of low-intensity endurance training and detraining on the mechanical and molecular properties of cardiomyocytes from spontaneously hypertensive rats (SHRs). Male SHRs and normotensive control Wistar rats at 16-weeks of age were randomly divided into eight groups of eight animals: NC8 and HC8 (normotensive and hypertensive control for 8 weeks); NT8 and HT8 (normotensive and hypertensive trained at 50-60% of maximal exercise capacity for 8 weeks); NC12 and HC12 (normotensive and hypertensive control for 12 weeks); NDT and HDT (normotensive and hypertensive trained for 8 weeks and detrained for 4 weeks). The total exercise time until fatigue (TTF) was determined by a maximal exercise capacity test. Resting heart rate (RHR) and systolic arterial pressure (SAP) were measured. After the treatments, animals were killed by cervical dislocation and left ventricular myocytes were isolated by enzymatic dispersion. Isolated cells were used to determine intracellular global $Ca^{2+}([Ca^{2+}]_i)$ transient and cardiomyocyte contractility $(1 \text{ Hz}; \sim 25 \text{ °C}).$ [Ca²⁺]_i regulatory proteins were measured by Western blot, and the markers of pathologic cardiac hypertrophy by quantitative real-time polymerase chain reaction (q-RT-PCR). Exercise training augmented the TTF (NC8, 11.4±1.5 min vs. NT8, 22.5±1.4 min; HC8, 11.7±1.4 min vs. HT8, 24.5±1.3 min; P<0.05), reduced RHR (NT8initial, 340 ± 8 bpm vs. NT8final, 322 ± 10 bpm; HT8initial, 369 ± 8 bpm vs. HT8final, 344 ± 10 bpm; P<0.05), and SBP in SHR animals (HC8, 178 ± 3 mm Hg vs. HT8, 161 ± 4 mm Hg; P<0.05). HC8 rats showed a slower $[Ca^{2+}]_i$ transient (Tpeak, 83.7±1.8 ms vs. 71.7±2.4 ms; T50% decay, 284.0±4.3 ms vs. 264.0±4.1 ms; P<0.05) and cell contractility (Vshortening, $86.1 \pm 6.7 \mu m/s$ vs. $118.6 \pm 6.7 \mu m/s$; Vrelengthening, $57.5 \pm$ 7.4 μ m/s vs. 101.3 \pm 7.4 μ m/s; P<0.05), and higher expression of ANF (300%; P<0.05), skeletal α -actin (250%; P<0.05) and a decreased α/β -MHC ratio (70%; P<0.05) compared to NC8. Exercise training increased [Ca²⁺]_i transient (NC8, 2.39 ± 0.06 F/F₀ vs. NT8, 2.72 ± 0.06 F/F₀; HC8, 2.28 ± 0.05 F/F₀ vs. HT8, 2.82 ± 0.05 F/F₀; P<0.05), and cell contractility (NC8, $7.4 \pm 0.3\%$ vs. NT8, $8.4 \pm 0.3\%$; HC8, $6.8 \pm 0.3\%$ vs. HT8, $7.8 \pm 0.3\%$; P<0.05). Furthermore, exercise normalized the expression of ANF, skeletal α -actin, and the α/β -MHC ratio in HT8 rats, augmented the expression of SERCA2a (NC8, 0.93 ± 0.15 vs. NT8, 1.49 ± 0.14 ; HC8, 0.83 ± 0.13 vs. HT8, 1.32 ± 0.14 ; P<0.05) and PLB_{ser16} (NC8, 0.89 ± 0.18 vs. NT8, 1.23 ± 0.17; HC8, 0.77 ± 0.17 vs. HT8, 1.32 ± 0.16; P<0.05), and reduced PLBt/SERCA2a (NC8, 1.21 ± 0.19 vs. NT8, 0.50 ± 0.21 ; HC8, 1.38 ± 0.17 vs. HT8, 0.66 ± 0.21 ; P<0.05). However, all these adaptations returned to control values within 4 weeks of detraining in both SHR and normotensive control animals. In conclusion, low-intensity endurance training induces positive benefits to left ventricular myocyte mechanical and molecular properties, which are reversed within 4 weeks of detraining, © 2013 Elsevier Ltd. All rights reserved.

Abbreviations: NC8 and HC8, normotensive and hypertensive control for 8 weeks; NT8 and HT8, normotensive and hypertensive trained for 8 weeks; NC12 and HC12, normotensive and hypertensive control for 12 weeks; NDT and HDT, normotensive and hypertensive trained for 8 and detrained for 4 weeks; MRS, maximal running speed; TTF, total exercise time until fatigue; RHR, resting heart rate; SAP, systolic arterial pressure; LIET, low-intensity endurance training; DET, detraining.

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1. Introduction

Hypertension is an independent risk factor for cardiovascular disease and a precursor of heart failure [1]. The hemodynamic overload imposed by hypertension results in a pathological pattern of concentric cardiac hypertrophy, which is commonly associated with upregulation of fetal genes, fibrosis, cardiac dysfunction, and apoptosis [2–4].

