Cardiac effects of acute exhaustive exercise in a rat model ☆

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A B S T R A C T

Background: The role of physical exercise in the prevention and treatment of cardiovascular diseases has been well described, however, elevations in cardionecrotic biomarkers after prolonged exercise (i.e. ultramarathon running) were observed. We aimed at understanding the biochemical, molecular biological, structural and functional alterations in the heart after exhaustive exercise in a rat model.
Methods: Rats of the exercise group were forced to swim for 3 h with 5% body weight (workload) attached to the tail, control rats were taken into the water for 5 min. After a 2-hour recovery period we performed left ventricular (LV) pressure-volume analysis to investigate LV function and mechanoenergetics. Additionally, blood and myocardium samples were harvested for biochemical and histological examinations. Gene expression changes were detected by qRT-PCR.
Results: When compared to controls, elevated plasma levels of cardiac troponin T and creatine kinase were detected after exhaustive exercise. Histological analysis showed sporadic fragmentation of myocardial structure and leukocyte infiltration in the exercised group. We observed increased end-systolic volume, decreased ejection fraction, impaired contractility (preload recruitable stroke work) and mechanoenergetics (ventriculoarterial coupling, mechanical efficiency) of LV after exercise. Myocardial expression of major antioxidant enzymes was increased along with increased myocardial nitro-oxidative stress. Bax/Bcl-2 ratio and TUNEL staining showed enhanced apoptotic signaling. Exhaustive exercise also resulted in the dysregulation of the matrix metalloproteinase system.
Conclusions: Excessive physical activity has an adverse effect on the heart. The observed functional impairment is associated with increased nitro-oxidative stress, enhanced apoptotic signaling and dysregulation of the matrix metalloproteinase system after exhaustive exercise.

1. Introduction

The role of regular physical activity is widely recognized in the prevention, management and treatment of several cardiovascular diseases, such as coronary heart disease [1,2]. Although regular exercise training reduces cardiovascular risk, recent studies have documented elevations in biomarkers consistent with cardiac damage (i.e. cardiac troponin (cTn)) after bouts of prolonged exercise in apparently healthy individuals without cardiovascular disease [3–6], which has raised concerns about cardiovascular health consequences of such exercise [7]. Prolonged and intensive physical activities such as ironman triathlon, cross country biking or ultramarathon running are becoming increasingly popular.

Numerous studies on humans [4,5] and animals [8,9] have reported elevated serum cardiac troponins — as highly specific and sensitive laboratory markers for biochemical detection of myocardial injury — after prolonged, exhaustive exercises that can exceed clinical cut-off value of myocardial infarction. Increased levels of reactive oxygen and nitrogen species, thus nitro-oxidative stress have been demonstrated after excessive physical exercise in human studies [10,11]. It is also well described that exhaustive exercise induced reactive oxygen species (ROS) generation leads to oxidative damage in rat myocardium [12]. Excessive ROS formation causes cellular dysfunction, protein and lipid peroxidation and DNA injury. These processes can lead to irreversible cell damage and death, which have been implicated in a wide range of pathological cardiovascular conditions [13].

Cardiac functional effects of acute bouts of exhaustive exercise have been investigated in human studies using echocardiography and MRI [14,15]. The observed transient impairment in left ventricular (LV)

function has been described as "exercise-induced cardiac fatigue" [16], however controversial data and study design differences result in uncertain conclusions [15,17]. Small animal studies also investigated exhaustive exercise induced cardiac functional changes by echocardiography [18,19] and although a few basic hemodynamic parameters were previously measured, they were largely dependent on loading conditions. Therefore, appropriate, hemodynamically validated standards are required for reliable investigation of exhaustive exercise induced cardiac functional changes. Pressure-volume (P-V) analysis is a useful approach for investigating several aspects of in vivo LV function independently from loading conditions. Advances of the last decade in the technical development and validation of miniature P-V catheters have made it possible to use this approach for studies in small animals [20].

The purpose of this study was to provide the first detailed in vivo characterization of left ventricular hemodynamic changes after an acute bout of exhaustive exercise using P-V analysis. For validation of our rat model we determined key markers of already described cellular and molecular mechanisms leading to myocardial injury as a consequence of excessive exercise.

2. Materials and methods

2.1. Animals

All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). All procedures and handling of the animals during the study were reviewed and approved by the Ethical Committee of Hungary for Animal Experimentation.

Forty-eight young adult (12-wk old, m = 325–375 g) male Wistar rats (Toxi-Coop, Dunakeszi, Hungary) were housed in a room with constant temperature of 22 ± 2 °C with a 12/12 h light–dark cycle and fed a standard laboratory rat diet ad libitum and free access to water.

2.2. Experimental design

A 150-l water tank was divided into 6 lanes with a surface area of 20×25 cm and a depth of 45 cm per lane and filled with tap water maintained at 30–32 °C to allow individual swim exercise. Attempting to minimize the general stress response, all rats were familiarized with swimming for 20 min 48 h before the experiments. Acute exercised rats (n = 26) were forced to swim for 3 h with a workload (5% of body weight) attached to the tail, as previously described [9]. A total of four rats needed to be rescued from the water and removed from the study due to an inability to complete the exercise protocol. Control rats (n = 22) were taken into the water for 5 min. After the exercise, rats were dried and twenty-four animals (12–12 exercised and control, group A) were sacrificed 2 h after swimming to collect blood and tissue samples. The 2 h resting period was chosen according to previous literature data [9]. The remaining twenty rats (10–10 exercised and control, group B) underwent hemodynamic measurements after the 2 h period of observation. In order to eliminate diurnal effects, the experiments were performed at the same time of the day.

2.3. Biochemical measurements

Twenty-four rats (group A) were anesthetized with a mixture of ketamine (100 mg/kg ip.) and xylazine (3 mg/kg ip.). The abdominal cavity was quickly opened, and blood samples were collected from the inferior vena cava in tubes pre-rinsed with EDTA. Blood samples were centrifuged and plasma cardiac troponin T (cTnT), creatine kinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and creatinine were measured by automated clinical laboratory assays on a Cobas Integra 400 (Roche Diagnostics, Mannheim, Germany) autoanalyzer.

