Cardiovasc Drugs Ther (2014) 28:125–135
DOI 10.1007/s10557-013-6503-8

ORIGINAL ARTICLE

AT_1 and Aldosterone Receptors Blockade Prevents the Chronic Effect of Nandrolone on the Exercise-Induced Cardioprotection in Perfused rat Heart Subjected to Ischemia and Reperfusion

Silvio Rodrigues Marques-Neto · Emanuelle Baptista Ferraz · Deivid Carvalho Rodrigues · Brian Njaine · Edson Rondinelli · Antônio Carlos Campos de Carvalho · Jose Hamilton Matheus Nascimento

Published online: 21 November 2013

Abstract
Purpose Myocardial tolerance to ischaemia/reperfusion (I/R) injury is improved by exercise training, but this cardioprotection is impaired by the chronic use of anabolic androgenic steroids (AAS). The present study evaluated whether blockade of angiotensin II receptor (AT_1-R) with losartan and aldosterone receptor (mineralocorticoid receptor, MR) with spironolactone could prevent the deleterious effect of AAS on the exercise-induced cardioprotection.
Methods and Results Male Wistar rats were exercised and treated with either vehicle, nandrolone decanoate (10 mg/kg/week i.m.) or the same dose of nandrolone plus losartan or spironolactone (20 mg/kg/day orally) for 8 weeks. Langendorff-perfused hearts were subjected to I/R and evaluated for the postischaemic recovery of left ventricle (LV) function and infarct size. mRNA and protein expression of angiotensin II type 1 receptor (AT_1-R), mineralocorticoid receptor (MR), and K_{ATP} channels were determined by reverse-transcriptase polymerase chain reaction and Western blotting. Postischaemic recovery of LV function was better and infarct size was smaller in the exercised rat hearts than in the sedentary rat hearts. Nandrolone impaired the exercise-induced cardioprotection, but this effect was prevented by losartan (AT_1-R antagonist) and spironolactone (MR antagonist) treatments. Myocardial AT_1-R and MR expression levels were increased, and the expression of the K_{ATP} channel subunits SUR2a and Kir6.1 was decreased and Kir6.2 increased in the nandrolone-treated rat hearts. The nandrolone-induced changes of AT_1-R, MR, and K_{ATP} subunits expression was normalized by the losartan and spironolactone treatments.
Conclusion The chronic nandrolone treatment impairs the exercise-induced cardioprotection against ischaemia/reperfusion injury by activating the cardiac renin-angiotensin-aldosterone system and downregulating K_{ATP} channel expression.

Keywords Myocardial ischaemia-reperfusion · Exercise-induced cardioprotection · Anabolic steroid · Nandrolone · Renin-angiotensin-aldosterone system · ATP-dependent potassium channels

S. R. Marques-Neto · E. B. Ferraz · J. H. M. Nascimento (✉)
Laboratório de Eletrofisiologia Cardíaca Antonio Paes de Carvalho, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, CCS, Bloco G, 21.941-902, Rio de Janeiro, Brazil
e-mail: jhmn@biof.ufrj.br

D. C. Rodrigues · E. Rondinelli
Laboratório de Metabolismo Macromolecular Firmino Torres de Castro, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

B. Njaine · A. C. Campos de Carvalho
Laboratório de Cardiologia Celular e Molecular, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Introduction

Cardiovascular disease is the leading cause of death worldwide [1, 2]. Physical inactivity is a main risk factor for cardiovascular disease, and the beneficial effect of regular physical activity on reducing cardiovascular disease risk in humans is well documented [3–7]. In animal models, experimental studies have provided evidence of the cardioprotective effect of exercise against ischaemia-reperfusion (I/R) injuries [8–10]. Different mechanisms have been proposed to explain the exercise-induced protection

against myocardial I/R injuries [11, 12]. Among these mechanisms, an important role for ATP-sensitive potassium (K_{ATP}) channels has been suggested by several studies [13–16].

There is a growing misuse of anabolic androgenic steroids (AAS) in association with exercise [17, 18]. Adverse cardiovascular effects of AAS, including cardiac hypertrophy, myocardial infarction and sudden cardiac death, have been reported [19–22]. Experimental studies in rats have demonstrated that the exercise-induced improvement of cardiac tolerance to I/R injuries is impaired by chronic AAS administration [23, 24]. The mechanism responsible for the AAS-mediated impairment of exercise-induced cardioprotection remains incompletely understood. However, some studies suggest that AAS increases pre- and postischaemic myocardial tumor necrosis factor-α (TNF-α) concentrations [23], impairs exercise-induced antioxidant enzyme activities [24], and activates the local renin-angiotensin system [25, 26]. Androgens induce cardiac hypertrophy [27], and cardiac hypertrophic remodelling is associated with the activation of the renin-angiotensin-aldosterone system (RAAS) [28–30]. Angiotensin II-induced cardiac TNF synthesis via the AT_1 receptor (AT_1-R) has been demonstrated in the mammalian heart [31], and both TNF and AT_1-R are involved in reactive oxygen species generation [32] and the regulation of K_{ATP} subunit expression [33]. Therefore, the present study was designed to 1) determine whether RAAS blockade with losartan (AT_1-R antagonist) and spironolactone (MR, mineralocorticoid receptor antagonist) would prevent the impairment of exercise-induced cardioprotection by nandrolone decanoate, a synthetic AAS; and 2) evaluate the effect of nandrolone-induced RAAS activation on K_{ATP} channel expression.

Material and Methods

Animals

The study followed the Principles of Laboratory Animal Care published by US National Institute of Health (NIH publication, revised in August 2002) and was approved by the local Institutional Animal Care and Use Committee (IBCCF 006). Male Wistar rats with initial weights of 200–250 g were used. The rats were housed in a temperature-controlled room (23±2 °C) on a 12:12 h dark/light cycle with free access to rat chow and water.

Exercise Training Protocol and Drug Treatment

Rats were randomly allocated into six experimental groups: vehicle-treated sedentary control (SC), vehicle-treated exercised control (EC), sedentary nandrolone-treated (SD), exercised nandrolone-treated (ED), exercised nandrolone-treated plus spironolactone (EDSP), and exercised nandrolone-treated plus losartan (EDLO). After 8 weeks of exercise training and treatments, rats were sacrificed and their hearts were excised, weighted and destined for in vitro I/R experiments ($n=5$ per group) or biochemical analysis ($n=10$ per group). All of the nandrolone-treated groups received a weekly intramuscular injection of the androgenic anabolic steroid nandrolone decanoate (10 mg/kg; Deca Durabolin, Organon, Brazil) for 8 weeks, as previously described [24]. Control animals (SC and EC) were treated with the same volume of vehicle on the same schedule (peanut oil with benzyl alcohol, 90:10, v/v). Spironolactone- (Espironolactona, EMS, Brazil) or losartan (Losartana potassica, Merck, Brazil)-treated rats received 20 mg/kg/day of each drug, administered via gavage. Exercised rats performed moderate intensity exercise training on a motor-driven treadmill (EP 131, Insight, Brazil) for 5 days per week over 8 weeks. During the first week, the exercise training was initiated at 10 m/min for 15 min per session to allow the animals to adapt to exercise stress. From the 2nd to the 5th week, the running speed and exercise duration were progressively increased to the target levels of 16 m/min and 60 min per session, after which they remained constant throughout the final 3 weeks.

To evaluate whether losartan and spironolactone alone induced cardioprotection against ischaemia/reperfusion injuries, additional control experiments were performed in 20 rats that were distributed into the following groups ($n=5$ per group): sedentary losartan-treated (SLO), sedentary spironolactone-treated (SSP), exercised losartan-treated (ELO), and exercised spironolactone-treated (ESP). The rats were treated daily by gavage with 20 mg/kg/day losartan or spironolactone for 8 weeks. After sacrifice, the hearts were evaluated in in vitro I/R experiments.

