Anabolic Steroid Associated to Physical Training Induces Deleterious Cardiac Effects

EVERTON CRIVOI DO CARMO, TIAGO FERNANDES, DANIEL KOIKE, NATHAN DANIEL DA SILVA JR., KATT COELHO MATTOS, KALEIZU TEODORO ROSA, DIEGO BARRETTI, STEPHANO FREITAS SOARES MELO, ROGERIO B. WICHI, MARIA CLAUDIA COSTA IRIGOYEN, and EDILAMAR MENEZES DE OLIVEIRA

1School of Physical Education and Sport, University of São Paulo, São Paulo, SP, BRAZIL; 2Human Movement Laboratory, São Judas Tadeu University, São Paulo, SP, BRAZIL; and 3Hypertension Unit, Heart Institute, Medical School, University of São Paulo, São Paulo, SP, BRAZIL

ABSTRACT

DO CARMO, E. C., T. FERNANDES, D. KOIKE, N. D. DA SILVA JR., K. C. MATTOS, K. T. ROSA, D. BARRETTI, S. F. S. MELO, R. B. WICHI, M. C. C. IRIGOYEN, and E. M. DE OLIVEIRA. Anabolic Steroid Associated to Physical Training Induces Deleterious Cardiac Effects. Med. Sci. Sports Exerc., Vol. 43, No. 10, pp. 1836–1848, 2011. Purpose: Cardiac aldosterone might be involved in the deleterious effects of nandrolone decanoate (ND) on the heart. Therefore, we investigated the involvement of cardiac aldosterone, by the pharmacological block of AT1 or mineralocorticoid receptors, on cardiac hypertrophy and fibrosis. Methods: Male Wistar rats were randomized into eight groups (n = 14 per group): Control (C), nandrolone decanoate (ND), trained (T), trained ND (TND), ND + losartan (ND + L), trained ND + losartan (TND + L), ND + spironolactone (ND + S), and trained ND + spironolactone (TND + S). ND (10 mg kg⁻¹ wk⁻¹) was administered during 10 wk of swimming training (five times per week). Losartan (20 mg kg⁻¹ d⁻¹) and spironolactone (10 mg kg⁻¹ d⁻¹) were administered in drinking water. Results: Cardiac hypertrophy was increased 10% by using ND and 17% by ND plus training (P < 0.05). In both groups, there was an increase in the collagen volumetric fraction (CVF) and cardiac collagen type III expression (P < 0.05). The ND treatment increased left ventricle–angiotensin-converting enzyme I activity, CYP11B2, and osteopontin induced by the ND treatment. Conclusions: We believe this is the first study to show the effects of ND on cardiac aldosterone. Our results suggest that these effects may be associated to TGFβ and osteopontin. Thus, we conclude that the cardiac aldosterone has an important role on the deleterious effects on the heart induced by ND. Key Words: ANABOLIC STEROID, ANGIOTENSIN II, ALDOSTERONE, CARDIAC HYPERTROPHY, CARDIAC COLLAGEN

Anabolic steroids induce adverse effects on the cardiovascular system, including myocardial structural changes and cardiac hypertrophy (15). Exercise training associated with anabolic steroid induces maladaptive remodeling and further deterioration in cardiac performance and causes loss of the beneficial effects in left ventricle (LV) function induced by exercising (27). However, the mechanisms of these processes are poorly understood.

Our previous findings indicate that the combination of physical training and nandrolone decanoate (ND) causes an increase in the heart collagen concentration and cardiac hypertrophy associated with activation of the cardiac renin–angiotensin system. These findings were reinforced by the fact that AT1 receptor antagonist prevented these effects induce by ND (27).

The renin–angiotensin system, via angiotensin II, plays an important role in regulating cardiac growth and in remodeling of the cardiac extracellular matrix (40). However, the angiotensin II effects on cardiac tissue have been associated with aldosterone actions (16). As the tissue angiotensin II, aldosterone synthesis is also detected in extra adrenal sites as cardiovascular tissue (33), being that the aldosterone synthase gene (CYP11B2) is a key enzyme in this process (33,37). An increase in CYP11B2 was associated with cardiovascular disorders, such as heart failure and myocardial infarction (37). The mineralocorticoid receptor and 11-β hydroxysteroid dehydrogenase 2 (11β-HSD2), which confers mineralocorticoid receptors selectivity to aldosterone target tissue, have also been detected in the heart (12). The increase in 11β-HSD2 seems to have deleterious effects on the heart, suggesting that the aldosterone has an important role in the cardiovascular system (24).

The production of aldosterone in the myocardium may stimulate the extracellular matrix due to the increase in cardiac fibrosis and the remodeling (26), leading to increased
myocardial stiffness and dysfunction, regardless of hemodynamic factors (5,26). These effects may be mediated by inflammatory factors as TGFβ and osteopontin that have a functional role in fibrosis and cardiac hypertrophy (31,28).

On the basis of our previous study, the cardiac renin–angiotensin system might be importantly involved in ND’s effects on cardiac hypertrophy and fibrosis when associated with physical training (27). However, the aldosterone effects are unknown. Therefore, our hypothesis was that the cardiac aldosterone might be involved in deleterious effects induced by ND treatment alone or associated with physical training.

For this purpose, initially, we evaluated the effects of ND treatment alone or associated with physical training on activation of the cardiac aldosterone synthesis, inflammatory markers, and left ventricular geometry and function in vivo, by M-mode echocardiography in rats. In the next step, we evaluated whether the pharmacological block of AT1 or mineralocorticoid receptors prevent the cardiac deleterious effects induced by ND and ND associated with physical training.