2.4. Histology

Myocardial samples of twenty rats (group B) were removed for histological processing immediately after completing the LV P-V analysis. Tissue samples were snap frozen or fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Transverse transmural slices of the ventricles were sectioned (5 μm) and processed conventionally for histological examination. One series of sections were stained with hematoxylin and eosin (HE). Light microscopic examination was performed with a Zeiss microscope (Axio Observer.Z1, Carl Zeiss, Jena, Germany) at a magnification of 400×, and digital images were captured by an imaging software (QCapture Pro 6.0, QImaging, Canada).

In situ detection of ROS was performed by using the oxidative fluorescent dye dihydroethidium (DHE; Sigma-Aldrich, St. Louis, USA). DHE is freely permeable to cell membranes and emits a red fluorescent signal when oxidized by ROS to ethidium, which is intercalating into DNA (typically nuclear localization). Fresh frozen LV myocardial

sections (16 μm) were incubated with 1 μM DHE (in PBS; pH 7.4) at 37 °C for 30 min in a dark humidified chamber [21]. Fluorescence in myocardial sections was visualized using a fluorescence microscope (Axio Observer.Z1, Carl Zeiss, Jena, Germany) with a 590 nm long-pass filter after background corrections to saline treated negative control. Eight images were taken randomly each of the slides and fluorescence area and intensity were analyzed using ImageJ (NIH, Bethesda, MD, USA) image analysis software.

To demonstrate nitrative stress, tyrosine nitration was detected in LV myocardial sections by immunohistochemistry [22]. Paraffin-embedded sections of myocardium were deparaffinized. After antigen retrieval (0.1 M citrate buffer, pH3, heating in microwave oven for 15 min) and quenching endogenous peroxidase with 0.3% H_2O_2 in 100% methanol for 15 min, slides were immunostained with a rabbit anti-nitrotyrosine antibody (1:200, Millipore, Bedford, MA, USA) overnight at 4 °C. Specific labeling was detected by incubation for 30 min at room temperature with a biotin-conjugated anti-rabbit goat antibody (Vector Laboratories, Burlingame, CA, USA) and amplified with an avidin–biotin peroxidase complex (Vector Laboratories). Nickel–cobalt enhanced diaminobenzidine (Vector Laboratories) was used as chromogen. Nitrotyrosine positive area was calculated using ImageJ.

Apoptosis in cardiomyocytes was determined with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. TUNEL staining was performed using DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Sections were counterstained by the red colored Nuclear Fast Red (Sigma-Aldrich, St. Louis, MO, USA). Thirty visual fields of heart sections were randomly selected in each animal, and TUNEL-positive cells were counted. Data are expressed as mean number of apoptotic cells per field.

2.5. Cardiac mRNA analysis

After the blood sample collection from twenty-four (group A) rats the chest cavity was quickly opened and the heart was removed. Left ventricular tissues were immediately snap frozen in liquid nitrogen, and stored at −80 °C. LV tissue was homogenized in RLT buffer, RNA was isolated from the ventricular samples by using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified by measuring optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio approximately 2.0. Reverse transcription reaction (1 μg total RNA of each sample) was completed by using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA) in triplicates of each sample in a volume of 10 μl in each well containing cDNA (1 μl), TaqMan® Universal PCR MasterMix (5 μl) and a TaqMan® Gene Expression Assay for the following targets (0.5 μl): atrial natriuretic factor (ANF; Rn00561661_m1), catalase (assay ID: Rn00560930_m1), glucose-6-phosphate dehydrogenase (G6PD; assay ID: Rn00566576_m1), gluthathione peroxidase 1 (GPX-1, Rn00577994_g1), glutathione reductase (GSR, assay ID: Rn01482159_m1), thioredoxin-1 (assay ID: Rn00587437_m1), superoxide dismutase 2 (SOD-2, assay ID: Rn00690587_g1), endothelial nitric oxide synthase (eNOS, assay ID: Rn02132634_s1), Bcl-2 associated X protein (Bax, assay ID: Rn02532082_g1), Bcl-2 (assay ID: Rn99999125_m1), matrix metalloproteinase-2 (MMP-2, assay ID: Rn01538170_m1), tissue inhibitor of metalloproteinase-2 (TIMP-2, assay ID: Rn00573232_m1), matrix metalloproteinase-9 (MMP-9, assay ID: Rn00579162_m1), tissue inhibitor of metalloproteinase-1 (TIMP-1, assay ID: Rn00587558_m1) and transforming growth factor β1 (TGF-β1, assay ID: Rn00572010_m1), all purchased from Applied Biosystems. Gene expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; reference gene; assay ID: Rn01775763_g1) and expression levels were calculated by the C_T comparative method $(2^{-\Delta CT})$. All results are expressed as values normalized to a positive calibrator (a pool of cDNA's from all samples of the control group).

2.6. Hemodynamic measurements — left ventricular pressure-volume (P-V) analysis

Twenty rats (group B) were anesthetized with pentobarbital (60 mg/kg ip.), tracheotomized, and intubated to facilitate breathing. Animals were placed on controlled heating pads, and the core temperature, measured via rectal probe, was maintained at 37 °C. A polyethylene catheter was inserted into the left external jugular vein for fluid administration. A 2-Fr microtip pressure-conductance catheter (SPR-838, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the ascending aorta and after stabilization for 5 min, mean arterial blood pressure (MAP) was recorded. After that, the catheter was advanced into the left ventricle under pressure control. After stabilization for 5 min, signals were continuously recorded at a sampling rate of 1000 samples/s using a P-V conductance system (MPVS-Ultra, Millar Instruments) connected to the PowerLab 16/30 data acquisition system (AD Instruments, Colorado Springs, CO, USA), stored and displayed on a personal computer by the LabChart5 Software System (AD Instruments). Hemodynamic parameters were computed and calculated as described before [20,23]. Briefly, with the use of a special P-V analysis program (PVAN, Millar Instruments), heart rate (HR), LV end-systolic pressure (LVESP), LV end-diastolic pressure (LVEDP), the maximal slope of LV systolic pressure increment (dP/dt_{max}) and diastolic pressure decrement (dP/dt_{min}), time constant of LV pressure decay [τ; according to the Weiss method and Glantz method], LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), stroke volume (SV), cardiac output (CO), ejection fraction (EF) and stroke work (SW) were computed and calculated. CO was normalized to body weight [cardiac index (CI)]. Total peripheral resistance (TPR) was calculated by the following equation: TPR = MAP/CO. Using P-V loops recorded during the transient compression of vena cava inferior (reducing preload) we measured load-independent

