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Anabolic steroids induce cardiac renin-angiotensin system and impair the beneficial effects of aerobic training in rats

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Rocha FL, Carmo EC, Roque FR, Hashimoto NY, Rossoni LV, Frimm C, Anéas I, Negrão CE, Krieger JE, Oliveira EM. Anabolic steroids induce cardiac renin-angiotensin system and impair the beneficial effects of aerobic training in rats. *Am J Physiol Heart Circ Physiol* 293: H3575–H3583, 2007. First published September 28, 2007; doi:10.1152/ajpheart.01251.2006.—We evaluated the effects of swimming and anabolic steroids (AS) on ventricular function, collagen synthesis, and the local renin-angiotensin system in rats. Male Wistar rats were randomized into control (C), steroid (S; nandrolone decanoate; 5 mg/kg sc, 2×/wk), steroid + losartan (SL; 20 $mg \cdot kg^{-1} \cdot day^{-1}$), trained (T), trained + steroid (T+S), and trained + steroid + losartan (T+SL; $n = 14$/group) groups. Swimming was performed 5 times/wk for 10 wk. Serum testosterone increased in S and T+S. Resting heart rate was lower in T and T+S. Percent change in left ventricular (LV) weight-to-body weight ratio increased in S, T, and T+S. LV systolic pressure declined in S and T+S. LV contractility increased in T ($P < 0.05$). LV relaxation increased in T ($P < 0.05$). It was significantly lower in T+S compared with C. Collagen volumetric fraction (CVF) and hydroxyproline were higher in S and T+S than in C and T ($P < 0.05$), and the CVF and LV hypertrophy were prevented by losartan treatment. LV-ANG I-converting enzyme activity increased (28%) in the S group (33%), and type III collagen synthesis increased (56%) in T+S but not in T group. A positive correlation existed between LV-ANG I-converting enzyme activity and collagen type III expression ($r^2 = 0.88$; $P < 0.05$, for all groups). The ANG II and angiotensin type 1a receptor expression increased in the S and T+S groups but not in T group. Supraphysiological doses of AS exacerbated the cardiac hypertrophy in exercise-trained rats. Exercise training associated with AS induces maladaptive remodeling and further deterioration in cardiac performance. Exercise training associated with AS causes loss of the beneficial effects in LV function induced by exercising. These results suggest that aerobic exercise plus AS increases cardiac collagen content associated with activation of the local renin-angiotensin system.

cardiac hypertrophy; collagen; renin-angiotensin system; ventricular function

ANABOLIC STEROIDS (AS) ARE synthetic derivatives of testosterone used in therapeutic dosages in medical practice (2). In addition, high doses of AS have been used by athletes to improve physical performance (14). In athletes, AS increases protein anabolism and, consequently, strength, potency, and muscle mass (14, 38). However, high doses of AS have adverse effects on the hepatic, endocrine, and cardiovascular systems. In the cardiovascular system, AS promotes myocardial structural changes (18, 33, 38). Previous studies suggest that high doses of AS associated with exercise training result in impaired lipid and lipoprotein metabolism, increased atherosclerosis, abnormal blood coagulation (13, 32), moderate cardiac hypertrophy with inadequate cardiac capillarization (39), increase in arterial blood pressure (26), apoptotic cell death of ventricular myocytes in vitro (46), and reduction in cardiac contractility in animals (27, 41) and athletes (42). However, the nature and the molecular mechanisms of these processes involving cardiac hypertrophy remain poorly understood.

A possible modulating mechanism for this hypertrophy is higher activity of the systemic renin-angiotensin system (RAS), which plays a role in regulating cardiac growth. It is well known that, in both humans and animals, RAS is of primary regulatory importance for the remodeling of cardiac extracellular matrix (7).

We investigated *1*) the effects of AS administration associated with or without swimming in the cardiac remodeling process and in cardiac function in rats; and *2*) the possible mediation of local RAS activity on cardiac remodeling promoted by AS administration in swimming-trained rats.

MATERIALS AND METHODS

Experimental Groups

Male Wistar rats (8–12 wk old; weighing 180–250 g; $n = 56$) were used. All protocols and surgical procedures were in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the Ethics Committee of the Medicine Faculty of the University of São Paulo. The rats were divided randomly into four groups, each with 14 rats: control (C), steroid (S), trained (T), and trained+steroid (T+S). Each group was subdivided into two groups: one for hemodynamic, biochemical, and molecular studies, and the other for morphological and histological studies. For pharmacological treatment, a new group was divided randomly in four groups, each with six rats: S, S treated with losartan [angiotensin type 1 (AT_1)-receptor blocker; 20 $mg \cdot kg^{-1} \cdot day^{-1}$ as drinking water] (SL), T, and T+S treated with losartan (T+SL). The animals were housed in standard cages and separated into groups (sedentary and trained). Food and water were provided ad libitum. Room temperature was kept at 23 ± 1°C. A 12:12-h light-dark cycle was maintained throughout the experiment. The rats were identified and weighed weekly. They were treated with the AS nandrolone decanoate (Decadurabolin; Organon, Roseland, NJ) administered subcutaneously twice a week, in a dosage of 5 mg/kg per injection, equaling 10 $mg \cdot kg^{-1} \cdot wk^{-1}$.

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Training Protocol

The swimming training was performed as described previously (31). The physical training was executed five times a week in a swimming system with warm water at 30–32°C, for 60 min, for 10 wk, with a gradual increase of work load [tail weight − %body weight (BW)] until it reached 5% of BW. This protocol is defined as a low-intensity, long training period, effective for promotion of cardiovascular adaptations and increase in muscle oxidative capacity (1).

Hemodynamic Measurements

Ventricular function. Rats were anesthetized with ketamine-xylazine-acepromazine (0.7:0.2:0.1 vol/vol/vol, 0.04 ml/kg im) and allowed to breathe room air spontaneously. A polyethylene catheter (PE-50, 8 cm, filled with heparinized saline) was introduced through the right carotid artery into the left ventricle (LV). During this procedure, arterial blood pressure and LV systolic and diastolic pressures and their first time derivatives (positive and negative, maximum dP/d*t* and minimum dP/d*t*, respectively) were recorded. For each rat, the initial and LV end-diastolic pressure were determined. Arterial and ventricular pressures were recorded continuously (Gould Statham P23XL transducer, Oxnard, CA) in an eight-channel recorder (Gould, model 5900, Oxnard, CA). Heart rate (HR) and dP/d*t* were determined by a biotechnician, triggered by pulse pressure, and recorded simultaneously with the other variables.