**METHODS**

**Experimental Groups**

Male Wistar rats (8–12 wk old, weighing 180–250 g; \( n = 112 \)) were used. All protocols were in accordance with the policy statement of the American College of Sports Medicine with experimental animals and were approved by the Ethics Committee of the School of Physical Education and Sports of the University of São Paulo. The rats were divided randomly into eight groups, each with 14 rats: control (C), nandrolone decanoate (ND), trained (T), trained ND (TND), ND + losartan (ND + L), ND + spironolactone (ND + S), trained ND + losartan (TND + L), and trained ND + spironolactone (TND + S). Each group was subdivided into two groups: one for biochemical and molecular studies and the other for hemodynamic and histological studies.

The AT1 receptor antagonist, losartan (Cozaar®; Merck Sharp, Whitehouse Station, NJ) and the mineralocorticoid receptors antagonist, spironolactone (Aldactone®; Pfizer S.R.L., New York, NY), were administered in the animals' water at doses of 20 and 10 mg·kg\(^{-1}\)·d\(^{-1}\), respectively. Because spironolactone is insoluble in water, it was first dissolved in ethanol at 25 mg·mL\(^{-1}\) and added to drinking water. The other groups received a vehicle (1% ethanol in drinking water) as described previously (16). The doses are described in the literature as sufficient to block the actions of angiotensin II and aldosterone, respectively, without interfering with the blood pressure (BP) of normotensive rats (5,11,27). The rats were treated with ND (Decadurabolin; Organon, Roseland, NJ) administered subcutaneously twice a week, in a dose of 5 mg·kg\(^{-1}\) per injection. This is a supraphysiologic dose and comparable to that frequently used by athletes in doping processes (700 mg·wk\(^{-1}\) or approximately 10 mg·kg\(^{-1}\)·wk\(^{-1}\)). This dose of ND is 100 times the therapeutic dosage (23). All the treatments began on the same day as the exercise training and continued thereafter.

Food and water were provided *ad libitum*. Room temperature was kept at 23°C ± 1°C. A 12:12-h light–dark cycle was maintained throughout the experiment. The rats were identified and weighed weekly.

**Training Protocol**

The swimming training was performed as described previously (14). The swimming training was performed during the dark cycle of the rat and consisted of 5 d·wk\(^{-1}\) of swimming sessions of 60 min in duration for 10 wk in a swimming system with water at 30°C–32°C. Exercise duration and workload were increased gradually until the rats were able to swim for 60 min wearing caudal dumbbells weighing 5% of their body weight. Thereafter, duration and workload were constant. Sedentary groups were placed in the swimming apparatus for 10 min, twice a week with no workload for the control of their being in the water. This protocol is defined as a low-intensity long training period, effective for the promotion of cardiovascular adaptations and increase in muscle oxidative capacity (14).

**Measurements and Procedures**

**Hemodynamic measurements.** *BP*. It was determined noninvasively using a computerized tail–cuff system. Rats were acclimatized to the apparatus during daily sessions for 6 d, 1 wk before starting the experimental period. The BP was determined before beginning the experimental protocol and was measured weekly until the end of the training to identify possible changes on BP that could interfere with the results. For BP recording, a rubber cuff was placed on the tail proximally and connected to a sphygmomanometer to gradually inflate and deflate the cuff from 0 to 250/300 mm Hg. In the most distal portion of the tail, a pneumatic transducer was attached to detect signs of passage of the pulse wave of BP in the artery and recorded on the system AT/CODAS (DataQ Instruments, Inc., Akron, OH) with sampling frequency of 1000 Hz. The BP was recorded at the time when the pressure on the tail became slightly less than the value of intra-arterial pressure, allowing the detection of the pulse pressure. For each animal, five measures of BP were taken, and the first and last steps were discarded to calculate the arithmetic mean of the remaining values.

**Echocardiography.** It was assessed in accordance with the recommendations of the American Society of Echocardiography (29). Transthoracic echocardiography was performed after the experimental period using Sequoia 512 equipment (ACUSON Corporation, Mountain View, CA) with a 10- to 14-MHz multifrequency linear transducer placed on the animals shaved chest (lateral recumbence). Rats were anesthetized with a mixture of xylazine (10 mg·kg\(^{-1}\)) and ketamine (90 mg·kg\(^{-1}\)). All measurements were performed by the same observer based on the average of three consecutive cardiac cycles. Wall thickness and LV dimensions were determined.
obtained from a short-axis view at the level of the papillary muscles. Two-dimensionally guided pulse Doppler recordings of LV transmitral flow were obtained from the apical four-chamber view. The systolic function was determined by shortening fraction (SF = ((LVd - LVs)/(LVd * 100), where LVd is LV end-diastolic diameter and LVs is the LV end-systolic diameter). The ejection fraction (EF) was calculated according to the Teichholz formula ($V = [7.0/2.4 + d] * D3$, where $V = \text{volume}$ and $D = \text{echocardiographically measured internal dimension}$) (34). The diastolic function was determined at peak velocity of $E$ wave, peak velocity of $A$ wave, $E/A$ ratio, and isovolumetric relaxation time (IVRT, was taken as the time from aortic valve closure to the onset of mitral flow).

**Tissue samples.** At the end of the experimental period, animals were killed by decapitation. The heart was removed from the thoracic cavity and dissected to separate the LV. The LV of one group was weighed and stored at $-80^\circ$C for biochemical and molecular analysis, and the LV of the other group, for histological analysis.

**Morphological and morphometric analysis.** Cardiac hypertrophy was determined by ratio of LV weight to animal body weight and cardiomyocytes diameter.

**Ratio of LV weight to animal body weight.** LV hypertrophy was calculated by LV weight (LVW, mg) normalized by BW (g) of the animals (LVW/BW, mg·g$^{-1}$).