The spontaneously hypertensive rat (SHR) is a widely used model of human essential hypertension. The compensated state of the SHR model has been reported as early as three months, while failure is reported at 18–24 months [5,6]. At the cellular level, in the compensated state of hypertension, it has been reported that left ventricular myocyte shortening increases, whereas the action potential duration and the time course of intracellular global Ca^{2+} ($[Ca^{2+}]_i$) transient, cell shortening and relaxation are prolonged in SHR [7–9].

Along with anti-hypertensive therapy, an active lifestyle is recommended for the management of high blood pressure (BP) in hypertensive individuals, and exercise training is an important hypotensive non-pharmacological therapeutic strategy [1,10–13]. Although high-intensity exercise training may lead to adverse remodeling and produce a substrate for cardiac arrhythmias [14], the recommended low-intensity exercise training improves whole heart inotropic performance, myocardial β -adrenergic responsiveness, phosphorylation of key $[Ca^{2+}]_i$ regulatory proteins (e.g., ryanodine receptor and phospholamban), and attenuates systolic dysfunction in the compensatory phase of hypertension in female SHRs [15–18]. Moreover, low-to-moderate exercise training improved midventricular shortening, myocardial capillary density, and decreased fibrosis and calcineurin activity in the myocardium of male SHRs [19].

Although previous studies have demonstrated that aerobic exercise training enhances single cardiomyocyte contractile function in normotensive rats [20–24] and that detraining reverses the improved cardiomyocyte contractile function to control levels in these animals [22], to date little is known about the effects of aerobic exercise training and detraining on the cardiomyocyte contractile function in hypertensive rats. Our group demonstrated that low-intensity exercise training induces beneficial effects to left ventricular myocytes' mechanical [25] and electrical [9] properties and that the mechanical adaptations are partially reversed within 4 weeks of detraining; however, the underlying mechanisms are not known.

This study was designed to investigate the effects of low-intensity endurance training (LIET) and detraining (DET) on the mechanical and molecular properties of cardiomyocytes from SHR animals in the compensated state of hypertension.

2. Materials and methods

2.1. Experimental animals

Four-month old male SHRs and normotensive Wistar rats were housed in collective cages under 12–12 h light/dark cycles in a temperature-controlled room (22 °C) and had free access to water and standard rodent chow. Eight experimental groups were allocated as follows: normotensive and hypertensive control for 8 weeks (NC8 and HC8); normotensive and hypertensive trained for 8 weeks (NT8 and HT8); normotensive and hypertensive control for 12 weeks (NC12 and HC12); and normotensive and hypertensive trained for 8 and detrained for 4 weeks (NDT and HDT).

Experimental protocols were approved by the Ethic Committee in Animal Use from Federal University of Viçosa (Protocol #48/2011) in accordance with the Guide for the Care and Use of Laboratory Animals/2011.

2.2. Exercise training protocol and detraining

The exercise training protocol was carried out on a motor-driven treadmill (Insight Equipamentos Científicos, Brazil), 5 days per week (Monday to Friday), 60 min/day, for 8 weeks. Before the beginning of the exercise-training program, the animals were placed on the treadmill for adaptation (10 min/day, 0% grade, 0.3 km/h) for 5 days. Forty-eight hours after the adaptation period, a test was performed to determine the maximal running speed (MRS) of each animal. The animals started running at 0.3 km/h, 0% grade, and the treadmill speed was increase by 0.18 km/h every 3 min until fatigue, which was defined as when the test was interrupted because the animals could no longer keep pace with the treadmill speed. The training intensity throughout the training period was monitored using the progressive increase of time and running speed, which reached 1 h/day, 0% grade, at 50-60% of MRS on the third week (adapted from Melo [26]). The MRS test was also performed at the end of the 4th week of training in animals from NT8, HT8, NDT, and HDT groups in order to update the training intensity. Forty-eight hours after the last training session, the MRS test was repeated in all animals, and after 4 weeks of detraining in animals from NC12, NDT, HC12, and HDT groups to evaluate their total exercise time until fatigue (TTF).

During the experimental period, the animals in control groups (NC8, NC12, HC12, and HC8) were handled every day and submitted to a short period of mild exercise (5–10 min, 0% grade, 0.3 km/h, 3 days/week). This exercise intensity and duration were below the levels required to evoke training adaptation [27]. The body weight (BW) from all rats was measured every week. The systolic arterial pressure (SAP) and resting heart rate (RHR) were recorded at the beginning and after 48 h of each experimental period by the tail-cuff methods previously described [19].