In Vitro I/R Experiments

Rats were heparinised (1,000 U/kg, i.p.) 10 min before being sacrificed by carbon dioxide inhalation and cervical dislocation. The hearts were rapidly excised following a mid-line thoracotomy, and the aorta was cannulated using a modified Langendorff apparatus, perfused retrogradely at constant flow (10 ml/min) with Krebs-Henseleit modified buffer (in mmol/L: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 glucose), and equilibrated with 95 % O_2/5 % CO_2 gas mixture at 36.5±0.5 °C. A water-filled latex balloon was placed into the left ventricle (LV) through the mitral valve and connected to a pressure transducer and PowerLab System (ADInstruments, Australia) for continuous LV pressure recording. The heart was kept immersed in a buffer-filled water-jacketed glass chamber, and the end-diastolic pressure (LVEDP) was adjusted to 10 mmHg. LV developed pressure (LVDP) was determined as the difference between the peak systolic and LVEDP. After stabilization (20–30 min) and basal

parameter recording, the hearts were subjected to normothermic no-flow global ischaemia for 30 min followed by reperfusion for 60 min, as described in previous studies [24, 34].

Infarct Size Measurement

At the end of the 60 min reperfusion period, the hearts were removed and sliced into 1.5 mm cross-sections from apex to base and incubated in 1 % triphenyl tetrazolium chloride for 4 min at 37 °C. The slices were then placed in a 10 % (v/v) formaldehyde solution for 24 h to improve the contrast between the stained (viable) and unstained (necrotic) tissues. The slices were placed between two glass slides and scanned (imaged). The infarct size was determined by planimetry using ImageJ software (version 1.22, National Institute of Health, USA). Infarct size was expressed as % of at risk (total) area.

Gene Expression

The mRNA levels of angiotensinogen, angiotensin converting enzyme (ACE), angiotensin II type 1 receptor (AT_1-R), mineralocorticoid receptor (MR), and K_{ATP} channel subunits (Kir6.1, Kir6.2 and SUR2a) were evaluated by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from frozen LV samples and treated with DNase using the RNeasy® Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared from 1 μg total RNA using random primers and a high-capacity reverse transcriptase kit (Applied Biosystems) according to the manufacturer's instructions. The amplification reactions were performed in a final volume of 25 μl in 96-well plates. Assays were performed with an ABI Prism 7500 (Applied Biosystems). The qRT-PCR mixture contained 1 μl 10× diluted cDNA, 12.5 μl 2× Power SYBR Master Mix (Applied Biosystems), and 150 nM each of forward and reverse primers. The following primers were used: (i) angiotensinogen: forward 5'-CACGGACAGCACCCTATTTT-3', reverse 5'-GCTGTTGTCCACCCAGAACT-3', amplicon length 100 bp; ACE: forward 5'-ACGGAAGCATCACCAAGGAG-3', reverse 5'-TGGCACATTCGCAGGAACG-3', amplicon length 140 bp; AT_1-R: forward 5'-ACACAACCCTCCCAGAAAG-3', reverse 5'-TGATGCTGTAGAGGGTAGGG-3', amplicon length 148 bp; MR: forward 5'-TCGCTTTGAGTTGGAGATCG-3', reverse 5'-ACGAATTGAAGGCTGATCTGG-3', amplicon length 363 bp; Kir 6.1: forward 5'-AGAAAGGCATCACGGAGAAG-3', reverse 5'-GAAGAGAAACGCAGAAGTGAATG-3', amplicon length 83 bp; Kir6.2: forward 5'-CAAGCCCAAGTTTAGCATCTC-3', reverse 5'-CCAGCACTCTACATACCGTAC-3', amplicon length 112 bp; SUR2a: forward 5'-CTTTGCCTCTCTGTCTCTCTTC-3', reverse 5'-CTGTCCTCGCCAATCTCATC-3', amplicon length 141 bp. The amplification program consisted of 55 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 30 s and 58 °C for 1 min. The qRT-PCR efficiency was evaluated using serial dilutions of the template cDNA, and melting curve data were collected to assess qRT-PCR specificity. Each cDNA was amplified in triplicate, and a corresponding sample without reverse transcriptase (no-RT sample) was included as a negative control. The expression of the chosen genes was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The relative quantities of gene-specific mRNA expression were determined by the comparative CT method as expressed by the equation $2^{-(\Delta Ct)}$ [35], where Ct is the "threshold cycle" determined for each plate by the 7500 real-time PCR system sequence detection software (Applied Biosystems). ΔCt was calculated as the difference between the target mRNA Ct and the endogenous control Ct (GAPDH). Relative mRNA levels were expressed as the fold change compared with the exercised control group.

Western Blot

Frozen LV samples were homogenised, and total protein was extracted in RIPA buffer (10 mmol/L Tris base- HCl, 150 mmol/L NaCl, 1 % NP-40, 1 % Triton X-100, 5 $mmol.L^{-1}$ EDTA, 0.1 % SDS, 1 % sodium deoxycholate) supplemented with 1× complete protease inhibitors (Roche). The protein concentrations of the lysates were determined using the Bradford assay. Proteins (30 μg) were separated on SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5 % non-fat dry milk and incubated with primary antibodies against AT_1-R (1:1000, sc-1173, Santa Cruz Biotechnology, Inc.), MR (1:500, sc-6861, Santa Cruz Biotechnology Inc.) Kir6.1 (1:250, sc-20808, Santa Cruz Biotechnology Inc.), Kir6.2 (1:250, sc-11228, Santa Cruz Biotechnology, Inc.), SUR2a (1:250, sc-32461, Santa Cruz Biotechnology, Inc.) followed by horseradish peroxidase-conjugated secondary antibodies (1:2000). Immunoblots were developed with ECL-plus (GE-Healthcare Life Sciences) according to the manufacturer's instructions, and the densitometry results were analysed with ImageJ freeware (version 1.22, National Institute of Health, USA) and normalised to β-actin as a loading control.

Statistical Analysis

The data are presented as the mean ± s.e.m. Statistical comparisons were performed by one-way ANOVA followed by a Newman-Keuls post-hoc test. Repeated-measures ANOVA was used to analyse the changes in LVDP and LVEDP over time. $P<0.05$ was considered to indicate statistical significance.

Results

Effect of AT_1-R and MR Blockade on Nandrolone-Induced Cardiac Hypertrophy

After 8 weeks of exercise training and nandrolone decanoate treatment, no significant differences in final body weight were observed among the groups (Fig. 1a). The heart weight to body weight (HW/BW) ratio of the EC rats was significantly increased by the exercise regimen ($P<0.01$ vs. SC group). The nandrolone-treated SD and ED groups demonstrated a marked increase in HW/BW ratio compared with the SC group ($P<0.001$). The combined effect of exercise and nandrolone on the ED group HW/BW ratio was greater than that of exercise alone ($P<0.05$ vs. EC) but similar to the effect of nandrolone alone on the SD group. Losartan and spironolactone treatments prevented the hypertrophic effect of nandrolone; thus, the HW/BW ratios of the EDSP ($P<0.05$) and EDLO ($P<0.01$) groups were diminished compared with the ED group but were not significantly different from those of the EC group (Fig. 1b).

