Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 87 (2003) 269–277
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Effects of prolonged stanozolol treatment on antioxidant enzyme activities, oxidative stress markers, and heat shock protein HSP72 levels in rat liver

Angel Pey [a], Ana Saborido [a], Isabel Blázquez [a], Jerónimo Delgado [b], Alicia Megías [b,*]

[a] *Department of Biochemistry and Molecular Biology I, Faculty of Chemistry, Complutense University, 28040 Madrid, Spain*
[b] *Department of Biochemistry and Molecular Biology I, Faculty of Biology, Complutense University, 28040 Madrid, Spain*

Received 28 February 2003; accepted 10 September 2003

Abstract

The abuse of anabolic-androgenic steroids (AAS) to enhance physical performance is widespread in sport communities despite their reported side effects. Since the biochemical bases for the hepatotoxic effects of these compounds are largely unknown, this investigation was aimed at testing whether prolonged (8 weeks) treatment with high doses (2 mg kg^{-1} body weight; 5 d wk^{-1}) of stanozolol (ST), either alone or in conjunction with treadmill-exercise training, induced changes in oxidative stress biomarker levels and antioxidant defence systems in rat liver. After ST oral administration, the mean values of serum parameters related to hepatic function were within normal ranges. No changes in protein carbonyl content and in the reduced to oxidized glutathione (GSH/GSSG) ratio were detected in liver homogenates of ST-treated rats, whereas thiobarbituric acid-reactive substances (TBARS) levels resulted increased ($P < 0.05$). Total superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) activities were higher ($P < 0.05$) in the liver of treated rats but mitochondrial SOD and glutathione reductase (GR) activities, and the 72 kDa heat shock protein (HSP72) level were not modified. Chronic exercise alone did not change any of the above parameters except for a remarkable enhancement of HSP72 expression; in no case training modified the effects of ST treatment. The present data show that 8 wk ingestion of ST, either with or without concurrent exercise training, can induce oxidative stress in rat liver despite the up-regulation of enzymatic antioxidant activities.

Keywords: Anabolic-androgenic steroids; Hepatotoxicity; TBARS; Heat shock proteins; Exercise training

1. Introduction

Stanozolol (ST) is a synthetic 17α-alkylated derivative of testosterone that exhibits a greater anabolic potency and a slower hepatic degradation than the natural male hormone. Due to these properties, ST, along with other synthetic derivatives of sexual steroids collectively referred to as anabolic-androgenic steroids (AAS), are used in medical practice in status of muscle wasting and to treat a variety of other conditions [1]. In addition to their therapeutic uses, AAS are employed at supraphysiologic doses in the sport context aimed at increasing muscular development, physical performance, aerobic capacity and tolerance to high-intensity training despite the fact that scientific evidences supporting these beneficial effects of AAS are not conclusive [2].

AAS therapy is associated with various adverse effects that are generally dose related; therefore, illicit use of the high doses taken by sportsmen carries substantial risks for health. A major side effect of 17α-alkylated AAS therapy is hepatotoxicity, including elevated levels of liver enzymes, cholestatic jaundice, peliosis hepatis, and various neoplastic lesions [1]. When hepatic function is monitored in healthy athletes abusing AAS, liver serum parameters show no changes or slight elevations, which revert to normal levels after discontinuing the drug [3,4]. However, conventional biochemical liver tests do not always reflect liver abnormalities particularly at the initial stages. In this regard, experimental evidences obtained in controlled studies on

Abbreviations: AAS, anabolic-androgenic steroids; CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HSPs, heat shock proteins; ROS, reactive oxygen species; SOD, superoxide dismutase; ST, stanozolol; TBARS, thiobarbituric acid-reactive substances

* Corresponding author. Tel.: +34-91-394-4267; fax: +34-91-394-4672.
E-mail address: amegias@bbm1.ucm.es (A. Megías).

0960-0760/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2003.09.001

rodents indicate that 17α-alkylated steroids can induce liver injury. ST, and other orally active AAS, has been shown to cause inflammatory or degenerative lesions in centrilobular hepatocytes, ultrastructural alterations in the canaliculi and degenerative changes in mitochondria and lysosomes [5,6]. Furthermore, in eugonadal male rats, prolonged ST-administration provokes an increase in the activities of liver lysosomal hydrolases and a decrease in some components of the microsomal drug-metabolizing system and in the activity of the mitochondrial respiratory chain complexes without modifying classical serum indicators of hepatic function [7,8].

Liver is a key organ actively involved in numerous metabolic and detoxifying functions. As a consequence, it is continuously exposed to high levels of endogenous and exogenous oxidants that are by-products of many biochemical pathways and, in fact, it has been demonstrated that intracellular oxidant production is more active in liver than in other rat tissues [9,10]. Under normal physiological conditions a major source of reactive oxygen species (ROS) is mitochondria because over 90% of the O_2 is consumed in the electron transport chain that produces a significant amount of ROS. Additional sources in generating ROS are peroxisomes, xanthine oxidase, NADPH oxidase, acyl-CoA oxidase and cytochrome P-450 [9,11]. Since prolonged administration of ST provokes dysfunction of mitochondrial respiratory chain complexes and mono-oxygenase systems [8,7], it would be possible that these alterations were accompanied by an increased ROS generation. Although liver is endowed with high levels of enzymatic and non-enzymatic antioxidant defences [12], an enhancement in ROS production exceeding the antioxidant defences and repair capacity could lead to oxidative stress and cell damage. This kind of risk should be higher if the consumption of O_2 were increased as occurring during exercise.