**Cardiomyocytes diameter.** For morphometric analyses, the hearts were stopped at diastole by perfusing with 14 mM KCl. After that, the LV was fixed at 6% formaldehyde and embedded in paraffin, cut into 5-μm sections at the level of the papillary muscle, and subsequently stained with hematoxylin and eosin for the visualization of cellular structures. Two randomly selected sections from each animal were visualized by light microscopy using an oil immersion objective with a calibrated magnification ($\times$400). Cardiomyocytes with visible nuclei and intact cellular membranes were chosen for diameter determination. The width of individually isolated cardiomyocytes displayed on a viewing screen was manually traced across the middle of the nuclei, with a digitizing pad, and determined by a computer-assisted image analysis system (QuantiMet 520; Cambridge Instruments, Woburn, MA). For each animal, ~20 visual fields were analyzed. Results were expressed as micrometers.

**Myocardial interstitial collagen volumetric fraction.** This was determined using direct and polarized light of the Picrosiris red prepared tissues, as reported previously (7). In short, 20 fields were selected from sections placed in a projection microscope ($\times$200), and interstitial collagen was determined by a computer-assisted image analysis system (QuantiMet 520; Cambridge Instruments). Collagen volumetric fraction (CVF) was calculated as the sum of all connective tissue areas divided by the sum of all muscle areas in all fields. Perivascular and patch cardiac (reparative fibrosis) were specifically excluded from this determination. Results were expressed as micrometers per area.

**Molecular and Biochemical Analysis**

**Angiotensin-converting enzyme I activity.** LV–Angiotensin-converting enzyme I (ACE) activity in rat tissue extracts were determined using Abz-FRK(Dnp)P-OH derivatives as substrates by continuously measuring the fluorescence according to Alves et al. (1). Tissue samples were quickly harvested, homogenized in Tris–HCl buffer, pH 7.6, containing 50 mM NaCl, and centrifuged at 1000g for 10 min. The assays were performed at 37°C in 0.1 M Tris–HCl buffer, pH 7.0, containing 50 mM NaCl and 10 μM ZnCl2. The hydrolysis rate of the intramolecularly quenched fluorogenic substrate Abz-FRK-(Dnp)p (10 mM) incubated with aliquots of tissue homogenate for 30 min at 37°C was assessed to obtain ACE enzymatic activity. Fluorescence increments along the time were read at 420-nm emission/320-nm excitation. Cardiac ACE activity was expressed as arbitrary fluorescence units (UF) per milligram of protein. The protein content was determined by the Bradford method (4).

**Cardiac protein expression.** Quantification of AT1 receptor was performed by Western blot analysis. The frozen ventricles were thawed and minced into small pieces and homogenized in cell lysis buffer containing 100 mM Tris–HCl, 50 mM NaCl, 10 mM EDTA, 1% Triton X-100, and a solution containing a mixture of protease inhibitors (potassium EDTA (25 mmol), o-phenanthroline (0.44 mmol), pepstatin A (0.12 mmol), and 4-chloromercuribenzoic acid (1 mmol)) to prevent the in vitro production and degradation of angiotensin peptides. Insoluble heart tissues were removed by centrifugation at 3000g at 4°C and 10 min. Samples were loaded and subjected to SDS–PAGE at 10% of polyacrylamide gels. After electrophoresis, proteins were electrotransferred to the nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Equal loading of samples (60 μg) and even transfer efficiency were monitored with the use of 0.5% of Ponceau S staining of the blot membrane. The blot membrane was then incubated in a blocking buffer (5% of nonfat dry milk, 10 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% of Tween 20) for 2 h at room temperature and then probed with a polyclonal antibody directed against AT1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature. The binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (1:2000), and improved chemiluminescence reagents (Amersham Biosciences) were used to visualize the autoradiogram that was later exposed to a photographic film. The film was developed, and the bands were analyzed using Scion Image software (Scion based on National Institutes of Health Image). α-Tubulin expression levels were used to normalize the results. Results were expressed as arbitrary units (AU).

**Cardiac gene expression.** The gene expression of collagen type I (sense GAGAGCATGACCGATGGA and antisense GAGTTTGCAGCTGTTGG), collagen type III (sense AAGGTCCACGAGGTGACAA and antisense AGGCCCTGGACTACCAACT), mineralocorticoid receptors (MR) (sense GCTTTGATGTTAGCTGCG and antisense
TGAGCACAATCCGGTAG), transforming growth factor β (TGFβ) (sense GCGGGTGTCCCTTTTGTA and antisense GCGGGTACTCTTTTGCC), osteopontin (sense GTC CTTCACTGCCAGCAC, and antisense GAATCTGG CAGCTGTCTGAT), CYP11B2 (aldosterone synthase gene) (sense GGTAGTCGACGAGGAACTC and antisense ATTAG TGCCTGCCAAATGTC) (36), and 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) (sense CCAGTTGTGACACTGG TTTTG and antisense GGGGTATGCACTGTCCTCTG) (13) were determined by reverse transcription–polymerase chain reaction (PCR).

Because of the very high similarity of sequence between CYP11B2 (aldosterone synthase) and CYP11B1, we chose to use a primer previously described in the literature as being specific for the aldosterone synthase (CYP11B2) (36). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense ATGGTGGTGAACACGGAA and antisense CGAGTA CTGGTGTCAGGTA) gene level was used to normalize the results. Results were expressed as AU.

Frozen tissue samples (150–200 mg) were homogenized in a guanidinium thiocyanate solution (4 M), and RNA was isolated according to the method described previously (6). Samples were quantified spectrophotometrically at 260 nm and checked for integrity by EtBr-agarose gel electrophoresis. RNA were primed with 0.5 µg·µL⁻¹ oligo(dT) (12–18 bp) (Invitrogen Life Technologies, Strathclyde, UK) to generate the first strand of DNA. Reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies).