Effect of AT_1-R and MR Blockade on Post-Ischaemic LV Function Recovery

All groups presented similar spontaneous heart rate values before ischaemia and after 60 min of reperfusion (data not shown). Initial LVEDP was established at 10 mmHg for all

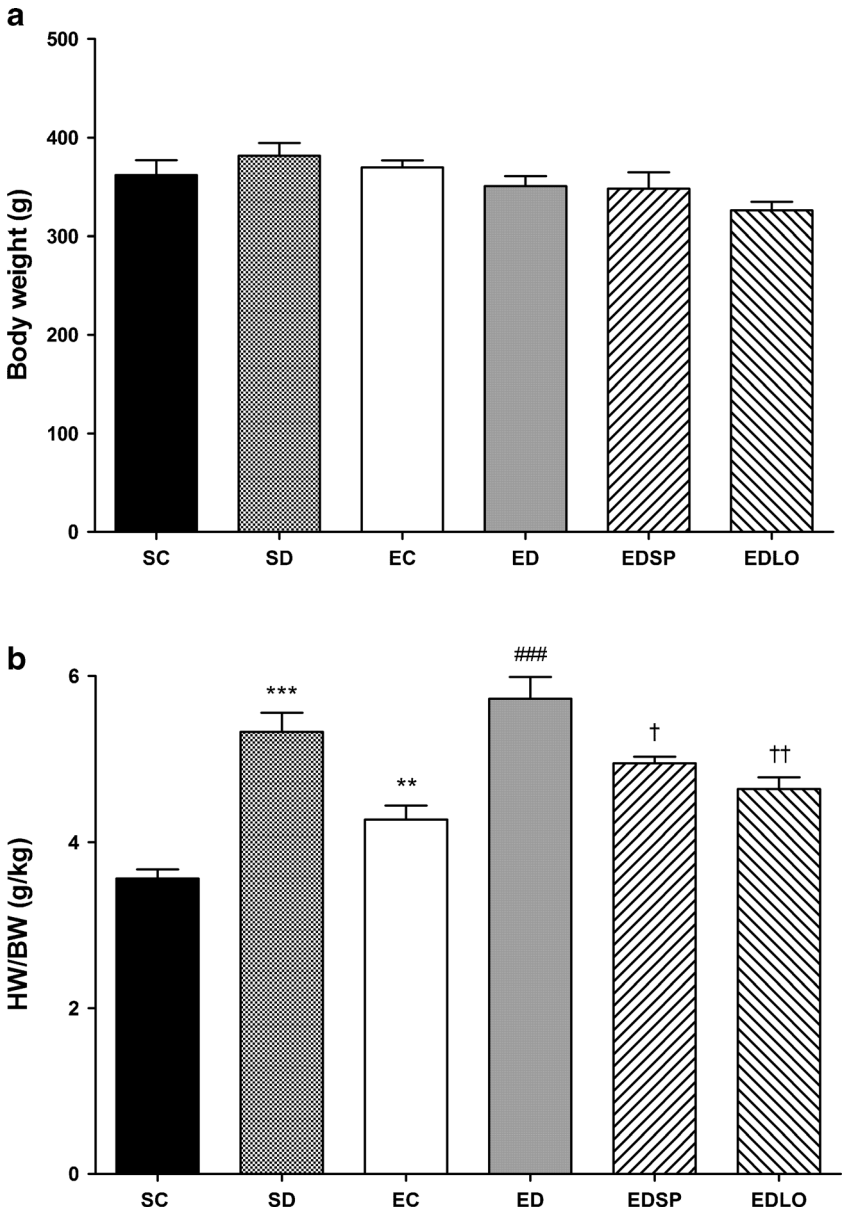

Fig. 1 Effects of 8 weeks of exercise and nandrolone treatment on (**a**) body weight and (**b**) the heart weight/body weight (HW/BW) ratio in sedentary control (SC), exercised control (EC), sedentary AAS-treated (SD), exercised and AAS-treated (ED), exercised and AAS plus spironolactone-treated (EDSP), and exercised and AAS plus losartan-treated (EDLO) rats. The data are expressed as the mean ± SEM ($n=5$/group). $^{**}P<0.01$, and $^{***}P<0.001$ vs. CS; $^{###}P<0.001$ vs. EC; $^{\dagger}P<0.05$ and $^{\dagger\dagger}P<0.01$ vs. ED

groups. Ischaemic contracture occurred during the global ischaemia period, but the increase in the LVEDP was attenuated in the EC, EDSP and EDLO groups (Fig. 2a and c). A further increase in LVEDP occurred during reperfusion in the SC, SD and ED groups but not in the EC, EDSP and EDLO groups (Fig. 2a and c). In comparison to the SC group, LVEDP level at the end of reperfusion was decreased in the EC (61.1 %), EDSP (50.1 %) and EDLO (58.9 %) groups but was increased in the SD (22.3 %) and ED (4.6 %) groups (Fig. 2e).

The basal (pre-ischaemia) LVDP was lower in the SD (95.2±3.5 mmHg) and ED (96.6±2.8 mmHg) compared to the EC (118.4±5.1 mmHg, $P<0.001$), EDSP (110.8±1.6 mmHg, $P<0.05$) and EDLO (112.8±3.8 mmHg, $P<0.01$) groups, but not significantly different from that in the SC (106.8±1.5 mmHg) group. The 30 min of global