contractility indexes: the slope (E_{es}, end-systolic elastance) of the LV end-systolic P-V relationship (ESPVR, according to the parabolic curvilinear model); preload recruitable stroke work (PRSW, the slope of the linear relation between SW and EDV) and the slope of the dP/dt_{max}-end-diastolic volume relationship (dP/dt_{max}-EDV). The slope of the LV end-diastolic P-V relationship (EDPVR) was calculated as a reliable index of LV stiffness. Arterial elastance (E_a) was calculated as LVESP/SV. To characterize LV mechanoenergetics stroke work (SW), pressure-volume area (PVA) and maximal power of LV work were computed, ventriculoarterial coupling (VAC) was determined by the quotient of E_a and E_{es}, while mechanical efficiency (Eff) was calculated as the ratio of SW and PVA. After completion of the hemodynamic measurements all animals were euthanized by exsanguination.

2.7. Statistical analysis

Statistical analysis was performed on a personal computer with a commercially available software (Origin 7G; OriginLab, Northampton, MA, USA). All data are expressed as means ± SEM. An unpaired two sided Student's t-test was used to compare parameters of exercised and control rats, after normal distribution of data was confirmed by Shapiro-Wilk test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Body weight and heart weight

The body weight did not differ before (368 ± 9 g exercised vs. 356 ± 8 g control, p = 0.3050) and after (350 ± 8 g exercised vs. 347 ± 7 g control, p = 0.7417) the acute exhaustive exercise. Body weight loss to baseline body weight ratio (4.69 ± 0.31% exercised vs. 2.41 ± 0.39% control, p < 0.0001) during the exercise protocol was significantly increased in exercised rats. Heart weight measured immediately after the hemodynamic measurements was similar in exercised and control animals (1.14 ± 0.04 g exercised vs. 1.13 ± 0.04 g control, p = 0.8777).

3.2. Biochemical parameters

When compared to the control group, serum cTnT concentrations increased significantly after exhaustive exercise (Table 1). Serum CK and LDH enzyme activity levels as well as that of AST were also markedly increased after exhaustive exercise. Serum creatinine did not differ between the groups (Table 1).

3.3. Histology

In contrast to control myocardium, sporadic fragmentation of myocardial fibers, leukocyte infiltration, tissue edema and cytoplasmic eosinophilia could be observed in the LV myocardium of acute exercised rats (Fig. 1A).

The red fluorescence signal intensity of DHE stained LV myocardial sections was markedly increased after exhaustive exercise suggesting a robust generation of ROS (Fig. 1B; area-intensity score: 38.2 ± 7.8 exercised vs. 5.7 ± 0.9 control, p < 0.0001).

Nitrotyrosine-staining showed increased tyrosine nitration in the myocardium of rats that underwent excessive exercise (Fig. 1C; fractional area: 16.7 ± 1.5% exercised vs. 8.6 ± 0.8% control, p = 0.0011).

The number of TUNEL-positive cardiomyocyte nuclei was significantly increased in the exercised group (Fig. 1D; mean TUNEL-positive cardiomyocyte nuclei/visual field: 1.26 ± 0.17 exercised vs. 0.59 ± 0.06 control, p < 0.01).
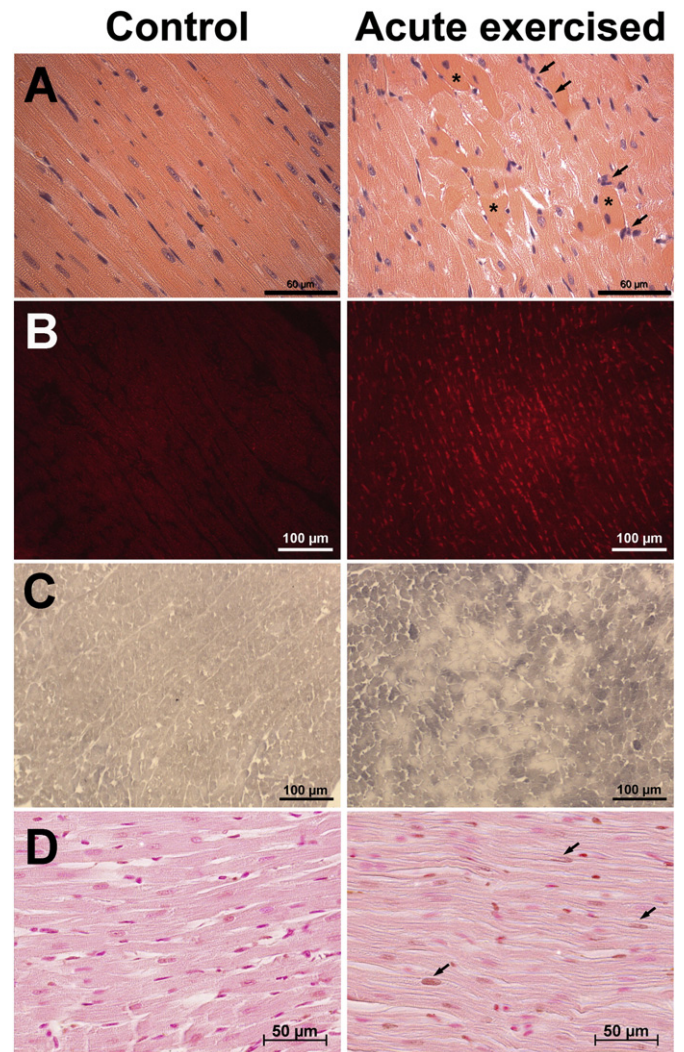

Fig. 1. Histological analysis of left ventricular (LV) myocardium. Panel A: Hematoxylin-eosin stained LV tissue sections showed sporadic fragmentation of myocardial fibers, interstitial edema, cytoplasmic eosinophilia (see star symbol) and leukocyte infiltration (see arrows) — signs of myocardial injury — in the LV myocardium of acute exercised rats compared to intact myocardium of control animals (magnification 400×, scale bar 60 μm). Panel B: Myocardial dihydroethidium staining showed more intense red fluorescent signal (typically nuclear position) in LV myocardium after exhaustive exercise indicating increased superoxide formation (magnification 200×, scale bar 100 μm). Panel C: Nitrotyrosine immunostaining revealed increased tyrosine nitration (dark gray color indicates nitrotyrosine positive area) in the myocardium of exercised group (magnification 200×, scale bar 100 μm). Panel D: Acute exercised rats showed increased number of TUNEL-positive cardiomyocyte nuclei (see arrows) compared to control, suggesting enhanced myocardial apoptotic activation (magnification 400×, scale bar 60 μm).