Arterial blood pressure and HR. Twenty-four hours after the last training session, the rats were anesthetized (ketamine 90 mg/kg and xylazine 10 mg/kg, intraperitoneally), and a catheter (PE-50) was inserted into the carotid artery, for direct measurement of arterial blood pressure and HR. The catheter was heparinized and filled with saline, and the external extremity was occluded. The catheter was placed subcutaneously and pulled out at the animal's back to facilitate the handling of awake animals.

For the arterial blood pressure register, animals were individually maintained in cages for at least 24 h before the experimental procedures. Forty-eight hours after the last training session, the arterial blood pressure was recorded for 30 min in quiet, conscious, unrestrained rats. The catheter was connected to a polyethylene tube (PE-100), and this to a pressure transducer (P23Db; Gould-Statham, Oxnard, CA) connected to an amplifier (General Purpose Amplifier-Stemtech). The arterial pressure was conveyed to an analog-digital system (Stemtech, Klamath Lake, OR), registered in real time in a microcomputer with a CODAS System and analyzed through a Windows-compatible system, with a sampling frequency of 1,000 Hz/channel. Through this program, the values of systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean blood pressure (MBP) were obtained beat to beat.

Serum and Tissues Samples

At the end of the experimental period, animals were killed, without prior anesthesia, by decapitation. Their blood was collected without an anticoagulant for angiotensin I (ANG I)-converting enzyme (ACE) assay. To determine plasma renin activity (PRA), the first 3 ml of trunk blood (a mixture of venous and arterial blood) were rapidly collected into chilled glass tubes containing a mixture of potassium EDTA (25 mM), *o*-phenanthrolene (0.44 mM), pepstatin A (0.12 mM), and 4-(chloromercuribenzoic acid; 1 mM). This mixture of protease inhibitors prevented the in vitro production and degradation of angiotensin peptides (25). The blood was centrifuged, and the plasma and serum were separated and stored at −20°C. In addition, tissues were harvested, and the heart was weighed and stored at −80°C until biochemical and molecular analysis. The intraperitoneal fat was weighed and normalized by total BW of the animal.

Cardiac Morphological and Morphometric Analysis

To measure cardiac mass, the heart was removed from the thoracic cavity and dissected to separate the LV. To measure cardiac mass, the LV was dissected corresponding to the remaining tissue upon removal of atria and the free wall of the right ventricle. The interventricular septum remained as part of the LV. To evaluate cardiac hypertrophy, the heart (left and right ventricle) weight (HW) was normalized by total BW of the animal (HW/BW in mg/g).

For morphometric analysis, another group of animals was trained. After blood was collected, the chest was opened, and the hearts were stopped at diastole by perfusion with 14 mM KCl. After the heart was weighed, the LV was fixed in 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 (×400). Myocytes 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.

Myocardial interstitial collagen volumetric fraction (CVF) was determined using direct and polarized light of the Picrosirius red prepared tissues, as reported previously (23). In brief, 20 fields were selected from sections placed in a projection microscope (×200), and interstitial collagen was determined by a computer-assisted image analysis system (Quantimet 520; Cambridge Instruments). 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.

Biochemical and Molecular Analysis

Hydroxyproline determination. LV collagen was quantified from the hydroxyproline concentration by using a modified method, as described previously (3). All tissue samples (approximate 100 mg wet wt) were taken from the same area of the LV wall.

The tissues were hydrolyzed in 1 ml of 8 N HCl at 115°C for 18 h under vacuum. Hydrolysis samples were filtered and vacuum dried. Oxidant (chloramine T; 0.5 ml) was added, vortexed, and allowed to

Table 1. *Body weight, heart weight, intraperitoneal fat, heart weight-to-body weight ratio, and myocyte diameter in untrained and trained rats, with and without anabolic steroids*

	n	Before BW, g	After BW, g	Intraperitoneal Fat, mg	HW, g	HW/BW, mg/g	Myocyte Diameter, μm
Control	6	240±26	396±26	2.6±0.8	1.00±0.03	2.5±0.1	6.7±0.2
Steroid	7	235±19	354±26	1.2±0.7*	0.96±0.1	2.7±0.1*	6.9±0.3
Trained	6	238±18	351±21	1.2±0.5*	1.01±0.1	2.9±0.2*	7.6±0.5*‡
Trained + steroid	7	235±20	321±22*	0.7±0.2*	0.99±0.1	3.1±0.1*†	7.6±0.6*‡

Values are means ± SD; *n*, no of rats. BW, body weight; HW, heart weight; HW/BW, ratio of HW by BW. Significant difference vs. *control, †trained + steroid, and ‡steroid: $P < 0.05$.

stand for 4 min. To this was added 1.0 ml Ehrlich reagent (3 ml of Ehrlich reagent plus 16 ml of isopropanol). The tubes were vortexed for 4 min and afterwards kept at 60°C for 21 min. The intensity of the coloration was measured at 558 nm after 1 h at room temperature. Hydroxyproline content was determined from duplicate samples of 150 μl using a calibration curve of 0.5–5 μg of 1-hydroxyproline. The data are expressed as milligrams per gram of hydroxyproline. Collagen mass was calculated by multiplying the amount of hydroxyproline by 7.46 (19).

ACE Activity

ACE activity was determined by a fluorometric assay based on the rate of generation of His-Leu by hydrolysis of Hippuryl-His-Leu substrate, as previously reported (35).

Protein Determination

The protein was analyzed by the colorimetric method in a spectrophotometer, with albumin as the standard (BSA, 1 m/ml) (5).