2.3. Cardiomyocyte isolation

Two days after the last MRS test, the rats were weighed and killed by cervical dislocation under resting conditions, and their hearts were quickly removed. Left ventricular myocytes were enzymatically isolated as previously described [28]. Briefly, the hearts were mounted on a Langendorff system and perfused for ~5 min with a modified Hepes-Tyrode solution of the following composition (in mM): 130 NaCl, 1.43 MgCl₂, 5.4 KCl, 0.75 CaCl₂,5.0 Hepes, 10.0 glucose, 20.0 taurine, and 10.0 creatine, pH 7.3 at 37 °C. The perfusion solution was changed for the calcium-free solution with EGTA (0.1 mM) for 6 min. Afterwards, the hearts were perfused for 15-20 min with a solution containing 1 mg/ml collagenase type II (Worthington, USA). The digested heart was then removed from the cannula, and the ventricles were removed and weighed. The left ventricle was separated, weighed, and cut into small pieces. The left ventricle tissues were placed into small conical flasks with collagenase-containing solution supplemented with 1% bovine serum albumin. The cells were dispersed by agitating the flasks at 37 °C for periods of 5 min. Then, single cells were separated from the non-dispersed tissue by filtration. The resulting cell suspension was centrifuged and resuspended in Hepes-Tyrode solution. Non-dispersed tissue was subjected to further enzyme treatment. The isolated cells were stored at 5 °C until use. Only calcium-tolerant, quiescent, rod-shaped cardiomyocytes showing clear cross-striations were studied. The isolated cardiomyocytes were used within 2-3 h of isolation.

2.4. Intracellular Ca^{2+} measurements

Intracellular calcium $([Ca²⁺]_i)$ transients were evaluated as previously described [29]. Briefly, freshly isolated cardiomyocytes obtained from resting left ventricles were loaded with 5 µM fluo-4 AM (Molecular Probes, Eugene, OR, USA) for 20 min at room temperature and then washed with an extracellular Hepes–Tyrode solution to remove excess dye. $[Ca²⁺]_i$ transients were elicited by field-stimulating cardiomyocytes through a pair of platinum electrodes with a 0.2 ms supra-threshold voltage square pulse. Cells were stimulated at 1 Hz to produce steady-state conditions. A Meta LSM 510 scanning system (Carl Zeiss GmbH,

Germany) with a × 63 oil immersion objective was used for confocal fluorescence imaging. Fluo-4 was excited at 488 nm (argon laser), and emission intensity was measured at 510 nm. For recording $[Ca^{2+}]_i$ transients, myocytes were scanned with a 512-pixel line positioned randomly along the longitudinal axis of the cell, although care was taken to avoid crossing the nuclei. Cells were scanned every 1.54 ms, and sequential scans were stacked to create two-dimensional images with time on the x-axis. Digital image processing was performed using routines custom-written in the Matlab® platform. Ca²⁺ levels were reported as F/F₀, where F is the maximal fluorescence intensity average measured at the peak of $[Ca^{2+}]_i$ transients, and F₀ is the baseline fluorescence intensity measured at the diastolic phase of $[Ca^{2+}]_i$ transients. The time to the peak of $[Ca^{2+}]_i$ transient and time from peak transient to half resting level of $[Ca^{2+}]_i$ were also determined.

2.5. Measurements of cell contractility

Cell contractility was evaluated as previously described [30]. Briefly, isolated cells were placed in a chamber with a glass coverslip base mounted on the stage of an inverted microscope (Nikon Eclipse -TS100, USA). The chamber was perfused with Hepes-Tyrode solution at room temperature. Steady-state 1 Hz contractions were elicited via platinum bath electrodes (Myopacer, Field Stimulator, Ionoptix, USA) with 5 ms duration voltage pulses and an intensity of 20 V. Cells were visualized on a PC monitor with a NTSC camera (Myocam, Ionoptix, USA) in partial scanning mode. This image was used to measure cell shortening (our index of contractility) in response to electrical stimulation using a video motion edge detector (IonWizard, Ionoptix, USA). The cell image was sampled at 240 Hz. Cell shortening was calculated from the output of the edge detector using an IonWizard A/D converter (Ionoptix, Milton, MA, USA). Cell shortening (expressed as a percentage of resting cell length), maximal velocity of contraction, and relaxation were calculated.

2.6. Western blots

Western blots were performed as previously described [31] with some modifications. Briefly, liquid-nitrogen-frozen left ventricles isolated from rats were homogenized in a buffer containing 50 mM potassium phosphate buffer (pH 7.0), 0.3 M sucrose, 0.5 mM DTT, 1 mM EDTA (pH 8.0), 0.3 mM PMSF, 10 mM NaF, and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich, USA). Samples were subjected to SDS-PAGE in polyacrylamide gels (6% or 8% depending on protein molecular weight). After electrophoresis, proteins were electrotransferred to nitrocellulose membrane (Amersham Biosciences, USA). Equal loading of samples (60 µg) and even transfer efficiency were monitored with the use of 0.3% Ponceau staining of the blot membrane. The blotted membrane was then blocked (5% nonfat dry milk, 10 mM Tris HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature and incubated with specific antibodies overnight at 4 °C. The following primary antibodies (Abcam, USA) were used: polyclonal antibodies for total phospholamban (PLBt, 1:1000), phosphorylated phospholamban at serine 16 (PLB_{ser16};1:1000), and sarcoplasmic reticulum Ca²⁺ ATPase (SERCA2a; 1:2500); monoclonal antibodies for Na⁺/Ca²⁺ exchanger (NCX;1:1000) and GAPDH (1:2000). Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (rabbit 1:2500; mouse 1:2000) depending on the protein, for 1:30 h at room temperature. Immunocomplexes were detected by chemiluminescent reaction (ECL kit; Amersham Biosciences, USA) followed by densitometric analyses with the software ImageJ. GAPDH expression levels were used to normalize the results.