3.4. Gene expression analysis

Myocardial gene expression analysis showed a significant increase of endogenous antioxidants glucose-6-phosphate dehydrogenase (G6PD), glutathione reductase (GSR), thioredoxin-1 and superoxide dismutase-2 (SOD-2), while catalase and glutathione peroxydase-1 (GPX-1) had a strong tendency toward higher expression values without reaching the level of statistical significance in rats after exhaustive exercise. Myocardial expression of endothelial nitric oxide synthase (eNOS) was increased in the exercised group (Fig. 2A).

The myocardial gene expression of proapoptotic mediator Bax significantly increased, while the antiapoptotic mediator Bcl-2 expression significantly decreased which led to a markedly significant augmentation of Bax/Bcl-2 ratio (Fig. 2B).

Table 1
Biochemical parameters measured from blood plasma in control and acute exercised rats.

	Control (n = 12)	Acute exercised (n = 12)	p value versus control group
Cardiac troponin T, ng/ml	0.025 ± 0.006	0.131 ± 0.022*	0.0001
CK, U/l	284.5 ± 37.5	2029.3 ± 461.4*	0.0011
LDH, U/l	541.5 ± 58.6	836.7 ± 130.1*	0.0011
AST, U/l	79.3 ± 2.7	247.6 ± 32.2*	<0.0001
Creatinine, μmol/l	34.6 ± 3.0	33.6 ± 1.9	0.7837

Values are means ± SEM. CK, creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase. *:p < 0.05 vs. controls.
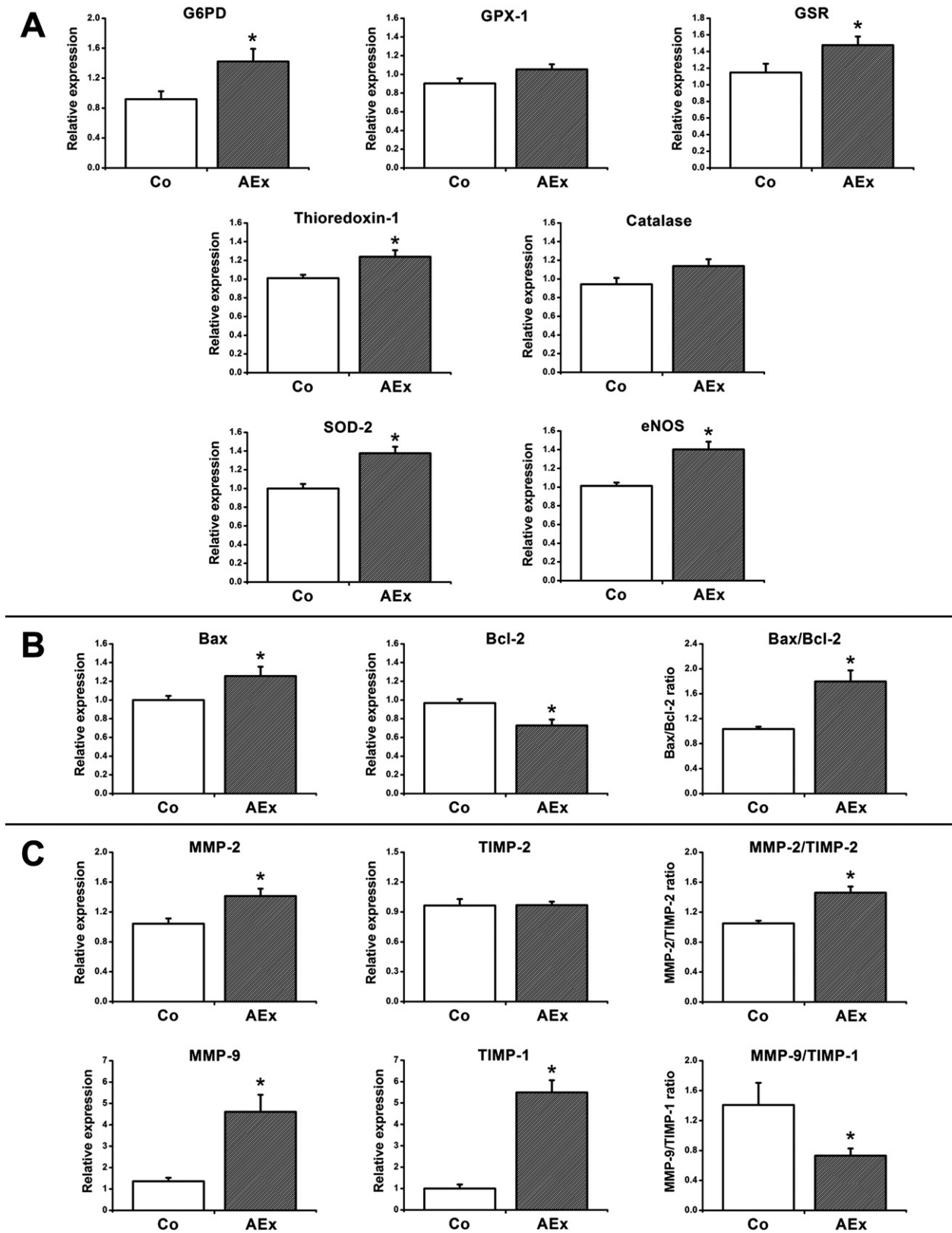
Fig. 2. Myocardial gene expression analysis. Relative myocardial expression of genes related to oxidative stress (Panel A: glucose-6-phosphate dehydrogenase (G6PD), glutathione perox-idase 1 (GPX-1), glutathione reductase (GSR), thiordeoxin-1, catalase, superoxide dismutase-2 (SOD-2) and endothelial nitric oxide synthase (eNOS)), apoptotic signaling (Panel B: Bax, Bcl-2 and Bax/Bcl-2 ratio) and extracellular matrix turnover (Panel C: matrix metalloproteinase (MMP)-2, tissue inhibitor of metalloproteinase (TIMP)-2, MMP-2/TIMP-2 ratio, MMP-9, TIMP-1, MMP-9/TIMP-1 ratio) in control (Co) and acute exercised (AEx) rats. *: p < 0.05 vs Co.