Amplification of cDNA segments was carried out under the following conditions: 5–7 µL of the reaction product of reverse transcription (cDNA), 2.5 µL of 10 × reaction buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl), 0.75 µL of MgCl₂ 50 mM, 2 µL of the mixture of 2.5 mM dNTP, 0.5 µL of each primer (12.5 µM), and 0.25 µL of Taq Platinum DNA polymerase (Gibco) (2.5 U of enzyme) and sterile H₂O to complete 25 µL. The PCR were performed in the MJ Research thermal cycler MiniCycler, following the conditions specified for each pair of primers. To analyze the formation of PCR products, aliquots of 10 µL of the reaction products were subjected to electrophoresis on agarose gel at 3%. The molecular weight marker used was the DNA ladder of 100 bp (Gibco-BRL).

The bands were visualized by the effect of ultraviolet radiation in a darkroom equipped with a transluminator and images acquired by the program Chemilimagor 5500 (Alpha Innotech, San Leandro, CA). The intensity of the bands obtained was analyzed using the analysis program Scion Image densitometry, provided free by National Institutes of Health online.

### Statistical Analysis

Results are represented as means ± SD. Statistical analysis was performed using the software BASIC STATISTIC, by two-way ANOVA. Statistical analysis was performed in three stages. Our first aim was to confirm the cardiac hypertrophy and fibrosis induced by ND and to observe the cardiac aldosterone synthesis in these groups. Thereafter, we compared the control groups (C and T) with the ND-treated groups (ND and TND). In a second step, we tested the hypothesis that the AT1 block has an important role on the cardiac effects induced by ND and cardiac aldosterone. We compared the groups treated with steroids (ND and TND) with the groups treated with ND plus losartan (ND + L and TND + L). In a third step, we tested the hypothesis that the mineralocorticoid block might be involved in cardiac effects induced by ND and cardiac aldosterone. Thereafter, we compared animals treated with ND (ND and TND) in relation to groups treated with ND and spironolactone (ND + S and TND + S). The analysis of BP before and after the experimental treatment was performed using ANOVA for repeated measures. If the F test was significant, we applied a post hoc Duncan to multiple comparisons. Correlation analysis was performed using the Pearson correlation coefficient (r). *P < 0.05* was accepted as statistically significant.

### RESULTS

#### Hemodynamic measurements

The data referring to BP and cardiac function are summarized in Table 1. We can observe that the ND did not increase the BP compared with...
control groups. We can also observe that none of the treatment used, losartan or spironolactone, altered the BP compared with the ND groups.

Regarding ventricular function, we observed that the ND treatment did not modify the ventricular function in the sedentary group. However, the peak velocity of E wave and E/A ratio was lower when ND treatment was combined with swimming training, suggesting diastolic dysfunction in this group.

When the TND group was treated with losartan (TND + L), the reduction in the peak velocity of E wave and E/A ratio was prevented. Similar results were also observed when the TND group was treated with spironolactone (TND + S). These results showed that both treatments were efficient in reverting the ND plus swimming training effects on diastolic function.

**Cardiac effects of ND.** Our first objective was to confirm ND’s effects on cardiac tissue and to analyze if cardiac aldosterone may be involved in this process.

Cardiac hypertrophy was observed in the ND, T, and TND groups when compared with C (2.3 ± 0.10, 2.2 ± 0.10, and 2.4 ± 0.12 mg·g⁻¹, respectively, vs 2.07 ± 0.09 mg·g⁻¹, P < 0.05) (Fig. 1A). Cardiac hypertrophy was exacerbated in TND compared with the ND and T groups (P < 0.05). The cardiomyocytes diameter was also significantly increased in the ND, T, and TND groups (10.86 ± 0.20, 11.39 ± 0.46, and 11.55 ± 0.33 μm, respectively, P < 0.05) compared with the C group (10.28 ± 0.14 μm) (Fig. 1B). Also, they were increased in T and TND groups compared with the ND group (P < 0.05).

CVF was significantly increased in ND and TND when compared with the C and T groups (2.32 ± 0.28 and 2.0 ± 0.11 vs 1.17 ± 0.17 and 1.33 ± 0.2 μm per area, respectively, P < 0.001) (Fig. 1C). Figure 1D shows the relative gene expression of collagen. The gene expression of collagen type III was significantly increased in the ND and TND groups when compared with the C and T groups (1.32 ± 0.32 and 1.26 ± 0.05 vs 0.73 ± 0.17 and 0.88 ± 0.12 AU, respectively, P < 0.05). The relative gene expression of collagen type I was not significantly different among the four groups.

To evaluate ND’s effects on renin-angiotensin system and cardiac aldosterone syntheses, we analyzed the LV-ACE activity, the AT1 receptor protein expression, and the mineralocorticoid receptors, aldosterone synthase (CYP11B2) and 11β-HSD2 gene expressions.

The LV-ACE activity was increased in the ND and TND groups compared with the C and T groups (2083 ± 161 and 1879 ± 75 vs 1445 ± 115 and 1377 ± 44 UF per milligram, respectively, P < 0.001) (Fig. 2A). The AT1 receptor protein expression was also increased in the ND and TND groups compared with the C and T groups (1.02 ± 0.2 and 1.15 ± 0.16 vs 0.66 ± 0.12 and 0.76 ± 0.06, respectively, P < 0.05) (Fig. 2B).

The mineralocorticoid receptors were not increased in the ND group (data not shown). However, the relative gene expressions of CYP11B2 and 11β-HSD2 were significantly increased in the ND (1.23 ± 0.2 and 1.24 ± 0.22) and TND groups (1.47 ± 0.27 and 1.49 ± 0.28) when compared with the C (0.85 ± 0.07 and 0.88 ± 0.08) and T groups (0.87 ± 0.15 and 0.56 ± 0.13), respectively (P < 0.05; Fig. 2C). Also, the relative gene expression of 11β-HSD2 was significantly decreased in the T group compared with the C group (P < 0.05). The CYP11B2 expression was positively correlated to the increase in collagen type III expression (r² = 0.7046, P < 0.05) (Fig. 2D).