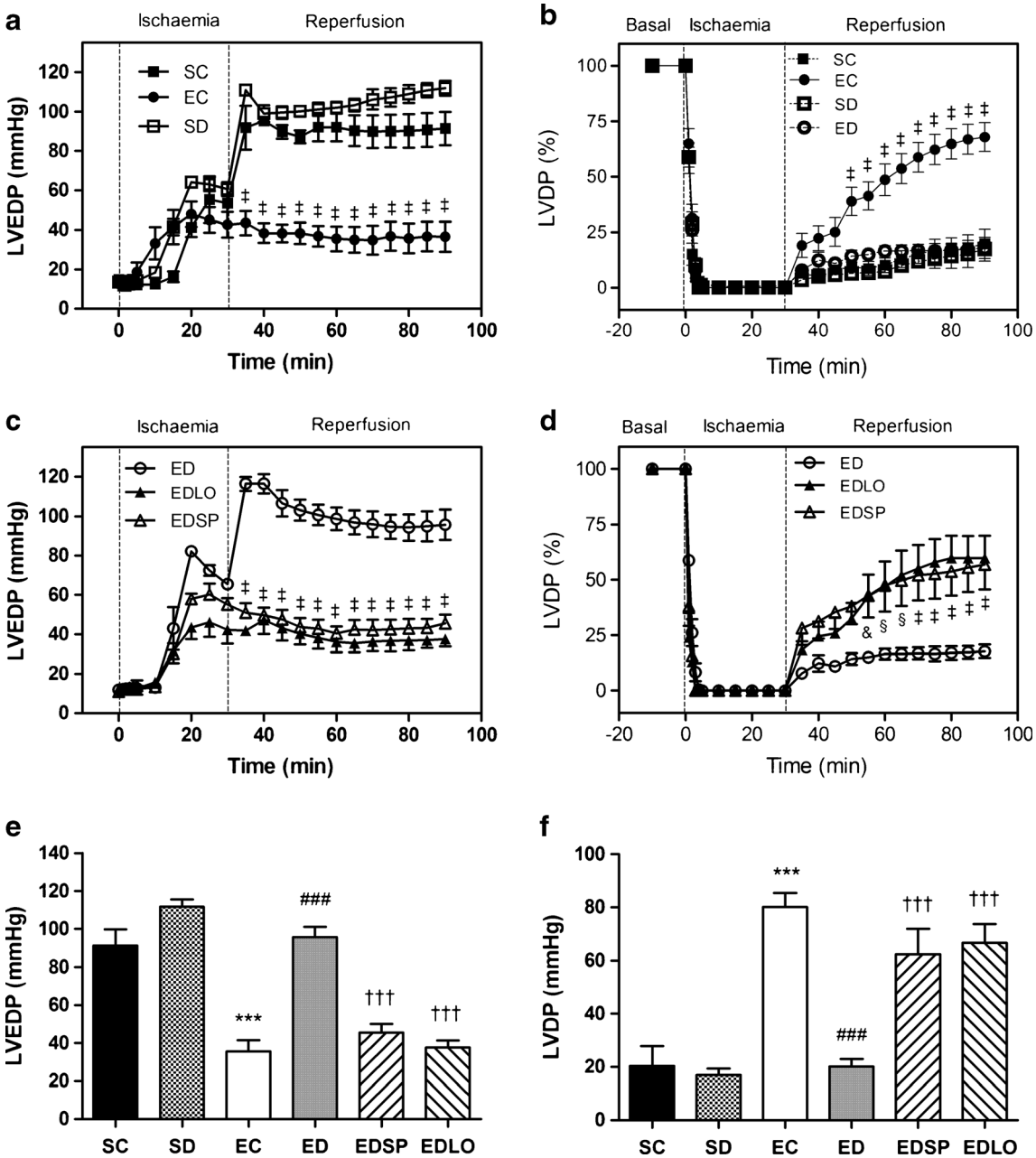

Fig. 2 Effects of 8 weeks of exercise and nandrolone treatment on postischaemic diastolic and systolic function recovery. Time course of changes in left ventricular end diastolic pressure (LVEDP: **a**, **c**) and developed pressure (LVDP: **b**, **d**) during the 30 min of global ischaemia and 60 min of reperfusion in ex-vivo perfused hearts. LVDP was expressed as percentual of variation relative to preischaemia period. LVEDP (**e**) and LVDP (**f**) measured at 60 min reperfusion. The data are expressed as the mean ± SEM ($n=5$ per group). &$P<0.05$, $^{\S}P<0.01$ and $^{\ddagger}P<0.01$ vs. SC and ED; ***$P<0.001$ vs. SC; $^{\#\#\#}P<0.001$ vs. EC; $^{\dagger\dagger\dagger}P<0.001$ vs. ED

ischaemia impaired the LV systolic function in hearts from all groups. Postischaemic recovery of LVDP, expressed as a percentage of the basal value, was significantly improved in hearts from exercised EC rats compared to SC sedentary rats, but not in exercised rats chronically treated with nandrolone decanoate (Fig. 2b). The nandrolone-induced impairment of

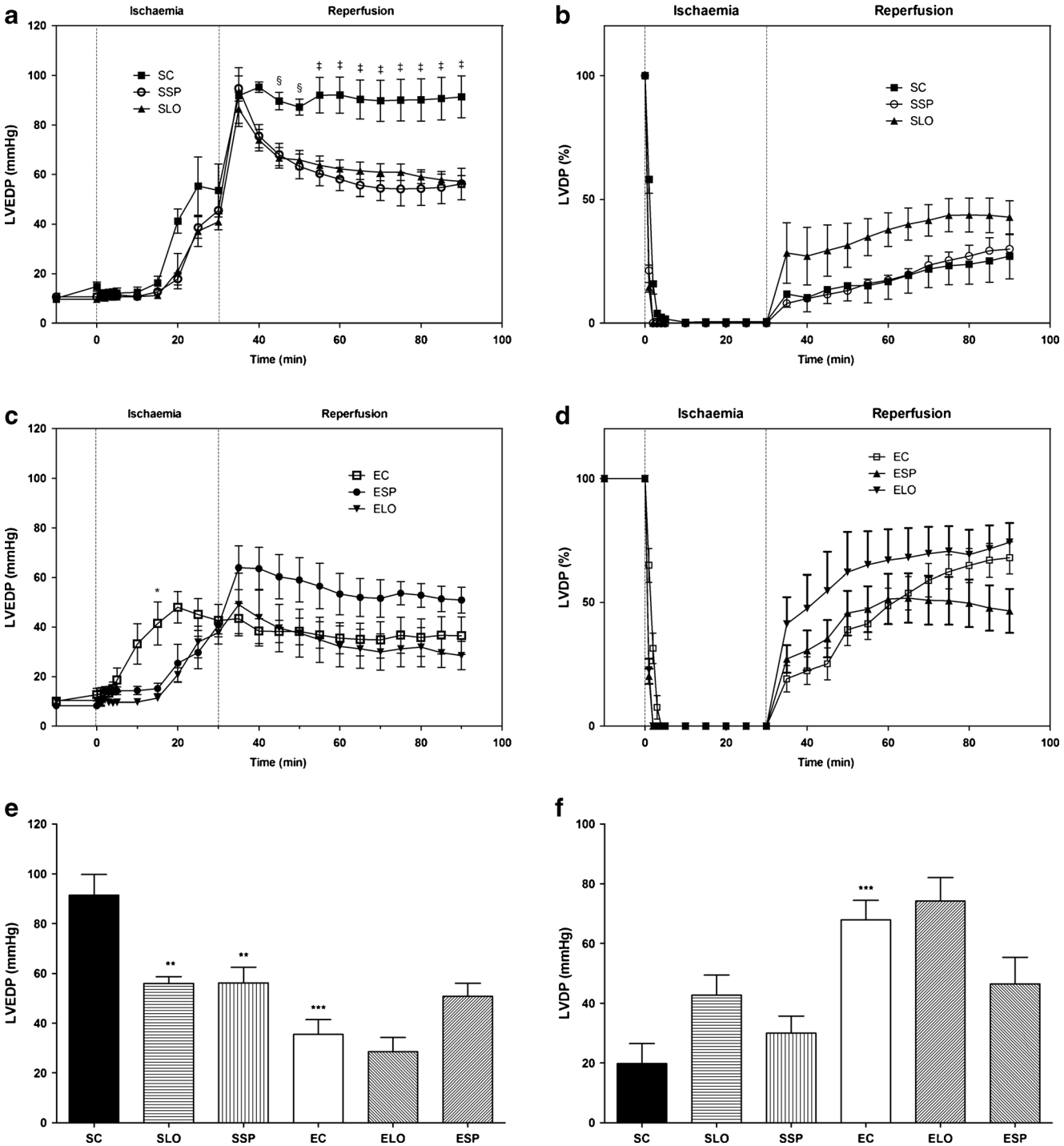

Fig. 3 Effects of spironolactone or losartan treatments on postischaemic recovery of diastolic and systolic function in sedentary and exercised rats. Time course of changes in left ventricular end diastolic pressure (LVEDP: **a**, **c**) and developed pressure (LVDP: **b**, **d**) during the 30 min of global ischaemia and 60 min of reperfusion in ex-vivo perfused hearts. LVDP was expressed as percentual of variation relative to preischaemia period. LVEDP (**e**) and LVDP (**f**) measured at 60 min reperfusion in sedentary control (SC), sedentary spironolactone-treated (SSP), sedentary losartan-treated (SLO), exercised control (EC), exercised spironolactone-treated (ESP), and exercised losartan-treated (ELO) rats. The data are expressed as the mean ± SEM ($n=5$ per group). $^{**}P<0.01$ and $^{***}P<0.001$ vs. SC; $^{\S}P<0.05$ and $^{\ddagger}P<0.001$ vs. SSP and SLO

the postischaemic recovery of LV function was prevented by the spironolactone (EDSP) and losartan (EDLO) treatments (Fig. 2d). The LVDP values at 60 min of reperfusion were increased by 293 % in EC compared to SC hearts. The LVDP value of the ED hearts was 74.8 % lower than that of the EC hearts but similar to those of the SC and SD hearts. The LVDP values of the EDSP and EDLO groups were 210 % and 231.8 % higher than that of the ED group, respectively, and did not differ from that of the EC group (Fig. 2f).

To evaluate whether AT_1-R and MR blockade alone had induced cardioprotection against ischaemia/reperfusion injuries, we treated sedentary (SSP and SLO) and exercised (ESP and ELO) rats with spironolactone or losartan, respectively, for 8 weeks. Chronic AT_1-R and MR blockade decreased the diastolic contracture in the hearts of sedentary rats (Fig. 3a and e), but did not significantly change the postischaemic LVEDP in exercised rats (Fig. 3c and e). Chronic AT_1-R and MR blockade did not significantly alter the postischaemic recovery of LVDP in hearts from sedentary SSP and SLO rats compared to SC rats (Fig. 3b and f), nor in hearts from exercised ESP and ELO rats compared to EC rats (Fig. 3d and f).