2.7. mRNA expression by quantitative real-time PCR

The relative gene expression of ANF (atrial natriuretic factor), skeletal α -actin, α -MHC (myosin heavy chain), and β -MHC were analyzed by quantitative real-time polymerase chain reaction (q-RT-PCR) as previously described [32]. Briefly, frozen left ventricles samples (100 mg) were homogenized in Trizol (1 ml) and RNA was isolated according to the manufacturer's instructions (Invitrogen Life Technologies, UK). Samples were quantified by spectrophotometer at 260 nm and checked for integrity by EtBr-agarose gel electrophoresis. RNA was primed with 0.5 µg/µl oligo (dT) (12–18 bp) (Invitrogen Life Technologies, UK) to generate the first strand cDNA. Reverse transcription (RT) was performed using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, UK).

Primers were designed using Primer 3 software (http://frodo.wi. mit.edu/primer3/). DNA sequence was obtained from GenBank ID: BA123456, and primers were made in separate exons to distinguish by size PCR products derived from cDNA from those derived from genomic DNA contaminants. The mRNA expression of pathological markers of cardiac hypertrophy were assessed by oligonucleotides primers as follows: for ANF,5'-CTT CGG GGG TAG GAT TGA C-3' and 5'-CTT GGG ATC TTT TGC GAT CT-3'; skeletal α -actin, 5'-ACC ACA GGC ATT GTT CTG GA-3' and 5'-TAA GGT AGT CAG TGA GGT CC-3'; α -MHC,5'-CGA GTC CCA GGT CAA CAA G-3' and 5'-AGG CTC TTT CTG CTG GAC C-3'; and β -MHC,5'-CAT CCC CAA TGA GAC GAA G-3' and 5'-AGG CTC TTT CTG CTG GAC A-3'.

Real-time quantification of the target genes was performed with a SYBRgreen PCR Master Mix, (Applied Biosystems, USA) using ABI PRISM 7700 Sequence Detection System (Applied Biosystems, USA). The expression of GAPDH (5'-GCT GAT GCC CCC ATG TTT G-3' and 5'-ACC AGT GGA TGC AGG GAT G-3') was measured as an internal control for sample variation in RT reactions. An aliquot of the RT reaction was used for 50-cycle PCR amplification in the presence of SYBRgreen fluorescent dye, according to a protocol provided by the manufacturer (Applied Biosystems, USA). PCR product generation was monitored by measuring the increase in fluorescence caused by the SYBRgreen binding to double-stranded DNA at each annealing phase. A dissociation curve was generated at the end of the reaction to verify that a single product was amplified. Each ventricle sample was analyzed in triplicate. Relative quantities of target gene expressions of rats groups were compared after normalization to the values of GAPDH [change in threshold cycle (Δ CT)]. Fold change in mRNA expression was calculated using the differences in Δ CT values between the two samples ($\Delta\Delta CT$) and the equation $2^{-\Delta\Delta CT}$.

2.8. Statistical analysis

Two-way ANOVA for repeated measurements with post-hoc testing by Tukey were used to compare the effects of training and hypertension on initial and final values of BW, SAP, and RHR. Two-way ANOVA with post-hoc testing by Tukey were used to compare the effects of training and hypertension on final values of BW, RHR, TTF, ventricular weight (VW), $[Ca^{2+}]_i$ transients, cellular contractility, and protein and gene expression. The relationship between $[Ca^{2+}]_i$ transients and TTF was assessed by linear regression. Results are means \pm SEM. A statistical significance level of 5% was adopted.

3. Results

3.1. General characteristics and physical capacity

General characteristics and physical capacity from each experimental group are summarized in Table 1. All animals gained BW from the beginning to the end of the experimental period (P<0.05). SHR animals had significantly elevated VW to BW ratio, and left ventricular weight (LVW) to BW ratio compared to normotensive rats,

Table 1

Body and ventricular weights, cardiovascular parameters and physical capacity.