Since no information is available on the effects of 17α-alkylated steroid treatment on hepatic antioxidant capacity, we designed a study to investigate in sedentary and trained rats whether a prolonged treatment with high doses of ST modified oxidative stress biomarker levels, redox status of glutathione and activities of the antioxidant enzymes in liver. In addition, the expression of the 70 kDa heat shock proteins (HSPs), stress proteins that may represent an important mechanism of protection against oxidative damage, was analyzed.

2. Materials and methods

2.1. Training program and steroid treatment

Thirty-two male Wistar rats (initial body weight, 155 ± 7 g) were obtained from Charles River (Barcelona, Spain). They were housed in an animal room at 22–24 °C and had free access to laboratory chow and tap water. The animals were adapted to an inverse 12:12 h light–dark cycle (dark period, 8:00–20:00) before the beginning of the exercise period. Rat care and handling and all the experimental procedures employed were in accordance with internationally accepted principles concerning the care and use of laboratory animals. Initially, animals were randomly divided between a sedentary (S) group ($n = 16$) and an exercise-trained (T) group ($n = 16$). The rats of the T group were exercised by running on a motor-driven treadmill (Columbus Instruments, Columbus, OH) 5 d wk^{-1} for 12 wk. During the first 4 wk, the speed and duration of the daily exercise sessions were progressively increased until the rats were capable of running continuously for 45 min at 25 m min^{-1}. At the beginning of the fifth training week, when maximal exercise intensity was reached, each group was arbitrarily subdivided into two groups: control ($n = 8$) and ST-treated ($n = 8$). The animals selected for stanozolol (17β-hydroxy-17α-methyl-5α-androst-2-eno[3,2-*c*]-pyrazole, Zambon, Barcelona, Spain) treatment received by gastric intubation 2 mg steroid per kilogram body weight as a homogeneous suspension in 1 ml of water, 5 d wk^{-1}, for 8 wk. The high level of ST was chosen in an attempt to simulate the massive doses of AAS used in athletics. During the 12 wk training period, sedentary rats performed weekly a single exercise session for 5 min at 15 m min^{-1} to familiarize themselves with treadmill exercise and handling.

Two weeks before the end of the training period, a treadmill endurance test was administered to all the sedentary and trained rats the day before the weekly rest of the trained group. During the test, animals ran at 25 m min^{-1} with a 5% slope until fatigue occurred, i.e., until they could no longer maintain the required running pace. Total exercise duration was recorded. After completion of the 12 wk exercise program, rats were not exercised for 48 h and received the last steroid dose 48 h before they were sacrificed (between 8:00 and 12:00 a.m. to minimize circadian fluctuation of hepatic GSH). Animals were killed by decapitation under ether anaesthesia. Blood was collected and the liver was rapidly excised, weighed, washed with cold saline, frozen in liquid nitrogen and stored at −80 °C until use. The *soleus* muscles were also removed, trimmed of connective tissue, weighed, fast frozen and stored at −80 °C.

2.2. Serum analyses

Serum was obtained by centrifugation at 3000 × *g* for 15 min. Fresh aliquots were diluted 1/2 with 2% (p/v) trichloroacetic acid (TCA), centrifuged at 18,000 × *g* for 10 min and the supernatant collected for GSH assay. The activities of the serum enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyltransferase (γ-GT) were analyzed on a Kodak Ektachem 500 analyzer by reflectance spectrophotometric procedures.

2.3. Preparation of liver homogenates

Procedures were carried out at 0–4 °C. Livers were cut into small pieces and homogenized with a Potter-Elvehjem tight-fitting glass-Teflon homogenizer in five volumes of 10 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, and 0.3 mM phenylmethylsulfonil fluoride. The homogenate was filtered through four layers of cheesecloth and aliquots were stored at −80 °C until assay. Protein concentration was determined by the method of Lowry et al. [13]. Small portions of livers (0.8 g) were homogenized with a Polytron PT-10 (Kinematica) two times for 5 s at speed setting 3 with a final 1 s burst at speed setting 7, in five volumes of 1% (p/v) TCA containing 2.5 mM butylated hydroxytoluene. The homogenate was centrifuged at $18,000 \times g$ for 10 min and the supernatant was diluted 1:200 with 1% TCA and used for glutathione assay as indicated below.

2.4. Assay methods

Lipid peroxidation was measured in serum aliquots (300 μl) and liver homogenates (300 μl) by the thiobarbituric acid (TBA) assay in the presence of 2.5 mM butylated hydroxytoluene essentially according to the method of Ohkawa et al. [14]. Thiobarbituric acid-reactive substances (TBARS) content was calculated based on a standard curve using 1,1,3,3-tetramethoxypropane as a standard. TBARS are expressed in micromol per liter of serum, or nmol per gram of tissue wet weight.

Total glutathione (T-GSH) was measured by the method of Tietzte [15] in the presence of 5,5′-dithiobis (2-nitrobenzoic) acid, NADPH and glutathione reductase (GR) using aliquots of 100 μl of serum or liver homogenates prepared as described above. GSSG was determined by the same method after derivatization of GSH with 2-vinylpyridine [16].