PRA

The PRA was measured by angiotensin radioimmunoassay, using a commercial kit (REN-CT2, CIS Bio International, Gif-sur-Yvette, France). This assay permits direct measurement of PRA. Results were quantified in a gamma counter, and the enzyme activity was expressed as nanograms of ANG I per milliliter per hour (nanograms per milliliter per hour of ANG I).

Testosterone Measurement

The testosterone was determined by radioimmunoassay in plasma using the *COAT-A-COAT Total Testosterone from Diagnostic Products* (human) kit (Los Angeles, CA).

mRNA Quantitation Using Real-Time PCR

The relative gene expression of collagen types I and III, enzyme-converting ANG I, and AT_{1a}- and AT_2-receptors were analyzed by real-time PCR.

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

Real-time PCR. Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). DNA sequence was obtained from GenBank, and primers were made in separate exons to distinguish by size PCR products derived from cDNA from those derived from genomic DNA contaminants. The mRNA expression of RAS components and type I/III collagen were assessed by oligonucleotides primers as follows: for ACE, 5′-CAg gAA CgT ggA ACT Tgg A-3′ and 5′-CTT TgA Cgg AAg CAT CAC C-3′; for COL1a1, 5′-AgA gAg CAT gAC CgA Tgg A-3′ and 5′-gAg gTT gCC AgT CTg TTg g-3′; for COL3a1, 5′-AAg gTC CAC gAg gTg ACA A-3′ and 5′-Agg gCC Tgg ACT ACC AAC T-3′; for AT1a, 5′-CAC AAC CCT CCC AgA AAg Tg-3′ and 5′-Agg gCC ATT TTg TTT TTC Tg-3′; for AT2, 5′-gCT ggg ATT gCC TTA ATg A-3′and 5′-CTT ggT CACA ggg TAA TTC Tg-3′. Real-time quantification of the target genes was performed with a SYBRgreen PCR Master Mix, (Applied Biosystem, PE, Foster City, CA) using ABI PRISM 7700 Sequence Detection System (Applied Biosystem). The expression of cyclophilin A (5′-AAT gCT ggA CCA AAC ACA AA-3′ and 5′-CCT TCT TTC ACC TTC CCA AA-3′) was measured as an internal control for sample variation in RT reaction. An aliquot of the RT reaction was used for 50 cycle PCR amplification in the presence of SYBRgreen fluorescent dye, according to a protocol provided by the manufacturer (Applied Biosystems). PCR product generation was monitored by measuring the increase in fluorescence caused by the SYBRgreen binding to double-stranded DNA at each annealing phase. A dissociation curve was generated at the end of the reaction to verify that a single product was amplified. Each heart sample was analyzed in triplicate. Relative quantities of target gene expressions of C rats vs.

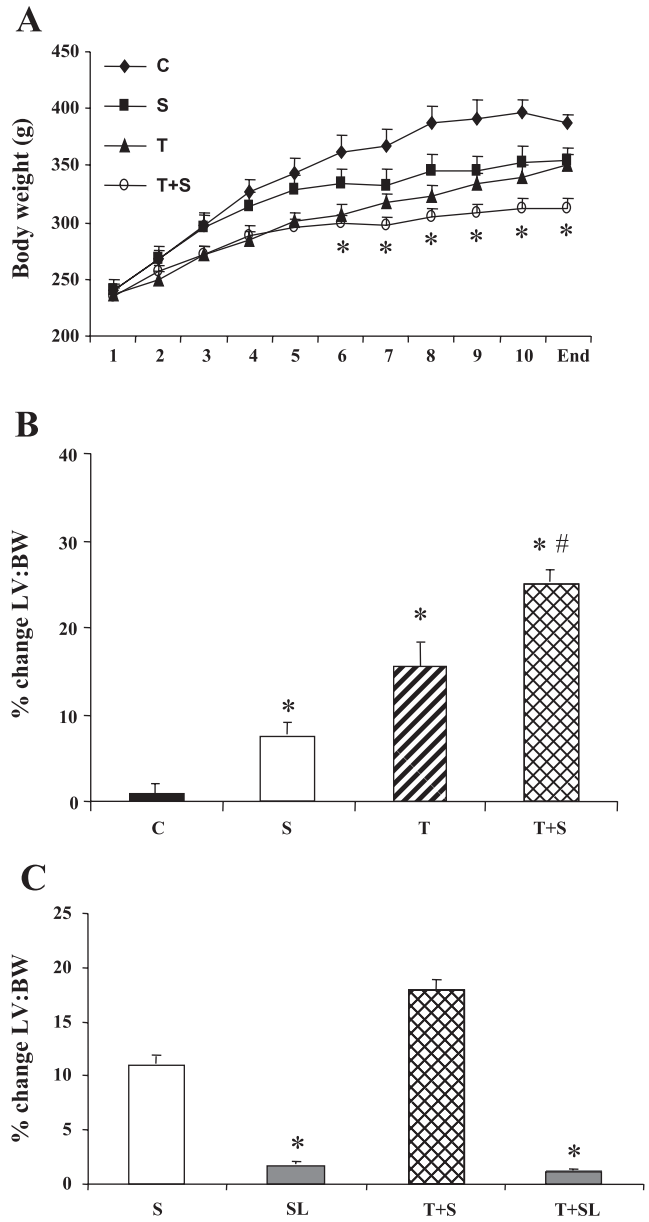

Fig. 1. Effect of anabolic steroids, swimming training, and swimming training associated with anabolic steroids on body weight (BW) and left ventricular (LV) hypertrophy. Groups are as follows: C, control; S, steroid; T, trained; T+S, trained and steroid; SL, steroid losartan treated; T+SL, trained plus steroid losartan treated. *A*: BW during the weeks of swimming training. *Significant difference vs. C group, $P < 0.05$. *B*: LV weight normalized by total BW change (%change LV/BW; mg/g). Significant difference vs. *C group, and #S and T groups: $P < 0.05$. *C*: LV weight normalized by total BW of S, SL, T+S, and T+SL groups (%change LV/BW; mg/g). LV weight normalized by total BW change of S and T+S was obtained compared with C group. *Significant difference vs. S and T+S groups, $P < 0.05$. Values are means ± SE.