Matrix metalloproteinase (MMP)-2 and MMP-9 expression values were both increased after intense exercise. The expression of tissue in-hibitor of metalloproteinase (TIMP)-2 did not differ between the groups, while TIMP-1 was significantly upregulated in exercised rats leading to increased MMP-2/TIMP-2 and decreased MMP-9/TIMP-1 ratio (Fig. 2C). Myocardial gene expression of transforming growth

Table 2
Hemodynamic parameters in control and exhaustive exercised rats.

	Control (n = 10)	Acute exercised (n = 10)	p value versus control group
HR, beats/min	418.6 ± 13.1	417.5 ± 8.0	0.9436
MAP, mmHg	112.5 ± 4.4	109.8 ± 3.5	0.6400
LVESP, mmHg	125.7 ± 4.4	125.1 ± 3.7	0.9272
LVEDP, mmHg	3.9 ± 0.6	4.4 ± 0.3	0.4208
LVEDV, μl	214.7 ± 15.5	211.9 ± 13.7	0.8943
LVESV, μl	100.1 ± 6.7	140.4 ± 8.5*	0.0015
SV, μl	139.1 ± 9.4	102.3 ± 11.3*	0.0226
CO, ml/min	58.48 ± 4.67	42.39 ± 4.53*	0.0235
CI, (ml/min)/100 g BW	17.31 ± 1.68	12.32 ± 1.22*	0.0271
EF, %	59.5 ± 1.8	44.1 ± 3.4*	<0.0001
SW, mmHg · ml	13.42 ± 0.55	9.11 ± 0.11*	0.0030
dP/dt_{max}, mmHg/s	8350 ± 465	7700 ± 458	0.3329
dP/dt_{min}, mmHg/s	11095 ± 741	10183 ± 693	0.3805
τ(Weiss), ms	7.8 ± 0.3	7.9 ± 0.3	0.6876
τ(Glanz), ms	10.9 ± 0.6	11.2 ± 0.6	0.7297
TPR, mmHg/(ml/min)	2.02 ± 0.16	2.84 ± 0.29*	0.0228
Slope of EDPVR, mmHg/μl	0.036 ± 0.004	0.041 ± 0.004	0.3133
Maximal power, mW	110.4 ± 16.5	58.7 ± 6.1*	0.0009

Values are means ± SEM. Hemodynamic parameters were measured by the Millar pressure-volume conductance catheter system. HR, heart rate; MAP, mean arterial pressure; LVESP, left ventricular (LV) end-systolic pressure; LVEDP, LV end-diastolic pressure; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume; SV, stroke volume; CO, cardiac output; CI, cardiac index; BW, body weight; EF, ejection fraction; SW, stroke work; dP/dt_{max} and dP/dt_{min} maximal slope of the systolic pressure increment and the diastolic pressure decrement, respectively; τ, time constant of LV pressure decay; TPR, total peripheral resistance; EDPVR, end-diastolic pressure-volume relationship. *:$p < 0.05$ vs. controls.

factor β1 (TGF-β1) showed a strong tendency toward higher values after exercise, however without reaching the level of statistical significance (relative expression: 1.36 ± 0.13 exercised vs. 0.98 ± 0.11 control, p = 0.0552). Atrial natriuretic factor (ANF) expression did not differ between the groups (relative expression: 1.71 ± 0.38 exercised vs. 1.59 ± 0.16 control, p = 0.7761).

3.5. Hemodynamic parameters

Baseline hemodynamic data. MAP, HR, LVESP, LVEDP, τ, dP/dt_{max} and dP/dt_{min} were not different in acute exercised animals compared to the control group (Table 2). Fig. 3 shows representative original steady-state P-V loops obtained from acute exercised and control rats. The decreased width of baseline P-V loops after exhaustive swimming reflects reduced SV along with unaltered LVEDV and increased LVESV. EF, CO and CI decreased significantly suggesting deteriorated systolic performance in rats after exhaustive exercise. TPR and E_a were increased in rats after intense exercise (Table 2).

Systolic and diastolic functional indexes derived from P-V analysis at different preloads. Fig. 4A shows representative original P-V loops recorded during transient occlusion of the inferior vena cava in exercised and control animals. The slope (E_{es}) of end-systolic P-V relationship (ESPVR) was less steep in exhaustive exercised than in control animals suggesting decreased contractility. The EDPVR did not differ between the groups indicating unchanged LV stiffness of myocardium after excessive exercise. Slope values of PRSW were significantly decreased after intense exercise indicating impaired contractility (Fig. 4B). As Fig. 4C shows, the linear relation of dP/dt_{max} and EDV was less steep in exhausted animals than in controls. The overall dP/dt_{max}-EDV slope values were significantly lower in exercised rats (Fig. 4C).

Cardiac mechanoenergetics. SW was decreased in acute exercised animals. P-V analysis revealed a significant decrease in E_{es} and an increase of E_a. Subsequently, ventriculoarterial coupling was significantly increased in exhaustively exercised rats suggesting contractility-afterload mismatch (Fig. 5A). Despite the decreased SW in exercised rats, pressure-volume area did not differ between the groups.
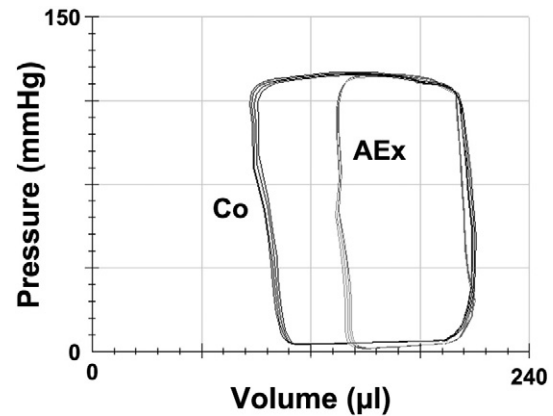

Fig. 3. Steady-state left ventricular pressure-volume loops. Original recordings of steady-state pressure-volume (P-V) loops in representative rats from control (Co) and acute exercised (AEx) groups. The decreased width of P-V loops in exercised animals indicate reduced stroke volume as a result of unaltered end-diastolic volume and increased end-systolic volume, thus decreased ejection fraction.