Protein oxidation was measured in liver homogenates aliquots (300 μl) by the carbonyl assay according to Levine et al. [17] using 10 mM 2,4-dinitrophenylhydrazine dissolved in 2.5 M HCl, accompanied by controls treated with 2.5 M HCl alone. The concentration of protein carbonyls was determined at 366 nm with an absorption coefficient of 22,000 M^{-1} cm^{-1}. Protein concentrations were determined on the HCl controls by measuring the absorbance at 280 nm using a BSA standard curve in 6 M guanidine-HCl and 20 mM potassium phosphate buffer (pH 2.3).

2.5. Enzyme activities

Citrate synthase (CS, EC 4.1.3.7) activity of *soleus* homogenates was measured at 37 °C in the presence of 0.2% Triton X-100 as previously described [18]. Total superoxide dismutase (SOD, EC 1.15.1.1) activity was measured in liver homogenates following at 25 °C the inhibition of the rate of pyrogallol auto-oxidation according to Marklund and Marklund [19]. Mn-SOD was determined in the presence of 1 mM KCN. One unit of SOD activity was defined as the amount required to inhibit the rate of pyrogallol oxidation by 50%. Catalase (CAT, EC 1.11.1.6) was assayed at 20 °C by the method of Aebi [20] slightly modified. Previously, liver homogenates aliquots were centrifuged at $1000 \times g$ for 10 min and the supernatants were used for the enzymatic assay. The reaction mixture contained 0.1 mM EDTA, 15 mM H_2O_2, 0.002% Triton X-100, a suitable amount of protein and 0.5 M potassium phosphate buffer, pH 7.0, in a final volume of 1 ml. The enzymatic activity is expressed as micromole (μmol) H_2O_2 decomposed per minute (min^{-1}) per milligram (mg^{-1}) tissue wet weight (wt.) Glutathione peroxidase (GPX, EC 1.11.1.9) was measured at 37 °C following NADPH oxidation at 340 nm in the presence of excess glutathione reductase, GSH and H_2O_2 as substrate [21]. In addition, the reaction mixture contained 1 mM sodium azide to inhibit catalase activity. Glutathione reductase (GR, EC 1.6.4.2) was assayed at 30 °C by measurement of the rate of NADPH oxidation at 340 nm in the presence of GSSG [22]. GPX and GR activities are expressed as micromole (μmol) NADPH per minute (min^{-1}) per gram (g^{-1}) tissue wet weight (wt.). All the enzymatic assays contained an adequate amount of liver homogenate (equivalent to 0.5, 0.02, 0.25 and 0.8 mg tissue wet wt. for SOD, CAT, GPX and GR, respectively).

2.6. Quantification of stress proteins

Liver homogenates were separated on 7.5% SDS-PAGE gels under standard denaturing conditions [23]. Slab-gels were loaded either with 60 or 20 μg of protein per slot for HSP72 or HSP73 detection, respectively. Resolved proteins were transferred to nitrocellulose membranes by semidry electroblotting. The blots were blocked overnight, washed, and incubated for 1 h with a 1:1000 dilution of the monoclonal antibodies anti-HSP72 (C92F3A.5, SPA-810, StressGen, Victoria BC) or anti-HSP73 (1B5, SPA-815, StressGen, Victoria BC). Peroxidase-conjugated polyclonal goat anti-mouse IgG antibody (NA931, Amersham Pharmacia Biotech) or goat anti-rat immunoglobulins (NA935, Amersham Pharmacia Biotech) were used as secondary antibodies at a 1:12000 dilution. The blots were washed, incubated with the ECL-detection reagent (Amersham Pharmacia Biotech) and exposed to X-OMAT AR X-ray films (Kodak Inc.). Quantification was performed in the linear absorption range using computerized densitometry with commercially available software (Sigmagel 1.0). For comparison between blots, one aliquot of the same homogenate was loaded as a standard in each gel to allow data normalization.

2.7. Statistical analysis

All samples were individually processed and measured in duplicate in the same assay. Results for each experimental group are reported as means ± S.D. Data were analyzed by

a two-way ANOVA test for the two main effects (exercise training and ST administration) and for the interaction between them, employing a standard computerized statistical program (Statgraphics Plus, Statistical Graphic Corp., US). When a significant F value was obtained a Scheffé post hoc analysis was performed to determine specific differences. A level of $P < 0.05$ was selected to indicate statistical significance.

3. Results

3.1. Body and liver weight, and effectiveness of training

After 12 wk of experimental period, final body weight was reduced significantly (two-way ANOVA, $P < 0.01$) in the trained groups in comparison with the sedentary ones (Table 1), a common finding for male rats [18]. Liver weight was reduced in trained rats but liver-somatic index was not modified (not shown). In addition to the decrease in body weight, both time to fatigue in the endurance test, and citrate synthase activity in *soleus* muscle homogenate, were increased in trained groups confirming the effectiveness of the training program. ST treatment had no significant effect on body and liver weight, exercise endurance capacity and CS activity in both sedentary and trained rats. Likewise, the protein content measured in liver homogenates was not influenced by either exercise training or ST administration.

3.2. Serum parameters

The determination of metabolites and enzyme activities in the serum can be of great value for the detection of liver alterations. In this regard, neither the endurance training nor the administration of ST modified significantly mean values of transaminases, alkaline phosphatase, and γ-glutamyltransferase activities (Table 2). Total glutathione serum levels and TBARS content were not different between the ST-treated and untreated groups and resulted also unaffected by exercise training.