	NC8	NT8	HC8	HT8	NC12	NDT	HC12	HDT
Initial BW (g)	395 ± 12	380 ± 11	364 ± 11	355 ± 10	369 ± 12	397 ± 12	347 ± 11	364 ± 13
Final BW (g)	$427\pm13^{\#}$	$423\pm12^{\#}$	$415 \pm 12^{\#}$	$392 \pm 11^{\#}$	$426 \pm 14^{\#}$	$458\pm14^{\#}$	$417 \pm 13^{\#}$	$441 \pm 15^{\#}$
VW (g)	1.69 ± 0.11	1.97 ± 0.10	1.93 ± 0.10	2.04 ± 0.10	1.68 ± 0.09	1.87 ± 0.08	1.83 ± 0.08	$2.12\pm0.10^{\ddagger}$
VW/BW (mg/g)	3.97 ± 0.24	$4.67 \pm 0.11^{\$}$	$4.63 \pm 0.11^+$	$5.36 \pm 0.11^{+\infty}$	4.00 ± 0.20	4.08 ± 0.20	4.41 ± 0.19	$4.83 \pm 0.21^+$
LVW (g)	1.32 ± 0.09	1.48 ± 0.08	1.48 ± 0.08	1.51 ± 0.08	1.33 ± 0.05	1.41 ± 0.05	1.39 ± 0.05	$1.59 \pm 0.06^{+\ddagger}$
LVW/BW (mg/g)	3.09 ± 0.18	$3.51 \pm 0.17^{\$}$	$3.56 \pm 0.17^+$	$3.95 \pm 0.16^{+\infty}$	3.15 ± 0.14	3.09 ± 0.14	3.33 ± 0.13	$3.64 \pm 0.15^+$
Initial SAP (mm Hg)	110.0 ± 4.3	112.0 ± 2.9	$176.6 \pm 3.9^+$	$176.2 \pm 4.8^+$	110.0 ± 4.0	110.7 ± 4.0	$182.0 \pm 4.8^+$	$178.7 \pm 5.3^+$
Final SAP (mm Hg)	107.0 ± 3.2	110.0 ± 3.2	$178.0\pm3.2^+$	$161.2 \pm 3.6^{\# + \infty}$	110.0 ± 4.4	114.3 ± 4.4	$183.0 \pm 5.2^+$	$182.5\pm5.9^+$
Initial RHR (bpm)	328 ± 8	340 ± 8	$359\pm8^+$	$369 \pm 8^+$	352 ± 10	356 ± 10	359 ± 9	373 ± 10
Final RHR (bpm)	330 ± 11	$322\pm10^{\#}$	352 ± 11	$344 \pm 10^{\#}$	364 ± 13	347 ± 13	$407 \pm 12^{#+}$	$393 \pm 13^{\#+}$
TTF (min)	11.36 ± 1.54	$22.45 \pm 1.42^{\$}$	11.65 ± 1.42	$24.48\pm1.33^{\circ\circ}$	11.39 ± 1.19	14.37 ± 1.10	11.67 ± 1.10	14.83 ± 1.10

Data are mean \pm SEM of 8 animals in each group. NC8, normotensive control for 8 weeks. NT8, normotensive trained for 8 weeks. HC8, hypertensive control for 12 weeks. HD7, hypertensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HD7, hypertensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HD7, hypertensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HD7, hypertensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HD7, hypertensive trained for 8 and detrained for 4 weeks. BW, body weight; VW, ventricular weight; LVM, left ventricular weight; SAP, systolic arterial pressure; RHR, resting heart rate; TTF, total exercise time until fatigue. #, significantly different from baseline value within the same group. +, significantly different from respective normotensive group. *, significantly different from HC8. § significantly different from HC12.

indicating ventricular hypertrophy caused by hypertension. The LIET induced ventricular/left ventricular hypertrophy in both SHRs and normotensive rats, and these adaptations were reversed within 4 weeks of DET (P<0.05). Resting SAP was higher (P<0.05) in SHRs compared to normotensive animals. SAP decreased (P<0.05) in the HT8 animals by the end of LIET when compared to HC8 animals, while DET reversed this adaptation. RHR was higher in SHRs compared to normotensive animals. LIET decreased (P<0.05) the RHR in animals from NT8 and HT8, which was reversed by DET. After 8 weeks of LIET, the TTF of the NT8 and HT8 groups were significantly longer than those of NC8 and HC8 groups, respectively. However, after 4 weeks of DET the TTF of normotensive and hypertensive exercised animals regressed to levels corresponding to those of untrained animals.

exhibited $[Ca^{2+}]_i$ transients amplitude $(2.72 \pm 0.06 \text{ F}/F_0 \text{ and } 2.82 \pm 0.05 \text{ F}/F_0$, respectively) higher than those from NC8 $(2.39 \pm 0.06 \text{ F}/F_0)$ and HC8 $(2.28 \pm 0.05 \text{ F}/F_0)$; however DET totally reversed this adaptation (P<0.05). No significant differences were found in the $[Ca^{2+}]_i$ transient amplitude between normotensive and SHR rats, in both LIET and DET conditions. It is noteworthy that no differences (P>0.05) were observed in the baseline fluorescence intensity average, measured between contractions at the diastolic phase of $[Ca^{2+}]_i$ transients among all experimental groups.

LIET decreased the time to peak of $[Ca^{2+}]_i$ transient (Fig. 2B) and time from peak to half resting level of $[Ca^{2+}]_i$ (Fig. 2C) in cardiomyocytes from NT8 and HT8 groups, although after DET these adaptations were reversed (P<0.05). These parameters were significantly longer in cardiomyocytes from SHRs compared to those from normotensive rats before and after LIET and DET, except for time from peak to half resting level of $[Ca^{2+}]_i$ which was similar between NT8 and HT8 after LIET.