Figure 3A shows the TGFβ and osteopontin gene expression. The expression of these genes was significantly increased in the ND (1089.21 ± 169 and 1381.60 ± 147,
respectively) and TND groups (1244.02 ± 113 and 1674.34 ± 326, respectively) when compared with the C (839.27 ± 122 and 777.68 ± 32, respectively) and T groups (858.29 ± 101 and 1067.103 ± 247, respectively) (P < 0.05). Moreover, the osteopontin gene expression was exacerbated in the TND group compared with the ND group (P < 0.01). CYP11B2 expression was positively correlated with the increase of TGFβ (r² = 0.8912, P < 0.05) (Fig. 3B) and osteopontin (r² = 0.8596, P < 0.05) (Fig. 3C). In addition, we observed a positive correlation between TGFβ and collagen type III expression (r² = 0.7927, P < 0.05) (Fig. 3D) and between osteopontin and collagen type III expression (r² = 0.6364, P < 0.05) (Fig. 3E).

Cardiac effects of ND and pharmacological block of AT1 receptor. Cardiac hypertrophy was inhibited by losartan treatment in ND + L and TND + L compared with the ND and TND groups (2.04 ± 0.16 and 2.13 ± 0.12 vs 2.3 ± 0.10 and 2.4 ± 0.12, P < 0.05, respectively) (Fig. 4A). Cardiomyocytes diameter was also prevented by the losartan treatment in the ND + L and TND + L groups (10.62 ± 0.32 and 11 ± 0.11 μm) when compared with the TND group (11.55 ± 0.33 μm).

CVF increase was prevented by the losartan treatment (Fig. 4C) in the ND + L and TND + L groups (1.49 ± 0.11 and 2.3 ± 0.17 μm per area) compared with ND and TND groups (2.3 ± 0.28 and 2.0 ± 0.11 μm per area, P < 0.05). Similar results were observed in relation to relative gene expression of collagen type III. The increase in the expression of collagen type III was prevented by the losartan (Fig. 4D) treatment in the ND + L and TND + L groups when compared with the ND and TND groups (0.90 ± 0.1 and 0.96 ± 0.14 vs 1.32 ± 0.32 and 1.26 ± 0.05 AU, respectively, P < 0.05). Moreover, the relative gene expression of collagen type I was decreased by losartan (Fig. 2E) treatment in the TND + L group (0.86 ± 0.12 AU) compared with the TND group (1.12 ± 0.12 AU, P < 0.05).

The LV-ACE activity increase was prevented by the losartan treatment in both groups (ND + L = 1682.3 ± 140 UF per milligram and TND + L = 1495.4 ± 187 UF per milligram vs ND = 2083 ± 161 UF per milligram and TND = 1879 ± 75 UF per milligram, P < 0.05) (Fig. 4E). On the other hand, the AT1 receptor increase was not prevented by the losartan treatment (ND + L = 0.95 ± 0.24 AU and TND + L = 1.02 ± 0.07 AU vs ND = 1.02 ± 0.2 AU and TND = 1.15 ± 0.16 AU; data not shown).

The relative gene expression of the CYP11B2 and 11β-HSD2 increases was prevented by the losartan treatment (Fig. 4F) in the ND + L (0.83 ± 0.17 and 0.97 ± 0.25) and TND + L groups (0.99 ± 0.14 and 0.90 ± 0.19) when compared with the TND group (1.47 ± 0.27 and 1.49 ± 0.28, respectively, P < 0.05) but not when compared with the ND group.

The relative gene expression of the TGFβ and osteopontin (Fig. 4G) increases was prevented by the losartan treatment in the ND + L (981.12 ± 148 and 899.31 ± 131) and TND + L groups (907.92 ± 83 and 996.14 ± 107), when compared with the ND (1089.21 ± 169 and 1381.60 ± 147 respectively, P < 0.05) and TND groups (1244.02 ± 113 and 1674.34 ± 326, P < 0.05), except when compared with the ND group to the TGFβ gene.
Cardiac effects of ND and pharmacological block of mineralocorticoid receptor. Unlike the results observed with losartan treatment, cardiac hypertrophy was not prevented by the spironolactone treatment in the ND + S and TND + S groups (2.17 + 0.09 and 2.3 + 0.14 mg g⁻¹) compared with the ND group (2.3 ± 0.10 and 2.4 ± 0.12) (Fig. 5A). Similar cardiac hypertrophy and cardiomyocytes diameter increases were also not prevented by the spironolactone treatment in the ND + S and the TND + S groups (10.76 ± 0.5 and 11.22 ± 0.40 μm) when compared with the ND and TND groups (10.86 ± 0.20 and 11.55 ± 0.33 μm, respectively) (Fig. 5B).

On the other hand, the CVF increase was prevented by the spironolactone treatment in the ND + S and TND + S groups (1.46 ± 0.53 and 1.37 ± 0.05 μm per area) when compared with the ND and TND groups (2.32 ± 0.28 and 2.0 ± 0.11 μm per area, P < 0.05) (Fig. 5C). Similar results were observed in gene expression of collagen type III where the increase induced by ND was prevented by the spironolactone treatment in the ND + S and TND + S groups (1.01 ± 0.07 and 0.85 ± 0.08 AU) compared with the ND and TND groups (1.32 ± 0.32 and 1.26 ± 0.05 AU, P < 0.05) (Fig. 5D). Moreover, the relative gene expression of collagen type I was decreased by the spironolactone treatment in the ND + S and TND + S groups (0.87 ± 0.09 and 0.88 ± 0.05 AU) when compared with the ND and TND groups (1.02 ± 0.06 and 1.12 ± 0.02 AU, P < 0.05) (Fig. 2F).