3.3. Glutathione status and oxidative stress biomarkers in liver

Hepatic glutathione status in the different experimental groups is shown in Table 3. In the non-treated animals, training induced no changes in GSH levels but reduced significantly ($P < 0.05$) GSSG content. As a result, the ratio of GSH to GSSG was significantly increased ($P < 0.05$) in the liver of trained non-treated rats. ST administration had no effect on the hepatic levels of GSH and GSSG in sedentary animals; however, ST-treated trained rats, showed an

Table 1
Effects of stanozolol administration and exercise training on body and liver weight, liver protein yield, *soleus* citrate synthase activity and exercise endurance capacity

Group	Body weight (g)	Liver weight (g)	Liver protein yield (mg protein g^{-1} wet wt.)	Endurance time (min)	CS activity ($\mu mol\ min^{-1}\ g^{-1}$ tissue)
Sedentary					
Control	473 ± 57	14.3 ± 1.9	265 ± 23	22 ± 2	31.1 ± 5.7
Stanozolol	458 ± 23	14.0 ± 1.2	293 ± 15	26 ± 5	32.7 ± 4.7
Trained					
Control	409 ± 27**	12.8 ± 0.4*	287 ± 20	198 ± 36***	41.1 ± 4.8**
Stanozolol	403 ± 38**	13.1 ± 0.9*	288 ± 13	195 ± 20***	39.0 ± 3.4**

Data are shown as means ± S.D. ($n = 8$). Endurance time is defined as the time rats kept pace with the treadmill at 25 m min^{-1} and 5% slope. CS activity: citrate synthase activity in *soleus* muscle homogenates. Two-way ANOVA analysis: a significant main effect for exercise training was observed for body weight, liver weight and CS activity ($P < 0.01$) and endurance time ($P < 0.001$).

* $P < 0.05$, significantly different from the corresponding control or stanozolol-treated sedentary group.

** $P < 0.01$, significantly different from the corresponding control or stanozolol-treated sedentary group.

*** $P < 0.001$, significantly different from the corresponding control or stanozolol-treated sedentary group.

Table 2
Effects of stanozolol treatment and exercise training on serum parameters

Group	AST ($U\ l^{-1}$)	ALT ($U\ l^{-1}$)	ALP ($U\ l^{-1}$)	γ-GT ($U\ l^{-1}$)	T-GSH (μM)	TBARS (μM)
Sedentary						
Control	135 ± 3	43 ± 8	91 ± 13	1.5 ± 0.4	6.38 ± 0.59	3.65 ± 0.68
Stanozolol	170 ± 9	52 ± 4	93 ± 20	1.4 ± 0.4	7.08 ± 1.25	3.46 ± 0.84
Trained						
Control	202 ± 15	55 ± 6	106 ± 11	1.2 ± 0.2	7.48 ± 1.25	3.16 ± 0.61
Stanozolol	161 ± 44	53 ± 13	97 ± 33	1.7 ± 0.5	6.99 ± 1.06	3.34 ± 0.48

Results are means ± S.D. ($n = 8$). AST, aspartate aminotransferase; ALT alanine aminotransferase; ALP, alkaline phosphatase; γ-GT, γ-glutamyltransferase; T-GSH, total glutathione; TBARS, thiobarbituric acid-reactive substances. There were no statistically significant differences between groups.

Table 3
Effects of stanozolol treatment and exercise training on hepatic glutathione status and liver content of protein carbonyls and TBARS

Group	GSH (μmol g^{-1} wet tissue)	GSSG (μmol g^{-1} wet tissue)	GSH/GSSG	Protein carbonyls (nmol mg^{-1} protein)	TBARS (nmol g^{-1} wet tissue)
Sedentary					
Control	5.4 ± 0.2	0.091 ± 0.008	59.8 ± 2.1	1.08 ± 0.19	56.4 ± 7.2
Stanozolol	5.6 ± 0.4	0.084 ± 0.009	65.7 ± 3.1	1.06 ± 0.25	69.0 ± 4.5†
Trained					
Control	5.5 ± 0.3	0.066 ± 0.007*	82.8 ± 4.1*	0.93 ± 0.20	54.0 ± 4.5
Stanozolol	6.7 ± 0.3*,†	0.079 ± 0.010	84.0 ± 3.2*	1.05 ± 0.19	67.9 ± 8.3†

Results are means ± S.D. ($n = 8$). Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; GSH/GSSG, reduced to oxidized glutathione ratio; TBARS, thiobarbituric acid-reactive substances. Two-way ANOVA analysis: a significant ($P < 0.05$) main effect for exercise training was observed for GSSG content and for the GSH/GSSG ratio; a significant ($P < 0.05$) main effect for stanozolol treatment was observed for TBARS content.

* $P < 0.05$, significantly different from the corresponding control or stanozolol-treated sedentary group.

† $P < 0.05$, significantly different from the corresponding sedentary or trained control untreated group.

increased liver content of GSH ($P < 0.05$) and a trend towards a higher GSSG levels versus control trained rats. As a consequence, the GSH-to-GSSG ratio showed no significant differences due to ST treatment.

Neither exercise training nor steroid treatment induced significant changes in protein carbonyl levels (Table 3). TBARS content was also unaffected by exercise training. Nevertheless, a significant main effect on hepatic TBARS levels was observed for ST treatment (two-way ANOVA, $P < 0.05$), indicating an enhancement in lipid peroxidation.