Table 2. *Hemodynamic parameters*

	n	Heart Rate, beats/min	SBP, mmHg	DBP, mmHg	MBP, mmHg
Control	6	328±16	116±8	97±6	107±5
Steroid	6	318±26	119±2	93±4	107±3
Trained	6	286±15*	118±14	88±10	103±11
Trained + steroid	5	268±19*†	108±6	81±5*	95±5

Values are means ± SD; *n*, no of animals. SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, means blood pressure. Significant difference vs. *control and †steroid: $P < 0.05$.

treated rats were compared after normalization to the values of cyclophilin [change in threshold cycle (ΔC_T)]. Fold change in mRNA expression was calculated using the differences in ΔC_T values between the two samples ($\Delta\Delta C_T$) and the equation $2^{-\Delta\Delta C_T}$.

Immunohistochemistry. Cardiac samples for ANG II immunohistochemistry were obtained from C, S, T, and T+S groups. LV sections were deparaffinized in xylene and rehydrated in ethanol. Retrieval of ANG II immunoreactivity was obtained by incubating sections in citrate buffer (0.01 M, pH 6.0) with further heating for 5 min in a microwave oven. The presence of ANG II was evaluated in LV tissue using anti-ANG II rabbit antiserum (1:400, Península, Belmont, CA). The antigen was marked by fast red dye, and the specificity of the secondary antibody was established in positive and negative controls. Images were obtained with a computer-assisted morphometric system (Leica Quantimet 500, Cambridge, UK). The number of positive signals in cardiomyocyte was evaluated and expressed as cells immunoreactive for ANG II per millimeter squared.

Statistical Analysis

Results are represented as means ± SD. Statistical analysis was performed using randomized two-way ANOVA, and to evaluate the BW during the weeks we used two-way ANOVA for repeated measures. Tukey's post hoc test was used for individual comparisons between means when a significant change was observed with ANOVA. $P < 0.05$ were accepted as statistically significant.

RESULTS

Cardiac Hypertrophy

The data referring to BW, intraperitoneal fat, HW, HW/BW, and myocyte diameter are summarized in Table 1. The BW before swimming training was similar among all groups. After the swimming training protocol, BW decreased significantly in T+S group, but not in the C, S, and T groups. The tendency of BW was to decrease during the weeks of swimming training in S and T groups; however, in the T+S group, it decreased significantly compared with that in the C group (Fig. 1*A*). This lower weight gain might be associated with a decrease of intraperitoneal fat observed in the S, T, and T+S groups (Table 1). Regarding the cardiac weight per se, no difference was seen among groups. The LV weight (LVW) normalized by total BW ratio, which was used as an index of hypertrophy (%change LVW/BW, mg/g), was significantly higher in S, T, and T+S (8, 16, and 24%, respectively) (Fig. 1*B*). Interestingly, the T+S group was also increased compared with S and T groups (15 and 7%, respectively). Losartan treatment prevents the LV hypertrophy in S and T+S groups. The LVW normalized by total BW ratio, which was used as an index of hypertrophy (%change LVW/BW, mg/g), was significantly higher in the S and T+S (2.3 ± 0.12 and 2.4 ± 0.13 mg/g) groups compared with SL and T+SL (2.0 ± 0.14 and 2.0 ± 0.19 mg/g), respectively (Fig. 1*C*). Swimming, regardless of AS treatment, significantly increased myocyte diameter, but no significant change in myocyte diameter was observed after AS treatment (Table 1).

Hemodynamic Parameters

Blood pressure and HR. Table 2 summarizes SBP, DBP, MBP, and HR results. SBP and MBP were not different among groups. DBP decreased in T+S but not in C. Exercise training significantly decreased resting HR.

LV function. Table 3 summarizes the results of LV function. LV systolic pressure was significantly higher in C and T than in S and T+S. LV end-diastolic pressure was not different among the groups, but LV initial diastolic pressure was significantly lower in T than in C. The +dP/d*t* was significantly higher in T than in C, S, and T+S. Similarly, −dP/d*t* was significant lower in S and T+S than in T. It was decreased in T+S compared with C.

Biochemical and Molecular Analysis

Testosterone measurement. As expected, the chronic AS administration causes a significant increase in serum testosterone concentration (ng/dl) in S (85.3 ± 19.3) and T+S (102.6 ± 18.3), but not in C (13.4 ± 6.5) and T (20.5 ± 9.9).

Collagen characteristics. Figure 2*A* shows representative histological sections of myocardium. The *top* panels show collagen fibers in the LV of the C and T groups. The *bottom* panels show the cardiac patch (reparative fibrosis), and the *inset* shows collagen fibers in the LV of the S and T+S groups. Figure 2*B* shows the quantitative analysis of collagen fibers in

Table 3. *Left ventricular function*

	n	LVSP, mmHg	LVIDP, mmHg	LVEDP, mmHg	+dP/d*t*, mmHg/s	−dP/d*t*, mmHg/s
Control	5	127±10	−5.6±2.5	3.5±1.3	4,780±924	4,333±785
Steroid	11	116±10*	−7.0±2.8	2.5±1.8	4,769±730	3,794±578
Trained	6	130±10	−8.9±2.8†	3.2±1.0	6,144±791‡	5,123±1,083§
Trained + steroid	8	111±7*	−7.8±1.6	2.8±1.7	4,054±653	2,972±631†

Values are means ± SD; *n*, no of animals. LVSP, left ventricular systolic pressure; LVIDP, left ventricular initial diastolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/d*t*, left ventricular contractility; −dP/d*t*, left ventricular relaxation. Significant difference vs. *control and trained ($P < 0.05$); †control ($P < 0.05$); ‡control ($P < 0.01$), steroid ($P < 0.05$), and trained + steroid ($P < 0.005$); §steroid ($P < 0.05$) and trained + steroid ($P < 0.0005$).