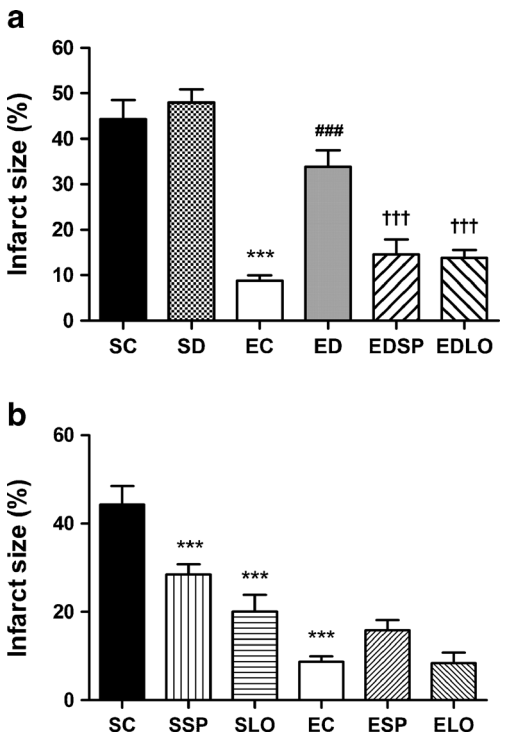

Fig. 4 **a** Spironolactone and losartan treatments preserved the exercise-induced myocardial infarct size reduction in nandrolone-treated rats. **b** Spironolactone and losartan treatment decreased the infarct sizes of sedentary (SSP, SLO) rats but did not alter the infarct sizes of exercised (ESP, ELO) rats. Infarct size was determined by TTC staining after 30 min of global ischaemia and 60 min of reperfusion in ex-vivo perfused hearts. Infarct size was expressed as a percentage of the risk area. The data are the mean ± SEM ($n=5$ per group). $^{***}P<0.001$ vs. SC; $^{###}P<0.001$ vs. EC; $^{†††}P<0.001$ vs. ED

Effect of AT_1-R and MR Blockade on the Ischaemia-Reperfusion Infarct Size

As demonstrated in Fig. 4a, 30 min global ischaemia induced a smaller infarct in EC hearts than in SC hearts. The SC and SD groups had similar infarct sizes. The exercise-induced infarct size reduction was attenuated by chronic nandrolone treatment, because the ED hearts had larger infarcts than did the EC hearts. However, the infarct size of the ED hearts was lower than those of the SC and SD hearts. Both losartan and spironolactone treatments prevented the nandrolone decanoate-mediated effect on the ischaemia-induced infarct because the infarcts of the EDSP and EDLO hearts were significantly smaller than those of the ED hearts. No significant differences in infarct size were observed among the EC, EDSP and EDLO hearts. To assess whether AT_1-R and MR blockade alone affected the ischaemia/reperfusion-induced cardiac infarct area, we evaluated heart infarct size in sedentary (SLO and SSP) and exercised (ELO and ESP) rats that were treated with losartan or spironolactone, respectively, for 8 weeks (Fig. 4b). AT_1-R and MR blockade reduced the infarct size in the hearts of the sedentary (SLO and SSP) rats. However, the infarct sizes in losartan- or spironolactone-treated exercised rats (ELO or ESP, respectively) were not significantly different than those in the EC group.

Nandrolone Treatment Increased AT_1-R and MR and Decreased K_{ATP} Expression

To address the potential molecular mechanism underlying the exercise training and nandrolone treatment-induced changes, we assessed angiotensinogen, ACE, AT_1-R, and MR expression. No significant differences in angiotensinogen or ACE mRNA levels were found among the groups (data not shown). However, as demonstrated in Fig. 5a and b, AT_1-R and MR mRNA levels were markedly higher in the ED group than in the EC group. Both spironolactone and losartan treatments prevented the nandrolone-induced elevation of AT_1-R and MR mRNA levels. Figure 5c demonstrates the AT_1-R protein expression profile. The AT_1-R levels in ED hearts were increased after chronic nandrolone administration compared with the EC group. Both losartan and spironolactone treatments prevented the increase in AT_1-R expression; thus, no differences in AT_1-R protein levels were observed among the EC, EDSP and EDLO groups. Similar results were observed for MR protein levels (Fig. 5d), which were elevated in the ED hearts compared with the SC and EC hearts. Exercise training itself increased MR expression; thus, the MR protein levels were higher in EC hearts than in the SC group hearts. Both losartan and spironolactone treatments also prevented the nandrolone-induced increase in MR protein level, which was similar among the SC, EDLO and EDSP groups.

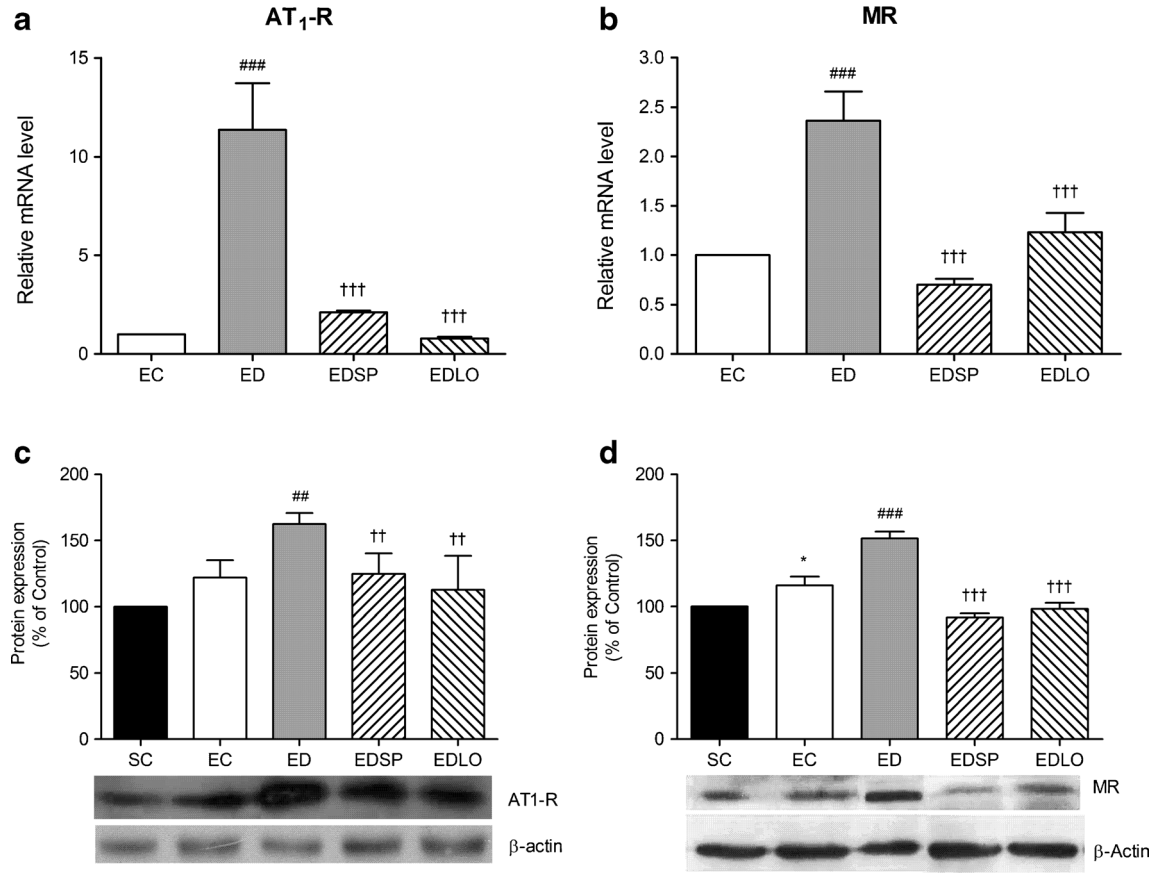

Fig. 5 Spironolactone and losartan treatments prevented the nandrolone-induced increases in cardiac type 1 angiotensin II (AT_1-R) and mineralocorticoid (MR) receptor mRNA (**a**, **b**) and protein (**c**, **d**) expression. mRNA transcript levels were determined by quantitative RT-PCR and normalized to the EC group mRNA value. AT_1-R and MR protein levels were determined by western blot analysis and normalised to the corresponding level in SC group. The data are expressed as the mean ± SEM (n = 4–10 per group). $^{*}P<0.05$ vs. SC; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs. EC; $^{\dagger\dagger}P<0.01$ and $^{\dagger\dagger\dagger}P<0.001$ vs. ED