The LV-ACE activity increase was prevented by the spironolactone treatment (1543 ± 145 and 1578 ± 100 UF per milligram) when compared with the ND and TND groups (2083 ± 161 and 1879 ± 75 UF per milligram, P < 0.05) (Fig. 5E). However, the AT1 receptor increase was not prevented by the spironolactone treatment compared with the ND and TND groups (1.10 ± 0.11 and 1.11 ± 0.12 vs 1.02 ± 0.2 and 1.15 ± 0.16 AU; data not shown).

FIGURE 3—Nandrolone decanoate effects and nandrolone decanoate associate to swimming training on inflammatory factors TGFβ (white bar) and osteopontin (black bar) gene expression (A). Control (C, n = 7), nandrolone decanoate (ND, n = 7), trained (T, n = 7), and trained ND (TND, n = 7). Representative blots of TGFβ and osteopontin are above A. Targeted bands were normalized to cardiac GAPDH. Data are presented as mean ± SD. †P < 0.05 versus C. #P < 0.05 versus T. §P < 0.05 versus ND. Correlation between CYP11B2 and TGFβ mRNA expression (r² = 0.89, P < 0.05; B) and osteopontin mRNA expression (r² = 0.86, P < 0.05; C). Correlation between TGFβ (r² = 0.79, P < 0.05; D) and osteopontin (r² = 0.64, P < 0.05; E) and collagen type III mRNA expression mRNA expression.
The relative gene expression of CYP11B2 and 11β-HSD2 increases was prevented by the spironolactone treatment (Fig. 5F) in the ND + S (0.82 ± 0.11 and 0.76 ± 0.16) and TND + S groups (0.99 ± 0.17 and 0.86 ± 0.25) when compared with the TND group (1.47 ± 0.27 and 1.49 ± 0.28, \( P < 0.05 \)) and also when compared with the ND group (1.23 ± 0.2 and 1.24 ± 0.22, \( P < 0.05 \)), except the TND + S group.

The relative gene expression of TGFβ and osteopontin (Fig. 5G) increase was prevented by the spironolactone treatment in the ND + S (833.37 ± 187 and 914 ± 07 ± 136, respectively) and TND + S groups (833.75 ± 80 and 935.28 ± 157, respectively) when compared with the ND (1089.21 ± 169 and 1381.60 ± 147, \( P < 0.05 \)) and TND groups (1244.02 ± 113 and 1674.34 ± 326, \( P < 0.05 \)).

**DISCUSSION**

The main findings of this study show for the first time that the cardiac aldosterone and inflammatory factor, TGFβ and osteopontin, participate in cardiac fibroses induced by ND with or without the association to physical training. These effects were reinforced by the fact that both AT1 receptor and mineralocorticoid receptors antagonist prevented the
increase of cardiac collagen and diastolic dysfunction observed in ND and ND trained groups.

**BP and cardiac effects of ND.** Our results show that ND treatment did not alter the BP; however, the effects of anabolic steroids on BP are still controversial in the literature. Some studies showed an increase in BP in anabolic steroid users (21). The mechanism involved can be related to the anabolic steroid action increasing the sympathetic nervous system activity, endothelial dysfunction, and baroreflex control (2). Other studies did not observe the same effect, where the anabolic steroid or ND treatment did not alter the BP (9,27), confirming our results.

As was observed in the ND-treated groups, the losartan or spironolactone treatment did not alter the BP because the doses used were described in the literature as sufficient to block the actions of angiotensin II and aldosterone without interfering with the BP of normotensive rats (5,11); however, we have shown that ND effects on cardiac hypertrophy and function were not associated with the systemic renin-angiotensin system but with its local effects.
Cardiac hypertrophy (CH) observed in the T group is a typical response of aerobic training by swimming and reflects physiological CH where an increase in cardiac mass without functional impairment occurs (14,20,27) as we showed by echocardiography. The swimming training protocol induced CH and the increase in cardiomyocyte diameter but not cardiac collagen (CVF or collagen type I and III expression). On the other hand, in the TND group, the diastolic dysfunction was observed, suggesting pathological CH. Similar results were observed in other studies where a decrease in peak velocity during early diastolic filling in anabolic steroid users was observed, by echocardiography (9). Diastolic dysfunction was observed in weight lifters who used anabolic steroids compared with those who did not (21). The results observed in this study corroborate this finding in anabolic steroids users.

The diastolic dysfunction has been strongly associated to increased interstitial collagen. Fibrillar collagens, types I and III, are the major structural proteins of the myocardial collagen matrix (9) and are responsible for distributing the forces working on the heart, exerting an important influence on ventricular compliance (38). We observed in a previous study that the exposure to supraphysiological ND doses might lead to tissue necrosis and diastolic dysfunction, resulting in structural changes similar to those observed in the early stages of heart failure (27). These data lead us to suggest that the diastolic dysfunction observed in TND group can be induced by the increases in CVF and expression of collagen type III.

The actions of angiotensin II on cardiac hypertrophy and fibrosis are well described in the literature (27,38); however, some actions attributed to angiotensin II may be directly related to the synthesis and release of aldosterone (26,39). The direct role of aldosterone as a mediator in cardiac hypertrophy and fibrosis has been observed, causing deleterious effects on the cardiovascular system. The aldosterone has been associated with inflammatory responses, inducing tissue damage, collagen synthesis, and cardiac remodeling (26), regardless of hemodynamic changes, suggesting local aldosterone effects (5).