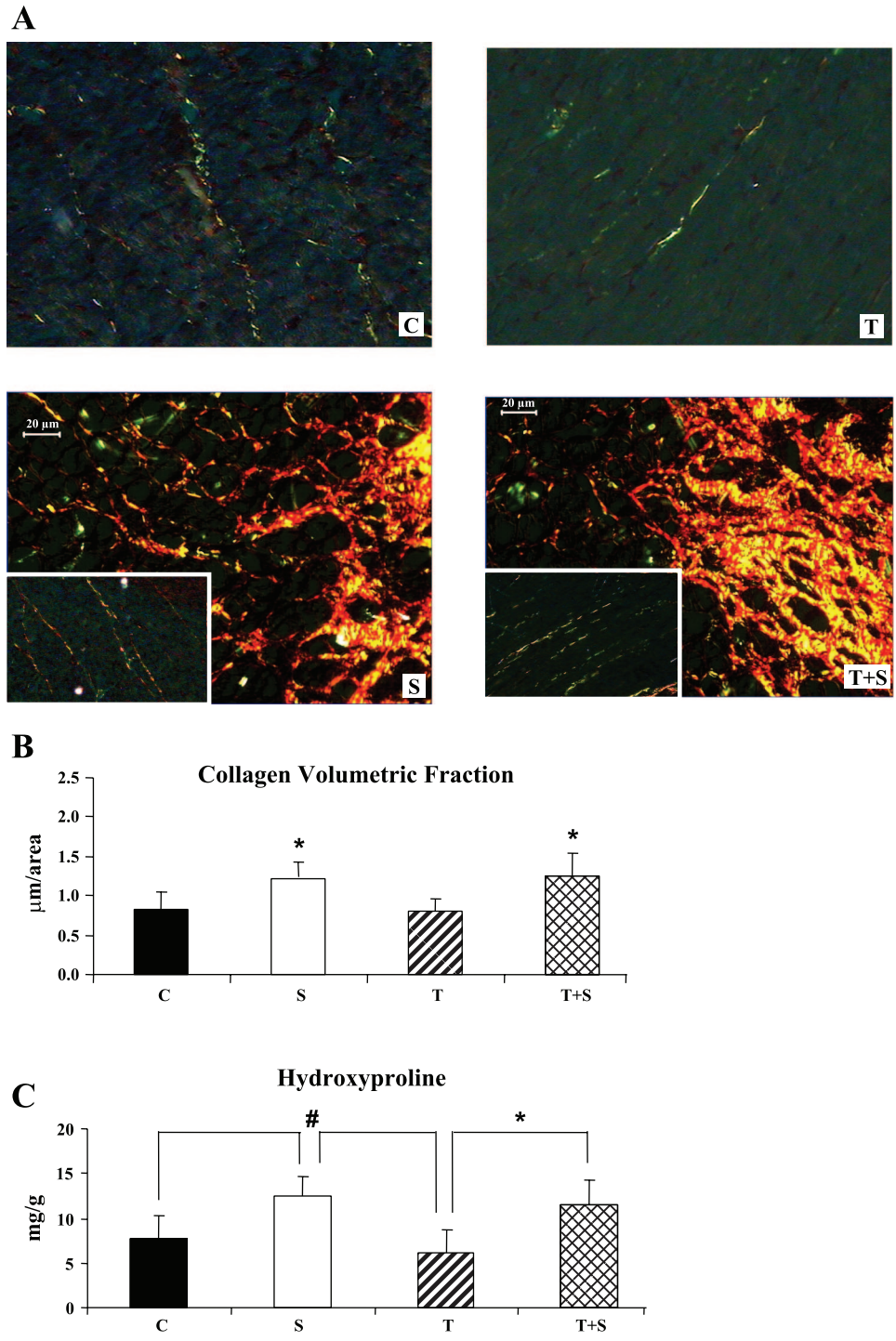

Fig. 2. Effects of anabolic steroids, swimming training, and swimming training associated with anabolic steroids on collagen fibers in the myocardium. *A*: representative histological sections of the myocardium under polarized light. Representative section of collagen fibers in the LV of C and T groups (*top*) and S and T+S groups (*bottom insets*) are shown. *Bottom*: regions with patched cardiac (reparative fibrosis) in the LV of the S and T+S groups. *B*: quantitative analysis of collagen fibers in myocardium. *Significant difference vs. C and T groups, $P < 0.05$. *C*: hydroxyproline concentration in the LV of C, S, T, and T+S groups. Significant difference vs. #C and T groups, and *T group: $P < 0.05$.

myocardium observed in Fig. 2*A*. The CVF was significantly increased in S and T+S compared with C and T groups, but not in C and T (1.20 ± 0.23 and 1.25 ± 0.30 vs. 0.84 ± 0.22 and 0.80 ± 0.17 μm/area, respectively; $P < 0.05$). Hydroxyproline concentration (Fig. 2*C*) was significantly higher in the S group than in C and T (12.4 ± 2.2 vs. 7.8 ± 2.5 and 6.2 ± 2.5 mg/g, respectively; $P < 0.05$). In addition, it was increased in T+S compared with the T group but was not increased in T (11.5 ± 2.8 vs. 6.2 ± 2.5 mg/g, respectively; $P < 0.05$). The collagen concentration calculated by hydroxyproline concentration was increased 59% (92.5 mg/g HW) in the S group than C (58.2 mg/g HW) group, which corresponded to ~8% of the cardiac hypertrophy observed in the S group compared with C group. The collagen concentration was increased 85% (85.8 mg/g HW) in the T+S group than T (46.3 mg/g HW) group, which corresponded to ~7% of the cardiac hypertrophy observed in the T+S group compared with T group.

Figure 3*A* shows representative histological sections of myocardium from groups treated with AT_1-receptor blocker (losar-

tan). The CVF increase was prevented by losartan treatment in SL and T+SL (0.89 ± 0.07 and 0.92 ± 0.22 μm/area) groups compared with S and T+S (1.2 ± 0.5 and 1.59 ± 0.38 μm/area; $P < 0.05$) groups (Fig. 3*B*).