Additionally, to evaluate the contributions of K_{ATP} channels to the effects of nandrolone, Kir6.1, Kir6.2 and SUR2a subunit expression was assessed (Fig. 6). Kir6.1 mRNA levels were decreased in ED hearts compared with EC hearts. Losartan but not spironolactone treatment prevented nandrolone-induced Kir6.1 downregulation; thus, the Kir6.1 mRNA content in EDLO hearts was higher than those in the ED, EC, and EDSP hearts (Fig. 6a). The increase in Kir6.1 mRNA levels after spironolactone treatment was not significantly different from that in the ED group, but the Kir6.1 mRNA level in the EDSP group was not significantly different from that in the EC group. Conversely, Kir6.2 mRNA levels were higher in the ED group than in the EC group. The nandrolone-induced Kir6.2 mRNA increase was prevented by the spironolactone and losartan treatments (Fig. 6b). Nandrolone treatment also downregulated SUR2a subunit expression (Fig. 6c). The ED group hearts exhibited significantly decreased SUR2a mRNA content compared with the EC group. Both losartan and spironolactone treatments prevented this effect; thus, EDSP and EDLO group hearts had greater SUR2a mRNA levels than did the ED group hearts. As shown in Fig. 6d, e and f, myocardial Kir6.1, Kir6.2 and SUR2a protein levels were elevated in the exercised EC hearts compared with the SC hearts. Chronic nandrolone treatment impaired the exercise-induced Kir6.1, Kir6.2 and SUR2a K_{ATP} channel subunit overexpression in the ED group. However, losartan (EDLO group) or spironolactone (EDSP group) treatment preserved the effects of exercise on Kir6.1, Kir6.2 and SUR2a K_{ATP} channel subunit expression.

Discussion

The major findings of this study are that nandrolone treatment abolishes exercise-induced cardioprotection via the downregulation of K_{ATP} channels, in association with cardiac AT_1 and mineralocorticoid receptor overexpression. The nandrolone-induced loss of cardioprotection by exercise, as well as the changes in AT_1-R, MR, and K_{ATP} channel expression, was prevented by AT_1 receptor blockade using losartan and MR blockade using spironolactone, suggesting a role for the renin-

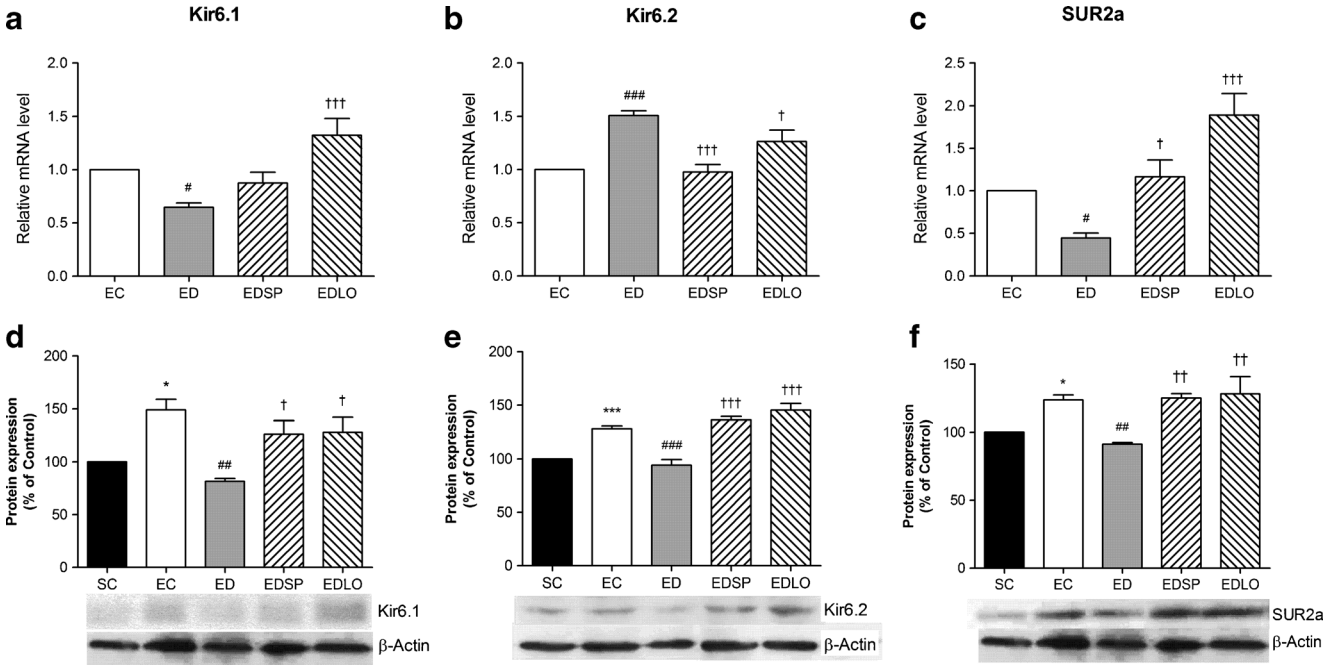

Fig. 6 Spironolactone and losartan treatments preserved the exercise-induced increases in K_{ATP} channel expression in the nandrolone-treated rat hearts. Cardiac K_{ATP} subunits (Kir 6.1, Kir 6.2, and SUR2a) mRNA (**a**, **b**, **c**) and protein (**d**, **e**, **f**) levels. mRNA transcript levels were determined by quantitative RT-PCR and normalised to the EC group mRNA value. Protein levels were determined by western blot analysis and normalized to the corresponding level in the SC group. The data are expressed as the mean ± SEM (n=4–10 per group). $^{*}P<0.05$ and $^{***}P<0.001$ vs. SC; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. EC; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, and $^{\dagger\dagger\dagger}P<0.001$ vs. ED

angiotensin-aldosterone system in regulating the deleterious effects of nandrolone.

As previously reported [24–26], we demonstrated significant cardiac hypertrophy after 8 weeks of nandrolone treatment. We also found that AT_1-R blockade with losartan and MR blockade with spironolactone effectively prevented the nandrolone-induced cardiac hypertrophy. These findings are consistent with those of Rocha et al. [25], who demonstrated cardiac hypertrophy with increased collagen deposition in trained nandrolone-treated rats. It was suggested that cardiac renin-angiotensin system activation was responsible for the nandrolone-induced cardiac remodelling because the AT_1-R blockade with losartan prevented the effects of nandrolone [25]. Consistent with these findings, other studies demonstrated that AT_1-R inhibition by losartan treatment prevented angiotensin II-induced fibrosis and hypertrophy and decreased AT_1-R expression [36, 37]. The participation of the RAAS in hypertrophic remodelling has been previously demonstrated in infarcted rats, in which angiotensin-activated myocardial aldosterone synthesis was associated with post-myocardial infarct remodelling [38]. Aldosterone increases AT_1-R mRNA and density [36, 39], and MR receptor blockade with spironolactone abolished collagen deposition and improved ventricular function in hypertrophic hearts from spontaneously hypertensive rats [40]. In our study, we did not demonstrate any significant changes in myocardial angiotensinogen or ACE mRNA levels, but AT_1-R and MR mRNA and protein expression were increased in the hearts of nandrolone-treated trained rats. Indeed, AT_1-R and MR blockade prevented the nandrolone-induced overexpression of AT_1-R and MR.