3.4. Antioxidant enzyme activity

Fig. 1 shows total SOD, CAT, GPX and GR activities measured for all the study groups. No differences were detected between sedentary and trained animals for any enzymatic activity. However a significant main effect was observed for stanozolol administration (two-way ANOVA, $P < 0.05$) on total SOD, CAT and GPX activities so that the enzymatic activities measured in the ST-treated groups were significantly higher than those determined in the respective untreated control groups ($P < 0.05$). In no case, there was interaction between training and ST treatment. GR activity was not significantly affected by the administration of ST. Mitochondrial SOD activity determined for the sedentary control group (0.56 ± 0.14 U mg^{-1} wet tissue) did not change significantly as a consequence of either training or ST treatment (not shown).

3.5. Expression levels of HSP72 and HSP73

To test the possibility that the hepatic antioxidant pathways may be complemented by the defence mechanism represented by heat shock proteins, we have analyzed the expression of 70 kDa HSPs in liver homogenates. Representative Western blot detection and the relative amounts of the inducible HSP72 isoform are presented in Fig. 2. HSP72 was undetectable in both the control and ST-treated sedentary groups whereas a strong accumulation of this protein was detected in liver samples from the trained groups. There seems to be, however, a marked heterogeneity in the individual expression levels of HSP72. Densitometric analysis of these immunoblots indicated that the combination of training and ST-treatment induced no statistically significant changes in the HSP72 content with respect to those elicited by exercise training alone. On the other hand, the level of the constitutive form HSP73 was unaffected by training, steroid administration or the combination of training and ST-treatment (not shown).

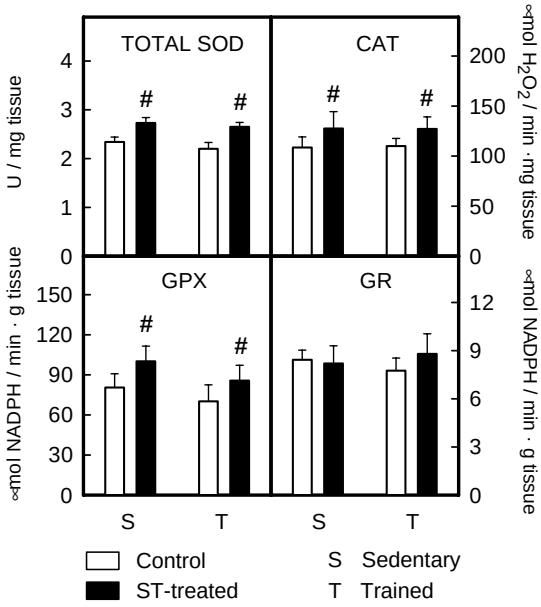

Fig. 1. Effects of stanozolol treatment and exercise training on rat liver antioxidant enzyme activities. Total SOD, total superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase. Results are presented as means±S.D. ($n = 8$). Two-way ANOVA analysis: a significant ($P < 0.05$) main effect for stanozolol administration was observed for total SOD, CAT and GPX activities. # $P < 0.05$, significantly different from the corresponding control group.

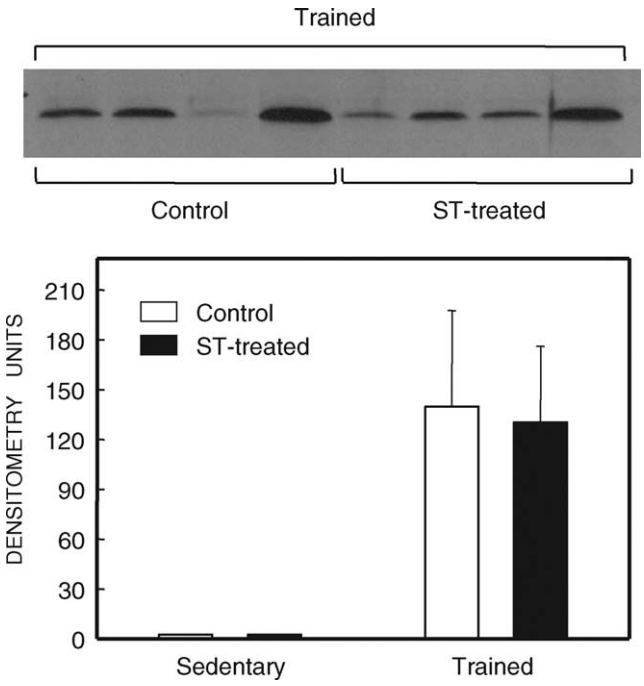

Fig. 2. Effect of stanozolol treatment and exercise training on HSP72 protein expression in rat liver. Homogenates (60 μg protein per well) were separated on 7.5% SDS-PAGE gels, transferred to nitrocellulose, and probed with a monoclonal antibody specific for HSP72 as described in Methods. Top: representative Western blot of liver samples from trained rats (four control and four ST-treated animals). Bottom: graphical representation of the values obtained from densitometric scanning of the corresponding Western blots for HSP72. No bands were detected in liver homogenates from control and ST-treated sedentary rats. Results are expressed as arbitrary densitometry units (means ± S.D., $n = 8$). There were no significant differences between the control and ST-treated trained groups.

4. Discussion

The main finding of the present study is that prolonged (8 wk) ingestion of ST induced a significant increase ($P < 0.05$) in the content of TBARS and in the activities of the antioxidant enzymes total SOD, CAT and GPX and did not modify the expression levels of HSP72 in rat liver. Simultaneous realization of exercise training did not alter the effects of ST administration.