Mechanical efficiency and maximum power of LV work were also impaired in exercised rats compared to the controls (Fig. 5B, Table 2).

4. Discussion

In the current study we validated our rat model of acute exhaustive exercise induced myocardial injury by confirming key mechanisms of cardiac damage. We provided the first detailed hemodynamic characterization and described several aspects of LV dysfunction after acute exhaustive exercise. Our results suggest that deteriorated cardiac performance (impaired contractility and mechanoenergetics) and myocardial injury might be associated with increased nitro-oxidative stress and enhanced proapoptotic activity in the LV myocardium.

4.1. Biomarkers of myocardial injury

In the present study an obvious myocardial injury was observed after exhaustive swimming exercise. The release of cTnT as well as non-specific cardiac enzymes CK, LDH and AST was markedly increased after exhaustive exercise (Table 1), which is in line with previous observations on animal models [8,9,19] and numerous human exercise studies [5,6]. In accordance with recent literature data our HE staining of LV myocardium showed signs of sporadic cardiomyocyte damage after exhaustive exercise [19] (Fig. 1A).

4.2. Cardiac dimensions and function

Although numerous human studies investigated cardiac function after a prolonged exercise both in nonelite subjects and in elite athletes by using echocardiography, there is controversial literature data about systolic and diastolic functional changes of LV [15]. Li et al. showed a significant impairment of cardiac function of experimental animals subjected to exhaustive physical activity both in vivo (echocardiography) and in vitro (Langendorff model) [19]. To the best of our knowledge, the present study is the first one that describes LV pressure and volume relations in detail and provides characterization of LV dysfunction in vivo after exhaustive exercise. P-V analysis clearly demonstrated significantly increased LVESV along with unchanged LVEDV, thus decreased SV and EF in rats that underwent our exhaustive exercise protocol (Fig. 3). These cardiac dimensions are consistent with previous experimental results [19] and correspond with human echocardiography data suggesting systolic impairment after exhaustive exercise [24,25].
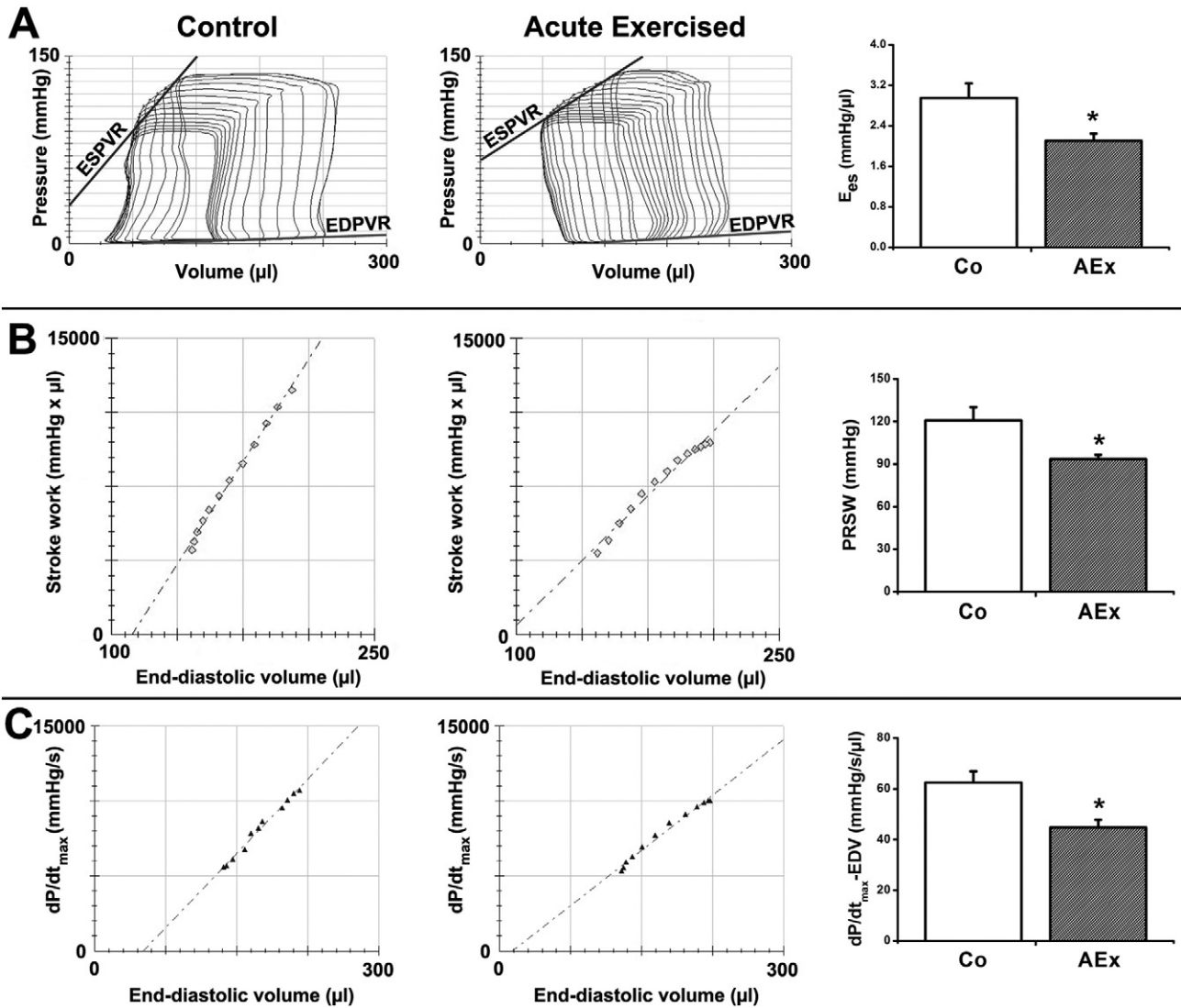
Fig. 4. Pressure-volume loop derived load independent left ventricular contractility parameters. The slope (E_{es}) of end-systolic pressure-volume relationship (ESPVR) (A); preload recruitable stroke work (PRSW), the slope of the relationship between stroke work (SW) and end-diastolic volume (EDV) (B); and maximal slope of the systolic pressure increment (dP/dt_{max}) − EDV relationship (dP/dt_{max}-EDV) (C) in representative rats from control (Co) and acute exercised (AEx) groups. As seen on the bar graphs, all of these contractility parameters are decreased in the AEx group, suggesting reduced systolic performance after exhaustive exercise. *: $p < 0.05$ vs. Co.