Previous studies from our group had demonstrated that hearts from exercise-trained rats that had been submitted to ischaemia/reperfusion exhibited a reduced infarct area and better left ventricular function recovery compared with sedentary rats [24]. However, chronically nandrolone-treated trained rats exhibited impaired exercise-induced cardioprotection, which was expressed as reduced left ventricular function recovery and increased infarct area, similar to the profile of sedentary rat hearts [24]. In the present study, we confirmed that cardioprotective effects had been induced by 8 weeks of treadmill exercise training and that these effects were impaired by chronic nandrolone decanoate administration. Conversely, AT_1-R and MR blockade prevented the attenuation of the beneficial effects of exercise on postischaemic cardiac function recovery by nandrolone, suggesting a role for RAAS activation by chronic AAS treatment in the impairment of exercise-induced cardioprotection. In our study hearts from sedentary rats chronically treated with MR and AT_1-R blockers presented reduced I/R-induced infarct size but without significant improvement in functional recovery. Spironolactone [41] and losartan [42, 43] have been reported to induce cardioprotection, however these effects were observed in conditions of short-time exposition to the drugs before I/R.

The mechanism underlying the nandrolone-mediated impairment of exercise-induced cardioprotection is unknown. However, we can suggest that the long-term effects of nandrolone treatment on exercise-induced cardioprotection are mediated by K_{ATP} channel expression regulation by the cardiac RAAS, because the exercised and nandrolone-treated rat hearts demonstrated Kir6.1, Kir6.2, and SUR2a subunit downregulation, which was prevented by AT_1-R and MR blockade. In the exercised and nandrolone-treated rat hearts, the expression of Kir6.2 protein was decreased despite the increase in the levels of mRNA. We do not know the mechanism of this dissociation between mRNA and protein expression, but we suggest that this dissociation could be due to independent translational and posttranscriptional mechanisms regulating Kir6.2 expression. A similar dissociation between Kir6.2 mRNA and protein expression was reported by Tsounapi et al. [44], who observed that rat testis subjected to ischaemia/reperfusion presented an up-regulation of Kir6.2 mRNA and a tendency for a decreased Kir 6.2 protein expression. The role of sarcolemmal K_{ATP} ($sarcK_{ATP}$) channels in exercise-induced cardioprotection was suggested by Brown et al. [13], who observed that $sarcK_{ATP}$ channel inhibition by HMR1098, but not mitochondrial K_{ATP} ($mitoK_{ATP}$) channel inhibition with 5HD, abolished training-induced cardioprotection in female rats. Rather, the authors observed exercise-induced increases in myocardial Kir6.2 and SUR2a K_{ATP} subunit protein expression. In the present study, the nandrolone-mediated impairment of exercise-induced cardioprotection was associated with the abrogation of the exercise-induced increases in Kir6.1, Kir6.2, and Sur2a expression. This finding is consistent with the observation of greater cardiac Kir6.2 and SUR2a protein expression and lesser susceptibility to ischaemia-reperfusion injury in female rats compared with male rats [14]. Such sex-dimorphic responses to ischaemia-reperfusion suggest an androgen-mediated downregulation of cardiac K_{ATP} channels. Taken together, these findings point to a role of $sarcK_{ATP}$ channels in the nandrolone-mediated impairment of exercise-induced cardioprotection. The ventricular $sarcK_{ATP}$ channel has been proposed to be composed by Kir6.2 and SUR2a subunits [45, 46], although Kir6.1 and SUR1 subunits are also expressed in ventricular myocytes [47]. On the other hand, there is yet no conclusive identification of the molecular constituents of $mitoK_{ATP}$ channel [48–50].

Our observation that AT_1-R and MR blockade prevented the nandrolone-mediated impairment of exercise-induced cardioprotection, AT_1-R and MR overexpression, and K_{ATP} channel downregulation suggest that the regulation of K_{ATP} channel expression by cardiac RAAS has a role in the long-term effects of AAS on exercise-induced cardioprotection. Tavares et al. [33] have demonstrated angiotensin II-mediated K_{ATP} channel expression remodelling in post-infarct heart failure. Angiotensin II-mediated K_{ATP} channel inhibition has also been reported in arterial smooth muscle cells [51, 52].

In summary, the present results demonstrated that exercise training improves myocardial tolerance to ischaemia-reperfusion injuries in male rats, but the association of exercise training and nandrolone treatment abolish this cardioprotection by a mechanism involving cardiac RAAS activation and downregulation of K_{ATP} channel expression.

Source of Fundings This work was supported by the FAPERJ–Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (grant E-26/110.342/2012). JHMN is a research fellow from CNPq. SRMN received a fellowship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Conflict of Interest None.

References

1. Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, et al. Heart Disease and Stroke Statistics–2008 Update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation. 2008;117:e25–e146.
2. World Health Organization. Cardiovascular diseases (CVDs). Fact sheet No. 317, 2011.
3. Shephard RJ, Balady GJ. Exercise as cardiovascular therapy. Circulation. 1999;99:963–72.
4. Myers J. Cardiology patient pages. Exercise and cardiovascular health. Circulation. 2003;107:e2–5.
5. Haskell WL, Lee IM, Pate RR, Powell KE, Blair SN, Franklin BA, et al. Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. Med Sci Sports Exerc. 2007;39:1423–34.
6. Nelson ME, Rejeski WJ, Blair SN, Duncan PW, Judge JO, King AC, et al. Physical activity and public health in older adults: recommendation from the American College of Sports Medicine and the American Heart Association. Circulation. 2007;116:1094–105.
7. Leung FP, Yung LM, Laher I, Yao X, Chen ZY, Huang Y. Exercise, vascular wall and cardiovascular diseases: an update (part 1). Sports Med. 2008;38:1009–24.
8. McElroy CL, Gissen SA, Fishbein MC. Exercise-induced reduction in myocardial infarct size after coronary artery occlusion in the rat. Circulation. 1978;57:958–62.
9. Bowles DK, Farrar RP, Starnes JW. Exercise training improves cardiac function after ischemia in the isolated, working rat heart. Am J Physiol Heart Circ Physiol. 1992;263:H804–9.
10. Yamashita N, Baxter GF, Yellon DM. Exercise directly enhances myocardial tolerance to ischemia-reperfusion injury in the rat through a protein kinase C mediated mechanism. Heart. 2001;85:331–6.
11. Gielen S, Schuler G, Adams V. Cardiovascular effects of exercise training–molecular mechanisms. Circulation. 2010;122:1221–38.
12. Frasier CR, Moore RL, Brown DA. Exercise-induced cardiac preconditioning: how exercise protects your achy-breaky heart. J Appl Physiol. 2011;111:905–15.
13. Brown DA, Chicco AJ, Jew KN, Johnson MS, Lynch JM, Watson PA, et al. Cardioprotection afforded by chronic exercise is mediated by sarcolemmal, and not the mitochondrial, isoform of the KATP channel in the rat. J Physiol. 2005;569:913–24.
14. Brown DA, Lynch JM, Armstrong CJ, Caruso NM, Ehlers LB,