To our knowledge, this is the first time that the in vivo effects of ST treatment on hepatic antioxidant defence systems and oxidative stress biomarker levels have been studied. Since the use of AAS as ergogenic aids is usually accompanied by exercise training, and exercise is known to affect a large number of physiological factors and biochemical systems, it was advisable to control the possible influence of exercise training on the effects of stanozolol on the liver. It is interesting to point out that all the measurements were performed on total homogenates rather than in isolated subcellular fractions to avoid erroneous interpretations because AAS treatment is known to induce hepatic structural and ultrastructural changes [5,6] that may cause modifications in the liver subcellular fractionation pattern of the ST-treated rats.

Training on its own did not modify hepatic levels of oxidative damage markers except for a significant reduction of the GSSG content leading to an improvement in the redox status of glutathione. Likewise, antioxidant enzymatic activities remained unchanged in liver of trained rats. Our results are in agreement with those previously reported showing that chronic exercise does not alter the liver content of protein carbonyls, nuclear DNA 8-hydroxy-2′-deoxyguanosine, and the lipid peroxidation marker malonaldehyde [24,25]. However, experimental evidence about the effects of exercise training on liver antioxidant enzymes is controversial since increases [26–28], decreases [29], or no changes [30–32] in their activities have been reported. The reason for these apparent discrepancies is unclear at present, but it could be related with differences in the intensity and/or duration of the exercise sessions employed in the aforementioned studies. Intense and/or prolonged sessions can cause oxidative stress in liver [25,26,33,34] and therefore repetition of the exercise stimulus along the training period could activate the synthesis of antioxidant enzymes as a long-term strategy to cope with the encountered oxidative stress during exercise sessions. The training program used in this work can be regarded as a moderate stimulus for normal rodent standards and hence, the individual sessions of exercise likely posses minimal oxidative stress to the liver likely owing to its high intrinsic antioxidant capacity.

A remarkable increase in the expression levels of the highly inducible HSP72 stress protein was detected in liver of trained rats. Other studies have previously shown that treadmill running can induce stress proteins in a variety of tissues [35]; however, the accumulation of HSPs was generally observed in the post-exercise period following a bout of intense exercise or after incremental programs of treadmill training in which either the speed or duration of exercise sessions were progressively increased. Therefore, the increase in HSPs levels have been regarded as a rapid and transient response required by cells to allow them cope or adapt to a new level of stress. It is worthy to note that the marked accumulation of HSP72 in liver of our trained rats was detected after several weeks of exercise at the same intensity and duration. This fact suggests that the daily stress of exercise sessions is the stimulus that continuously induces and maintains HSP72 at elevated levels conferring to liver cells protection that extends at least up to 48 h after cessation of exercise. The exact component responsible for the increase of HSP72 observed in the current study is unclear since a variety of conditions known to induce synthesis of stress proteins [35] may be simultaneously occurring during treadmill running. It seems unlikely that an augmented exercise-linked ROS production was the signal that triggers the synthesis of HSP72 since, in liver of control trained rats we did not detect changes in oxidative stress biomarker levels as well as in hepatic antioxidant enzymatic activities, as could be expected if

ROS overproduction occurred. Therefore, other physiological stressful conditions associated with exercise such as hemodynamic changes, hypoxia, hyperthermia, metabolic challenges, and perhaps molecular signals proceeding from the exercising muscles, as interleukin-6 [36] may be the stimulus causally related with the liver response to exercise stress.

On the other hand, HSP72 content was not increased in liver of either sedentary or trained rats after ST treatment. Stress proteins are involved in the protection of cells against different types of proteotoxic insults [35]. Therefore the lack of changes in HSP72 expression levels may be related with the protein carbonyl content, a marker of oxidative damage to proteins, that remained unchanged in steroid-treated rats. However, the possibility that other stress proteins are up-regulated by ST administration cannot be discarded.

The increase of total SOD, CAT and GPX activities detected in liver from both sedentary and trained ST-treated animals is consistent with the fact that oxidative stress occurred in some extent. We found augmented levels of the lipid peroxidation marker TBARS in liver homogenates from treated animals 48 h after receiving the last steroid dose. Interestingly, no changes in TBARS content were detected in serum (as shown in this work), as well as in skeletal muscles and heart (unpublished results). Thus, the continuous and prolonged ingestion of ST appears to provoke a local and sustained oxidative stress state in liver that could lead to increased expression of antioxidant enzymes through free radical-mediated induction of redox sensitive signal cascades. In this respect, 4-hydroxy-2-nonenal, the major end product of oxidized fatty acid metabolism has been reported to activate signal transduction mechanisms and to modulate the expression of various genes [37]. How oral ST treatment could be associated with an excessive free radical production is unknown at present but available experimental evidence points toward the involvement of the mitochondrial electron transport chain and/or the cytochrome P450 oxidase systems. Previous studies of our group have shown that 8 wk ingestion of ST and other orally active AAS provokes a decrease in the activity of the mitochondrial respiratory chain complexes as well as degenerative changes of the mitochondria; moreover, AAS inhibited in vitro the mitochondrial electron transport chain [6,8]. Thus, a dysfunction of this chain could result in overproduction of ROS exceeding antioxidant defences. However this hypothesis does not fit well with the absence of changes in mitochondrial SOD activity that would be expected to increase as an immediate defence mechanism against oxidative damage to mitochondrial membranes particularly susceptible to lipid peroxidation. Another putative source of free radicals are hepatic cytochrome P450 (CYP) isoforms that have been shown to release ROS during their catalytic cycles contributing significantly to the total cellular production of reactive oxygen in rat liver even under basal conditions [11]. Therefore, metabolism by cytochrome P450 mono-oxygenases of the high doses of ST continuously administered to the rats would increase greatly the production of ROS and the resulting oxidative stress could, on one hand, up-regulate the activity of the antioxidant enzymes shown in this work and, on the other hand, repress the expression of cytochrome P450 isoenzymes genes [38]. This could explain the marked decrease in cytochrome P450 levels and mono-oxygenase activities previously detected in male rat liver after prolonged treatments with ST and other 17-α alkylated AAS [5,7].