4.3. Systolic function and cardiac contractility

In our rat model exhaustive exercise was associated with unchanged dP/dt_{max} and LVESP, but markedly decreased EF (Table 2). Although dP/dt_{max} and EF have been widely used as cardiac contractility parameters, it is well recognized that they are dependent on loading conditions, and therefore cannot reliably be used to assess LV contractile function in models where loading is altered [26]. The method of simultaneous LV pressure and volume analysis performed by the miniaturized pressure-conductance catheter allowed us to calculate highly sensitive load-independent indexes of LV contractility.

The slope of ESPVR (E_{es}) has been described as a fairly load-insensitive index of LV contractility, which was significantly decreased after intense exercise, indicating a deteriorated inotropic state of the LV myocardium (Fig. 4A). However, because this relation can be altered not only by changes in the contractile state but also by changes in chamber geometry and other diastolic factors, we also calculated other reliable contractility parameters [26].

PRSW represents the slope of the linear relation between SW and EDV. It has been described as a sensitive LV contractility index

independent of chamber size and mass. PRSW was also significantly decreased in exhaustive exercised rats compared to controls (Fig. 4B). Although this parameter integrates data from the entire cardiac cycle, it is influenced most of all by the systole [26].

Analysis of dP/dt_{max}-EDV relationship allowed us to compare dP/dt_{max} values at a given LVEDV between the experimental groups. A previous investigation has demonstrated [27] that the slope of the relation between dP/dt_{max} and EDV, another P-V loop-derived index, represents a less load-dependent parameter of LV contractility. This sensitive contractility index was also impaired after exhaustive exercise compared to controls (Fig. 4C).

4.4. Diastolic function

Indices of LV stiffness (LVEDP and slope of EDPVR) were not significantly different between exercised and control animals, as well as unchanged ventricular relaxation has been observed after intense exercise (as indicated by τ and dP/dt_{min}; Table 2). The observed unchanged diastolic function is in line with recent findings on isolated rat hearts [28]. Nevertheless there are several human studies describing a
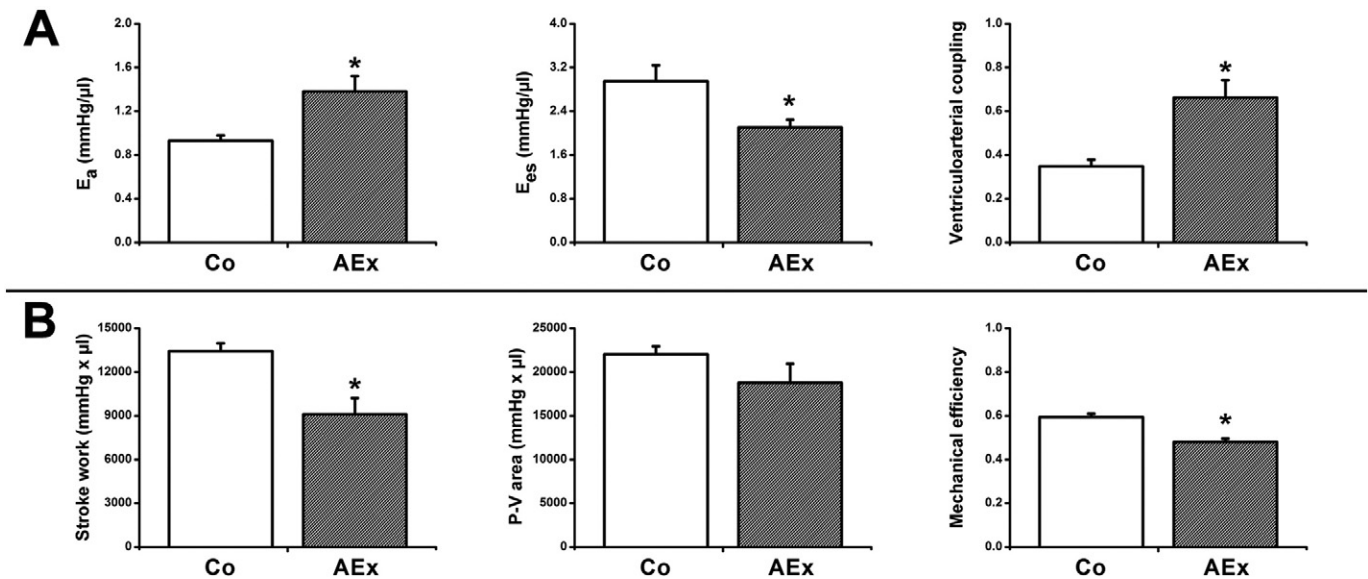
Fig. 5. Cardiac mechanoenergetics. Panel A: Decreased end-systolic elastance (E_{es}) and increased arterial elastance (E_a) resulted in deteriorated ventriculoarterial coupling (E_a/E_{es}) in acute exercised (AEx) rats compared with controls (Co). Panel B: Decreased stroke work (SW) along with unaltered pressure-volume area (P-V area) led to reduced mechanical efficiency (SW/P-V area) in the swimming group. All of these parameters suggest impaired LV mechanoenergetics after exhaustive exercise. *: $p < 0.05$ vs. Co.

transient diastolic dysfunction (decreased early to late diastolic ratio assessed by echocardiography; E/A) after exhaustive exercise [15]. Further experimental reports are required for appropriate comparison of human and animal diastolic values.

4.5. Mechanoenergetics

P-V analysis was used to characterize mechanoenergetic changes after exhaustive exercise. We determined E_{es} and E_a, which are relative load-independent indices of LV contractility and vascular loading, respectively. E_a is an integrative index that incorporates the principal elements of arterial load, including peripheral vascular resistance, total arterial compliance and characteristic impedance [29]. Ventriculoarterial coupling (E_a/E_{es}) is an important determinant of net cardiac performance and energetics [30]. The parallel decrease of myocardial contractility (E_{es}) and the increase of E_a led to a significant impairment of the ventriculoarterial coupling ratio in acute exercised animals (Fig. 5A). Impaired ventriculoarterial coupling in the exercised group reflects an inadequate matching between LV contractility and afterload after exhaustive exercise, which results in a suboptimal transfer of blood from the LV to the periphery with more excessive changes in pressure.