Gene expression. Figure 4*A* shows the relative gene expression of collagen types I and III and AT_{1a}-receptor. The relative gene expression of collagen type I was increased in T+S group compared with T group ($P < 0.076$). However, the relative gene expression of collagen type III was significantly increased in T+S group compared with T group, but was not increased in T (0.60 ± 0.17 vs. 0.37 ± 0.01, arbitrary units; $P < 0.05$). Moreover, the relative gene expression of AT_{1a}-receptor was significantly increased in S and T+S (1.59 ± 0.16 vs. 1.61 ± 0.1 arbitrary units; $P < 0.05$) groups compared with C (1.0 ± 0.05 arbitrary units) group and in T+S group compared with T (0.74 ± 0.05 arbitrary units; $P < 0.05$) group. The relative gene expression of AT_2-receptor was not detected by the methodology used. The relative gene expression of ACE to C, S, T, and T+S (1.0 ± 0.19, 1.21 ± 0.34, 1.18 ± 0.2 and 1.21 ± 0.13 arbitrary units, respectively) was not significantly different among the four groups.

Cardiac ACE activity and ANG II quantification. Figure 4*A* shows the LV-ACE activity (Fig. 4*B*) was significantly increased in S and T+S groups compared with C and T groups (2.8 ± 0.69 and 2.9 ± 0.4 vs. 2.18 ± 0.49 and 2.1 ± 0.34 nM His-Leu·min^{-1}·mg^{-1}, respectively; $P < 0.01$). The ANG II quantification by immunohistochemistry methodology was significantly increased in S and T+S groups compared with C group (0.0044 ± 0.00043 and 0.0038 ± 0.00056 vs. 0.0027 ± 0.000135 ANG II/mm^2, respectively; $P < 0.05$), and T group (0.0033 ± 0.00047 ANG II/mm^2) was not significantly different from the C group. However, the PRA in C, S, T, and T+S (1.55 ± 0.59, 1.59 ± 0.76, 0.86 ± 0.25, and 1.05 ± 0.38 ng ANG I·ml^{-1}·h^{-1}, respectively) groups was not significantly different among the four groups.

Interestingly, considering the data from all groups, a close, positive correlation existed between LV-ACE activ-

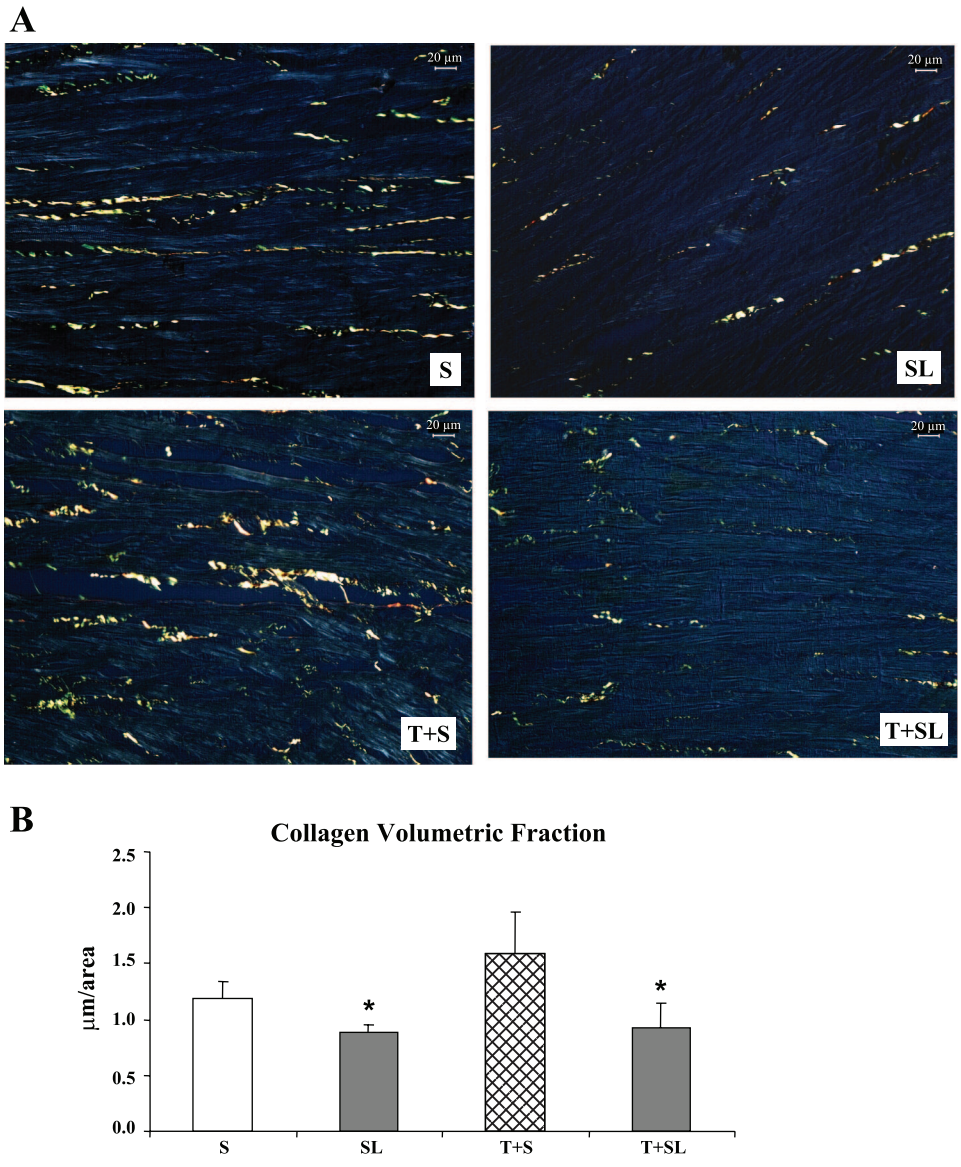

Fig. 3. Effects of losartan treatment on collagen fibers in the myocardium induced by chronic anabolic steroid use. *A*: representative histological sections of the myocardium under polarized light. Representative section of collagen fibers in the LV of S and SL groups (*top* panels), and T+S and T+SL groups (*bottom* panels) are shown. *B*: quantitative analysis of collagen fibers in myocardium of S, SL, T+S, and T+SL groups. Results are presented as means ± SD. *Significant difference vs. S and T+S groups, $P < 0.05$.