Johnson MS, et al. Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. J Physiol. 2005;564:619–30.
15. Chicco AJ, Johnson MS, Armstrong CJ, Lynch JM, Gardner RT, Fasen GS, et al. Sex-specific and exercise-acquired cardioprotection is abolished by sarcolemmal KATP channel blockade in the rat heart. Am J Physiol Heart Circ Physiol. 2007;292:H2432–7.
16. Quindry JC, Schreiber L, Hosick P, Wrieden J, Irwin JM, Hoyt E. Mitochondrial KATP channel inhibition blunts arrhythmia protection in ischemic exercised hearts. Am J Physiol Heart Circ Physiol. 2010;299:H175–83.
17. Hartgens F, Kuipers H. Effects of androgenic-anabolic steroids in athletes. Sports Med. 2004;34:513–54.
18. Sj qvist F, Garle M, Rane A. Use of doping agents, particularly anabolic steroids, in sports and society. Lancet. 2008;371:1872–82.
19. Sullivan ML, Martinez CM, Gennis P, Gallagher EJ. The cardiac toxicity of anabolic steroids. Prog Cardiovasc Dis. 1998;41:1–15.
20. Di Paolo M, Agozzino M, Toni C, Luciani AB, Molendini L, Scaglione M, et al. Sudden anabolic steroid abuse-related death in athletes. Int J Cardiol. 2007;114:114–7.
21. Fineschi V, Baroldi G, Monciotti F, Reattelli LP, Turillazzi E. Anabolic steroid abuse and cardiac sudden death: a pathologic study. Arch Pathol Lab Med. 2001;125:253–5.
22. Urhausen A, Albers T, Kindermann W. Are the cardiac effects of anabolic steroid abuse in strength athletes reversible? Heart. 2004;90: 496–501.
23. Du Toit EF, Rossouw E, Van Rooyen J, Lochner A. Proposed mechanisms for the anabolic steroid-induced increase in myocardial susceptibility to ischemia/reperfusion injury. Cardiovasc J South Afr. 2005;16:21–8.
24. Chaves EA, Pereira-Junior PP, Fortunato RS, Masuda MO, Carvalho ACC, Carvalho DP, et al. Nandrolone decanoate impairs exercise-induced cardioprotection: role of antioxidant enzymes. J Steroid Biochem Mol Biol. 2006;99:223–30.
25. Rocha FL, Carmo EC, Roque FR, Hashimoto NY, Rossoni LV, Frimm C, et al. Anabolic steroids induce cardiac renin-angiotensin system and impair the beneficial effects of aerobic training in rats. Am J Physiol Heart Circ Physiol. 2007;293:H3575–83.
26. Do Carmo EC, Fernandes T, Koike D, Da Silva Jr ND, Mattos KC, Rosa KT, et al. Anabolic steroid associated to physical training induces deleterious cardiac effects. Med Sci Sports Exerc. 2011;43: 1836–48.
27. Marsh JD, Lehmann MH, Ritchie RH, Gwathmey JK, Green GE, Schiebinger RJ. Androgen receptors mediate hypertrophy in cardiac myocytes. Circulation. 1998;98:256–61.
28. Iwai N, Shimoike H, Kinoshita M. Cardiac rennin-angiotensin system in the hypertrophied heart. Circulation. 1995;92:2690–6.
29. Barauna VG, Magalhaes FC, Krieger JE, Oliveira EM. AT1 receptor participates in the cardiac hypertrophy induced by resistance training in rats. Am J Physiol Regul Integr Comp Physiol. 2008;295:R381–7.
30. Zhang AD, Cat AND, Soukaseum C, Escoubet B, Cherfa A, Messaoudi S, et al. Cross-talk between mineralocorticoid and angiotensin II signaling for cardiac remodeling. Hypertension. 2008;52: 1060–7.
31. Kalra D, Sivasubramanian N, Mann DL. Angiotensin II induces tumor necrosis factor biosynthesis in the adult mammalian heart through a protein kinase C–dependent pathway. Circulation. 2002;105:2198–205.
32. Nakamura K, Fushimi K, Kouchi H, Mihara K, Miyazaki M, Ohe T, et al. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-α and angiotensin II. 1998;98:794–9.
33. Tavares NI, Philip-Couderc P, Baertschi AJ, Lerch R, Montessuit C. Angiotensin II and tumour necrosis factor α as mediators of ATP-dependent potassium channel remodeling in post-infarction heart failure. Cardiovasc Res. 2009;83:726–36.
34. Serejo FC, Rodrigues-Junior LF, Tavares KCS, Campos de Carvalho AC, Nascimento JHM. Cardioprotective properties of humoral factors released from rat hearts subject to ischemic preconditioning. J Cardiovasc Pharmacol. 2007;49:214–20.
35. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 2008;3:1101–8.
36. Robert V, Heymes C, Silvestre JS, Sabri A, Swynghedauw B, Delcayre C. Angiotensin AT1 receptor subtype as a cardiac target of aldosterone: role in aldosterone-salt-induced fibrosis. Hypertension. 1999;33:981–6.
37. Takeda Y, Yoneda T, Demura M, Usukura M, Mabuchi H. Calcineurin inhibition attenuates mineralocorticoid-induced cardiac hypertrophy. Circulation. 2002;105:677–9.
38. Silvestre JS, Heymes C, Oubénaïssa A, Robert V, Aupetit-Faisant B, Carayon A, et al. Activation of cardiac aldosterone production in rat myocardial infarction. Circulation. 1999;99:2694–701.
39. Ullian ME, Schelling JR, Linas SL. Aldosterone enhances angiotensin II receptor binding and inositol phosphate responses. Hypertension. 1992;20:67–73.
40. Mill JG, Milanez MC, Rezende MM, Gomes MGS, Leite CM. Spironolactone prevents cardiac collagen proliferation after myocardial infarction in rats. Clin Exp Pharmacol Physiol. 2003;30:739–44.
41. Chai W, Garrelds IM, Arulmani U, Schoemaker RG, Lamers JMJ, Danser AHJ. Genomic and nongenomic effects of aldosterone in the rat: why is spironolactone cardioprotective. Br J Pharmacol. 2005;145:664–71.
42. Sato M, Engelman RM, Otani H, Maulik N, Rousou JA, Flack III JE, et al. Myocardial protection by preconditioning of heart with losartan, na angiotensin II type 1-receptor blocker: implication of bradykinin-dependent and bradykinin-independent mechanisms. Circulation. 2000;102(Supl 3):346–51.
43. Flynn JD, Akers WS. Effects of the angiotensin II subtype 1 receptor antagonist losartan on functional recovery of isolated rat hearts undergoing global myocardial ischemia-reperfusion. Pharmacotherapy. 2003;23:1401–10.
44. Tsounapi P, Saito M, Dimitriadis F, Kitatani K, Kinoshita Y, Shomori K, et al. The role of K_{ATP} channels on ischemia-reperfusion injury in the rat testis. Life Sci. 2012;90:649–56.
45. Seharaseyon J, Sasaki N, Ohler A, Sato T, Fraser H, Johns DC, et al. Evidence against functional heteromultimerization of the KATP channel subunits Kir6.1 and Kir6.2. 275. J Biol Chem. 2000;23: 17561–5.
46. Flagg TP, Kurata HT, Masia R, Caputa G, Magnuson MA, Lefer DJ, et al. Differential structure of atrial and ventricular KATP: atrial KATP channels require SUR1. Circ Res. 2008;103:1458–65.
47. Morrissey A, Rosner E, Lanning J, Parachuru L, Chowdhury PD, Han S, et al. Immunolocalization of KATP channel subunits in mouse and rat cardiac myocytes and the coronary vasculature. BMC Physiol. 2005;5:1.
48. Seharaseyon J, Ohler A, Sasaki N, Fraser H, Sato T, Johns DC, et al. Molecular composition of mitochondrial ATP-sensitive potassium channels probed by viral Kir gene transfer. J Mol Cell Cardiol. 2000;32:1923–30.
49. Cuong DV, Kim N, Joo H, Youm JB, Chung J-Y, Lee Y, et al. Subunit composition of ATP-sensitive potassium channels in mitochondria of rat hearts. Mitochondrion. 2005;5:121–33.
50. Foster DB, Rucker JJ, Marbán E. Is Kir6.1 a subunit of mitoKATP? Biochem Biophys Res Commun. 2008;366:649–56.
51. Kubo M, Quayle JM, Standen NB. Angiotensin II inhibition of ATP-sensitive K^+ currents in rat arterial smooth muscle cells through protein kinase C. J Physiol. 1997;503:480–96.
52. Sampson LJ, Davies LM, Barrett-Jolley R, Standen NB, Dart C. Angiotensin II-activated protein kinase C targets caveolae to inhibit aortic ATPsensitive potassium channels. Cardiovasc Res. 2007;76:61–70.