3.2. $[Ca^{2+}]_i$ transients

Fig. 1 shows typical line-scan images recorded from fieldstimulated rat cardiomyocytes loaded with the Ca^{2+} indicator fluo 4-AM. As shown in Fig. 2A, cardiomyocytes from NT8 and HT8 groups Furthermore, the relationship between the $[Ca^{2+}]_i$ transient amplitude, time to peak of $[Ca^{2+}]_i$ release, time from peak to half resting level of $[Ca^{2+}]_i$, and the TTF attained during the progressive test in normotensive and SHR rats were established. To this end, the peak



Fig. 1. Representative line-scan images recorded from field-stimulated cardiomyocytes loaded with the Ca^{2+} indicator fluo 4-AM (5 μ M). Ca^{2+} signal is shown as fluorescence ratio (F/F₀), with the fluorescence intensity (F) normalized to intensity at rest before stimulation (F₀). NC8, normotensive control for 8 weeks. NT8, normotensive trained for 8 weeks. HC8, hypertensive control for 8 weeks. HT8, hypertensive trained for 8 weeks. NC12, normotensive control for 12 weeks. NDT, normotensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HDT, hypertensive trained for 8 and detrained for 4 weeks.



Fig. 2. Cardiomyocyte calcium transients. (A) Amplitude of transients. (B) Time to peak. (C) Time from peak transient to half resting value. NC8, normotensive control for 8 weeks. NT8, normotensive trained for 8 weeks. HC8, hypertensive control for 8 weeks. HT8, hypertensive trained for 8 weeks. NC12, normotensive control for 12 weeks. NDT, normotensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HD7, hypertensive trained for 8 and detrained for 4 weeks. Data are mean \pm SEM of 70–80 cells in each group. *, significantly different from NC8. *, significantly different from NC12. *, significantly different from NDT.

of the $[Ca^{2+}]_i$ transient, time to peak of $[Ca^{2+}]_i$ and time from peak to half resting level of $[Ca^{2+}]_i$ for each rat was plotted against the individual point of fatigue (TTF; Fig. 3). As shown in Fig. 3, the TTF was strongly correlated (P<0.05) with these parameters.

3.3. Contractile activity

LIET increased cell shortening in NT8 (13.5%) and HT8 (14.7%) compared with NC8 and HC8, respectively, whereas DET reversed such adaptations (P<0.05; Figs. 4 and 5A). No significant differences were found in cell shortening between normotensive and SHR rats in both LIET and DET conditions, except for the HDT group which showed lower cell shortening compared with NDT and HC12 groups (P<0.05).

As shown in Figs. 5B and C, cardiomyocytes from NT8 and HT8 animals exhibited higher maximal velocity of shortening (14.5% and 25.3%, respectively) and maximal velocity of relengthening (20.5% and 53.4%, respectively) compared with those from NC8 and HC8, respectively. However, these adaptations were completely reversed within 4 weeks of DET (P<0.05). These shortening and relengthening velocities were significantly slowed in cardiomyocytes of the SHRs compared with normotensive rats before and after LIET and DET, except for maximal velocity of shortening, which was similar between NC12 and HC12 after DET.

3.4. Calcium regulatory proteins

LIET induced a significant increase in the left ventricle expression of SERCA2a and PLB_{ser16} and decreased the PLBt/SERCA2a ratio of animals from NT8 and HT8 groups, although these changes were reversed to control values within 4 weeks of DET in both groups (Fig. 6). The left ventricle contents of PLBt and NCX were not affected by either LIET or DET in both normotensive and SHR rats (P>0.05). No significant differences were found in the expression of PLBt, PLB_{ser16}, SERCA2a, or NCX, or in the PLB/SERCA2a ratio between normotensive and SHR rats after LIET and DET (Fig. 6).

3.5. Molecular markers of pathological cardiac hypertrophy

Pathological cardiac hypertrophy is characterized by the induction of genes normally expressed during fetal development, such as ANF, and pathological cardiac hypertrophy markers such as skeletal α -actin and decreased α/β -MHC ratio. Accordingly, as shown in Fig. 7, hypertension increased (P<0.05) the expression of ANF (300%), skeletal α -actin (250%), and decreased the α/β -MHC ratio (70%) in the left ventricle of SHRs compared to normotensive rats. However, LIET was able to normalize these parameters in SHRs, whereas within 4 weeks of DET all these benefits were abolished (P<0.05). No significant changes were found in the expression of ANF, skeletal α -actin, or α/β -MHC ratio in the left ventricles of normotensive rats after either LIET or DET (Fig. 7).

4. Discussion

The aim of the present study was to verify the effects of LIET and DET on the mechanical and molecular properties of cardiomyocytes isolated from spontaneously hypertensive rats.

LIET increased the exercise capacity (i.e., TTF) in normotensive and hypertensive rats and was efficient in decreasing the SAP in SHR animals, although this was reversed within 4 weeks of DET. This hypotensive effect of regular exercise in hypertensive individuals is well-established in the literature [1,11,12,33]. It is noteworthy that exercise intensity influences its pressure-lowering effect, inasmuch as larger reductions are detected at lower exercise training intensities [13,26,34–36].

The exercise regime used here decreased the RHR in both SHRs and normotensive rats, although 4 weeks of DET reversed these adaptations. These results show that aerobic conditioning improved physiological variables relevant to the assessment of cardiovascular health, since RHR is associated with increased life expectancy and decreased occurrence of cardiovascular events [37,38]. Epidemiological data show that a high RHR is associated with increased cardiovascular morbidity and mortality in the whole population, and also it has been associated with poor prognosis in patients with cardiovascular disease [38–40].