On the basis of these facts, we investigated the hypothesis that cardiac aldosterone may be associated with deleterious effects induced by treatment with ND and its association to swimming training. Thus, we investigated the mineralocorticoid receptors CYP11B2 and the 11β-HSD2 enzyme expression on the heart. The expression of mineralocorticoid receptors was not modified by ND treatment; however, aldosterone synthase gene (CYP11B2) and 11β-HSD2 enzyme expression was increased by ND treatment. These results suggest that ND treatment may lead to an increase in cardiac aldosterone synthesis because the increase in CYP11B2 gene expression was related to high aldosterone concentrations, as has been well demonstrated in the literature (37).

Increased expression of the CYP11B2 gene is also related to increased in cardiac collagen synthesis (37), confirming the results presented in this study that showed a positive correlation between the expression of CYP11B2 and the expression of collagen type III in animals treated with ND.

The effects of aldosterone are modulated by binding to the mineralocorticoid receptors, but aldosterone actions depend on various factors, including the glucocorticoids concentrations, which act as mineralocorticoid receptors antagonist in the heart by inhibiting the aldosterone actions (24). 11β-HSD2 enzyme inactive metabolites binding to mineralocorticoid receptors contributing to increased aldosterone action on these receptors (18). 11β-HSD2 enzyme expression was increased with ND (40% and 121.5%) and TND (68.6% and 167%) treatments compared with the C and T groups, respectively. These results suggest that, in addition to an increase in aldosterone syntheses, the ND treatment also altered the mineralocorticoid receptor selectivity, providing a major action of aldosterone.

Our results also suggest that aerobic training is associated with lower aldosterone actions because 11β-HSD2 enzyme expression was lower in the T group compared with the C group. However, more studies are needed to understand the effects of physical training on 11β-HSD2 enzyme expression and aldosterone levels.

The aldosterone actions on cardiac fibrosis were suggested to be related to inflammatory factors TGFβ and osteopontin where, in characteristic features of hyperaldosteronism, increased expressions of inflammatory factors in the heart plus interstitial and perivascular fibrosis are observed (28). An interesting result of the present study was the higher expression of inflammatory factors TGFβ and osteopontin and their positive correlation with the increase of CYP11B2 expression observed with ND treatment. Moreover, the increased inflammatory factors’ expression was positively correlated with collagen type III expression increased in the ND-treated groups. These results suggest that the aldosterone effects on cardiac collagen induced by ND can be related to high TGFβ and osteopontin expression.

The role of osteopontin on cardiac fibrosis has been confirmed in mice with osteopontin deficiency, where the aldosterone actions on cardiac fibrosis were inhibited (31). The aldosterone actions on myocardial fibrosis and cardiac remodeling mediated by osteopontin have also been shown in studies with angiotensin II and aldosterone blockers that were effective in inhibiting the osteopontin expression in the infarcted area of the myocardium, reducing collagen and preventing cardiac hypertrophy (39). The expression of TGFβ has been increased by the activation of AT1 receptors (3). TGFβ exerts its actions on cardiomyocytes, leading to tissue necrosis, and on fibroblasts, increasing the expression of proteins related to fibrosis (3). TGFβ has also been shown to be released by aldosterone, leading to an increase in the expression of growth factors and connective tissue (32). These observations corroborate the results observed in this study.

Effects of losartan and spironolactone treatment. The AT1 receptor antagonist treatment (losartan) prevented increasing the CYP11B2 and 11β-HSD2 expression. The activation of AT1 receptors is involved on the
pathological conditions of cardiac aldosterone synthesis and their blockade prevents the increase of aldosterone synthase expression (8,39), confirming the results observed in this study; however, these mechanisms are not fully understood. Because we observed that the increase in CYP11B2 expression was directly correlated to the increase in collagen type III expression and inflammatory factors, TGFβ and osteopontin, we can suggest that the effects observed with Losartan treatment on cardiac collagen may be associated with lower cardiac aldosterone action.

Considering that cardiac aldosterone can be related to the deleterious effects induced by ND, a mineralocorticoid receptor antagonist was administered, spironolactone, to analyze these effects. An important point to be discussed regarding the pharmacologic treatments is the action of spironolactone on androgen receptors. Spironolactone is structurally a steroid and can act as an antiandrogen, decreasing the production and blocking the effect of androgens in the target tissue (25). However, the effects of spironolactone on the blockade of androgen receptors seem to be dose-dependent and occur when administered at high doses (100 mg d⁻¹) (25). In the present study, the rats were treated with 10 mg kg⁻¹ d⁻¹ of spironolactone, approximately 6 mg d⁻¹, which is an extremely low dose to induce antiandrogenic effects, however inhibited the cardiac actions of aldosterone. Thus, this result leads us to suggest that the effects observed by spironolactone treatment were not by the androgen receptors blocking. These results are corroborated in a study where spironolactone was administered at the dose of 80 mg kg⁻¹ d⁻¹ in spontaneously hypertensive rats, and it was observed that its effects on cardiac tissue were not related to the blockade of androgen receptors (35).

We showed that spironolactone and Losartan treatments were effective in preventing collagen expression. Spironolactone prevented CVF in the ND + S and TND + S groups compared with their respective controls. Also, it prevented the increase in collagen type I and type III expression in the ND + S and TND + S groups compared with the TND group and in collagen type I and III expression in the TND + S group compared with the ND group, confirming the involvement of mineralocorticoid receptors and aldosterone in the cardiac collagen increase induced by ND.