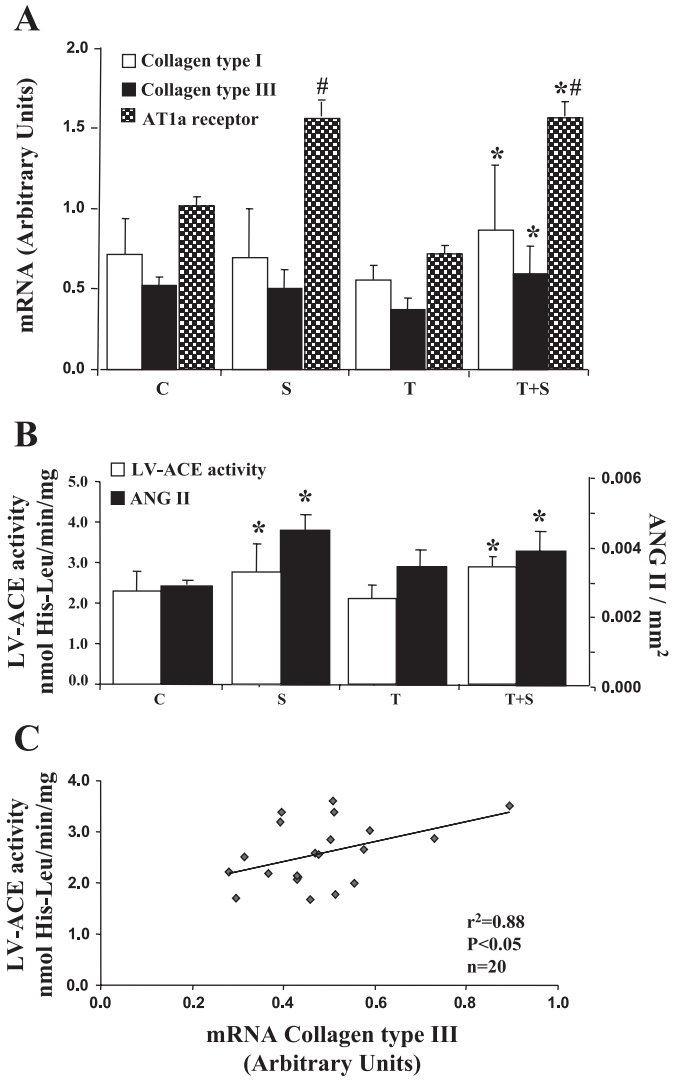

Fig. 4. Effect of anabolic steroids, swimming training, and swimming training associated with anabolic steroids on mRNA collagen types I and III, angiotensin type 1a (AT_{1a})-receptor, and LV-angiotensin I-converting enzyme (ACE) activity. *A*: effects on gene relative expression to collagen types I and III and AT_{1a}-receptor of C, S, T, and T+S groups. *Significant difference vs. T for: collagen type III ($P < 0.05$), collagen type I ($P = 0.076$), and AT_{1a}-receptor ($P < 0.05$). #Significant difference vs. C for AT_{1a}-receptor ($P < 0.05$). *B*: effect on LV-ACE activity (nmol His-Leu·min^{-1}·mg^{-1}) of C, S, T, and T+S groups. *$P < 0.01$ vs. C and T groups. Effects on angiotensin II (ANG II) quantification (ANG II/mm^2; $n = 4$/group) are shown for C, S, T, and T+S groups. *$P < 0.05$ vs. C group. *C*: direct correlation between LV-ACE activity and gene relative expression to collagen type III ($r^2 = 0.88$; $P < 0.05$). *n*, No. of animals.

ity and collagen type III expression ($r^2 = 0.88$; $P < 0.05$) (Fig. 4*C*).

DISCUSSION

The main findings of the present study are as follows. *1*) Swimming training associated with supraphysiological doses of AS causes exacerbated cardiac hypertrophy with interstitial fibrosis. This cardiac hypertrophy is associated with the cardiac RAS activation. *2*) The improvement in LV function promoted by exercise training is lost by AS treatment.

The present study showed that chronic AS administration causes a significant increase in the serum testosterone concentration of S and T+S rats. These results are consistent with effects previously described in rats that underwent chronic AS treatment showing serum testosterone concentration higher than that in C rats (41).

The reduction in BW in the T+S groups is compatible with a reduction in intraperitoneal fat. These findings may reflect a higher body fat metabolism achieved by aerobic training and AS administration. A similar observation was described in previous studies in which the excess of androgen caused an increase in fat metabolism in adipose tissue (37). Yu-Yahiro et al. (45) reported a similar decrease in BW and showed that steroid treatment blunted appetite, resulting in less weight gain in rats. Beutel et al. (4) showed that chronic treatment with low and high doses of steroid decrease the BW of treated animals. In contrast, other studies show neither change (30) nor BW gain (24).

The resting bradycardia confirms the effectiveness of exercise training in this study, as our laboratory reported previously (31). In addition, this exercise training adaptation was maintained with AS treatment.

Endurance training is known to cause eccentric cardiac hypertrophy in which the adaptive responses are distributed across the LV wall. However, many factors influence exercise-induced cardiac hypertrophy. Some are the mode, the intensity, the duration, and the frequency of the exercise regimen (17). In this study, the absolute weight of cardiac mass was not significantly different among groups. However, the swimming training protocol was efficient in increasing the HW/BW at 16% compared with that in the C group. This index was followed by an increase in myocyte diameter of 13% compared with that in the C group. This response is typical of aerobic training by swimming and has been called physiological cardiac hypertrophy (17, 22, 31). Moreover, for the physiological cardiac hypertrophy, the increase in cardiomyocyte volume results from the increase in the amount of contractile protein synthesis, which results in an increase predominantly in myofibril length. An increase in sarcoplasmic reticulum volume and mitochondria was observed (44). In this study, cardiac hypertrophy was followed by beneficial adjustments in the cardiovascular system, such as resting bradycardia and improvement in contractile performance of LV by increasing the positive derivative rate of the rise in pressure (+dP/d*t*). Previous results show that this swimming training protocol can increase the myosin ATPase activity in LV and increase the percentage of V1 isoforms of cardiac myosin, improving the systolic function in hypertensive rats (36). Other findings that deserve attention are the reduction of the initial diastolic pressure and the increased −dP/d*t* in the T group. This is an indication that lusitropism improved in trained hearts.