SHR shows an increased sympathetic drive directed to the heart and blood vessels even in resting conditions thus contributing to sustain high blood pressure levels in this rat strain [41,42]. Long term aerobic exercise reduces the sympathetic drive to periphery and contributes to reduce heart rate and blood pressure [34,43]. Despite no direct assessment of the sympathetic drive, heart rate reduction at



Fig. 3. Relationship between total exercise time until fatigue (TTF) and $[Ca^{2+}]_i$ transient amplitude, time to peak and time from peak transient to half resting in normotensive (A, C and E) and SHR (B, D and F) animals. Open/filled triangles and circles represent the means of individual SHR and normotensive rats. NC8, normotensive control for 8 weeks. NT8, normotensive trained for 8 weeks. HC8, hypertensive control for 8 weeks. HT8, hypertensive trained for 8 weeks. NC12, normotensive control for 12 weeks. NDT, normotensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HDT, hypertensive trained for 8 and detrained for 4 weeks.

rest suggests attenuation of sympathetic activity in the exercised animals. This may explain the improvement of mechanical and molecular properties of left ventricular myocytes.

The exercise training protocol increased VW/BW and LVW/BW ratios in normotensive animals which were reversed within 4 weeks of DET. Exercise-induced cardiac hypertrophy is reversible and is characterized by normal cardiac morphology (i.e., no fibrosis or apoptosis) and normal or enhanced cardiac function [4,44]. In SHR animals, the exercise training superimposed to hypertension increased VW/BW and LVW/BW ratios, which were also reversed by DET. Pathological cardiac hypertrophy is associated with cell death, cardiac dysfunction, reduced contractility, increased interstitial fibrosis, decreased vascularization, and re-expression of fetal genes (i.e., ANF, skeletal α -actin, and β -MHC) [4,45,46]. Our data confirm this type of cardiac hypertrophy in SHRs by showing a slower cardiomyocyte [Ca²⁺]_i transient and contractility, an increased expression of cardiac ANF and skeletal α -actin, as well as a decreased α/β -MHC ratio, as compared to normotensive rats. However, the LIET applied was able to hasten and increase the cardiomyocyte $[Ca^{2+}]_i$ transient and contractility in SHR left ventricles. In addition, the LIET normalized the expression of ANF, skeletal α -actin, and the α/β -MHC ratio in the SHR left ventricles, supporting the idea that endurance training impacts beneficially on cardiac function, even in the presence of pathological cardiac hypertrophy. Nevertheless, within 4 weeks of DET such improvements returned to control levels.

Most important, our results demonstrate that LIET induced lusitropic and inotropic benefits to the cardiomyocyte function in SHR animals. For example, an enhanced systolic function was observed as it leaded to an increased cardiomyocyte $[Ca^{2+}]_i$ transient and shortening, and faster rates of $[Ca^{2+}]_i$ release and contraction in SHRs and normotensive animals. Moreover, LIET improved the diastolic function as it leaded to faster rates of cardiomyocyte relengthening due to a faster $[Ca^{2+}]_i$ transient decay, in both hypertensive animals. However, within 4 weeks of



Fig. 4. Representative records of cardiomyocytes contractions. Values are percentages of resting cell length (% r.c.l.). NC8, normotensive control for 8 weeks. NT8, normotensive trained for 8 weeks. HC8, hypertensive control for 8 weeks. HT8, hypertensive trained for 8 weeks. NC12, normotensive control for 12 weeks. ND7, normotensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HD7, hypertensive trained for 8 and detrained for 4 weeks.

DET, these beneficial adaptations regressed to values corresponding to those of the untrained hypertensive and normotensive animals.

The cardiomyocyte $[Ca^{2+}]_i$ transient, contraction and relaxation are modulated by regulatory calcium handling proteins [47–49]. Despite the slower $[Ca^{2+}]_i$ transient and contractility in SHR cardiomyocytes, our results demonstrate that hypertension did not affect the left ventricle expression of PLBt, PLB_{ser16}, SERCA2a, or NCX in these animals. Similar results have been reported previously [15,16,50]. However, LIET increased the expression of SERCA2a and PLB_{ser16}, and reduced the PLBt/SERCA2a ratio without changes in NCX and PLBt levels in both hypertensive and normotensive rats. The reduced PLBt/SERCA2a ratio would increase the Ca²⁺ sensitivity and the activity of SERCA2a, since PLB is the primary inhibitor of SERCA2a [51]. Therefore, as SERCA2a expression increases with no change in PLBt expression, the decreased PLBt/SERCA2a ratio induced by LIET indicates that SERCA2a is less inhibited.

Increased left ventricle phosphorylation levels of phospholamban at serine 16 in both normotensive and hypertensive exercised groups suggest increased sympathetic drive to the heart. Stimulation of β -adrenergic receptors increases phospholamban phosphorylation at serine 16 by a cAMP/PKA mediated process as has been reported in the literature [47,52]. This mechanism, however, seems to be unlike in our study. Other subcellular cascades also modulating phospholamban phosphorylation/dephosphorylation [47,53] may account for this unexpected finding. Further studies, however, are necessary to explore this subject.