An interesting result was observed in cardiac hypertrophy and cardiomyocytes diameter. Unlike the results observed with the Losartan treatment, the spironolactone treatment did not modify the cardiomyocytes diameter and cardiac hypertrophy. These data might be the result of the action of angiotensin II on cardiomyocytes. The actions of angiotensin II through AT1 receptors are responsible for activating a variety of intracellular signaling pathways, increasing the expression of genes related to hypertrophy (40). Thus, blocking AT1 receptors with Losartan would be expected to prevent the effects of angiotensin II on cardiac hypertrophy and cardiomyocytes diameter. In a previous study, we showed similar results, where Losartan prevented theses effects induced by swimming training (20).

Losartan and spironolactone prevented the LV-ACE activity increase in the ND and TND groups. The ACE activity increase may have contributed to the development of heart fibrosis because there are high levels of ACE activity in the fibrotic area of the heart (19). In our earlier study, we also showed that the increase in cardiac ACE activity and angiotensin II concentrations was correlated with collagen concentration (27). The LV-ACE activity may also be related to the concentration of aldosterone.

Besides the increase in angiotensin II, the LV-ACE activity may also be related to the concentration of aldosterone. The relation between ACE and aldosterone has been shown in studies with ACE inhibitors, where ACE inhibitors suppress aldosterone production. On the other hand, plasma aldosterone concentrations may rise over time during long-term treatment with ACE inhibitors in dogs with congestive heart failure. This phenomenon is called aldosterone escape, and it showed that the aldosterone might have an important role on cardiac fibroses even when ACE activity is blocked or decreased (22).

In addition, aldosterone increases angiotensin II binding, upregulates the expression of AT1, increases the ACE activity, and potentiates angiotensin II–stimulated intracellular signaling and proliferation in peripheral cardiovascular tissues (16,17,30,36). Thus, our results suggest that a low LV-ACE activity can be one of the factors responsible for the decrease in cardiac collagen with Losartan or spironolactone treatment; however, we cannot discard the possibility that the effects of LV-ACE could be related to the aldosterone action because the increase in aldosterone action increased LV-ACE activity and was responsible for ND’s effects on the heart. However, more studies are necessary to understand these mechanisms.

Therefore, spironolactone treatment in the TND and ND groups prevented the CYP11B cardiac and 11β-HSD2 expression increase. Both CYP11B2 and 11β-HSD2 are directly associated to cardiac fibrosis (24). Thus, the lower expression of these genes observed with spironolactone therapy can also be responsible for the minor cardiac fibrosis found, confirming the participation of aldosterone on cardiac fibroses induced by ND treatment.

As previously reported, the effects of aldosterone on cardiac fibrosis can be related to an increase in inflammatory factors TGFβ and osteopontin. Both Losartan and spironolactone treatments decreased the CYP11B2 expression and, consequently, the expression of inflammatory factors. These results may explain the similar effects observed with both treatments, contributing to the beneficial effects observed in these groups.

In the present study, we showed similar effects with blockers of AT1 or mineralocorticoid receptors on cardiac aldosterone syntheses, inflammatory factors, and preventing deleterious cardiac effects induced by ND. The effects of angiotensin II and aldosterone on cardiac fibrosis were well demonstrated in the literature and can stimulate cardiac effects by distinct pathways (5,16,17,18,30,36). However,
the interplay between aldosterone and angiotensin II at the target tissue level is very complex and still poorly understood.

Recently, a mechanism of crosstalk between the mineralocorticoid receptors and the AT1 receptors has been proposed, in which when angiotensin II and aldosterone act at the same time on their respective receptors, there were different effects from those observed when only one of them is acting (10,17). It was demonstrated that signaling pathways such as ERK1/2, JNK, and NF-κB to be activated need aldosterone and a functional AT1 receptor, suggesting that the effects of aldosterone in cardiac fibroses depended in part on the activation of an angiotensin II–AT1 receptor–mediated pathway (10). In addition, it has been shown that angiotensin II induced ventricular damage mediated by a mechanism dependent on signaling via the mineralocorticoid receptor aldosterone (10,30) and that the aldosterone block restores or prevents cardiac inflammation and remodeling caused by renin–angiotensin enhancement (30). These results showed that the aldosterone and angiotensin II could synergistically promote deleterious cardiac effects via the interaction of the AT1 and mineralocorticoid receptors.

These data can explain the results observed in the present study where both losartan and spironolactone were effective in reducing cardiac fibroses induced by ND. The ND treatment increased the LV-ACE activity and CYP11B2 expression, suggesting the increase in both angiotensin II and aldosterone. Angiotensin II and aldosterone increase may have activated the crosstalk mechanisms, and when the angiotensin II or aldosterone action were blocked, this mechanism was also blocked. However, we did not perform any experiment to confirm the crosstalk between AT1 and the mineralocorticoid receptor. More studies are necessary to understand the exact mechanism involved in renin–angiotensin aldosterone system regulation and the effects induced by ND treatment. Here, we have shown that ND treatment or its association with swimming training increased the LV-ACE activity and CYP11B2 expression, suggesting the increase in both angiotensin II and aldosterone and promoting cardiac dysfunction.

Finally, we believe that this is the first study to show the effects of ND on cardiac aldosterone because an increase in aldosterone synthase and the 11β-HSD2 enzyme gene expressions was observed. Our results still suggest that the effects of aldosterone on cardiac tissue are associated to inflammatory mediators such as TGFβ and osteopontin. In conclusion, ND and its association to swimming training promote cardiac deleterious effects increasing interstitial collagen by cardiac aldosterone action.

The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for the E. C. Carmo fellowship for the present investigation and Dr. M. I. Phillips for critically reading the article. E. M. de Oliveira received scholarships from CNPq (process No. 307591/ 2009-3), Brazil. The results of this study do not constitute endorsement by the American College of Sports Medicine.

REFERENCES

20. Oliveira EM, Sasaki MS, Cerencio M, Barauna VG, Krieger JE. Local renin–angiotensin system regulates LV hypertrophy induced...