In the present work, no significant changes that could be attributed to ST hepatotoxicity were detected in classical serum parameters related to hepatic function. Our results are consistent with those of previous studies in rodents [5–8] and in athletes who self-administered high doses of AAS in which observed serum alterations are minor and infrequent [3,4]. Nevertheless, it has been demonstrated that chronic treatment of rats with ST and other 17α-alkylated AAS provoked histopathological lesions in liver, exerted a proliferative effect on liver cells as indicated by increased mitosis and binucleation, caused ultrastructural abnormalities of hepatocytes, and induced changes in the activity of molecular components responsible for key processes as drug biotransformation and mitochondrial electron transport [5–8]. It must be kept in mind that the standard liver function tests routinely employed, in spite of their name, do not measure liver function in any quantitative sense, as they are concerned rather with severe liver damage. Therefore, cellular and molecular alterations on the borderline of being overtly cytotoxic to the liver would be no reflected in the conventional methods used for monitoring hepatic function.

Our results show that prolonged ST treatment can cause an oxidative stress situation in rat liver as indicated by enhanced lipid peroxidation extent. Interestingly, ROS overproduction and lipid peroxidation have been implicated in the pathogenesis of many types of liver injury and especially in the hepatic damage induced by several toxic drugs [37]. Oxidative stress has been also recently implicated in hormone-induced prostate carcinogenesis [39]. Thus, it is tempting to speculate with the possibility that the observed changes in prooxidant/antioxidant status could be causally related with the adverse effects of ST on liver. Mitochondria might be an important cellular target for oxidative damage since the mitochondrial membrane is rich in polyunsaturated fatty acids; alteration in the lipid environment of respiratory chain complexes may cause a decrease in their activity leading, in turn, to a perturbation of energetic metabolism. On the other hand, lipid peroxidation-derived aldehydes are generally stable and can diffuse within the cell [37]; hence they could attack targets far from the site of the original free radical-initiated event expanding oxidative damage to a wide range of molecules. Thus, lipid peroxidation might constitute an initial event of a multi-step process leading finally to liver injury. Further work is necessary to establish the relationship between changes in oxidative stress and ST-induced

liver damage and to elucidate the sequential mechanisms involved.

Acknowledgements

The authors are grateful to Angel Enrique Díaz (Consejo Superior de Deportes, C.N.I.C.D., Spain) for his collaboration in the measurement of serum enzyme activities, and to Zambon S.A. (Barcelona) for kindly providing us with stanozolol. This work was supported by grant no. PB98-0822 from the Dirección General de Enseñanza Superior e Investigación Científica (D.G.E.S.I.C.), Spain.