Another interesting finding was the higher cardiac hypertrophy index (8%) observed in the untrained AS group compared with that in the C group, which was proportional to the collagen concentration increase. The AS by itself alone produced cardiac hypertrophy. However, the diameter of the myocytes did not increase compared with those in the C group. In fact, a previous study reported the presence of androgen receptors in cardiac cells in both humans and animals (29). Moreover, evidence exists of the presence of endogenous pathways of androgen actions in cardiac hypertrophy development and a higher androgen receptor expression in hypertrophied hearts, in humans and rats (28). In this study, cardiac

hypertrophy was due to higher collagen concentration, as determined by histological quantification of the CVF and hydroxyproline methods. The physical exercise associated with AS treatment increased the cardiac hypertrophy ratio to 24, 15, and 7% compared with that in C, S, and T groups, respectively. These results on cardiac mass are similar to results previously reported (15, 39). However, this apparent cardiac hypertrophy was not detected in the diameter of the myocytes. The myocyte diameter was similar in the T+S and T groups. This result shows that the AS were not involved in the hypertrophy of the myocytes, suggesting that another component contributed to the increase in cardiac mass. The cardiac hypertrophy and collagen syntheses induced by steroid treatment and by physical exercise associated with AS treatment were totally prevent by losartan (AT_1-receptor blocker) treatment.

Hemodynamic and nonhemodynamic factors might be involved in the disequilibrium between myocyte growth and collagen turnover. Fibrillar collagens, types I and III, are the major structural proteins of the myocardial collagen matrix. Type I collagen is usually present in the form of thick fibers, and its concentration determines the stiffness of the myocardium. Type III collagen forms fine reticular networks and is more distensible than type I collagen (9). In this study, when the cardiac collagen of the T+S group was investigated, a higher amount of collagen was found, as determined by the hydroxyproline method and histological quantification of the CVF. This group also had an increase in collagen type I ($P = 0.076$) and type III ($P < 0.05$) cardiac expression compared with these in the T group. These findings may contribute to the larger cardiac hypertrophy in this group. Thus these factors explain the loss of benefits of aerobic physical training on ventricular function index (+dP/d*t*, −dP/d*t*, and LV initial diastolic pressure) in the T+S group compared with that in the T group. The abundance of interstitial collagen observed in S and T+S groups contributed to decreased LV systolic pressure and −dP/d*t*, a finding that was more exacerbated in the T+S group. Thus the combined diastolic and systolic dysfunction of the LV occurs as the result of myocardium fibrosis, and this is relevant for the development of heart failure. Reactive fibrosis occurs in the absence of myocyte necrosis and initially is an adaptive response that preserves the force-generating capacity of the hypertrophied myocardium. Later in hypertrophy, a reparative (or replacement) fibrosis occurs in response to cell loss. Cardiac fibrosis can be accompanied initially by diastolic and ultimately by systolic ventricular dysfunction. Evidence suggests a clear association between such adverse structural remodeling and activation of the RAS (44).

An interesting feature of the present study was the higher local RAS activation. An important result observed was the higher local ACE activity found in the hearts from the S and T+S groups vs. C and T groups and the close, positive correlation between LV-ACE activity and collagen type III ($r^2 = 0.88$; $P < 0.05$). Moreover, the AT_{1a}-receptor expression was increased ~60% in the hearts from the S and T+S groups vs. C group and ~120% in T+S group vs. T group, and the ANG II was increased in the hearts from the S (60%) and T+S (40%) groups vs. C group. This is evidence of the local RAS participation in cardiac hypertrophy development observed in this model. Other studies showed that the cardiac ANG II concentration contributes to the hyperplasia of cardiac fibroblasts and to the development of cardiac fibrosis (44), and the ACE activation might be contributing to the development of heart fibrosis, as previously reported, showing that the fibrotic area in the heart has high levels of ACE activity (11, 34). Several experimental studies provide evidence that circulating and local RAS promote the development of myocardial fibrosis (6, 7), and ANG II can mediate myocardial fibrosis independently of the mechanical load (20). Other results demonstrated in cultured adult rat cardiac fibroblasts that ANG II increases collagen synthesis and inhibits matrix metalloproteinase activity, the key enzyme of interstitial collagen degradation (8, 9). In fact, several results suggest that ANG II exerts an important role in cardiac hypertrophy development throughout enhanced ACE activity. These findings are reinforced by the fact that ACE inhibitors and AT_1-receptor antagonists promote cardiac hypertrophy regression in experimental animals (43) and humans (16) and in hypertensive patients (7, 15). ANG II stimulates collagen synthesis by both AT_1-receptor and AT_2-receptor activation in cultured adult rat cardiac fibroblasts, and ANG II-induced inhibition of collagenase activity was specifically mediated by AT_2-receptor (10). In support of these findings, the abolition of ANG II-induced cardiac fibrosis in mice lacking the AT_2-receptor gene was demonstrated (21). On the other hand, considering that ACE also inactivates bradykinin, the increased ACE activity in the S and T+S groups increased the breakdown of bradykinin, which can reduce nitric oxide production, independent of the regulation of ANG II production (40). Recently, it was demonstrated that exercise training induces enhancement of nitric oxide production; however, AS (nandrolone decanoate) treatment blocks this effect promoted by physical exercise, probably because this hormone damages the endothelial function of trained animals (13). These findings reveal the important influence of the RAS on the regulation of interstitial collagen and on cardiac function. The local RAS activation in heart remodeling induces direct consequences on cardiac function, reducing the benefits of physical training by the steroid association in the T group.

In conclusion, this study showed that supraphysiological doses of AS exacerbated cardiac hypertrophy in exercise-trained rats. The AS alone or in combination with exercise training induces maladaptive remodeling and further deterioration of cardiac performance. The exercise training associated with AS causes loss of the beneficial effects of LV function induced by exercise training. We believe this is the first study to show that the combination of exercise and AS causes an increase in the heart collagen concentration associated with activation of the cardiac RAS.

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