Stroke work is determined as the area within the P-V loop and represents the external mechanical work performed by the left ventricle during a single heart cycle. P-V area (PVA) has been described as an index of LV total mechanical energy and is linearly related to total myocardial oxygen consumption [31]. Decreased SW along with unaltered PVA leads to decreased mechanical efficiency in exhaustive exercised animals compared with controls, suggesting a deterioration of metabolic efficiency after such exercise: decreased mechanical work along with similar myocardial oxygen consumption (Fig. 5B). According to our knowledge the present study is the first to demonstrate decreased mechanical efficiency after exhaustive exercise.

4.6. Oxidative stress

Even though the exact mechanisms responsible for exercise-induced myocardial injury are still not well understood, there have been accumulated evidence indicating that exhaustive exercise causes imbalance between ROS and antioxidant defense, resulting in oxidative stress in the myocardium [12]. Increased formation of ROS (by the mitochondrial electron transport chain, NAPDH and xanthine oxidases [32]) activates a

broad variety of hypertrophy signaling kinases and transcription factors and induces apoptosis by DNA and mitochondrial damage and activation of proapoptotic signaling kinases [33]. A robust generation of ROS and thus increased oxidative stress was observed by DHE-staining in the myocardium of exhaustive exercised rats compared to controls (Fig. 1B). A recent experimental study of exhaustive exercise showed the key role of Nrf2, the primary transcriptional regulator of antioxidants, including G6PD, GPX-1, GSR and catalase, which are upregulated as a compensatory reaction to ROS overproduction [12]. Correspondingly we found significantly increased myocardial gene expressions of endogenous antioxidants, such as G6PD, GSR as well as thioredoxin-1 after exhaustive exercise. In accordance with Muthusamy et al. [12], we observed an upregulation of mitochondrial superoxide dismutase-2 (SOD-2) in the myocardium, suggesting mitochondrial superoxide generation as a result of exhaustive exercise (Fig. 2A). Increased superoxide concentration reduces the bioavailability of nitric oxide (NO) by chemical inactivation to form toxic peroxynitrite. A markedly increased protein nitration was observed by nitrotyrosine staining (Fig. 1C), suggesting increased nitrative stress, which could be the consequence of peroxynitrite formation [32]. Peroxynitrite can also "uncouple" eNOS to become a dysfunctional superoxide-generating enzyme that contributes to vascular oxidative stress [34]. In our study the myocardial expression of eNOS increased in response to oxidative stress, which might reflect upregulation as a reaction to decreased NO bioavailability and eNOS uncoupling. Moreover, nitration of several myocardial proteins can have a potential deleterious effect on myocardial contractility and increased peroxynitrite formation can induce apoptosis as well as matrix metalloproteinase activation [32].

4.7. Apoptosis

The ratio of proapoptotic and antiapoptotic proteins (e.g. Bax/Bcl-2) regulates myonuclei integrity and cell survival by controlling mitochondrial membrane permeability [35]. According to our results exhaustive exercise resulted in a markedly increased Bax/Bcl-2 ratio, thus enhanced apoptotic signaling in the myocardium. This finding has further been supported by the increased number of TUNEL positive cardiomyocyte nuclei, suggesting increased DNA fragmentation after acute exercise (Fig. 1D).

4.8. Oxidative stress induced dysregulation of MMPs

ROS can stimulate cardiac fibroblast proliferation and activate matrix metalloproteinases, which play a key role in the homeostasis of extracellular matrix in the myocardium. Sustained MMP activation (increased expression of MMPs or downregulation of their endogenous inhibitors, TIMPs) might influence the structural properties of the myocardium by providing an abnormal extracellular environment, which the cardiomyocytes interact with [13,36]. As recent investigations showed, expression and activity of MMP-2 and MMP-9 are increased in the skeletal muscle after a single bout of exercise [37]. The observed significant changes of MMP-2/TIMP-2 as well as MMP-9/TIMP-1 ratio suggest MMP dysregulation in myocardium of exercised rats. These findings raise the possibility that enhanced oxidative stress can be a stimulus for myocardial MMP activation, which might play an important role in the development of exhaustive exercise-induced cardiac dysfunction. TGF-β1 is a pleiotropic cytokine, which is involved in cardiac injury, repair and fibrotic remodeling [38]. A strong tendency toward upregulation of TGF-β1 expression after exhaustive exercise suggests the activation of reparative and profibrotic mechanisms after myocardial injury induced by prolonged exercise.

4.9. Study limitations

The interpretation of results from the present study is limited to young male rats. The possible influence of gender, age or species should be assessed in future studies. Swimming with weight attached to the tail has been suggested to be effective to induce exhaustive exercise induced cardiac injury and oxidative stress in animals [9] even though swimming is associated with an important stress response [39]. In order to minimize the influence of stress all rats were familiarized with swimming 48 h before the formal swimming protocol.

Our current data on increased myocardial gene expression of MMPs and TIMPs within 2 h suggest initiation of matrix degradation and profibrotic activity, however these phenomena are more relevant in a longer period after myocardial insult.

This study was designed to observe hemodynamic alterations induced by one bout of exhaustive exercise, however impact of the observed molecular and functional changes on the longer term remains unanswered. Further studies are warranted to elucidate the possible reversibility of myocardial dysfunction described in the present work.

5. Conclusions

In conclusion we confirmed exhaustive exerciseinduced cardiac injury and nitro-oxidative stress in our rat model and for the first time we described in vivo cardiac dysfunction in detail by using the P-V conductance catheter system. Based on reliable load-independent indices we demonstrated systolic functional deterioration (reduced contractility) in exhaustive exercised animals along with unchanged diastolic function and impaired mechanoenergetics (decreased efficiency, ventriculo-arterial mismatch). Elevations in cardiac enzymes, sporadic cardiac injuries and possible impaired myocardial function along with the activation of proapoptotic and profibrotic activity raise the question whether prolonged endurance exercise could induce persistent myocardial damage and dysfunction.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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