References

[1] N.T. Shahidi, A review of the chemistry, biological action, and clinical applications of anabolic-androgenic steroids, Clin. Therapeutics 23 (2001) 1355–1390.
[2] F. Celotti, P.N. Cesi, Anabolic steroids: a review of their effects on the muscles, of their possible mechanism of action and of their use in athletics, J. Steroid Biochem. Mol. Biol. 43 (1992) 469–477.
[3] R.C. Hickson, K.L. Ball, M.T. Falduto, Adverse effects of anabolic steroids, Med. Toxicol. Adverse Drug Exp. 4 (1989) 254–271.
[4] F. Hartgens, H. Kuipers, J.A. Wiinen, H.A. Keizer, Body composition, cardiovascular risk factors and liver function in long-term androgenic-anabolic steroids using bodybuilders three months after drug withdrawals, Int. J. Sports Med. 17 (1996) 429–433.
[5] L.D. Boada, M. Zumbado, S. Torres, A. López, B.N. Diaz-Chico, J.J. Cabrera, O.P. Luzardo, Evaluation of acute and chronic hepatotoxic effects exerted by anabolic-androgenic steroid stanozolol in adult male rats, Arch. Toxicol. 73 (1999) 465–472.
[6] R. Gragera, A. Saborido, F. Molano, L. Jiménez, E. Muñiz, A. Megías, Ultrastructural changes induced by anabolic steroids in liver of trained rats, Histol. Histopathol. 8 (1993) 449–455.
[7] A. Saborido, F. Molano, A. Megías, Effect of training and anabolic-androgenic steroids on drug metabolism in rat liver, Med. Sci. Sports Exerc. 25 (1993) 815–822.
[8] F. Molano, A. Saborido, J. Delgado, M. Morán, A. Megías, Rat liver lysosomal and mitochondrial activities are modified by anabolic-androgenic steroids, Med. Sci. Sports Exerc. 31 (1999) 243–250.
[9] L.L. Ji, Antioxidants and oxidative stress in exercise, Proc. Soc. Exp. Biol. Med. 222 (1999) 283–292.
[10] J. Bejma, P. Ramires, L.L. Ji, Free radical generation and oxidative stress with ageing and exercise: differential effects in the myocardium and liver, Acta Physiol. Scand. 169 (2000) 343–351.
[11] T.P. Dalton, H.G. Shertzer, A. Puga, Regulation of gene expression by reactive oxygen, Annu. Rev. Pharmacol. Toxicol. 39 (1999) 67–101.
[12] L.L. Ji, Exercise and oxidative stress: role of the cellular antioxidant systems, Exerc. Sports Sci. Rev. 23 (1995) 135–166.
[13] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[14] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
[15] F. Tietzte, Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues, Anal. Biochem. 27 (1969) 502–522.
[16] O.W. Griffith, Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine, Anal. Biochem. 106 (1980) 207–212.
[17] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltiel, E.R. Stadtman, Determination of carbonyl content in oxidatively modified proteins, Methods Enzymol. 186 (1990) 464–478.
[18] J. Delgado, A. Saborido, M. Morán, A. Megías, Chronic and acute exercise do not alter Ca^{2+} regulatory systems and ectonucleotidase activities in rat heart, J. Appl. Physiol. 87 (1999) 152–160.
[19] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474.
[20] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.
[21] L. Flohe, W.A. Gunzler, Assays of glutathione peroxidase, Methods Enzymol. 105 (1984) 114–121.
[22] I. Carlberg, B. Mannervik, Glutathione reductase, Methods Enzymol. 113 (1985) 484–490.
[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[24] A.Z. Reznick, E. Witt, M. Matsumoto, L. Packer, Vitamin E inhibits protein oxidation in skeletal muscle of resting and exercised rats, Biochem. Biophys. Res. Commun. 189 (1992) 801–806.
[25] J. Liu, H.C. Yeo, E. Overvik-Douki, T. Hagen, S.J. Doniger, D.W. Chu, G.A. Brooks, B.W. Ames, Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants, J. Appl. Physiol. 89 (2000) 21–28.
[26] P. Venditti, S. Di Meo, Antioxidants, tissue damage, and endurance in trained and untrained young male rats, Arch. Biochem. Biophys. 331 (1996) 63–68.
[27] M.M. Kanter, R.L. Hamlin, D.V. Unverferth, H.W. Davies, A.J. Merola, Effect of exercise training on antioxidant enzymes and cardiotoxicity of doxorubicin, J. Appl. Physiol. 59 (1985) 1298–1303.
[28] D.O. Wilson, P. Johnson, Exercise modulates antioxidant enzyme gene expression in rat myocardium and liver, J Appl. Physiol. 88 (2000) 1791–1796.
[29] H. Hong, P. Johnson, Antioxidant enzyme activities and lipid peroxidation levels in exercised and hypertensive rat tissues, Int. J. Biochem. Cell Biol. 27 (1995) 923–931.
[30] C. Leeuwenburgh, J. Hollander, S. Leichtweis, M. Griffiths, M. Gore, L.L. Ji, Adaptations of glutathione antioxidant system to endurance training are tissue and muscle fiber specific, Am. J. Physiol. 272 (1997) R363–R369.
[31] K. Duncan, S. Harris, M. Ardies, Running exercise may reduce risk for lung and liver cancer by inducing activity of antioxidant and phase II enzymes, Cancer Lett. 116 (1997) 151–158.
[32] C. Nakao, T. Ookawara, T. Kizaki, S. Oh-ishi, H. Miyazaki, S. Haga, Y. Sato, L.L. Ji, H. Ohno, Effects of swimming training on three superoxide dismutase isoenzymes in mouse tissues, J. Appl. Physiol. 88 (2000) 649–654.
[33] S. Asami, T. Hirano, R. Yamaguchi, Y. Tsurudome, H. Itoh, H. Kasai, Effects of forced and spontaneous exercise on 8-hydroxydeoxyguanosine levels in rat organs, Biochem. Biophys. Res. Commun. 243 (1998) 678–682.
[34] L.L. Ji, C. Leeuwenburgh, S. Leichtweis, M. Gore, R. Fiebig, J. Hollander, J. Bejma, Oxidative stress and aging. Role of exercise and its influences on antioxidant systems, Ann. N. Y. Acad. Sci. 854 (1998) 102–117.
[35] M. Locke, The cellular stress response to exercise: role of stress proteins, Exerc. Sport Sci. Rev. 25 (1997) 105–136.
[36] M.A. Febbraio, P. Ott, H.B. Nielsen, A. Steensberg, C. Keller, P. Krustrup, N.H. Secher, B.K. Pedersen, Hepatosplanchnic clearance of interleukin-6 in humans during exercise, Am. J. Physiol. 285 (2003) E397–E402.

[37] K. Uchida, M. Shiraishi, Y. Naito, Y. Torii, Y. Nakamura, T. Osawa, Activation of stress signaling pathways by the end product of lipid peroxidation. 4-Hydroxy-2-nonenal is a potential inducer of intracellular peroxide production, J. Biol. Chem. 274 (1999) 2234–2242.

[38] Y. Morel, R. Barouki, Repression of gene expression by oxidative stress, Biochem. J. 342 (1999) 481–496.

[39] N.N. Tam, S. Ghatak, S.M. Ho, Sex hormone-induced alterations in the activities of antioxidant enzymes and lipid peroxidation status in the prostate of Noble rats, Prostate 55 (2003) 1–8.