The increase in SERCA2a expression also may affect inotropy, leading to prolonged ventricular filling time and higher loading of Ca^{2+} in the sarcoplasmic reticulum [54]. Increased expression of SERCA2a and PLB_{thr17} without alteration of PLBt levels in response to highintensity exercise training has being shown elsewhere [24,54]. In hypertensive animals, data in the literature are controversial since an increased myocardium expression of SERCA2a with no changes in the NCX levels was reported [19] and also that increased phosphorylated levels of PLB_{ser16} with no changes in SERCA2a was observed in the myocardium after LIET [15]. Someone could argue that a higher effective SERCA activity leads to a greater SR Ca^{2+} content that provides a possible arrhythmogenic substrate under some conditions. Resting ECG recordings were obtained in all animals at the end of



Fig. 5. Cardiomyocyte contractile function. (A) Cell shortening expressed as % of resting cell length. (B) Maximal velocity of shortening. (C) Maximal velocity of relengthening. NC8, normotensive control for 8 weeks. NT8, normotensive trained for 8 weeks. HC8, hypertensive control for 12 weeks. NT8, hypertensive trained for 8 weeks. NC12, normotensive control for 12 weeks. ND7, normotensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HDT, hypertensive trained for 8 and detrained for 4 weeks. Data are mean \pm SEM of 90 cells in each group. *, significantly different from NC8. *, significantly different from NC12. *, significantly different from NC12. *, significantly different from NC12. *, significantly different from NC12.

treatments and we observed premature ventricular beats in 2 rats of the HC8 group only (data not shown).

Therefore, the increased left ventricle expression of PLB_{ser16} , and SERCA2a, and reduced PLBt/SERCA2a ratio are likely explanations for the increased cardiomyocyte $[Ca^{2+}]_i$ transient amplitude and shortening, faster $[Ca^{2+}]_i$ transient decay and relengthening observed here in cardiomyocytes from hypertensive and normotensive trained animals. Nevertheless, all these improvements were reversed within 4 weeks of DET.

We also verified the associations of the cardiomyocyte function with the exercise capacity (i.e., TTF). Larger improvements of TTF correlated rather closely with different changes in cellular features, such as improved systolic contraction (i.e., larger $[Ca^{2+}]_i$ transient amplitude and reduced time to the peak of $[Ca^{2+}]_i$) and enhanced



Fig. 6. Protein expression in the left ventricle. (A) Total phospholamban (PLBt). (B) Phosphorylated phospholamban at serine 16 (PLB_{ser16}). (C) Sarcoplasmic reticulum Ca²⁺ ATPase (SERCA2a). (D) Na⁺/Ca²⁺ exchanger (NCX). (E) PLBt/SERCA2a ratio. Targeted bands were normalized by GAPDH expression. NC8, normotensive control for 8 weeks. NT8, normotensive trained for 8 weeks. HC8, hypertensive control for 8 weeks. HT8, hypertensive trained for 8 weeks. NC12, normotensive control for 12 weeks. NDT, normotensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HDT, hypertensive trained for 8 and detrained for 4 weeks. Data are mean \pm SEM of 6–7 animals in each group. *, significantly different from NC8. #, significantly different from HC8.

diastolic filling (i.e., reduced time to the $[Ca^{2+}]_i$ decay) as a function of LIET as well as DET, when these improvements were reversed.

5. Conclusion

In conclusion, LIET improves left ventricular myocyte contractile function in SHR animals, which is associated with improved Ca^{2+} handling via upregulation of SERCA2a and PLB_{ser16} expression and

reduction of the PLB/SERCA2a ratio. Our exercise regime also minimized the increase of pathological cardiac hypertrophy. All these mechanical and molecular beneficial adaptations induced by LIET were reversed within 4 weeks of DET.

These findings have clinical relevance to the design of therapeutic strategies based on regular LIET for the prevention of heart failure as a consequence of hypertension-induced cardiac hypertrophy progression. It also indicates the benefits resultant from the maintenance of a physically active lifestyle.



Fig. 7. Gene expression in the left ventricle. (A) Atrial natriuretic factor (ANF). (B) Skeletal α -actin. (C) α/β -myosin heavy chain (MHC) ratio. Targeted genes were normalized by GAPDH mRNA. NC8, normotensive control for 8 weeks. NT8, normotensive trained for 8 weeks. HC8, hypertensive control for 8 weeks. HT8, hypertensive trained for 8 weeks. NC12, normotensive control for 12 weeks. NDT, normotensive trained for 8 and detrained for 4 weeks. HC12, hypertensive control for 12 weeks. HDT, hypertensive trained for 8 and detrained for 4 weeks. Data are mean $\pm\,\text{SEM}$ of 5-6 animals in each group. #, significantly different from NC8. *, significantly different from HC8.[†], significantly different from NC12.[‡], significantly different from NDT

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Disclosures

None.

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